

-1-

**PREDICTING THE SUCCESS OF  
PANCREATIC ISLET ISOLATION: A STUDY  
OF PANCREATIC PRESERVATION AND  
ISLET CELL APOPTOSIS**

**BY**

**MR DANIEL M. RIDGWAY  
MB.CHB. MRCS.**

**DECEMBER 2008**

**A Thesis submitted to the University of  
Leicester for the Degree of Doctor of  
Medicine.**

UMI Number: U525594

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U525594

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

## ***Table of Contents.***

<b><i>Table of Contents.</i></b> .....	<b>2</b>
<b><i>Statement of Originality.</i></b> .....	<b>5</b>
<b><i>Dedication.</i></b> .....	<b>6</b>
<b><i>Acknowledgements.</i></b> .....	<b>7</b>
<b><i>Publications.</i></b> .....	<b>8</b>
<b><i>Abstract.</i></b> .....	<b>10</b>
<b><i>List of Tables.</i></b> .....	<b>11</b>
<b><i>List of Figures.</i></b> .....	<b>13</b>
<b><i>Abbreviations.</i></b> .....	<b>18</b>
<b><i>Part I. Literature Reviews.</i></b> .....	<b>22</b>
<b><i>Chapter 1. The Clinical Problem: Diabetes Mellitus.</i></b> .....	<b>23</b>
1.1 Introduction.....	23
1.2 Classification of diabetes mellitus. ....	23
1.3 Clinical Features and Diagnosis.....	24
1.4 Incidence and prevalence.....	25
1.5 Pathology of IDDM. ....	26
1.6 Genetic aetiology of IDDM. ....	28
1.7 Autoimmune basis of IDDM. ....	28
1.8 Environmental basis of IDDM.....	29
1.9 Costs and complications of IDDM. ....	31
1.10 Prevention of IDDM. ....	32
1.11 Treatments for diabetes.....	33
1.12 Conclusion. ....	34
<b><i>Chapter 2. Pancreas and Islet Cell Transplantation for Diabetes Mellitus.</i></b> .....	<b>36</b>
2.1 Introduction.....	36
2.2 Vascularised pancreas and clinical islet transplantation.....	36
2.3 Technical aspects of islet cell transplantation.....	49
2.4 Conclusion. ....	68
<b><i>Chapter 3. Management of the Donor Pancreas.</i></b> .....	<b>70</b>
3.1 Introduction.....	70
3.2 The pre-preservation phase. ....	70
3.3 Preservation of the donor pancreas. ....	75

3.4 Practical pancreas preservation methods. ....	80
3.5 Conclusion. ....	90
<b>Chapter 4. Apoptosis in Pancreas and Islet Cell Transplantation. ....</b>	<b>91</b>
4.1 Introduction. ....	91
4.2 Morphology of apoptosis. ....	91
4.3 Induction of apoptosis. ....	96
4.4 Bcl-2 genes controlling apoptosis and anoikis. ....	100
4.5 The Bioenergetics of Apoptosis and Pancreas Preservation. ....	102
4.6 Conclusion. ....	106
<b>Part II. Experimental Chapters. ....</b>	<b>188</b>
<b>Chapter 5. Development of Novel Methodology. ....</b>	<b>109</b>
5.1 Introduction. ....	109
5.2 Preliminary experiments using the luciferin assay. ....	109
5.3 Preliminary experiments to determine sample cellularity. ....	116
5.4 Preliminary experiments on immunohistochemistry of apoptotic proteins. ....	120
5.5 Conclusion. ....	132
<b>Chapter 6. Studies in the Rodent Model. ....</b>	<b>133</b>
6.1 Introduction. ....	133
6.2 Aims. ....	133
6.3 Methods. ....	133
6.4 Statistical Methodology. ....	151
6.5 Results. ....	152
6.6 Conclusion. ....	171
<b>Chapter 7. Studies in the Porcine Model. ....</b>	<b>174</b>
7.1 Introduction. ....	174
7.2 Aims. ....	174
7.3 Methods. ....	174
7.4 Statistical Methodology. ....	186
7.5 Results. ....	186
7.6 Factor Analysis. ....	193
7.7 Conclusion. ....	193
<b>Chapter 8. Studies in the Human Donor Pancreas. ....</b>	<b>194</b>
8.1 Introduction. ....	194
8.2 Aims. ....	194

8.3 Methods.....	194
8.4 Statistical Methodology.....	201
8.5 Results.....	201
8.6 Conclusion.....	204
<b>Chapter 9. Discussion, Limitations and Future Work.....</b>	<b>205</b>
9.1 Introduction.....	205
9.2 Islet Yields.....	205
9.3 Gradient purity.....	208
9.4 In vitro islet function.....	210
9.5 Apoptotic gene expression.....	212
9.6 Apoptotic immunohistochemistry.....	214
9.7 Adenine nucleotide ratios.....	216
9.8 Factor Analysis.....	219
<b>Bibliography.....</b>	<b>222</b>

## ***Statement of Originality.***

This thesis has been produced from my own individual work at the University of Leicester Department of Surgery from 2001 to 2004.

Mr Daniel M. Ridgway

## ***Dedication.***

For my Mum and Dad, with thanks for their enduring love and support in all that I do....

....and dedicated to the memory of my Grandparents.

## *Acknowledgements.*

The work to prepare this thesis would not have been possible without the guidance and assistance of the laboratory and clinical staff of the University of Leicester. In particular I would like to thank Dr Sue Swift, Head of Islet Isolation Laboratory, for her invaluable guidance in the early stages and with many of the laboratory methods.

Dr Roger James provided expert tuition and guidance in rodent islet isolation and viability experiments. Miss Rachel Kimber provided the tissue, islet yield and functionality data for the porcine experiments. Mr Danish Kanji assisted with developing the luciferin-luciferase assay, and Mr Naufil Alam with the TUNEL, Bax and Bcl-2 assays. Miss Helen Waller and Dr Gareth Bicknell assisted in the development of PCR methodology, and Miss Punam Metha with IHC semi-quantification.

I would also like to thank the surgical and laboratory members of the Human Islet Programme who gave their time and expertise, often at the most anti-social of hours, to procure and process the human donor pancreata.

I must also thank my Head of Department, Professor Michael Nicholson, for allowing this work to take place in the Transplant Laboratory facilities; and my Supervisor Mr Steve White for his encouragement, criticism and guidance throughout the work and preparation of the manuscript.

Finally I must thank my family and friends, especially Rachel, who have persevered with and supported me to ensure the completion of the work.

## ***Publications.***

### PEER REVIEWED PUBLICATIONS.

---

**Ridgway D.** White S.A, Swift S, Kimber R and Nicholson M.L. Pancreatic islet cell transplantation in the treatment of diabetes mellitus. *Update* 2001 (10):304-307.

**Ridgway D.** Nicholson ML and White SA. Islet cell transplantation: progress in the clinical setting. *Treat Endocrinol* 2003; 2(3):173-189.

### POSTER PRESENTATIONS TO MEETINGS AND LEARNED SOCIETIES.

---

9<sup>th</sup> April 2003      The British Transplant Society 6<sup>th</sup> Annual Congress, Commonwealth Institute, Kensington, London, U.K.

**Ridgway DM.** Swift SM, White SA and Nicholson ML. Pancreatic cold ischaemia: the influence upon islet yield – 5 years experience in human islet isolation.

Kimber RM, White SA, **Ridgway DM.** Swift SM and Nicholson ML. The effect of two-layer organ preservation on islet yield after warm ischaemic injury.

Swift SM, Alam NA, Simpson EL, **Ridgway DM.** James RFL, Pringle H, White SA and Nicholson ML. Cell death in the pancreas during islet isolation.

Swift SM, Seare NJ, Simpson EL, **Ridgway DM.** Brook NR, Lewis GRR, White SA and Nicholson ML. Counting the cost of clinical grade human islets.

20-24<sup>th</sup> Sept 2003      European Society of Organ Transplantation – ESOT 2003, Venice, Italy.

Swift SM, Seare NJ, Simpson EL, **Ridgway DM.** Brook NR, Lewis GRR, White SA and Nicholson ML. Counting the cost of clinical grade human islets.

Swift SM, Alam NA, Simpson EL, **Ridgway DM.** James RFL, Pringle H, White SA and Nicholson ML. Cell death in the pancreas during islet isolation.

26<sup>th</sup>-28<sup>th</sup> Jan 2004      23<sup>rd</sup> Workshop of the AIDPIT Study Group, Igls, Austria.

## ***Publications.***

### PEER REVIEWED PUBLICATIONS.

---

**Ridgway D.** White S.A, Swift S, Kimber R and Nicholson M.L. Pancreatic islet cell transplantation in the treatment of diabetes mellitus. *Update* 2001 (10):304-307.

**Ridgway D.** Nicholson ML and White SA. Islet cell transplantation: progress in the clinical setting. *Treat Endocrinol* 2003; 2(3):173-189.

### POSTER PRESENTATIONS TO MEETINGS AND LEARNED SOCIETIES.

---

9<sup>th</sup> April 2003      The British Transplant Society 6<sup>th</sup> Annual Congress, Commonwealth Institute, Kensington, London, U.K.

**Ridgway DM.** Swift SM, White SA and Nicholson ML. Pancreatic cold ischaemia: the influence upon islet yield – 5 years experience in human islet isolation.

Kimber RM, White SA, **Ridgway DM.** Swift SM and Nicholson ML. The effect of two-layer organ preservation on islet yield after warm ischaemic injury.

Swift SM, Alam NA, Simpson EL, **Ridgway DM.** James RFL, Pringle H, White SA and Nicholson ML. Cell death in the pancreas during islet isolation.

Swift SM, Seare NJ, Simpson EL, **Ridgway DM.** Brook NR, Lewis GRR, White SA and Nicholson ML. Counting the cost of clinical grade human islets.

20-24<sup>th</sup> Sept 2003      European Society of Organ Transplantation – ESOT 2003, Venice, Italy.

Swift SM, Seare NJ, Simpson EL, **Ridgway DM.** Brook NR, Lewis GRR, White SA and Nicholson ML. Counting the cost of clinical grade human islets.

Swift SM, Alam NA, Simpson EL, **Ridgway DM.** James RFL, Pringle H, White SA and Nicholson ML. Cell death in the pancreas during islet isolation.

26<sup>th</sup>-28<sup>th</sup> Jan 2004      23<sup>rd</sup> Workshop of the AIDPIT Study Group, Igls, Austria.

**Ridgway DM**, Swift SM, White SA and Nicholson ML. The role of ATP bioluminescence during pancreatic storage and islet isolation.

**Ridgway DM**, Swift SM, Nakamura T, Wada H, Nicholson ML and White SA. Improvement of islet yield by pancreas preservation using trehalose containing solution.

27<sup>th</sup> -29<sup>th</sup> Jan 2008

27th Workshop of the AIDPIT Study Group, Igls, Austria.

**D.M.Ridgway**, R.M.Kimber, S.M.Swift, M.L.Nicholson and S.A.White. Pancreatic ADP:ATP ratio predicts islet yield after porcine pancreas preservation.

**D.M.Ridgway**, S.M.Swift, M.L.Nicholson and S.A.White. Pancreatic ADP:ATP ratio predicts acinar tissue expression of Bcl-2 after rodent pancreas static cold storage.

**D.M.Ridgway**, S.M.Swift, M.L.Nicholson and S.A.White. Preservation of the porcine pancreas: an assessment using ADP:ATP ratio.

**D.M.Ridgway**, S.M.Swift, M.L.Nicholson and S.A.White. Human pancreatic ADP:ATP ratio correlates with cold ischaemic preservation injury.

## ***Abstract.***

Islet cell transplantation offers a potential cure for diabetes. However, procurement of a high quality donor pancreas, maintenance of islet viability during pancreatic preservation and isolation of a high yield of viable and functioning islets are critical for successful islet cell transplantation.

This thesis used a novel method of assessing donor pancreatic adenosine nucleotide concentrations under different preservation conditions and throughout the isolation process. These were correlated with conventional measures of islet yield, viability, function and novel immunohistochemical markers of pancreatic apoptosis.

In a rodent model, high tissue ADP:ATP ratios were correlated with low islet yields ( $R=-0.67$ ), low *in vitro* islet function assessed by static incubation ( $R=-0.61$ ), high levels of gradient contamination ( $R=0.73$ ) and positive immunostaining for the pro-apoptotic protein Bax in islet ( $R=0.77$ ) and acinar tissues ( $R=0.80$ ).

In preserved porcine pancreata, higher pancreatic ADP:ATP ratios were obtained after machine perfusion (1.78) compared to either two-layered method (0.99), cold stored groups (1.19) and unpreserved pancreas (0.73); and were seen as the duration of preservation increased. High ADP:ATP ratios were correlated with a reduced islet yield ( $-0.51$ ) but showed no correlation with other *in vitro* measures of gradient purity, islet function or apoptotic index.

In human pancreata, preserved using University of Wisconsin cold storage, higher mean ADP:ATP ratios were obtained as the duration of cold ischaemia increased ( $F(4,32)=19.8$ ;  $p<0.001$ )

Rapid luciferin-luciferase determination of ADP:ATP ratios provides a means to predict islet yield in rodent and porcine models after pancreas preservation. Additionally, the ratio predicts islet purity and *in vitro* function of rodent islets. It provides a rapid means of assessing tissue viability during pancreas preservation, including cold ischaemic injury in the human pancreas, and may predict the likelihood of subsequent successful islet isolation.

## ***List of Tables.***

Table 1-1. Clinical features of diabetes mellitus. ....	24
Table 1-2. ADA diagnostic criteria for diabetes(2) .....	25
Table 2-1. Long-term outcomes of SPK from University of Wisconsin(97). ....	43
Table 4-1. Morphology of necrotic and apoptotic cell death.....	91
Table 6-1. Experimental protocols for the rodent studies. (n) per group. ....	135
Table 6-2. Primer and probe sequences used for real time PCR analysis.....	145
Table 6-3. Serial section staining protocol for Bax and Bcl-2 immunohistochemistry..	146
Table 6-4. Significant differences in islet IEQ yields from preservation groups. ....	153
Table 6-5. Significant differences in gradient contamination between preservation groups. .....	154
Table 6-6. Differences in mean stimulation index assessed by static incubation.....	155
Table 6-7. Significant differences in mean Bax immunohistochemistry staining across preservation groups.....	159
Table 6-8. Significant differences in Bcl-2 immunohistochemical staining across preservation groups.....	160
Table 6-9. ADP:ATP ratios in pre-preservation pancreata of each group. ....	163
Table 6-10. Significant differences in post-preservation ADP:ATP ratios between preservation groups.....	164
Table 6-11. Significant differences in digest ADP:ATP ratios between preservation groups.....	164
Table 6-12. Significant differences in ADP:ATP ratios in pre-culture islets between preservation groups.....	165
Table 6-13. Significant differences in ADP:ATP ratios of post-culture islets between preservation groups.....	165
Table 6-14. ATP:DNA ratios in pre-preservation pancreata of each group. ....	168
Table 6-15. Significant differences in post-preservation ATP:DNA ratios between preservation groups.....	169
Table 6-16. Significant differences in digest ATP:DNA ratios between preservation groups.....	169
Table 6-17. Significant differences in ATP:DNA ratios in pre-culture islets between preservation groups.....	170

Table 6-18. Significant differences in ATP:DNA ratios of post-culture islets between preservation groups.....	170
Table 6-19. Correlations of ADP:ATP ratio with other tests of islet function and viability. ....	171
Table 6-20. Correlation of ATP:DNA ratios with other tests of islet function and viability. ....	171
Table 7-1. Preservation groups used in porcine studies.....	175
Table 7-2. Serial staining protocol for TUNEL assay. ....	182
Table 7-3. Differences between mean islet yields in different preservation groups.....	187
Table 7-4. Differences between islet cleavage indices in different preservation groups.	187
Table 7-5. Significantly increasing levels of gradient contamination between preservation groups.....	188
Table 7-6. Increasing perfusion stimulation indices by preservation method. ....	189
Table 7-7. Differences in mean TUNEL apoptotic index between preservation methods. ....	190
Table 7-8. Mean porcine pancreas ADP:ATP ratios by preservation method.....	191
Table 7-9. Mean porcine ADP:ATP ratios with increasing duration of preservation. ...	191
Table 7-10. Mean ATP:DNA ratios between different preservation groups. ....	192
Table 7-11. Mean ATP:DNA ratios with increasing duration of preservation preservation ....	192
Table 7-12. Correlations of ADP:ATP ratio with other tests of islet function and viability. ....	193
Table 8-1. Human pancreas donor and digest data. ....	201

## *List of Figures.*

Figure 1-1. The geographical incidence of diabetes. ....	26
Figure 1-2. Time dependent pathogenesis of Type 1A diabetes(14). ....	27
Figure 2-1. SPK with vesical and systemic iliac drainage. ....	40
Figure 2-2. SPK with enteric and portal venous drainage. ....	40
Figure 2-3. The COBE 2991 cell processor. ....	52
Figure 2-4. Density gradient purification on the COBE processor. ....	52
Figure 3-1. Schematic illustration of TLM. ....	86
Figure 4-1. Morphology of apoptosis and necrosis. ....	93
Figure 4-2. Extrinsic and intrinsic apoptotic induction pathways(719). ....	97
Figure 4-3 The luciferase reaction to generate bioluminescence. ....	105
Figure 4-4. Conversion of ADP to ATP using ADP converting reagent. ....	105
Figure 4-5. Modes of cell death and output from the ATP assay. ....	105
Figure 5-1. Logarithmic transformation of ATP standard luminescence. ....	111
Figure 5-2. Cumulative results of luminometer ATP standard calibrations. ....	111
Figure 5-3. Variation of ATP concentration with number of assayed pancreas sections. .....	112
Figure 5-4. Variation of ATP concentration with number of assayed digest sections. ..	113
Figure 5-5. Variation of ATP concentration with width of assayed pancreas section....	113
Figure 5-6. Variation of ATP concentration with width of assayed digest section. ....	113
Figure 5-7. Comparable levels of ATP from three pancreatic areas. ....	114
Figure 5-8. Variation of ATP concentration with section storage temperature and duration. ....	114
Figure 5-9. Variation of ATP concentration with number of pre-cultured islets assayed. .....	115
Figure 5-10. Variation of ATP concentration with number of post-culture islets assayed. .....	115
Figure 5-11. ADP:ATP ratios for each phase of isolation in fresh pancreata. ....	116
Figure 5-12. Variation in rapid DNA concentration with increasing number of sections assayed. ....	118
Figure 5-13. Variation in rapid DNA concentration with increasing width of section assayed. ....	118

Figure 5-14. Variation in rapid DNA concentration with increasing number of islets assayed. ....	119
Figure 5-15. Scatter plot demonstrating correlation between rapid and conventional DNA assays. ....	119
Figure 5-16. ATP:DNA ratios for each phase of isolation in fresh pancreata. ....	120
Figure 5-17. Staining of positive controls after 10 minutes of antigen presentation. ....	122
Figure 5-18. Staining of positive controls after 20 minutes of antigen presentation. ....	123
Figure 5-19. Staining of positive controls after 30 minutes of antigen presentation. ....	123
Figure 5-20. Staining of negative controls after 10 minutes of antigen presentation. ....	124
Figure 5-21. Staining of negative controls after 20 minutes of antigen presentation. ....	124
Figure 5-22. Staining of negative controls after 30 minutes of antigen presentation. ....	125
Figure 5-23. Staining of positive controls after 90 minutes tertiary antibody incubation. .....	125
Figure 5-24. Staining of positive controls after 60 minutes tertiary antibody incubation. .....	126
Figure 5-25. Staining of positive controls after 30 minutes tertiary antibody incubation. .....	126
Figure 5-26. Staining of negative controls after 90 minutes tertiary antibody incubation. .....	127
Figure 5-27. Staining of negative controls after 60 minutes tertiary antibody incubation. .....	127
Figure 5-28. Staining of negative controls after 30 minutes tertiary antibody incubation. .....	128
Figure 5-29. Staining of positive controls after 10 minutes antigen presentation. ....	128
Figure 5-30. Staining of positive controls after 20 minutes antigen presentation. ....	129
Figure 5-31. Staining of positive controls after 30 minutes antigen presentation. ....	129
Figure 5-32. Staining of negative controls after 10 minutes antigen presentation. ....	130
Figure 5-33. Staining of negative controls after 20 minutes antigen presentation. ....	130
Figure 5-34. Staining of negative controls after 30 minutes antigen presentation. ....	131
Figure 5-35. Staining of positive controls after 60 minutes tertiary antibody incubation. .....	131
Figure 5-36. Staining of negative controls after 60 minutes tertiary antibody incubation. .....	132
Figure 6-1. Rodent dissection demonstrating cannulation of the pancreatic duct. ....	134

Figure 6-2. UW and ET-K solutions compared during rodent experiments.....	135
Figure 6-3. Collagenase and collagenase distension of the preserved rodent pancreas..	136
Figure 6-4. Apparatus for rodent TLM preservation. ....	136
Figure 6-5. Discontinuous BSA density gradients for rodent islet purification. ....	138
Figure 6-6. Rodent islets stained with dithizone - insulin granules within $\beta$ cells staining red. ....	139
Figure 6-7. Apparatus for BSA continuous mini-gradients and measurement of gradient purity. ....	140
Figure 6-8. Cumulative insulin and amylase content in a freshly processed pancreas mini-gradient. ....	141
Figure 6-9. Thermocycler used for RT-PCR. ....	144
Figure 6-10. Gel electrophoresis used for PCR product analysis. ....	144
Figure 6-11. The indirect sandwich system of Bax and Bcl-2 immunohistochemistry..	148
Figure 6-12. Luminometer cassette for ATP:ADP assay.....	150
Figure 6-13. Chart recorder tracing of ATP:ADP luminescence.....	150
Figure 6-14. Luminometer used for DNA assay.....	151
Figure 6-15. Stacked bar chart demonstrating islet yield and sizes from pancreata using different preservation methods. ....	152
Figure 6-16. Calculated % amylase contamination at a 60% insulin content by different storage methods. ....	153
Figure 6-17. Islet stimulation indices by static incubation achieved after differing methods of preservation. Table 6-6 shows increasing mean SI from static incubations and a lack of subset overlap again denotes a significant difference between preservation methods. ....	154
Figure 6-18. Example agarose gel of $\beta$ actin amplification from 3 preservation groups.	155
Figure 6-19. Representative real time PCR amplification plot of Bcl-2 expression. ....	156
Figure 6-20. Variation in Bax and Bcl2 mRNA expression between storage groups assessed by real time PCR. ....	157
Figure 6-21. Variation in expression of the endogenous control beta actin between storage groups.....	157
Figure 6-22. Variation in Bax positive staining amongst acinar and islet tissues in different preservation groups. ....	158
Figure 6-23. Variation in Bcl2 positive staining in acinar and islet tissues in different preservation groups.....	159

Figure 6-24. ADP:ATP ratios in the pre-stored pancreas across different preservation groups.....	161
Figure 6-25. ADP:ATP ratios in the post-storage pancreas across different preservation groups.....	161
Figure 6-26. ADP:ATP ratios in the pancreatic digest across different preservation groups.....	162
Figure 6-27. ADP:ATP ratios in pre-cultured islets across preservation groups.....	162
Figure 6-28. ADP:ATP ratios in post-culture islets across preservation groups. ....	163
Figure 6-29. ATP:DNA ratios in pre-stored pancreata using different preservation methods. ....	166
Figure 6-30. ATP:DNA ratios in post-storage pancreata using different preservation methods. ....	166
Figure 6-31. ATP:DNA ratios in pancreatic digest using different preservation methods. ....	167
Figure 6-32. ATP:DNA ratios in pre-cultured islets using different preservation methods. ....	167
Figure 6-33. ATP:DNA ratios in post-culture islets using different preservation methods. ....	168
Figure 7-1. The RM Waters renal perfusion machine used for perfusive preservation..	176
Figure 7-2. Human liberase used for porcine islet isolation. ....	177
Figure 7-3. Semi automated chamber for pancreatic digestion. ....	177
Figure 7-4. Example perfusion plot from porcine islets. ....	181
Figure 7-5. The TUNEL technique of immunohistochemistry.....	184
Figure 7-6. Mean islet yields after pancreas preservation by different methods. ....	186
Figure 7-7. Mean islet cleavage indices after pancreatic preservation by different methods. ....	187
Figure 7-8. Minigradient contamination at 60% islet yield after different preservation methods. ....	188
Figure 7-9. Perfusion stimulation indices after different methods of pancreas preservation.....	189
Figure 7-10. Variation in TUNEL staining in islets and acinar tissues after different preservation methods. ....	190
Figure 7-11. ADP:ATP ratios in the porcine pancreas preserved using different methods. ....	191

Figure 7-12. ATP:DNA ratios by different preservation methods. ....	192
Figure 8-1. En bloc retrieval of the human donor pancreas.....	195
Figure 8-2. Pancreatic digest prior to COBE purification. ....	197
Figure 8-3. Digest yields from human pancreata.....	202
Figure 8-4. Purified islet yields from human pancreata.....	202
Figure 8-5. Post-culture islet yields from human pancreata. ....	203
Figure 8-6. Islet stimulation indices from human pancreata.....	203
Figure 8-7. ADP:ATP ratios obtained after controlled periods of cold ischaemia in the human pancreas.....	204

## *Abbreviations.*

°C	degrees celsius
μl	microlitre
μm	micrometer
μmol	micromols per litre
AD	anno domini
ADA	American Diabetes Association
AIF	apoptosis inducing factor
ALS	anti-lymphocyte serum
APC	antigen presenting cell
ATG	anti-thymocyte globulin
BC	before Christ
BMI	body mass index
BSA	bovine serum albumin
CD	cluster determinant
CITR	Collaborative Islet Transplant Registry
cm	centimetre
CMV	cytomegalovirus
CO <sub>2</sub>	carbon dioxide
CPP	cryoprecipitated plasma
CsA	cyclosporin A
DAISY	Diabetes Autoimmune Study in the Young
DCCT	Diabetes Control and Complications Trial
DGF	delayed graft function
DM	diabetes mellitus
DNA	deoxyribose nucleic acid
DPPRG	Diabetes Prevention Programme Research Group
DXR	delayed xenograft rejection
EC	Euro-Collins solution
ECM	extracellular matrix
ELISA	enzyme linked immunosorbant assay
ERK	extracellular signal-regulated kinase

ESRF	end stage renal failure
ET-K	extracellular type University of Kyoto solution
FAK	focal adhesion kinase
FSD	Ficoll-Sodium diatrizoate
g	gram
GAD	glutamic acid decarboxylase
GBSS	Geys balanced salt solution
GFR	glomerular filtration rate
GLP-1	glucagon like peptide 1
GPx	glutathione peroxidase
GTT	glucose tolerance test
HAR	hyperacute rejection
HbA1c	glycosylated haemoglobin
HBSS	Hanks balanced salt solution
HES	hydroxyethyl starch
HLA	human leucocyte antigen
HMHO	Her Majesty's Home Office
HO-1	hemeoxygenase-1
HOC	hyperosmolar citrate solution
HTK	histidine tryptophan ketoglutarate solution
IAK	islet after kidney transplant
IAP	endogenous apoptosis inhibitor
IBMIR	instant blood mediated inflammatory reaction
ICA	islet cell autoantibodies
ICAM	intercellular adhesion molecule
IDDM	insulin dependent diabetes
IEQ	standard islet equivalents
IFN $\alpha$	interferon alpha
IFN $\gamma$	interferon gamma
IHC	immunohistochemistry
IITR	International Islet Transplant Registry
IL-1 $\beta$	interleukin 1 beta
ILK	integrin linked kinase

IPTR	International Pancreas Transplant Registry
ITA	islet transplant alone
JNK	c-Jun NH2-terminal kinases
K <sup>+</sup>	potassium
LFA	lymphocyte functional antigen
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
MEM	minimal essential medium
mg	milligram
MHC	major histocompatibility complex
MHz	megahertz
MI	myocardial infarction
ml	millilitre
mm	millimetre
MMF	mycophenolate mofetil
MPO	myeloperoxidase
mV	millivolts
Na <sup>+</sup>	sodium
NF-κB	nuclear factor kappa beta
NGS	normal goat serum
NHBD	non-heartbeating donor
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
NIDDM	non insulin dependent diabetes
NIH	National Institute for Health
nm	nanometre
NOD	non obese diabetic
NPH	neutral protamine Hagedorn
PAK	pancreas after kidney transplant
PEP	phosphoenolpyruvate
PFC	perfluorochemical
PNF	primary non function
PT	mitochondrial permeability transition pore

PTA	pancreas transplant alone
PTLD	post-transplantation lymphoproliferative disease
RNA	ribose nucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium 1640
RR	risk ratio
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immunodeficiency disease
SGFP	silica gel filtered plasma
SI	stimulation index
SIK	simultaneous islet and kidney transplant
SPK	simultaneous pancreas and kidney transplant
SPSS	Statistical Package for Social Sciences
TAN	total adenine nucleotides
TBA	thiobarbituric acid
TBS	TRIS buffered saline
TLM	two layer method
TNFR	tumour necrosis factor receptor
TNF $\alpha$	tumour necrosis factor alpha
TRIGR	Trial to Reduce Insulin dependent diabetes in the Genetically at Risk
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
UK	United Kingdom
UNOS	United Network for Organ Sharing
US	United States
USRDS	United States Renal Data System
UTI	urinary tract infection
UW	University of Wisconsin solution
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen
VPT	vascularised whole pancreas transplant
$\beta$ cells	beta cells
$\Delta\phi_M$	mitochondrial membrane potential

***Part I- Literature Reviews.***

# ***Chapter 1. The Clinical Problem: Diabetes Mellitus.***

## ***1.1 Introduction.***

Diabetes mellitus has been a clinical entity since ancient times and the Egyptian Ebers Papyrus dated to 1550BC provides the earliest description of a diabetes like illness. The term 'diabete' is Ionian Greek for a siphon (*dia*- 'through' and *bainein*- 'to go') and was first used by Aretaeus of Cappodcia (81-138 AD); while 'mellitus' is Latin meaning honied. Aretaeus described diabetes as 'a mysterious affection...melting down of the flesh and limbs into urine...life is short, disgusting and painful, thirst unquenchable, death inevitable...' This graphically describes the three main symptoms of the disease: polydipsia, polyuria and weight loss.

In 1674 Thomas Willis(1621-1675) described the sweet nature of diabetic urine but attributed this to urinary salts and acids rather than sugar. Experiments carried out by Joseph Freiherr von Mering (1849-1908) and Oscar Minkowski (1859-1931) on pancreatectomised dogs first showed the relationship between the pancreas and diabetes. In 1869 Paul Langerhans (1847-1888) described pancreatic acinar tissue and islet cells, and in 1900 Opie (1873-1971) discovered that it was the destruction of these islets that caused diabetes.

In 1921 insulin was extracted from canine pancreata by Frederick G. Banting (1891-1941) and Charles H. Best (1899-1978) for which they, along with MacLeod and Collip, won the Nobel Prize for Medicine and Physiology. In 1922, a fourteen year old boy, Leonard Thompson, became the first to be treated with an extract of sheep pancreas containing insulin.

## ***1.2 Classification of diabetes mellitus.***

Diabetes is a syndrome of metabolic disorders characterised by alterations in carbohydrate, fat and protein metabolism, and chronic hyperglycaemia due to relative insulin deficiency, resistance or both.

Two major sub-groups are described, insulin dependent or Type 1, and non-insulin dependent or Type 2 diabetes mellitus. Insulin dependent diabetes mellitus (IDDM) is a chronic disorder caused by the autoimmune destruction of insulin producing pancreatic beta ( $\beta$ ) cells in genetically susceptible individuals(1).

Non- insulin dependent diabetes (NIDDM) is more common than IDDM and is associated with a lack of sensitivity of tissue insulin receptors in the face of normal levels of insulin production – so called ‘insulin resistance’. This most commonly occurs in middle age and may be sub-classified into obese and non-obese NIDDM.

Other important, but less common types of diabetes include gestational diabetes, where glucose intolerance first appears during pregnancy, mature onset diabetes of the young, and malnutrition related diabetes. There are several secondary causes of diabetes including pancreatic diseases such as chronic pancreatitis, endocrine disorders such as Cushings disease, acromegaly and phaeochromocytoma, and drug induced diabetes.

The American Diabetes Association (ADA) committee recommends the term Type 1A diabetes is used for immune mediated diabetes with destruction of the islet  $\beta$  cells of the pancreas, whilst non immune mediated diabetes with severe insulin deficiency is termed Type 1B. In this thesis the terms ‘diabetes’, ‘diabetes mellitus (DM)’, ‘Type 1 diabetes’ and ‘IDDM’ are synonymous and refer to Type 1A diabetes(2).

### ***1.3 Clinical Features and Diagnosis.***

IDDM classically presents as a triad of symptoms, namely polydipsia, polyuria and substantial weight loss, though other symptoms may also occur at presentation.(Table 1-1)

<b>POLYDIPSIA</b>	<b>MYOPIA</b>
<b>POLYURIA</b>	<b>DRY MOUTH</b>
<b>WEIGHT LOSS</b>	<b>NOCTURIA</b>
<b>BALINITIS</b>	<b>WASTING</b>
<b>FATIGUE/TIREDNESS</b>	<b>PURITIS</b>
<b>VULVAE</b>	

**Table 1-1. Clinical features of diabetes mellitus.**

Diagnosis of diabetes must be made by the demonstration of hyperglycaemia, by three possible methods, which must then be confirmed on a subsequent day. These diagnostic criteria are presented in Table 1-2.

<b>SYMPTOMS OF DIABETES PLUS RANDOM PLASMA GLUCOSE CONCENTRATION OF <math>\geq</math> 11.1MMOL/L</b>
<b>OR</b>
<b>FASTING PLASMA GLUCOSE OF <math>\geq</math> 7.0MMOL/L AFTER AT LEAST 8 HOURS</b>
<b>OR</b>
<b>2 HOUR PLASMA GLUCOSE OF <math>\geq</math> 11.1MMOL/L DURING AN ORAL GLUCOSE TOLERANCE TEST COMPRISING A GLUCOSE LOAD OF 75G ANHYDROUS GLUCOSE DISSOLVED IN WATER.</b>

Table 1-2. ADA diagnostic criteria for diabetes(2)

#### ***1.4 Incidence and prevalence.***

Diabetes is a common disorder affecting approximately 143 million people worldwide with the number expected to reach 333 million by 2025(3). The UK also has a rising incidence and 18 to 20 per 100,000 children are diagnosed each year(4). Furthermore, recent epidemiological studies now show a comparable incidence in adults and that another peak incidence is seen in people aged 65-74 years(5).

Geographical variation exists in the incidence of diabetes. A child in Finland is almost 40 times more likely to develop the disease than a child in Japan(6), and almost 100 times more likely to get it than a child in the Zunyi region of China(4).

The EURODIAB collaborative study, involving 44 countries in Europe, indicates an annual rate of increase in incidence of 3-4%, with a larger increases in some central and Eastern European countries especially amongst children aged 0-4 years(7).

There appears to be a gradient of incidence from North to South across Europe. However this is not as simple as it first appears, as incidence rates in Iceland are similar to those found in Spain, and Sardinia has rates approaching those found in Finland(6) (Figure 1-1).



**Figure 1-1. The geographical incidence of diabetes.**

In the UK, Scotland has the highest incidence of diabetes; rising from 13.8/100,000 between 1968 and 1976, to 23.9/100,000 in 1993(8) whilst the Republic of Ireland has the lowest incidence (6.8/100,000)(9). Additionally, it is significantly more common in non Caucasian UK ethnic communities compared with the Caucasian population: up to five times more common in people of Asian descent and up to four times as common in people of African and Afro-Caribbean descent. Though there is no difference between sexes and the peak age specific incidence, of 22/100,000 occurs at 12 years of age(9).

### ***1.5 Pathology of IDDM.***

Islets of Langerhans are discrete, rounded clusters of cells scattered throughout the pancreas and making up approximately 2-5% of the pancreatic mass in adults. There are four endocrine cell types within the islets. Approximately 80% are insulin secreting beta ( $\beta$ ) cells, 15% glucagon secreting alpha ( $\alpha$ ) cells, 4% somatostatin secreting delta ( $\delta$ ) cells and 1% are F cells containing pancreatic polypeptide. In a person with long standing IDDM the pancreas contains islet remnants with normal numbers of  $\alpha$ ,  $\delta$  and F cells but an absence of  $\beta$  cells. However, there is a long prodromal phase in IDDM and approximately 80% of the  $\beta$  cell mass must be lost before the disease is clinically apparent. So at the time of diagnosis there are three morphologically distinct types of islets in the pancreas which represent different stages of the disease process. i.e. before, during and after destruction of  $\beta$  cells.(10). These are:

- Insulin deficient islets - which form the majority .
- Islets containing  $\beta$  cells infiltrated by chronic inflammatory cells (insulinitis)

- Histologically normal islets with a normal complement of  $\beta$  cells.

The hall mark of Type 1 diabetes is the selective destruction of insulin producing cells in the pancreas; termed 'insulinitis' and is characterised by the presence of certain genetic and immunological markers. Examination of islet tissue in pancreatic biopsies from patients with recent onset Type 1 diabetes confirms insulinitis, and the presence of an infiltrate composed of  $CD4^+$  and  $CD8^+$  T lymphocytes, B lymphocytes and macrophages, suggesting that these cells have a role in the preferential destruction of the  $\beta$  cells(11). Early studies in mice showed that using anti- $CD3^+$  treatment prevented diabetes(12), and a trial using humanized anti- $CD3^+$  antibody in patients with new onset Type 1 diabetes showed maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group. They also had reduced glycosylated haemoglobin (HbA1c) levels and insulin doses. These clinical responses were associated with a change in the ratio of  $CD4^+$  T cells to  $CD8^+$  T cells at 30 and 90 days(13).

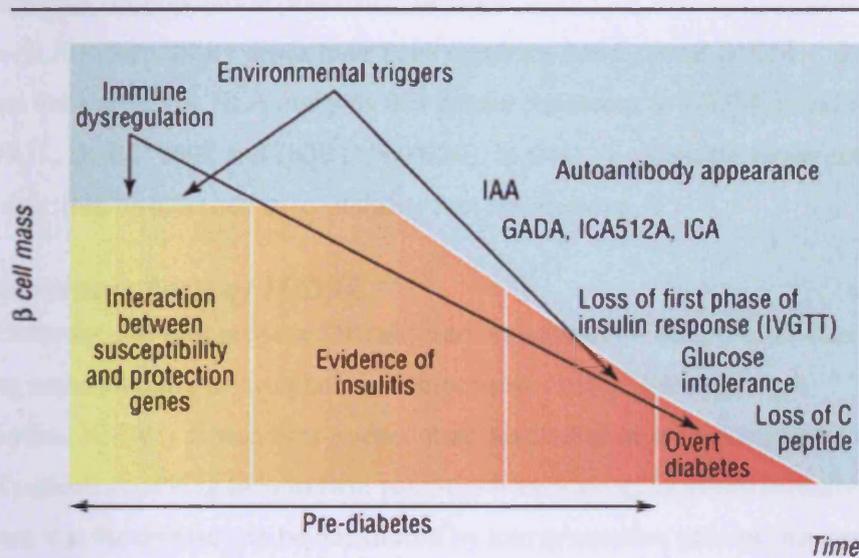


Figure 1-2. Time dependent pathogenesis of Type 1A diabetes(14).

There seems to be a phased progression towards the development of overt diabetes (Figure 1-2). An initial interaction of genes and environmental triggers precipitate an immune mediated response and insulinitis, with the concomitant appearance of autoantibodies, followed eventually by the loss of first phase insulin response. The progression to overt diabetes, resulting in significant  $\beta$  cell destruction, is triggered by the development of a more aggressive autoreactive T cell phenotype with cell surface expression of CD95 ligand (FasL). Concurrent is a change in the Th1/ Th2 balance of cytokine expression towards a more pro-inflammatory milieu comprising interleukin 1

beta (IL-1 $\beta$ ), tumour necrosis factor alpha (TNF $\alpha$ ) and gamma interferon (IFN $\gamma$ ).

Examination of islets during insulinitis suggests that CD95 mediated apoptosis occurs(15).

This is a potent mechanism of  $\beta$  cell destruction which may arise from impaired  $\beta$  cell mitochondrial function(16).

### ***1.6 Genetic aetiology of IDDM.***

Concordance for Type 1 diabetes is 30-40% for monozygotic twins, and the risk to a first degree relative is approximately 5%(17). The major genetic determinant for susceptibility to Type 1 diabetes lies within the human leucocyte antigen (HLA) region of the major histocompatibility complex (MHC) on the short arm of chromosome 6; termed IDDM 1. More than 90% of patients who develop Type 1 diabetes have either DR3, DQ2 or DR4, DQ8 haplotypes at this region, whereas fewer than 40% of normal controls have these haplotypes(18). DR3-DR4 heterozygosity is highest in children who develop diabetes before age 5 (50%) and lowest in adults presenting with Type 1 diabetes (20-30%), compared with a US population prevalence of 2.4%.

Four non-HLA susceptibility genes have been confirmed and named IDDM 4, 5, 8 and 12(19) and there are some HLA antigens that confer resistance to IDDM including HLA-DR11, DR15, DQB1\*0602 and DQB1\*0603(20). In total 17 candidate genes have thus far been described as inherited susceptibility loci for diabetes.

### ***1.7 Autoimmune basis of IDDM.***

In 1974 Bottazzo *et al* first proposed the autoimmune nature of IDDM after discovering circulating autoantibodies to cytoplasmic components of islet cells (islet cell autoantibodies, ICA)(1). It was later noticed that the disease process recurred in the graft of IDDM patients receiving twin to twin pancreas transplants(21). More recently it has been shown that the disease can be transferred by transplantation of bone marrow from a diabetic donor to an immunosuppressed non-diabetic recipient, and in the non-obese diabetic (NOD) mouse model it has been shown that T cells have the ability to 'adoptively transfer' the disease(22). In the NOD mouse the autoimmune process is CD4+ T cell mediated(22) and it is thought that this subset is critical in the early stages of the human disease(23). However the majority of human  $\beta$  cell destruction arises from a T cell mediated autoimmune process(22) where CD8+T cells predominate.

Autoantibodies are present in 70-80% of newly diagnosed Type I diabetics(23), frequently years before the clinical onset of the disease(24). This supports the idea of a

long pre-clinical phase, during which the autoimmune destruction of the  $\beta$  cells occurs and sub clinical disease can be detected(1).

Several target epitopes for autoantibodies are implicated in the pathogenesis of IDDM. Studies of young children from birth suggest that the appearance of autoantibodies is a major risk factor for the future development of diabetes and that anti-insulin autoantibodies are often the first to develop(25). Nevertheless, in the Diabetes Auto Immunity Study in the young (DAISY Study), high risk children with positive autoantibodies, but without any intervention, seemed to have less severe diabetic ketoacidosis, lower HbA1c, and a lower rate of admission to hospital at time of diagnosis(26).

The development of the disease, in relatives of patients with Type 1 diabetes, can now be predicted with reasonable accuracy by the detection of islet related autoantibodies. Detection of two or more autoantibodies (GAD, IA-2, or insulin autoantibodies) in relatives of patients with Type 1 diabetes has a positive predictive value for the development of diabetes exceeding 90%(27). Furthermore the presence of multiple diabetes related autoantibodies seems to be similarly predictive in the general population(28).

However, the role of autoantibodies in the pathogenesis of Type 1 diabetes has not yet been established and the development of Type 1 diabetes in a patient with X linked agammaglobulinaemia suggests that they are not needed for either the initiation or progression of the disease(29).

Type 1 diabetes is associated with other autoimmune conditions. The most common association is with thyroid disease(30) and the Belgian Diabetes Registry indicates that the prevalence of thyroid peroxidase autoantibodies is 22% in patients with Type 1 diabetes. Approximately 1 in 10 patients with Type 1 diabetes express transglutaminase IgA autoantibodies, and more than half of these patients have coeliac disease on intestinal biopsy. Recent studies from Europe and North Africa have documented prevalence rates of coeliac disease in Type 1 DM patients of 8.3% and 10.3% respectively(31, 32). Whilst approximately 1 in 50 people with Type 1 diabetes have 21-hydroxylase autoantibodies and around 25% of these progress to Addison's disease.

### ***1.8 Environmental basis of IDDM***

Two major environmental hypotheses account for the increased incidence of Type 1 diabetes. The first 'hygiene' hypothesis proposes that environmental factors alter the

development of autoimmunity. As an oversimplification, our environment for young infants is far too clean, leading to a deficiency in immunoregulation such that 'Th2' diseases (for example asthma) and 'Th1' diseases (such as Type 1 diabetes) are increasing dramatically(33, 34).

The second hypothesis suggests that exposure to an environmental factor may be responsible, and is supported by the seasonality, increasing incidence, and epidemics of Type 1 diabetes demonstrated in cross sectional and retrospective studies(35). The low concordance between monozygotic twins also implies an environmental role, as in a purely genetic condition concordance would be approaching 100%. Other evidence comes from migration studies, such as that of the Indian population now living in the UK. Though the incidence of IDDM in the Indian subcontinent is extremely low; studies from Leicester and Bradford with large migrant Indian populations show the incidence of IDDM in the populations is fast approaching that of the indigenous Caucasian population(36).

Many associations with viral infection have been found in Type 1 diabetes, though only congenital rubella syndrome has been conclusively associated with the disease(37). Post mortem studies show a tropism for islet cells amongst enteroviruses, some of which use established cell surface molecules as receptors(38). Coxsackie B2, B3, B4 and B5 enteroviruses have been isolated from the sera of patients with newly diagnosed IDDM and B4 virus may be present in 30% of serum from recent onset IDDM patients compared with 5% of controls(10). Coxsackie virus has been cultured from the pancreas of a child who died of diabetes; whilst the mumps virus and cytomegalovirus have also been suggested to be involved in triggering IDDM.

Several cases of patients developing anti-islet autoantibodies and Type 1 diabetes after treatment with interferon alpha (IFN $\alpha$ ) are reported(39) and IFN $\alpha$  can generate insulinitis and diabetes in animal models. Induction of this cytokine by viral infection has been proposed as a mechanism by which Type 1 diabetes is initiated(40) and neutralizing this cytokine may potentially prevent the disease(39).

The immature gut immune system has been implicated in the pathogenesis of diabetes and exposure of the gut to bovine insulin in cow milk formulas may initiate the development of  $\beta$  cell specific autoantibodies in genetically susceptible children(41). Though the DAISY study, found that early ingestion of cereal or gluten may increase the risk of Type 1 diabetes(42, 43) no evidence that bovine milk ingestion, enteroviral infection, or vaccination contributed to the risk of diabetes was established(44). Hence

the TRIGR study in Finland is under way to determine if elimination of cow's milk from infants' diet can prevent Type 1 diabetes.

### ***1.9 Costs and complications of IDDM.***

Diabetes has both direct and indirect costs to the NHS and society. Indirect costs are difficult to quantify but include early retirement, absenteeism and early death. The diabetic population certainly carries the burden of excessive mortality. Soedamah-Muthu *et al* compared the all-cause mortality data of 7,713 patients with diabetes with those of 38,518 age and sex matched controls for the period 1992 to 1999 and found a mortality rate hazard ratio of 3.7 between patients with and without diabetes(45).

The direct costs of diabetes refer to the expense of treatment and services. Using the latest ADA criteria, even patients with abnormal fasting glucose incur increased health care costs compared to normoglycemic individuals(46). It is estimated that, once overt diabetes is present, it accounts for 9% of acute NHS expenditure and bed days; and that the presence of diabetic complications increases costs to social services four fold(47). Per capita, adjusting for age, sex and ethnicity, the health costs incurred by a population with diabetes are 2.4 times those of a non diabetic population(48).

Patients with diabetes have a high baseline rate of diabetic complications. McAlpine *et al* studied the patients with diabetes in Tayside, Scotland during 1997. 942 patients had established Type 1 diabetes and 29 were newly diagnosed patients. The incidence per 1000 patients of diabetes complications were angina 8.8; myocardial infarction 8.6; cerebrovascular accident 1.1; lower limb amputation 3.2; peripheral vascular disease 5.5; blindness 1.1 and ESRF 6.4 with overall mortality of 14.6 per 1000 patients(49). Roberts *et al* studied the mortality risk amongst patients under 30 years of age with Type 1 diabetes by analysis of hospital admission data. Though death in the population was uncommon, over a three year period following a hospital admission it was nine times more frequent than in the general population(50) Cusick *et al* confirmed the association between the presence of diabetic complications and subsequent mortality in 1440 patients with Type 1 diabetes. The 5 year estimate for mortality from all causes was 5.5%, with amputation and poor visual acuity being the strongest predictors of mortality in patients with Type 1 diabetes (hazard ratios 5.08 and 1.74 respectively)(51)

A large study, the Diabetes Control and Complications Trial, confirmed that the complications of IDDM are associated with poor control of blood glucose levels. The study concluded that intensive insulin therapy, using thrice daily injections, delayed the

onset and slowed the progression of microvascular complications by between 35 and 90% compared to conventional insulin treatment. The risks of developing retinopathy and nephropathy were proportional to the mean HbA<sub>1c</sub> during the preceding period and the intensive regimen was most effective when begun early, prior to the development of complications, aiming to achieve an HbA<sub>1c</sub> concentration of 7% or less. However, the major drawback in adopting such an intensive insulin regimen was the tripling in frequency of severe and potentially life threatening hypoglycaemic episodes(52, 53).

### ***1.10 Prevention of IDDM.***

Most studies using drug treatments have failed to prevent the development of diabetes. The US Diabetes Prevention Trial failed to slow the progression to diabetes, in relatives at high and moderate risk, using antigen based treatment with oral or parenteral insulin(54). Whilst the European Nicotinamide Diabetes Intervention Trial also found no difference in the development of diabetes when participants were assigned to receive either the oral B complex vitamin nicotinamide or placebo treatment(55).

More recently, the Diabetes Prevention Programme Research Group (DPPRG) found a reduction in the incidence of Type 2 diabetes of 31% over 2.8 years using metformin(56), and studies have suggested that metformin might improve the glycaemic control of Type 1 diabetes patients who are overweight, receiving large doses of insulin, or have an HbA<sub>1c</sub> > 8%(57). The co-existence of insulin resistance in patients with Type 1 diabetes is a new area of interest and it is possible that metformin may also slow the progression to overt Type 1 diabetes.

The recently published Diabetes Reduction Assessment with ramipril and rosiglitazone medication (DREAM) study showed a significant reduction in the incidence of diabetes over 3 years with rosiglitazone compared to placebo (11.6 vs 26%); whilst ramipril therapy showed no reduction in the incidence of diabetes in patients with impaired fasting glucose, glucose intolerance or both (58). Though the use of thiazolidinediones, such as rosiglitazone, may appear beneficial in preventing diabetes; this class of drug is associated with increased fluid retention, heart failure and liver toxicity in some patients(59).

Lifestyle alterations have had more impact upon the prevention of diabetes. The DPPRG study showed a reduction in incidence of diabetes by 58%, using a 7% weight loss and 150 minutes of physical activity lifestyle alteration, over a 2.8 year period(56) and a similar study from Finland again showed a risk reduction of 58% in 522 at risk individuals(60). Despite the impressive results of clinical studies, the labour intensive and expense burden of replicating these interventions make them impractical for most

healthcare providers. However, over reliance upon drug therapies is likely to detract from research to find simpler and reliable lifestyle alterations which will prevent a population at risk from becoming reliant upon potentially harmful medication(61).

### **1.11 Treatments for diabetes.**

Insulin is the mainstay of treatment for Type 1 diabetes. Endogenous insulin is comprised of two chains, the 21 amino acid  $\alpha$  chain and the 30 amino acid  $\beta$  chain, which are connected by two disulphide bridges. It is synthesized in pancreatic  $\beta$  cells as proinsulin, an 86 amino acid polypeptide which is incorporated into granules at the Golgi complex. Inside the granules, proinsulin undergoes proteolysis to form insulin and a cleaved peptide(C peptide). Insulin and C peptide are released in equimolar quantities by calcium dependent exocytosis. There are two types of insulin secretion; basal, which occurs even during fasting, and stimulated, in response to raised blood glucose concentration. Insulin has many effects on carbohydrate, lipid and protein metabolism. However, its principal role is in the regulation of blood glucose concentration.

Broadly there are two types of exogenous insulins; short and long acting. Short acting (soluble) insulin acts rapidly within an hour of administration. Its peak action is at about 3-4 hours and lasts about 8 hours. The actions of insulin can be prolonged, by complexing it with zinc or proteins in crystalline form, to produce neutral protamine Hagedorn (NPH) type insulins. These long acting insulins have a variable duration, usually between 12 and 20 hours. IDDM patients usually inject a mixture of both short and long acting insulin, which they can either mix themselves or use a ready mixed formulation. The primary objective of insulin therapy is to keep blood sugar levels as close to physiological levels without causing hyper or hypoglycaemia, but this level of control is difficult to maintain. Since January 2006 inhaled insulin, with approximately 10% of the activity of injected insulin, has been available. It acts rapidly after 30 to 90 minutes but has a prolonged action compared to subcutaneous insulin(62). Furthermore it has a more pronounced effect on peripheral glucose disposal and reduced effect on hepatic glucose production than intravenous infused insulin in animal studies(63). Though overall glycaemic control is similar with subcutaneous and inhaled insulin(64, 65) the inhaled version is likely to be popular amongst patients who relinquish multiple daily injections for inhaled pre-prandial insulin and a single long acting evening insulin injection(66, 67). However the cost is likely to exceed that of injectable insulin and NICE has currently declined funding for inhaled insulin in the NHS(68, 69).

A more physiological pattern of basal and stimulated insulin level is achieved using modern engineered very long acting and rapid acting insulins and insulin analogues. These can provide control and convenience similar to that obtained with insulin pumps, reduce the variability of insulin absorption and allows insulin therapy even in very young children(70). The insulin analogue glargine is produced by recombinant DNA technology and at physiological pH forms amorphous microprecipitates which slowly release insulin into the circulation over 24 hours. It functions as a very long acting insulin mimicking peakless basal insulin secretion(71, 72). Lepore *et al* demonstrated glargine administration, at 0.3u/kg by subcutaneous injection, to closely mimic continuous subcutaneous insulin infusion; the current gold standard of basal insulin replacement(73); while Rossetti *et al* demonstrated a significant improvement in HbA1c concentration, a reduction in the number of severe hypoglycaemia episodes and reduction in total basal insulin dose when glargine was added to a multiple short acting subcutaneous injection regimen(74). Similar improvements in HbA1c and hypoglycaemia have been achieved by the addition of once daily glargine to NPH insulin regimens(75). Large open labelled randomized controlled trials comparing NPH insulin with glargine showed that basal insulin therapy with glargine to be as safe as NPH, and as effective as NPH insulin twice a day in maintaining glycemic control in Type 1 diabetes(76, 77). The majority of studies in children and adolescents confirm that glargine gives comparable or improved glycaemic control, and reduced incidence of nocturnal or severe hypoglycaemia, compared to regimens of NPH insulin(78-80). Glargine is now recommended by NICE for patients with diabetes who require assistance with their insulin regimen, whose lifestyle is restricted due to recurrent hypoglycaemia and for those who would otherwise require twice daily insulin in combination with oral hypoglycaemic drugs(81).

### ***1.12 Conclusion.***

Diabetes is a common disease with a rising incidence. Though the precise aetiology is unknown; a combination of genetic and environmental factors result in the autoimmune mediated destruction of  $\beta$  cells within the Islets of Langerhans. The chronic complications give rise to a significant clinical burden and incur vast expenditure for health care providers. Whilst intensive insulin therapy and strict glycaemic control can delay such complications, it risks life-threatening hypoglycaemia. No strategy has yet proven wholly effective in preventing diabetes, and conventional treatment is centred on exogenous insulin administration. Though novel agents and therapeutic targets are

becoming established, their long-term efficacy upon diabetic complications is unknown. Therefore, the only recognized means of attaining physiological glucose control and euglycaemia is by restoring *in vivo* insulin secretion from  $\beta$  cells(82). This is most readily achieved by whole pancreas or isolated islet cell transplantation.

## ***Chapter 2. Pancreas and Islet Cell Transplantation for Diabetes Mellitus.***

### ***2.1 Introduction.***

Historically vascularised whole pancreas transplantation (VPT) has been undertaken to restore a functioning  $\beta$  cell mass which provides physiological endogenous insulin secretion to attain insulin independence(83). However, this poses considerable risks to the recipient with high rates of surgical morbidity and mortality, and the need for maintenance immunosuppression(84, 85). Therefore, VPT remains an unrealistic treatment option for most young patients with diabetes or those unfit for major surgery.. Simplifying and restricting transplantation to only the islets of Langerhans has been realized but has not become widespread in clinical practice. Until recently the success of islet transplantation has not been comparable to that of VPT, but with improvements in islet isolation and immunosuppression this may change(86). Herein the current status of VPT and the recent progress, rationale and indications for human islet cell transplantation are reviewed.

### ***2.2 Vascularised pancreas and clinical islet transplantation.***

Vascularised pancreas transplantation is usually considered for medically suitable patients with Type 1 diabetes who are either suitable for concomitant renal transplantation from the same donor (simultaneous pancreas and kidney; SPK); or in patients already in receipt of a well functioning renal transplant with stable maintenance immunosuppression (pancreas after kidney; PAK); or in selected patients with severe hypoglycaemic unawareness (pancreas transplant alone; PTA).

Islet cell transplantation also has the potential to restore a functioning  $\beta$  cell mass, physiological endogenous insulin secretion and attain insulin independence(87). When compared to VPT this is achieved with relative technical ease and minimal potential morbidity; the transplant itself being performed under local anaesthesia as a potential day case procedure(88, 89). Though recipients still require a regimen of lifelong immunosuppression, the use of a 'cellular transplant' means there is great potential to develop tolerogenic protocols and graft immunomodulation with the long-term aim of withdrawing immunosuppression.

### **2.2.1 Demographics of pancreas transplantation.**

The International Pancreas Transplant Registry (IPTR) at the University of Minnesota has data regarding VPT activity from December 1966 to June 2004, during which time 23,000 pancreas transplants have been performed. Of the 15333 transplants performed in the US between October 1987 and June 2004; 78% have been SPK, 16% PAK and 7% PTA. Of the 5017 cases performed outside the US in the same period; 91% have been SPK, 5% PAK and 4% PTA(90).

Between 2000 and 2004 the patient 1 year survival rates, amongst US Type 1 diabetic recipients of cadaveric pancreas allografts, were SPK 95%, PAK 95% and PTA 98%, whilst 1 year graft survival rates were 85%, 78% and 76% respectively(90). In the largest published single centre series of 1194 recipients from December 1966 to March 2000 a progressive increase in 1 year graft survival rates was seen from 64% to 79% for SPK; 76% to 98% for PAK and 67% to 88% for PTA(91). The low graft survival rates in PTA may reflect the high rates of rejection (30%) especially amongst young recipients of PTA(90). Knoll *et al* reported similar 1 year graft and patient survival rates of 72% and 94% for PAK and 71% and 98% for PTA(92).

Graft function is a strong predictor of patient survival. A Cox regression analysis of SPK outcomes has shown that loss of the pancreas and renal allograft components were associated with a hazard ratio of later patient death of 3.51 and 14.99 respectively. For PAK recipients these hazard ratios were 4.17 and 9.17 respectively.

VPT has also been performed for Type 2 diabetes in 7.2% of SPK, 4.9% of PAK and 4.3% of PTA between 2000 and 2004. There were no significant differences in SPK graft survival between Type 1 and 2 recipients, though it may be reduced in recipients with very early onset diabetes(90). Nath *et al* reported the outcomes of VPT in 17 recipients with Type 2 diabetes. One year patient and graft survival rates were both 94% and after a mean 4.3 year follow up the patient survival rate remained high at 71%(93).

### **2.2.2 Technical aspects of pancreas transplantation.**

VPT is a technically challenging operation. Refinements to the surgical techniques have largely concentrated on the method of draining pancreatic exocrine secretions and optimizing pancreatic venous drainage.

#### 2.2.2.1 Management of exocrine secretions.

From the early 1990's VPT with bladder drainage of exocrine secretions was preferred, as enteric drainage was associated with a high rate of intrabdominal infection, enteric leaks and graft failure (Figure 2-1 and Figure 2-2)(94, 95).

From 1987 to 1996 over 90% of US pancreas transplants were performed with bladder drainage of the exocrine secretions. Analysis of the IPTR data for SPK shows a one year pancreas graft survival rates of 87% with bladder drainage vs 85% for enteric drainage. Renal graft survival was 91% and 93% respectively, and overall patient one year survival was 94% and 96% respectively. For PAK one year pancreas graft survival rates for bladder and enteric drainage were 80% and 77% respectively and one year patient survival rates were 96% and 95% respectively. For PTA one year graft survival rates for bladder and enteric drainage were 79% and 72% respectively and one year patient survival rates were 97% and 98% respectively(90).

In recent years the complications of bladder drainage and the need for conversion to enteric drainage has reduced the number of primary bladder drained grafts. Between 2000 and 2004, 9% of bladder drained grafts were converted to enteric drainage at 1 year and 17% by three years. Increasing numbers of grafts now have primary enteric drainage. In 2002-2003, 82% of SPK, 72% of PAK and 57% of PTA were enterically drained(90).

Series reporting long-term patient, kidney or pancreas graft survival are equivalent when enteric or bladder drainage are compared(96-103). Sollinger *et al* have reported the largest series of VPT using both methods of drainage. Of 388 bladder drained grafts 62.5% had urinary tract infection (UTI), 17.7% haematuria, 15.4% anastomotic leakage, 2.8% urethral strictures and 2.5% urethral disruption. Of 112 enterically drained grafts 11.7% had UTI and 8% anastomotic leak. Rates of fungal and cytomegalovirus (CMV) infection were higher in bladder drained grafts and 23.8% of bladder drained grafts were later converted to enteric drainage(97).

Bloom *et al* reported a higher incidence of leak, ascites, acute rejection, graft pancreatitis, UTI and other infections in bladder drained recipients; enterically drained grafts had a higher incidence of abscess formation and hypertension. This series also demonstrated the phenomenon of intravascular volume depletion and loss of bicarbonate from bladder drained grafts. Consequently a conversion rate of 18.7% from bladder to enteric drainage was reported(99).

Hakim *et al* reported 20% of patients having complications arising from the duodenal vesical anastomosis including leaks, haematuria, recurrent UTI and bladder stones(103).

Other urological complications such as duodenal stump rejection, stump rupture with haematuria and urine extravasation in bladder drained recipients are unusual but have been described(104, 105). Del Pizzo *et al* reported a series of 140 bladder drained grafts. Half had significant urological problems requiring invasive corrective procedures and 21% were converted to enteric drainage(106). In a similar series Gettman described significant urological complications in 79% of recipients(107) and Kuo *et al* report the rate of UTI in bladder and enterically drained grafts was 52% and 2% respectively(98). Notably two cases of cancer in bladder drained grafts have also been reported(108, 109). Although primary or conversion to enteric drainage can reduce the infective complications it has its own unique problems including a reported 8% leak rate from the duodenal anastomosis(110).

#### 2.2.2.2 Management of venous drainage.

Historically, VPT involved a systemic venous anastomosis to an iliac vein. This results in relative systemic hyperinsulinaemia(111-114) and, to attempt to replicate a more physiological pattern of graft insulin secretion, Gaber advocated venous drainage into the portal circulation (Figure 2-1 and Figure 2-2)(115).

Stratta *et al* conducted a randomized trial of venous vs systemic drainage and showed no significant differences in patient or graft survival after a mean 17 month follow up(102). A similar study by Petruzzo *et al* reported no differences in graft survival and other physiological parameters such as creatinine, HbA1c, and fasting glucose, insulin and C peptide concentrations(116). Using UNOS data from 1994-2001, Troppmann *et al* report a one year renal allograft rejection rate of 48% and 37% respectively in systemic and portally drained SPK. Though these high rates of rejection may reflect the historical use of older calcineurin inhibiting immunosuppressants, it also illustrates disparate rates of graft rejection between systemically and portally drained grafts(117). A 3 year follow up study by Philosophe *et al* found a significantly higher rate of rejection amongst systemically drained grafts, and 3 year graft survival rate of 79% vs 65% for portal and systemic drainage respectively.(118). However, according to IPTR data, of primary pancreas grafts with enteric drainage in the period 2000- 2004, 23% of SPK, 27% of PAK and 44% of PTA had portal venous drainage. There were no significant differences in one year graft survival or technical failure rates between portal and systemically drained grafts. Moreover there is now some evidence that portal drainage allows antigen delivery to the liver to achieve a state of donor specific tolerance more readily than in systemically drained grafts(119)

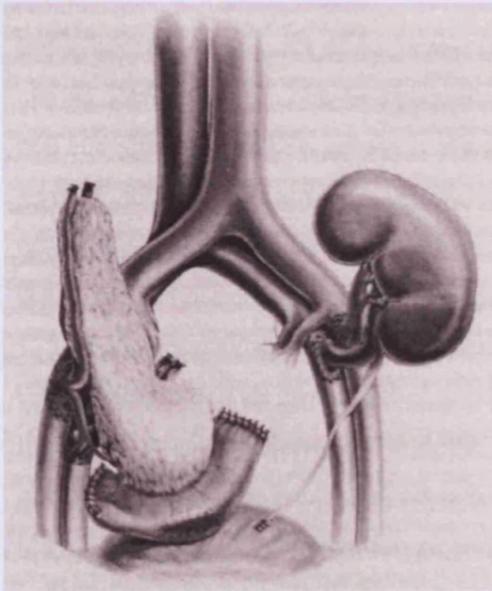


Figure 2-1. SPK with vesical and systemic iliac drainage.

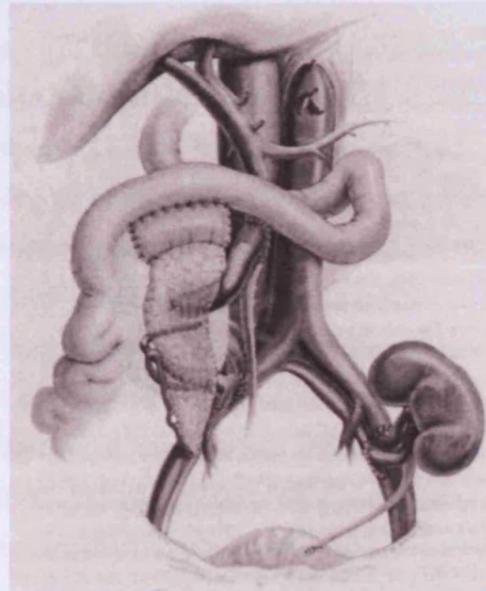


Figure 2-2. SPK with enteric and portal venous drainage.

### ***2.2.3 Graft failure in pancreas transplantation.***

Primary non function (PNF) refers to those who never attained insulin independence and is a rare cause of VPT graft failure in 3 to 5% of recipients. More common are 'technical failures' which constitutes graft arterial or portal thrombosis, bleeding, infection, graft pancreatitis or anastomotic leak leading to graft pancreatectomy. This is the major cause of graft loss in the first 6 months but technical failure rates for all VPT categories have decreased with time. In 1988 12.4 % of SPK, 13% of PAK and 24.3% of PTA grafts encountered technical failures. In 2002 the rates were 6.4%, 7.7% and 7.4% respectively. Alloimmune mediated graft loss arises due to acute and chronic rejection. Acute rejection is seen in 1 to 25% of recipients and occurs most frequently between 7 and 12 months. Low rates of graft loss due to acute rejection occur in enterically drained SPK and PAK (2.3% and 6.6% respectively) but the 1 year rejection rate in enterically drained PTA is significantly higher (14.6%). Chronic rejection occurs after 12 months in 2 to 33% of recipients and, after technical failure, is the most frequent cause of graft loss. Risk factors for chronic rejection include previous acute rejection, isolated pancreas transplantation, CMV infection, re-transplantation and 1 or 2 antigen mismatch at the B locus(120). Recurrent autoimmune mediated selective destruction of graft  $\beta$  cells can occur in 6 to 12 weeks(21, 121) and grafts show T lymphocytic and monocytic infiltration. Even where excellent haplotype matching is achieved, such as in living donor pancreas

transplantation, recurrent diabetes and the presence of circulating islet cell antibodies can be demonstrated(122). Autoimmune recurrence is characterized by the return of islet cell and glutamic acid decarboxylase autoantibodies, but the correlation of the return of serological autoimmunity with the pathological demise of allograft  $\beta$  cells is not yet well established. It is not known if adjustments to immunosuppressive regimens, in light of the serological findings, may alter the course of recurrent autoimmune disease(121, 123-125). Pancreatic allografts carried out with new immunosuppressive regimes demonstrate some resistance to recurrent diabetes mellitus. In a large series reporting the results of needle biopsies of pancreatic allografts, Drachenberg *et al* found no islet inflammation occurring outside the context of acute graft rejection. Indeed such insulinitis was restricted only to high grade acute rejection suggesting the absence of an islet specific cell mediated inflammatory response in these grafts(126).

Finally allografted  $\beta$  cells are subjected to the physiological stress of post-transplantation Type 2 diabetes which occurs in the recipient. This arises from the toxic effects of steroids and/or tacrolimus based immunosuppression on islet cells(127, 128) and the induction of a state of insulin resistance in the periphery. Indeed hyperglycaemia and normal to elevated C peptide secretion profile characteristic of Type 2 diabetes has previously been demonstrated in pancreas transplant recipients(129).

#### **2.2.4 Monitoring pancreas graft function.**

Techniques for monitoring graft rejection vary according to the type of VPT performed. Where the graft is bladder drained, urinary amylase and bicarbonate content(130) or cystoscopic biopsy of the pancreas and duodenum(131) can be performed. Alternatively, in SPK, the concomitant renal graft can be biopsied if serum creatinine begins to rise as a surrogate marker of pancreas rejection. For enterically drained VPT a pancreas graft biopsy is required(132) but is difficult to perform with the intra-peritoneally positioned graft. Despite ultrasound guidance, graft biopsy is associated with a significant failure (12%) and complication (2.8%) rate. A high proportion of biopsy complications require surgical intervention(133, 134) despite attempts to improve the yield and safety of the procedure using computerized tomography guidance, laparoscopic and percutaneous techniques(127, 135, 136). The severity of rejection is graded according to a standard histological grading scale after Drachenberg *et al*(137) which correlates well with other measures of graft rejection such as serum lipase and response to supraphysiological steroid immunosuppressive therapy(138).

Less invasive functional assessments, including IVGTT, are used for established allografts. A decline in the rate of glucose utilization is 88.7% sensitive and 91% specific for underlying graft rejection(139). Graft failure within 12 months can be predicted with 93% sensitivity and 100% specificity where fasting glucose are elevated to 128mg/dl, and rejection within 4 years where change in basal insulin secretion was less than 32 uU/ml during IVGTT (75% sensitivity and 75% specificity)(140). Poor post operative outcomes, in systemically venous drained grafts, may also be predicted by impaired glucose tolerance and poor stimulated insulin secretion after oral glucose tolerance but its value in graft monitoring is yet to be established(141).

### ***2.2.5 Immunosuppression for pancreas transplantation.***

Immunosuppressive protocols can be divided into combinations of long-term maintenance and short term induction regimens. Most recent maintenance protocols have used combinations of 5 main agents. Prior to 2000, cyclosporin A (CsA) with mycophenolate (MMF) was the most frequent regimen but this is now rarely used. In 2000-2004 over 60% of VPT received tacrolimus and MMF based regimens. When combined with anti-T cell induction agents, 1 year graft survival rates were 83 to 88% with the highest in SPK recipients. Sirolimus with tacrolimus is another synergistic regimen, though sirolimus is associated with poor wound healing in the early post-operative period. However, graft survival rates of 83 to 87% comparable to tacrolimus and MMF have been achieved, though the follow up duration for these recipients is short. Multivariate analysis has demonstrated that tacrolimus with either MMF or sirolimus based regimens are independently associated with a reduced graft failure rate, whilst tacrolimus and MMF is associated with a reduced risk of technical failure.

Hariharan *et al* showed an 80% PAK graft survival rate where tacrolimus based long-term immunosuppression was combined with induction monoclonal antibody agents(142). Now more than 75% of VPT recipients receive some form of induction agent which can be divided into three main types: T cell depleting polyclonal (thymoglobulin, anti-thymocyte globulin, ATG), T cell depleting monoclonal (OKT3, Campath) antibodies, and non depleting anti-CD25 monoclonal antibodies (Zenapax, Simulect).

IPTR data shows that the use of non depleting induction agents, depleting agents and no induction agent resulted in a one year graft survival rates of 91%, 85% and 86% respectively in technically successful SPK under a tacrolimus and MMF maintenance regimen. For PAK, using the same maintenance regimen, graft survival rates are highest

(85%) in those receiving depleting induction agents and lowest for those receiving no induction; whilst graft survival rates for PTA do not differ for any combination of induction agents

### **2.2.6 Long-term results in pancreas transplantation.**

The long-term results of VPT are now known and the majority of published series concentrate on SPK recipients(91, 96-99, 143-146) with comparisons between enterically and bladder drainage(91, 96-99, 143, 144).

Data from UNOS and IPTR between 1988 and 2003 show 5 and 10 year graft survival rates for SPK, PTA and PAK of 69% and 46%; 58% and 17%; 58% and 17% respectively(147).

The results of the largest single centre series of 500 SPK performed from 1986 are shown in Table 2-1.

	<b>PANCREAS GRAFT SURVIVAL (%)</b>	<b>RENAL GRAFT SURVIVAL (%)</b>	<b>PATIENT SURVIVAL (%)</b>
<b>1 YEAR</b>	<b>87.5</b>	<b>88.6</b>	<b>96.4</b>
<b>5 YEAR</b>	<b>78.1</b>	<b>80.3</b>	<b>88.6</b>
<b>10 YEAR</b>	<b>67.2</b>	<b>66.6</b>	<b>76.3</b>

**Table 2-1. Long-term outcomes of SPK from University of Wisconsin(97).**

Of 53 patients deaths, 25 still had graft function and causes of death in decreasing order of frequency were cardiac (38%), cerebrovascular (5.5%), infectious (17%) and malignancy (9%)(97). Nankivell *et al* have reported 1, 5 and 10 year patient survival after SPK of 96.7%, 94% and 84.4%, and graft survival rates of 87.4%, 86.5% and 86.5% respectively and cardiovascular death accounted for 64.3% of mortality(148).

The United States Renal Data system (USRDS) indicate a 10 year patient survival of 67% with SPK, with 33.4% of deaths arising from cardiovascular disease, 7.1% from cerebrovascular disease, 21.5% from infective causes and 3.3% from malignancy(149). A similar analysis of the UNOS data from 1987 to 1996 reported an 8 year patient survival of 72% and a similar profile of mortality(150).

Though not the major cause of patient death, recipients of SPK are at greater risk of developing post-transplant malignancy than other recipient groups. This arises due to more aggressive immunosuppression(151), pre-existing malignancy in the pancreas graft(152) or

as a complication of bladder drainage(108, 109). Additionally pancreas transplant recipients have a higher incidence, lower survival and shorter duration to diagnosis and death from post transplantation lymphoproliferative disease (PTLD) compared to other recipient groups(153).

### **2.2.7 Pancreas transplantation and diabetic complications.**

With improving rates of graft and patient survival, the impact of VPT upon long-term diabetic complications can be assessed.

#### *2.2.7.1 Effect upon diabetic nephropathy.*

SPK confers a survival advantage over other therapies for diabetic ESRF. Tyden *et al* showed a 20% 8 year survival in isolated renal transplant recipients but 80% survival in SPK recipients; whilst loss of the pancreas component reduced 10 year survival to only 33%(154). Ojo *et al* determined an advantage of SPK over both live and cadaveric donor renal transplants alone through a 67% vs 65% vs 46% ten year survival respectively(149); whilst Rayhill *et al* and Becker *et al* demonstrated annual recipient mortality rates in SPK of 1.5% compared to the 6.27% after cadaveric donor renal transplantation alone(155, 156). Studies using analytical models, incorporating potential complications and adverse transplant outcomes, have concluded that SPK yields a greater life expectancy and improvement in quality of life when compared to either cadaveric donor renal transplantation or dialysis(92, 157).

Fioretto *et al* conclusively demonstrated that PTA can reverse histological diabetic nephropathy following five years of post-transplant normoglycaemia(158). However, this comes at the expense of a reduction in glomerular filtration rate (GFR) caused by calcineurin inhibitor based immunosuppression and it is recommended that baseline renal function is accounted for in selecting patients for PTA(159, 160).

#### *2.2.7.2 Effect upon diabetic retinopathy.*

The impact of VPT on diabetic retinopathy is difficult to assess as many recipients undergo ophthalmic procedures during their post-transplantation follow up(161, 162). Pearce *et al* confirmed the high rates of photocoagulation therapy amongst VPT recipients but showed no progression of diabetic retinopathy after transplantation(163). Koznarova *et al* showed significantly improved visual acuity at three years in recipients with functioning SPK grafts compared to renal transplants alone or SPK recipients after rejection of the pancreatic component(164) and Chow *et al* showed reduction in photocoagulation treatments from 83% prior to transplantation to 14% with a functioning SPK and 19% with a non functioning renal component SPK(165). However, others have

failed to show a significant impact upon progressive retinopathy in long-term follow up studies, suggesting that the effects of retinopathy may be too far advanced to be reversed by restoring euglycaemia(166).

#### 2.2.7.3 *Effect upon diabetic neuropathy.*

VPT has a beneficial effect on diabetic neuropathy which some attribute to improved glycaemic control and correction of uraemic neuropathy with concomitant renal transplantation(167-170). Neurological scores and motor/sensory conduction are significantly improved compared to recipients of renal transplants alone or following VPT graft failure after a 10 year follow up, though residual neurological deficits remained in all recipients(171). Others have also reported an initial improvement in nerve conduction velocity from 6 months(172) to 2 years(143), and a slower improvement with axonal recovery in 59 recipients of kidney-pancreas transplants over 8 years though again velocities never returned to normal(172). Improvements in autonomic neuropathy are also reported(171) particularly in orthostatic hypotension(173) and diabetic gastroparesis(174-176).

#### 2.2.7.4 *Effect upon cardiovascular disease.*

VPT has variable effects on cardiovascular disease. LaRocca *et al* reported a significantly higher ejection fraction and reduced diastolic function in 42 SPK recipients compared to 26 kidney alone recipients. They also reported a lower incidence of acute MI (2.4% vs 17.6%) and a reduced number of episodes of angina(177). A further study in a larger cohort confirmed the decreased incidence of MI (3% vs 20%) but showed no difference in frequency of angina(178). Radionuclide ventriculography has been used to compare left ventricular function in SPK recipients and isolated renal transplant recipients at 4 years follow up with a significant improvement in SPK recipients(179); whilst an angiographic study of recipients with and without functioning pancreas grafts showed regression of coronary atherosclerosis in 38% of those with a functioning pancreas transplant after a 3.9 year follow up(180). The mechanism of cardiac protection is unclear but likely relates to reduction in cardiac risk factors by lowering triglycerides, homocysteine and von Willebrand factor in VPT recipients(181).

VPT has less convincing effects on hypertension. Though short term studies demonstrate improved blood pressure control(179, 182, 183), long-term follow up results are less conclusive. Fiorina *et al* report a drop in rates of hypertension in SPK recipients which did not reach statistical significance compared to kidney alone recipients at 4 years(184).

Naf *et al* also reported a fall in the proportion of SPK recipients with hypertension before and 5 years after transplantation (88% vs 49%)(185) though La Rocca *et al* found no significant difference in rates of hypertension after two years follow up(178).

VPT does not impact upon diabetic non coronary vascular disease with a limb amputation, angioplasty or bypass grafting rate of 0.11 events per patient per year amongst VPT recipients(186). Kalker *et al* reported a 19% incidence of lower limb amputation with no significant difference between SPK and kidney alone recipients(187) after a 56 month follow up(188). Indeed there is some evidence that carotid atheromatous disease progresses after pancreas transplantation(189, 190) and cerebrovascular events are a prominent cause of death in 7.1% of SPK recipients(149).

### **2.2.8 Current results in clinical islet cell transplantation.**

The Collaborative Islet Transplant Registry (CITR) holds data regarding islet cell transplants performed at 31 centres in North America. Between 1999 and 2005, 593 islet infusions have been performed in 319 recipients, and detailed data is available on 225 recipients (71%) and 425 infusions (72%). The median age of an islet transplant recipient is 42.3 years with a median duration of diabetes of 29 years. 38% of recipients were maintained using an insulin pump prior to transplantation and 92.6% either had pump therapy or three or more insulin injections a day. 89% had basal C peptide secretion of less than 0.5ng per ml and 78% had an HbA1c of 6.5% or greater(191).

Like VPT recipients, those receiving islet cell allografts can be categorised. The recent majority are Type 1 diabetics who have received an islet transplant alone without a previous kidney transplant (ITA, n=203). In such recipients the benefit of restoring euglycaemia and risks of lifelong immunosuppression outweigh the risk of death caused by their severe hypoglycaemic unawareness. The increase in ITA numbers reflects the efforts by islet transplant centres to replicate the success of the Edmonton group in attaining insulin independence using a steroid free immunosuppressive protocol(88). Analysis of CITR data shows half of ITA recipients have some insulin independence within 6 months, and 49.4% within 1 year, of their first islet infusion. Those receiving two islet infusions achieve higher rates of insulin independence; 60.2% at 6 months and 54.4% at 1 year after their last infusion. So insulin independence is not yet sustained in the majority of recipients, and only 67.5% of those attaining any period of insulin independence remain independent at 1 year, decreasing to 43.3% at 2 years.

Despite a lack of sustained insulin independence, there is a significant decline in the number of patients reporting severe hypoglycaemic episodes following the first islet infusion (85% pre-transplant vs 2.6% post-transplant). The rate of hypoglycaemic episodes remain low up to six (3.8%) and 12 months (4%) after transplantation, and all are reported by patients requiring exogenous insulin.

Following initial islet infusion; fasting glucose, HbA1c and C peptide secretion improve. Those achieving a period of insulin independence had mean fasting blood glucose concentration of 6.13 mmol/l, HbA1c of 6% and basal C peptide secretion levels of 1.1ng/ml. This compares to 6.74 mmol/l, 6.5% and 0.41ng/ml in those who failed to attain any insulin independence at 1 year(191).

Individual centres have reported results since the advent of the Edmonton protocol. Ryan *et al* updated the original Edmonton series to report 54 transplants performed in 30 recipients. Seventeen patients became insulin independent after infusion of at least 9000 islet equivalents (IEQ) per kg body weight (*vide infra*). Of 15 consecutive recipients followed up for 1 year, 12 (80%) were insulin independent at 1 year(192). The National Institute for Health (NIH) experience was reported by Hirshberg *et al*. Six C peptide negative female patients with hypoglycaemic unawareness received intraportal islet transplants under a daclizumab, sirolimus and tacrolimus based immunosuppressive regimen. No patient required more than two islet infusions and, during a follow up period between 17 to 22 months, all patients reported less frequent and less severe episodes of hypoglycemia and half achieved insulin independence at 1 year(193).

Shapiro *et al* recently reported the results of an international multicentre study to demonstrate the feasibility and reproducibility of the Edmonton protocol. Of 36 recipients transplanted in 9 centres, 16 (44%) achieved insulin independence with adequate glycaemic control at 1 year. Though only 5 (14%) had maintained insulin independence at 2 years, islet graft function provided protection from severe hypoglycaemia and improved levels of glycosylated haemoglobin(194).

The CITR report a smaller number of recipients of islet after kidney transplants (IAK, n=22). These are performed to improve glycaemic control in conjunction with renal replacement therapy for ESRF arising from diabetic nephropathy. 86.4% of recipients were either using an insulin pump, or having three or more daily injections of insulin, with a mean daily insulin requirement of 35.7 units before the first islet infusion.

Recipients of a single infusion (n=5) have received a mean 12860 IEQ/kg body weight, those having 2 infusions (n=14) have received mean 15257 IEQ/kg and with 3 infusions

(n=3) have had mean 22524 IEQ/kg body weight. 45.5% of ITA recipients had daclizumab, sirolimus and tacrolimus based immunosuppression. At six months and 1 year after first islet infusion, 46.7% and 42.9% respectively had achieved insulin independence. Of the small number who have completed six month (n=7) and 1 year (n=4) follow up, 46.7% and 33.3% respectively achieved insulin independence. There was a marked reduction in the frequency of severe hypoglycaemic episodes (54% before vs 4.5% after first islet infusion), reduced total insulin requirement (85% reduction at 6 months and 71% reduction at 1 year), improved HbA1c titre (mean 8% pre-transplant vs 6% at 6 months and 1 year) and increased C peptide secretion (mean 0.3 ng/ml pre-transplant vs 1.8ng/ml by 6 months and 1 year)(191). Lehmann *et al* previously reported the largest single centre series of simultaneous islet with kidney (SIK) transplants. After a median follow up of 2.3 years, 80% of recipients of 2 or more islet infusions (n=6) had sustained insulin independence using a steroid free immunosuppressive protocol. This supports the concept that such regimens can prevent islet allograft rejection in patients with more advanced diabetic complications such as ESRF(195).

Prior to 2001, SIK (n=101) or IAK (n=59) were the most frequent type of islet transplants performed and the International Islet Transplant Registry (IITR) in Giessen, Germany is the most comprehensive source of data relating to this period. The University of Giessen has the most extensive single centre experience with SIK (n=35) and IAK (n=21), achieving 77% and 43% islet graft survival respectively. Mean HbA1c levels decreased from 7.8% to 7% and 8.5% to 6.9% in SIK and IAK recipients; and insulin requirements were reduced by a mean of 18U/day and 33U/day respectively. Insulin independence was achieved in ten patients, and no severe hypoglycaemic episodes occurred in the follow up period(196). The Giessen peritransplant protocol included the use of donor pancreata with less than 6 hours of cold ischaemia; a 1:1 donor to recipient ratio of intraportal infused islets; induction immunosuppression with anti T cell agents; the use of parenteral CsA; isolation media including antioxidants; transplantation of a minimum islet mass of 6000 IEQ/kg recipient mass, and finally islet cell culture to reduce islet immunogenicity. Finally, strict post operative glycaemic control was achieved using intravenous insulin and total parenteral nutrition. Where all these criteria were met 47% of recipients showed persistent graft function, with 29% attaining insulin independence at one year(196).

A small number of recipients of combined liver and islet cell allografts have also been reported to the IITR(196). These patients have been rendered diabetic after total pancreatectomy as part of upper abdominal exenteration for extensive gastrointestinal

malignancy. Co-transplantation with the liver offers a theoretical immunological advantage by increasing islet graft survival and enhancing the potential for tolerance(197). The largest reported series is from Miami (n=11) where islets were isolated from either the pancreas of the liver donor (n=5) or from a third party (n=6) to establish an adequate islet mass. A mean islet mass of 7938 IEQ/kg was transplanted, and insulin independence was achieved in seven patients for an average of 514 days. However tumour recurrence caused the death of some recipients (n=4), despite them remaining insulin independent. Survivors with insulin independence had received a mean of 6668 IEQ/kg under tacrolimus immunosuppression(198). Between 1990 and 1999, IITR data from all centres performing islet and liver co-transplantation shows 47% of recipients gaining insulin independence at one year(196). Furthermore, the high risk of portal vein thrombosis and tumour recurrence after liver and islet co transplantation has rendered the technique of historical interest and a surgical idiosyncrasy rather than established best practice.

The IITR has reported 21 islet cell transplants performed for insulin treated type 2 diabetes or diabetes arising from liver cirrhosis, haemachromatosis or cystic fibrosis(196). The Miami group have reported a series of liver and islet co-transplants (n=7) in insulin resistant, hyperinsulinaemic patients with cirrhosis to improve insulin resistance and restore normoglycaemia. Five recipients received an additional donor bone marrow stem cell infusion of CD34<sup>+</sup> cells, and all recipients received tacrolimus and steroid based immunosuppression. All showed a reduction in their insulin requirements and 2 recipients achieved insulin independence. However all recipients were C peptide positive prior to transplantation and had similar pre and post transplant C peptide levels. This infers that the islet grafts contributed minimally to endogenous insulin production and it is likely that the improved glucose homeostasis was due to their improved hepatic function as a result of the liver component of the graft(199).

## ***2.3 Technical aspects of islet cell transplantation.***

### ***2.3.1 Islet cell isolation.***

Islet isolation begins with the enzymatic digestion of the pancreas. This is achieved by intraductal distension of the pancreas with collagenase(200-202), an enzyme mixture filtered from bacterial cultures of *Clostridium histolyticum*(203). Crude collagenase is a mixture of at least 12 component enzymes, of which collagenase forms the major active constituent, and hence its production is subject to considerable batch to batch

variation(204). Attempts have been made to purify collagenase and engineer the precise mixture of enzymes required for pancreas digestion(205, 206) but it appears that synergy exists between the collagenase and neutral protease components of the mixture in order to attain optimal digestive activity. Purified collagenases lacking neutral proteases result in poorer islet yields compared to the use of crude unpurified preparations(206); whilst enzyme blends with high neutral protease activity improve islet yield but are detrimental to islet viability and function(207, 208). For human islet isolation the semi-purified enzyme blend Liberase<sup>®</sup> achieves consistently superior islet yields(209). Liberase<sup>®</sup> has a low lipopolysaccharide endotoxin content(210, 211) and, prior to its introduction, reagents with a high endotoxin content, such as Ficoll and Collagenase P, probably contributed to the high levels of islet allograft primary non-function(212). Studies have confirmed that endotoxin has a detrimental effect upon islet cell engraftment(213), activates proinflammatory cytokine gene expression(214), complement(215) and nitric oxide mediated allograft damage(216, 217). In a recent study Balamurguran *et al* demonstrated that the duration of islet exposure to exogenous enzymes including Liberase HI correlated with decreased insulin secretory capacity, increased expression of adhesion molecules implicated in the development of post transplant peri-islet inflammation and activation of apoptotic pathways(218). The need to further improve the enzyme blend, control batch to batch variability and achieve consistently low endotoxin content continues to generate research interest in the optimal enzyme for human pancreas digestion(219). Whilst recently concerns regarding the transmission of prion diseases have arisen as enzymes such as Liberase<sup>®</sup> contain bovine growth factors. The pressure of pancreatic ductal distension with collagenase influences islet yield(220). High pressure distension disrupts the integrity of the islets allowing collagenase to enter the islet cells(221) and the use of controlled perfusion devices, to maintain a constant ductal pressure, are now advocated by experienced islet isolation laboratories(222). The timing of collagenase administration, where pancreata are subjected to periods of preservation, also influences islet yields(223). In a porcine model, pancreata pre-loaded with collagenase prior to preservation had improved *in vitro* islet function, but reduced *in vivo* function compared to islets from post-loaded or unstored pancreata (224). The use of alternative solutions and adjustment of pH for intraductal administration of collagenase has also influenced the success of islet isolation(225). Intraductal collagenase, dissolved in conventional media results in a reduced islet yield, function and viability, compared to

dissolution of collagenase in oxygen carrying media specifically designed for intraductal administration(226).

After ductal distension, pancreas digestion occurs in an automated 'Ricordi' chamber(227). This allows progressive controlled mechanical dispersal of the pancreas by steel spheres which collide within the chamber; disrupting the pancreas and yielding free islets. This involves either manual or mechanical agitation of the chamber, though efforts to improve the isolation efficiency, by redesigning the chamber and incorporating automated agitation of the apparatus, have not significantly improved islet yields(228, 229). So the use of the Ricordi chamber, by technical staff experienced in the isolation procedure, remains part of the 'gold standard' for optimal human islet isolation.

The mechanical and enzymatic rigor of islet isolation, and inherent islet fragility, has led to many efforts to modify the digestion process. Some groups have advocated the use of organ preservation solutions for dissolution of collagenase, and utilise their cytoprotective properties to protect and improve the function of the isolated islets.

However, collagenase is a calcium dependent enzyme, and many preservation solutions are calcium free, high sodium, low potassium media, which may be toxic to islets at the temperatures required for isolation(230). Other attempts to rationalize the digestion phase, simplify and reduce the cost of the procedure have been attempted but the yields and functionality of islets from such studies have not been superior to conventional methods(231). However, the addition of antioxidants to isolation media may abrogate the detrimental effects of islet exposure to inflammatory cytokines(232). The Geissen group first demonstrated a clinical benefit by including antioxidants in their peri-transplant protocol and Ichii *et al* recently reported an increase in clinically transplantable islet yields from UW and TLM preserved pancreata when nicotinamide was supplemented in processing media(233).

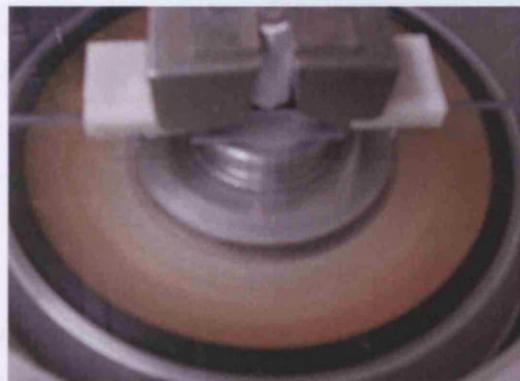
### ***2.3.2 Islet cell purification.***

Not all islet transplants require purification of the islet digest and islet autotransplantation is frequently performed without purification to maximize the islet mass. Where the infused tissue mass is a very close haplotype match or is from a scarce donor source, such as a living related donor, islet purification may be forgone to achieve expedient transplantation of the maximal islet mass(234). However, most groups agree that islet purification is appropriate for the majority of islet cell allotransplants(235). Advocates of islet purification argue that infusing a small volume of highly pure cells makes intraportal

infusion safer, and much of the evidence for this is inferred from the adverse results of some intraportal islet autotransplants. Disseminated intravascular coagulation(236), portal hypertension(237), visceral infarction(238), thrombosis(239) and death(236, 237) have all been reported following islet autotransplantation but fortunately with improved isolation methods and the use of endotoxin free Liberase e<sup>®</sup> these are now rare. However, further support for islet purification arises from the low rates of graft survival from unpurified preparations, containing high levels of endotoxin and xenoprotein from unrefined collagenase blends(212).

Islet cell purification has been performed in all recent clinical series of islet allotransplants and has changed little in the last decade(88, 240-242). It relies on the principle of density gradient separation using the COBE 2991 cell processor (Figure 2-3 and Figure 2-4)(243).

**Figure 2-3. The COBE 2991 cell processor.**



**Figure 2-4. Density gradient purification on the COBE processor.**

This allows cellular components to migrate to their isodense point(244); the islets being less dense and therefore more buoyant in the gradient than exocrine contaminant. To achieve good separation of islets from acinar tissue there has to be good cleavage between them as any cellular attachment will affect density and purity. However, swelling of the acinar tissue, a consequence of pancreatic preservation injury, allows non-islet cells to become less dense and contaminate the purest islet fractions of the gradient(245). Storage of the pancreas and pancreatic digest in University of Wisconsin (UW) solution prior to islet purification reduces acinar contamination and improves islet

yield(246-248) and higher islet purity can also be achieved by using a continuous linear density gradient rather than a stepwise discontinuous gradient which was the original described technique(249). Important factors to consider for selecting gradient media are viscosity, osmolarity and endotoxin content(250, 251) and in the future media will have to be strictly non-toxic and endotoxin free to meet the stringent quality control criteria required for clinical transplantation.

Several gradient media have been evaluated but the most commonly used are Ficoll based; for example Ficoll(252), Euro-Ficoll(253) and Ficoll-Sodium diatrizoate (FSD)(254). Newer non-Ficoll agents such as Nycodenz(254), Percoll(255) and Optiprep (iodixanol in UW solution)(256) have also been evaluated for islet purification. Recently Huang *et al* improved islet purification using Biocoll, a commercially prepared Ficoll derived gradient solution with very low levels of endotoxin, diluted in UW solution to create linear density gradients of 1.065 to 1.095 g/mL. Human islets purified using the new medium were increased in yield (435,318 vs 218,000) and viability (92.1% vs 65.4%). More than 95% of islets were present in discrete gradient fractions, the volume of which increased from 20ml to 60ml. Islets showed good physiologic responses to insulin secretagogues and restored normoglycemia in a streptozotocin-induced diabetic severe combined immunodeficiency disease (SCID) mouse model(257). In our centre Biocoll gradients are now used for human islet purification and, where purification yields are low, additional 'rescue gradient' purifications are performed. Islets isolated from such gradients are comparable in viability, *in vitro* and *in vivo* function to conventional purified islets, and can increase the isolated islet mass from a donor pancreas by upto 28%(258).

Not all centres performing islet isolation consistently produce transplantable islet preparations. Indeed upto 30% of isolates remain un-transplantable and the lack of reproducible consistently successful islet isolation remains a barrier to the widespread application of islet transplantation(259). It is well known that good technique and practised expertise are critical to successful islet isolation. Organisation into isolation teams and networks allows such expertise to be maintained and shared; and can expand both the number of transplantable islet preparations and recipient population(260).

### **2.3.3 Islet cell culture.**

The majority (56%) of recent clinical islet cell transplants have used a median 28 hour period of cell culture between islet isolation and transplantation. Under careful monitoring of pH, pCO<sub>2</sub> and pO<sub>2</sub>(261), and using cell culture medium(262), islets can be

'culture purified'. Critical to successful islet culture has been a low islet density within the culture vessel. This reduces islet hypoxia but means that up to 30 culture flasks are required for each pancreas processed. Recent modifications to culture flasks incorporating an oxygen permeable silicone rubber membrane base have allowed islets from a whole pancreas to be successfully maintained at high culture densities for up to 48 hours with no decrease in islet viability(263).

During islet culture residual non viable acinar tissue, remaining within the purified islet fraction, dies allowing the islet fractional purity to increase. This period also allows pooling of islets from multiple donors to achieve an optimal islet mass and the potential for islet modification by gene and protein transduction methods to alter islet phenotype and immunogenicity(264, 265). Furthermore there is increasing evidence that *ex vivo* islet expansion can be achieved to potentially increase the islet yield beyond that of the original donor pancreas and allow transplantation of multiple recipients from single donor pancreata(266-268). Hering *et al* demonstrated clinical success by transplanting cultured islets from two layer method (TLM) preserved pancreata (*vide infra* 3.4.4) using single donors. Under induction immunosuppression with a humanized anti-CD3 monoclonal antibody (hOKT3<sub>g-1</sub>) and maintenance therapy with sirolimus and low dose tacrolimus; 4 out of 6 recipients achieved insulin independence with normal HbA<sub>1c</sub> levels and no episodes of hypoglycaemia(269). Similarly, Froud *et al* recently reported insulin independence rates of 79%, 43% and 43% at 12, 18 and 33 months after 1 or 2 infusions of cultured human islets under a steroid free immunosuppression regimen (270).

### **2.3.4 Islet cell viability.**

Current methods of islet viability assessment and quality control do not accurately quantify islet viability prior to transplantation though several techniques have been investigated.

Brazda *et al* described the increasing concentration of amylase in preservation solutions as a valid 'surrogate' marker of whole pancreas graft viability. High concentrations were a strong indicator of exocrine tissue destruction and showed good correlation with graft viability assessed by acridine-orange staining. They demonstrated differences in pancreas viability following Euro-Collins (EC) and UW solution preservation with significantly lower amylase concentrations following 4 and 8 hour UW cold storage (6.45 IU/g vs. 2.2 IU/g and 11.5 IU/g vs. 3.58 IU/g respectively). The authors of the study suggested that the time and cost saving implications of such a test, as a surrogate marker of subsequent islet viability, could be considerable(271). Dye testing for membrane integrity(272-274)

and colorimetry(275) assays can distinguish dead from living isolated islets but give no information about islet function. Similarly confocal microscopy(276, 277) and laser scanning cytometry(278) show the integrity of islet cells but give no functional assessment, and are time consuming and expensive. A pre-isolation histomorphometric assessment of islet density and size in the pancreas has been shown to correlate well with yield and successful islet xenotransplantation; but again is time consuming, labour intensive and, as yet, untested in the human clinical donor pancreas(279).

*In vitro* functional measures such as perfusion(280) and static glucose incubations imply information about islet function but give no measure of *in vivo* graft function(281, 282). To assess *in vivo* function there is reliance upon 'bioassays' using xenotransplantation into diabetic athymic nude rodent models(283-285). These do not necessarily reproduce the situation in the human allotransplantation and are impractical when there is an urgency to transplant fresh islet cells to optimize graft function(286).

Papas *et al* recently described a technique to predict the *in vivo* function of transplanted islets by *ex vivo* measurement of islet oxygen consumption. This was 89% sensitive and 77% specific for predicting successful reversal of diabetes in the nude mouse model and the authors advocated its use in place of this standard bioassay of islet viability and function(287). The same group used nuclear magnetic resonance spectroscopy to show alterations in oxidative metabolism during simulated islet transportation and storage(288). Armann *et al* recently described the use of a luminol based assay to determine the production of reactive oxygen species (ROS) by isolated islets.

Comparison with conventional measures of necrotic cell death, mitochondrial membrane, ATP:ADP and insulin secretory capacity confirmed a correlation between ROS production with the percentage of apoptotic cells and their functional potency *in vivo*. The authors proposed the new assay as a further surrogate measure of islet quality prior to transplantation(289). However, technical expertise and resources to allow such assessments prior to clinical islet transplantation is beyond the capacity of the standard isolation laboratory.

Islet viability and function after transplantation have historically been determined by the recipients ongoing insulin requirement, C peptide secretion and improved glycaemic control. The 'beta score', a more formal clinical assessment of post-transplant function which incorporates all of these factors, has been found to correlate well with graft response to a glycaemic challenge(290).

To date, clinical human islet transplantation lacks a suitable early marker of graft rejection. Though the liver is accessible to allow biopsy, identification and monitoring of

intraportal islet grafts if needed (291, 292). Such biopsies are not used routinely for the diagnosis of rejection as it risks bleeding and a poor yield of islet tissue in the biopsy sample in ITA recipients. Recently a promising 'surrogate marker' of islet graft rejection has been detection of insulin mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) in the peripheral circulation. This coincides with the release of  $\beta$  cells into the circulation during acute rejection of intraportal islet allografts and may abrogate the need for invasive graft biopsies(293). Another method has used real time PCR analysis of mRNA transcripts for the cytotoxic lymphocyte genes granzyme B, perforin and FasL. Elevated mRNA transcript levels in peripheral blood, particularly of granzyme B, were predictive of islet graft rejection up to 197 days prior to the appearance of hyperglycaemia and increased insulin requirements in a non human primate model(294). Similar analysis of mRNA transcripts in human (n=8) islet allograft recipients showed elevation 25 to 203 days before the onset of hyperglycaemia, and may be a means to predict islet allograft failure at a subclinical stage(295).

### ***2.3.5 Islet transplant site and the inflammatory response.***

The intraportal site is the commonest site for clinical islet cell transplantation. Both CITR and IITR data show that all recipients attaining insulin independence received intraportal islet transplants(191, 196). The intraportal site is used by default, in the absence of another alternative site, but has important clinical advantages and disadvantages. Cannulation of the portal vein for islet transplant infusion is achieved easily under local anaesthesia with fluoroscopic guidance(89). Intraportal islet grafts secrete insulin into the portal sinusoids mimicking the physiological pancreatic secretion of insulin into the portal system seen *in vivo*(296, 297). However, localized high insulin concentrations around transplanted islets can cause glycogen deposition and microvesicular steatosis in the periportal region(87, 298). The consequence of this on islet viability and long-term function is unknown, though embolisation of islets into liver sinusoids also induces focal hepatic necrosis which contributes to islet graft failure(299). Additionally, studies of intraportal islet auto transplants show a normal albeit blunted biphasic insulin response to glucose(300, 301) with less hepatic insulin extraction(301) and possibly overspill of insulin into the systemic circulation(297). Hypoglycaemia induced glucagon responses have also been suboptimal with intraportal islet grafts in both dogs(302) and humans(300, 301, 303, 304).

The composition of portal blood has implications for intraportal grafted islets. They become exposed to supra-physiological concentrations of potentially damaging cytokines

released from the gut(305, 306) and intraportal nitric oxide(217) which can mediate islet damage. Oral administration of immunosuppressive drugs also means that intraportally grafted islets are exposed to elevated and potentially toxic levels of immunosuppression compared to systemic blood levels(307).

In VPT, completion of arterial and venous anastomoses mark the end of ischaemic hypoxia but this is then followed by an injurious period of reperfusion. However, as a cellular transplant, islets have a more protracted period of ischaemia with no direct blood flow during the immediate post transplant period. Indeed, in the first 48 hours following transplantation the capillary bed of the islet collapses and is expelled from the graft(308) leaving minimal or no donor endothelial tissue(309). At this point islet perfusion is dependent upon portal sinusoid endothelial vasoconstriction and vasodilatation. Within 24-48 hours, expression of vascular endothelial growth factor (VEGF) is upregulated(310). This appears critical to establishing islet neo-vascularisation and the presence of exogenous VEGF enhances the oxygenation of islet grafts, inhibits the generation of the allogeneic immune response and prevents islet necrosis and apoptosis(311). Indeed islet adenoviral transfection to increase the endogenous expression of VEGF is a potent means of inducing resistance against inflammatory cytokine induced apoptosis whilst retaining *in vitro* glucose stimulated insulin secretion and islet viability(312). Perfusion of the graft is not achieved until the process of neo-angiogenesis has established new capillary networks of host origin(313). This begins as early as 7 days, and is well established after 10 to 14 days, after transplantation(314, 315). The most obvious consequence of delayed arterial reperfusion is hypoxic injury to islets which causes islet death and dysfunction(316, 317). Normally the oxygen saturation and tension of hepatic arterial and portal venous blood are 96% and 15.2 kPa, and 85% and 13.2 kPa respectively(318). Experimental studies have shown significantly reduced oxygen tensions in intrahepatically transplanted islets compared to native islets or whole pancreas transplants(319-321) though, interestingly, this hypoxia lasts up to 9 months; way beyond the time required for revascularization of the graft(322). Centrally located  $\beta$  cells may be more at risk from hypoxia due to limited diffusion of oxygen and nutrients(323) and the administration of hyperbaric oxygen improves graft function in animal models of islet transplantation by abrogating this hypoxic injury(324, 325). Islets remain at risk, even in circumstances when graft oxygenation can be rapidly restored, from hypoxic-reoxygenation injury. *In vitro* experiments have shown a significant reduction in apoptotic cell death when islets are co- cultured with agents to abrogate H-R injury(326).

The intra-portal microenvironment dictates the components of the early innate and antigen specific immune responses to which islets are exposed. This comprises of controlling the peri-islet inflammatory response, through the mobilisation of leucocytes, activation of intrahepatic Kupffer cells and activation of 'resident macrophages'.

Mobilisation of leucocytes into islet grafts is achieved by the release of leucocyte rolling receptors from the sinusoidal endothelium(327) and islet expression of adhesion and co-stimulatory molecules which cause leucocyte adherence, diapedesis and infiltration into the graft(328). Indeed, islets may be predisposed to early destruction by infiltrating host leucocytes as they express adhesion molecules as a result of cytokine upregulation, hypoxia or after cell culture following isolation(329-332). Blockage of adhesion molecules on islets by antibodies or antisense oligonucleotides is known to prevent islet graft rejection(333) and improves the early function of islet allografts(334).

Of relevance to intrahepatic islet transplantation is activation of Kupffer cells, the resident macrophages of the liver which injure cells through the release of free radicals or secretion of inflammatory cytokines(335). Bottino *et al* confirmed Kupffer cell activation following intrahepatic islet transplantation by elevated levels of Kupffer cell secreted TNF $\alpha$  and interleukin 6 (IL-6). This trend was reversed after hepatic macrophage depletion(336). Endotoxin contamination of islets, due to persisting residual collagenase, causes Kupffer cell activation and pro-inflammatory cytokine production(212) whilst necrosis of co-transplanted acinar tissue releases inflammatory mediators which further activate Kupffer cells, cause foreign body giant cell reaction in islet grafts and decrease islet viability(337).

Islets themselves contain 'resident macrophages' of donor origin which are important potential mediators of islet injury and destruction(338-340). They are an inraislet source of IL-1 $\beta$ (341) and iNOS(340) and respond to proinflammatory stimuli such as TNF $\alpha$ , IFN $\gamma$  and LPS(342). Culture conditions which are toxic to resident macrophages result in decreased cytokine stimulated iNOS expression and NO production from islets(341, 343). After 7 days in such conditions, IFN $\gamma$  exposure causes no cytotoxicity whereas islets not depleted of resident macrophages are destroyed(344).

Clinically immunosuppressive regimens have been designed to reduce the production of inflammatory cytokines arising from islet grafts and in response to intrahepatic engraftment. Froud *et al* report insulin independence rates, comparable with using the Edmonton steroid free protocol, after 1 or 2 infusions of cultured human islets under an anti-TNF $\alpha$  monoclonal antibody (Infliximab) induction regimen(270).

Intrahepatic islets exist in a pro-thrombotic milieu. Expression of TNF $\alpha$  and IL-1 $\beta$ , by both islets and hepatic Kupffer cells, results in increased expression of endothelial cell factor VII, thromboplastin and platelet activation factor. Additionally tissue factor, a membranous glycoprotein which activates the extrinsic clotting cascade, is expressed by Kupffer cells, hepatic sinusoidal endothelium and islets after stimulation with IL-1 $\beta$ , TNF $\alpha$ , LPS or immune complexes(345, 346). Moberg *et al* confirmed an increase in tissue factor expression which caused production of thrombin, platelet activation and aggregation and the development of a fibrin capsule around the islets(347, 348); a process now termed the 'instant blood mediated inflammatory reaction' (IBMIR)(349). The systemic administration of low molecular weight dextran showed a dose-dependent inhibition of IBMIR with inhibition of coagulation and complement activation and reduced consumption of platelets. Though this treatment can be easily monitored using the APTT ratio it exposes the recipient to the risk of bleeding(350). Similarly Ozmen *et al* successfully prevented IBMIR *in vitro* using a specific thrombin inhibitor. This showed a dose dependent reduction of active coagulation and complement components, reduced leucocyte graft infiltration and maintained islet morphology(351). Cabric *et al* recently reported a method to conjugate heparin complexes to the surface of islets. This successfully prevented IBMIR *in vivo* and avoids the need for systemic administration of anticoagulants(352). There is close correlation between the duration of donor pancreatic warm ischaemia and the expression of tissue factor. Tissue factor levels can be reduced by administration of the anti-oxidant nicotinamide(353) and may be a useful surrogate marker to predict IBMIR in islet grafts prior to islet isolation(354).

Alternative sites for islet transplantation have been extensively studied. Canine autotransplantation studies using the intrasplenic site show a normal or exaggerated hypoglycaemic glucagon response(355). Similarly intrasplenic islet transplants in non-human primates have a capacity for inducing prolonged normoglycaemia(356, 357). However, experience with human intrasplenic islet autotransplantation has occasionally resulted in infarction or thrombosis requiring splenectomy; and intrasplenic islet grafts cannot be biopsied safely using percutaneous techniques. The renal subcapsular site is frequently used in small animal models of islet cell transplantation(358) but is unlikely to be adopted clinically. Human islet recipients have a preponderance of diabetic renal microangiopathy which could jeopardize renal subcapsular neo-angiogenesis. Additionally the renal subcapsule results in low islet cell oxygen tension(319) and the systemic secretion of insulin from this site does not mimic the physiological situation. Comparable graft function is attained using an omental pouch which allows the islet graft

to be transplanted, and explanted if required, with relative ease(359). Immune privileged sites, such as the thymus(360) and testis(361), have been studied for islet transplantation in the hope that a state of donor specific tolerance may be achieved. Though successful, with intrathymic islet injection, in the animal model(362, 363) it has yet to be reproduced clinically.

### **2.3.6 Adequacy of islet mass.**

It is difficult to determine the number of islets required to achieve insulin independence. Much of the information is inferred from patients having partial pancreatectomy, however islets are distributed unevenly with high numbers of small islets in the head, and moderate numbers of larger islets in the tail of the gland(364). Inherently, such pancreata are pathological and the number and distribution of islets therein may not accurately reflect those in a healthy donor pancreas. Nevertheless, seventy two percent of patients having resection of greater than 80% of the pancreatic mass, and 32% where a less aggressive resection is performed, develop post-operative diabetes(365). Donors for living related pancreas transplantation have a less extensive segmental pancreatectomy with a 15% risk of developing impaired glucose tolerance as a result of the loss of this islet mass(366). Using the tail of the resected human pancreas, Matsumoto *et al* achieved an islet yield sufficient for transplantation(367) and have now reported the first successful live donor clinical human islet transplantation using 8100 IEQ/kg(234). Using more selective donor criteria including a supra-physiological insulin response to arginine stimulation, it is hoped that rates of glucose intolerance in donors can be reduced. Variation in islet size, function, viability and engraftment makes comparison of absolute islet numbers between recipients and studies largely uninformative. To standardize the measure of transplanted islet mass, infusions are defined according to the number of islet equivalents per kilogram of recipient weight, based on a mean islet diameter of 150  $\mu\text{m}$ . Autotransplantation with an islet mass of only 1000 IEQ/kg can achieve insulin independence(368) and a series from Leicester reported a median islet requirement of 2020 IEQ/kg to achieve a low HbA1c concentration (6.6%) and C peptide secretion (0.66mg/ml) following autotransplantation(369). However, the addition of allogeneic and autoimmune responses in Type 1 diabetes results in increased islet cell loss and decreased engraftment because of rejection and recurrent insulinitis. Consequently the islet mass requirements for SIK and IAK allotransplants were originally estimated to be 5000 IEQ/kg and 8000 IEQ/kg respectively(196). The original series of allografts under the Edmonton protocol deemed this an underestimate and advocated infusion of at least 11000IEQ/kg in ITA recipients(88).

IITR data comparing recipients of less than 6000IEQ/kg (n=78) and greater than 6000IEQ/kg (n=146) show C peptide secretion in 67% vs 78% (n=52 vs 114) and one year insulin independence in 5% vs 22% (n=4 vs 32)(196). Similar CITR data, relating graft function to infused islet mass, show that 69% of those infused with more than 5500 IEQ/kg had C peptide secretion and/or insulin independence by 6 months, but only 60% of those infused with less than 5500 IEQ/kg achieved this. By 1 year 72% of recipients having a higher transplant islet mass had sustained C peptide secretion whilst only 42% of those with a lower islet mass remained C peptide positive(191).

Some authors view IEQ as wholly inaccurate for measuring the transplanted islet mass, arguing that dithizone which is used to stain insulin positive particles may non specifically stain non-insulin containing cell types. A variable amount of exocrine contamination then overestimates the IEQ size of the graft. Equally, under culture conditions, non-endocrine tissue can take up dithizone whilst  $\beta$  cells lose dithizone staining through insulin degranulation (370). Kemeulen *et al* recently published the results of ' $\beta$  cell transplants' using cultured islets with an anti-thymocyte globulin (ATG) induction protocol and MMF and tacrolimus based maintenance regimen. Non uraemic patients with Type 1 diabetes received between 0.5 and  $5.0 \times 10^6$  insulin positive  $\beta$  cells/kg body weight via intraportal infusions. Sixteen of 17 patients receiving more than  $2 \times 10^6$   $\beta$  cells had graft function at 2 months but 0 of 5 who received fewer  $\beta$  cells had graft function. Of 20 recipients who had at least 1 infusion of  $>2 \times 10^6$   $\beta$  cells, 17 had maintained graft function with improved metabolic glycaemic control 1 year after transplantation. This supports the concept that the number of infused  $\beta$  cells is closely correlated with metabolic graft function, and a minimum of 2 million  $\beta$  cells/kg body weight is required for prolonged graft function(371).

Hyperglycaemic clamp studies have demonstrated that the insulin secretory capacity of functioning islet grafts is only 25% that of age and sex matched controls(371) and, despite an adequately infused cell mass and insulin independence, the  $\beta$  cell reserve in the face of potential allo and autoimmune mediated rejection is considerably reduced. Ryan *et al* used a Kaplan –Meier survival analysis on the Edmonton series of recipients which also showed a progressive decline in islet graft function (372). So it is likely that the islet mass required to sustain long-term insulin independence without islets 'burning out' is considerably higher than originally proposed.

Clearly transplantation of an adequate islet mass is crucial to the success of islet cell transplantation and this currently appears to be only achieved by 'sequential' transplants with islets isolated from more than one donor pancreas. However, recently there have

been successful islet transplants using single donor pancreata. Hering *et al* used a humanized anti CD3 monoclonal antibody (hOKT3<sub>g-1</sub>) induction agent, TLM pancreas preservation method and islet cell culture to achieve long-term insulin independence (n=3) and freedom from hypoglycaemic unawareness in all recipients 5 out of 6 recipients(269).

### **2.3.7 Immunosuppression and rejection.**

Allorejection of islet grafts arises from expression of MHC Class I antigens on the islet cells themselves, and MHC Class II antigens on 'passenger leucocytes' within the islet graft(373). A T cell mediated alloreactive response occurs either by 'indirect' antigen processing and presentation by host antigen presenting cells (APC); by 'direct' stimulation and T cell clonal expansion in regional draining lymph nodes mediated by donor APCs(374, 375) or by 'antibody dependent cellular cytotoxicity' mediated by the presence of alloantibodies in previously sensitized patients(376).

The initiators and modifiers of the allogeneic response are cytokines secreted from the APCs and T lymphocytes. These mediate islet graft PNF and rejection(377, 378) and are directly detrimental to glucose stimulated insulin release from islets(379, 380).

Manipulation of the cytokine response following using monoclonal antibodies, antioxidants and phosphodiesterase inhibitors have produced conflicting and inconsistent results(381).

An alternative approach is to attempt to induce donor specific tolerance to islet grafts in the recipient. Immunomodulation was historically used to modify islet immunogenicity by deletion of passenger leucocytes from the islet graft(382) and techniques have included islet low temperature culture(309, 383), gamma(384, 385) and ultraviolet irradiation(386-388) and the use of depleting monoclonal antibodies to delete or mask donor dendritic cell HLA antigens(389-391). Although tolerance has been achieved in small animal models, it is dependant upon the animal strain combinations used, and most experiments have required immunosuppressive drugs as well as leucocyte depleting immunomodulation of the graft to prevent rejection.

Conventional immunosuppressants such as CsA(392), azathioprine(393) and prednisolone(394) are unsatisfactory for islet transplantation. They are toxic to  $\beta$  cells(395), impair insulin secretion(393, 396) and induce a state of insulin insensitivity in peripheral tissues(397). Their mandatory use during the lifetime of the graft incurs risks including malignancy, nephropathy, infection and hypertension. Nevertheless historical immunosuppressive regimens for islet transplantation were based around a 'triple

therapy' combination of azathioprine, prednisolone and CsA with either ATG or OKT3 induction agents(383, 398, 399).

It is hoped that alternative agents may improve immunosuppression regimens. Based on achievements after renal, hepatic and thoracic transplantation(400, 401) tacrolimus (FK506) was soon introduced for islet transplantation. However, tacrolimus is known to be more diabetogenic than CsA(402) through the direct inhibition of insulin mRNA transcription(403). Indeed upto 20% of renal transplant recipients receiving tacrolimus based immunosuppressive regimens are rendered diabetic. Deoxyspergualin, which has inhibitory effects upon macrophage activity(404, 405), has also been used with success after clinical islet transplantation(406) and is associated with reduced  $\beta$  cell inhibition in both human and rodent models(407). Other agents with low  $\beta$  cell toxicity include MMF and the enteric coated derivative Myfortic(408, 409), leflunomide(410, 411) and FTY720(412). Hering *et al* recently reported successful long-term islet xenotransplantation using a combination of FTY720, everolimus and leflunomide combined with anti-CD25 and CD154 monoclonal antibody induction. However the use of such potent combined immunosuppression in a clinical setting remains untested(413). The most recent, and most successful, agent in clinical islet transplantation has been sirolimus(88, 414) used in conjunction with low dose tacrolimus. These agents act synergistically, allowing the dose of potential nephrotoxic, neurotoxic and diabetogenic calcineurin inhibitors to be reduced, whilst preventing acute allojection(415, 416). Despite a side effect profile including myelosuppression, hyperlipidaemia and predisposition to infection; sirolimus (trough concentration 12 to 15 ng/ml for 3 months then 7 to 10 ng/ml thereafter) with reduced dose tacrolimus (trough concentration 3 to 6 ng/ml) formed the maintenance regimen for the Edmonton protocol. Shapiro *et al* first reported the successful use of this steroid free immunosuppressive protocol to achieve short term insulin independence in seven recipients of intraportal islet transplants. Though the novel immunosuppressive regimen undoubtedly contributed to their success, the peritransplant protocol also included early harvesting of the pancreas from strictly selected donors, minimization of pancreatic cold ischaemia time, the use of endotoxin free human liberase for pancreas digestion, induction immunosuppression with a humanized interleukin 2 receptor antibody (Daclizumab) and sequential allografting with a donor to recipient ratio of at least 2:1 to achieve an islet mass of at least 11000 IEQ/kg(88).

CITR data show that 61.1% of recent recipients have received a regimen that replicated the Edmonton protocol but a number of other induction agents including infliximab

(8.4%), alemtuzumab (4.4%), 15-deoxyspergualin (2.5%) and ATG (1.5%) have been used. Of note only 4.5% have received steroid immunosuppression as induction or maintenance agents. Furthermore a significant number of recipients are maintained on tacrolimus and MMF (6.1%), sirolimus and MMF (3.7%) or a combination of all three agents (8.0%); though information on the maintenance regimen in 25.8% of recipients is not known. Notably there is recent evidence to suggest that sirolimus may also be harmful to islets and it is imperative that future immunosuppressive regimens be totally devoid of calcineurin inhibitors to prevent their diabetogenic effects(417).

### **2.3.8 Autoimmunity and islet cell transplantation.**

Recurrent autoimmune mediated islet graft loss resembles the insulinitis of early Type 1 diabetes(418). Stegall *et al* reported three cases of islet allograft autoimmune destruction in identical twin sibling recipients without immunosuppression, and a further case in an HLA identical non twin sibling allograft treated with low dose CsA(419). Jaeger *et al* observed the persistence and recurrence of autoantibodies in immunosuppressed recipients of islet transplants. This suggests that immunosuppression sufficient to prevent allorejection is inadequate in preventing autoimmune mediated islet graft loss in some recipients(420). Where recipients are autoantibody negative prior to transplantation and subsequently seroconvert; the islet allograft is potentially exposed to autoimmune mediated rejection. In such situations serological monitoring of recipients for the presence of autoantibodies may be a means of determining islet grafts at risk(421). Conventional immunosuppressants can alter the progression to diabetes, and new agents may yet prevent both autoimmune recurrence *and* allorejection. Patients with early type 1 diabetes are occasionally treated with calcineurin inhibitors. Though it seems absurd to expose a failing residual islet mass to a drug known to decrease insulin secretion, promote insulin resistance and directly cause  $\beta$  cell damage; the beneficial effect of these drugs upon the course of autoimmune mediated  $\beta$  cell damage is well known(422, 423). Cyclosporin, administered in the early phase of Type 1 diabetes, delays the progression towards insulin dependence and is associated with a reduction in islet autoantibodies and maintenance of normal C peptide levels(424). However, double blinded, placebo controlled studies show no beneficial effect of CsA in preserving  $\beta$  cell function, and have highlighted concerns over the administration of such nephrotoxic agents to patients with potentially impaired renal reserve. Similarly the onset and severity of autoimmune diabetes in animal models can be prevented using sirolimus(425, 426), FTY720(427) and leflunomide(428), though no large long-term trials of their efficacy in clinical autoimmune diabetes are available.

### ***2.3.9 Stem cells and islet cell transplantation.***

The widespread application of islet cell transplantation is limited by the low number of donor pancreata. As the techniques of both VPT and islet transplantation improve there is likely to be competition between the two modalities for donor pancreata, an increasing number of potential recipients but a static donor organ pool. It is necessary, therefore, to explore alternative sources of insulin secreting cells for transplantation(429).

The concept of artificially engineering an insulin secreting  $\beta$  cell at low cost, in unlimited numbers and in a pathogen free environment is not new. Groups have been successful in producing such cells from tumour(430-432) and genetically transformed cell lines(433-436) arising from both pancreatic and neuroendocrine tissues(437). Although clonal proliferation of large numbers of cells is observed, their use for transplantation has several drawbacks. Firstly such transformed cells retain a potentially neoplastic phenotype and consequently pose a theoretical risk to the recipient. Secondly, the function of such cell lines may not exactly mimic the exquisite physiological responses seen in islets. A maximal response at lower threshold glucose concentration, together with a progressive loss of insulin secretion and synthesis has been observed in engineered  $\beta$  cell lines(438).

It is possible to harness the innate proliferation of human fetal tissues to produce a genetically modified clonal cell line which shows a glucose sensitive insulin secretion(439-441). This has been most successfully reproduced in neonatal islets cells in sufferers of persistent hyperinsulinaemic hypoglycaemia in infancy which show an insulin secretion within the normal range(442).

A topical and exciting area lies in the development of insulin secreting cells from embryonic stem cells. The molecular mechanisms controlling the embryology of the pancreas by various transcription factors can be controlled at several levels(443, 444).

The first stage of development under the influence of the embryonic notochord, and the second stage under the control of mesenchyme derived factors, can be influenced to allow insulin secreting cell production. This has been achieved using both embryonic pluripotential stem cells(445) and adult islet progenitor ductal cells(446).

The use of stem cells has several disadvantages. Firstly, the 'directed differentiation' of embryonic stem cells into insulin secreting cells is not easily achieved. Endodermal derivatives such as pancreas, liver, and intestine have proved difficult tissues to produce, requiring precise and controlled differentiation protocols(447). Furthermore such differentiation yields a mixture of cell types requiring further purification. Purification is based on using cell surface markers and fluorescence activated cell sorting or genetic

selection using marker genes to confer antibiotic resistance(444, 448, 449). Despite cell purification, the insulin producing cells may require a period of maturation before their phenotype is adequately established. This is certainly a feature of those derived from embryonic stem cells which display variable characteristics according to the presence of growth and differentiation factors(450, 451), and the surrounding glucose concentration(452). Concerns have been made regarding the stability of the phenotypes of such insulin secreting cells. Prior to use in transplantation, the cells would need to demonstrate a consistent and reproducible phenotype with no potential for malignant transformation or teratoma formation. It would also be advantageous to manipulate the immunogenicity of engineered cells before transplantation. Through the use of stem cell derivatives it may be possible to generate a clonal cell line mimicking the recipients own  $\beta$  cells. This would effectively make the procedure an 'autologous' transplant, and allow immunosuppression to be discontinued. Gaur *et al* recently published a report of successful withdrawal of immunosuppression where CD34+ enriched/CD18+ depleted stem cells were co-infused with allogeneic islets to graft function and survival in a non human primate model(453).

A further advance in stem cell research has been the advent of nuclear transfer of adult DNA into enucleated embryonic stem cells(454), and the ability to reverse the phenotypes of adult tissues towards their pluripotential stem cell stage(455). Both these techniques have the potential to allow the generation of autologous cellular grafts for transplantation. The latter technique to generate 'adult stem cells' has further advantages in avoiding the bioethical issues surrounding the use of embryonic tissues for stem cell research. Indeed Todorov *et al* recently reported the isolation of pluripotential progenitor cells from the discarded tissue following islet isolations(456).

### ***2.3.10 Islet Xenotransplantation.***

Much research has examined the use of islet tissue from other species for human 'xenotransplantation'. This has important implications as it may provide a potentially unlimited source of donor islet tissue. Despite some success in vascularised whole organ xenotransplantation, the use of cellular xenografts such as islets raises some unique problems that need to be addressed(457).

For whole organ xenotransplantation there is a need to choose a donor species with morphologically and physiologically similar characteristics to the recipient. Where a cellular transplant is performed the gross morphology of the original organ is a less important consideration. This would initially be seen as advantageous, allowing a large

number of potential donor species to be considered. Cellular xenotransplantation, however, is still hindered by the considerable immunological barriers between some donor and recipient species combinations. Studies of vascularised xenotransplants have demonstrated complement mediated hyperacute rejection (HAR) to be the first insult to the graft(458). This arises due to the binding of xenoreactive preformed natural antibodies (PFNAs) to antigens present on the graft vascular endothelium(459-461), and results in endothelial activation(458) with graft haemorrhage, oedema, inflammation and thrombosis(458). Cellular xenotransplants, however, are avascular and as such contain no donor endothelial tissue(462, 463). As a result they are relatively resistant to HAR but are destroyed by a later process of cell mediated delayed xenograft rejection (DXR)(464). DXR arises due to 'direct' and 'indirect' presentation of xenoantigens to host T cells by donor and host APCs respectively(465). During 'direct' presentation, donor APCs within the graft express xenoantigen-MHC II complexes. It is the recognition of these by host CD4<sup>+</sup> T cells which induces the production of effector molecules and later clonal T cell activation against the presented antigen. Pivotal to this method of antigen presentation is the recognition of donor MHC as foreign. Where a large species disparity exists this recognition may be inefficient resulting in a less aggressive T cell response and prolonged xenograft survival(466). Conversely, homology between donor and recipient MHC will also fail to induce an aggressive T cell response, and it is thought that this may explain the survival of porcine to human xenografts(467). Furthermore, strategies to immunomodulate islet xenografts and deplete donor APCs should theoretically allow increased graft survival by preventing direct antigen presentation(468). Unfortunately extended xenograft survival has not proved so straightforward.

The ideal for xenotransplantation is to establish immune tolerance allowing immunosuppression to be stopped. This has been achieved in rodent models through donor lymphoid cell infusion with and without total body irradiation(469, 470). However, such irradiation is not without side effects and successful xenogeneic bone marrow engraftment in large animals has proved difficult to achieve. Even where xenogeneic chimerism has been established the survival of islet grafts has been brief(471).

Attempts to prolong islet xenograft survival through encapsulation in biocompatible membranes have produced varying success amongst discordant models in both small(472, 473) and large animals(474, 475). A feature of most failed encapsulated grafts is the formation of pericapsular fibrotic tissue. This forms as a result of the bioincompatibility of the alginates within the capsule, and the indirect cell mediated response that cannot be abrogated by encapsulation. Consequently recipient anti CD4 monoclonal antibody

therapy is required for successful long term engraftment of encapsulated islet xenografts(476).

The recent interest in transgenic animals for human transplantation has widespread implications for vascularised organ xenotransplants. Such animals are engineered with deliberate genomic alterations, deletions and inclusions of human cDNA. These nuclear changes are phenotypically expressed as absence of xenoantigens or complement regulating protein production(477). As xenoantigen recognition and complement activation plays the pivotal role in the HAR response to xenotransplantation, it is hoped that this aggressive response can be dampened by transplanting organs from transgenic animals(220, 478-481). Such grafts are still subject to DXR and islet cellular xenografts from such animals may still be subject to this form of cell mediated rejection. A recent study of adult and fetal islets from transgenic pigs has showed a reduced expression of human decay accelerating factor (hDAF), a regulator of complement activity. *In vitro* protection against complement mediated cell lysis was seen when such transgenic porcine islets were exposed to fresh human serum(240).

The potential transmission of zoonotic disease when performing xenotransplantation will need to be addressed as the technique develops. The risk of viral transmission is of particular concern when non-human primates are the donor species. Many unclassified simian viruses exist, with unknown potential to infect the immunocompromised human host(482). The use of porcine donors reduces the potential number of infecting viruses although several pathogens are thought to be transmissible(483). More concerning is infection by porcine endogenous retroviruses which replicate after permanent incorporation into the host genome(484). Although transmission has been demonstrated *in vitro*, it is thought that this happens less readily *in vivo*(485). Human recipients of porcine islet xenografts have not demonstrated retroviral infection(486). However the routine screening of potential xenografts shall be required to minimize the risks of cross infection.

#### **2.4 Conclusion.**

Vascularised whole pancreas transplantation is an established treatment that restores endogenous insulin secretion, achieves insulin independence and physiological euglycaemia in suitable recipients. It has recognised long-term benefits for some of the complications of diabetes but also has potentially lifethreatening surgical and immunosuppressive complications.

Islet cell transplantation is a less established treatment modality but has achieved clinical success in restoring endogenous insulin secretion and short-term insulin independence. The impact of islet transplantation upon the long-term complications of diabetes is not yet known.

Both modalities rely upon the scarce resource of donor pancreata. Whilst VPT uses a single pancreas, islet transplantation usually requires sequential islet transplants from multiple donors to attain an adequate islet mass. However, improvements in islet isolation, purification and cell culture techniques mean that successful single donor islet transplantations are clinically feasible. There is, therefore, an urgent need to maximize the efficiency with which donor pancreata are used. By optimizing cadaveric pancreas preservation, and utilizing marginal and live donor pancreata, the donor pool can be expanded for both VPT and islet transplantation.

An increasing number of laboratories are now proficient in islet isolation but wide variation in islet yield exists both between and within facilities. Several factors contribute to the unpredictable outcome of the procedure; the most notable being the variation in islet density after the pancreatic preservation and digestion phases.

All current methods of pre-transplant islet viability and functional assessment have required the isolation process to be virtually completed before islets can be procured for testing. There is an advantage in making these assessments as early as possible. The ideal test would allow the success of islet isolation and islet viability to be predicted from a simple assay prior to islet isolation. If this could be performed during pancreas procurement or preservation the isolation could be stopped when there was little chance of producing a high yield of viable functioning islets for transplantation. This could help to achieve consistently high quality islet isolates from good quality pancreata, whilst less suitable donor organs could be excluded from the time consuming and costly process of unsuccessful islet isolation.

## ***Chapter 3. Management of the Donor Pancreas.***

### ***3.1 Introduction.***

Both VPT and islet transplantation rely on procurement of a good quality donor pancreas, but procurement and preservation processes cause pancreatic cellular injury that ultimately affect recipient outcomes and islet viability.

This chapter briefly reviews the pathophysiology that occurs before and during donor pancreas preservation. Developments in organ preservation technology are also discussed with an emphasis on static cold storage, perfusive and persufflative methods of pancreas preservation.

### ***3.2 The pre-preservation phase.***

#### ***3.2.1 Donor selection.***

The first stage of successful VPT or islet transplantation is the identification of a suitable high quality donor pancreas. Currently no 'donor test' can accurately predict the outcome of transplantation(487); but recently donor parameters have been used to construct scoring systems as surrogate markers of pancreas quality. These are now used to identify suitable organs for VPT and islet isolation(488, 489).

Beneficial factors for islet isolation appear to be the use of young, lean donors with a short period of ITU admission, no evidence of prolonged periods of hypotension or requirement for inotropic support(490-492). Nano *et al* retrospectively studied 437 pancreata used for islet isolation. Univariate analysis showed that islet yield was significantly associated with donor age, donor BMI, good macroscopic condition of the donor pancreas (i.e. no evidence of pancreatitis or calcification) and pancreas weight; whilst multivariate analysis found high donor BMI and pancreas weight significantly contributed to islet yield(493). Historically, extremely young(494), overweight or obese donors(495) have been underutilised but have now proven to be a potential source of islets suitable for transplantation.

Successful VPT and islet transplantation using 'marginal donors' is now reported. Pancreata procured from non-heart beating donors (NHBD) have demonstrated good immediate function with a warm ischaemia time of 20-30 mins with 16 hours of cold

storage(496). Furthermore, NHBD pancreata with a warm ischaemic period of 30 to 40 minutes have been successfully transplanted following UW preservation(497).

Markmann *et al* have demonstrated comparable recovery of islets from NHBD and cadaveric donors. Such islets demonstrate equivalent *in vitro* and *in vivo* function, and an islet transplant of 8500 IEQ/kg body weight was successful in achieving insulin dependence in a Type 1 diabetic recipient(498). A series of 5 recipients of NHBD islets have demonstrated C peptide secretion, significant improvement in HbA1c and reduced insulin requirements proving the clinical validity of using this 'marginal donor' source(499).

Live donors provide the optimal conditions for organ procurement by minimising both warm and cold ischaemia times. Matsumoto *et al* first proposed the potential for live donor islet transplantation by isolated sufficient islets from the segmentally resected pancreas(367) and the same group have now reported a successful islet transplant using a segmentally resected live donor pancreas(234).

### **3.2.2 Donor optimization.**

Conventionally the pancreas is procured from a cadaveric heart-beating donor.

Manipulation of donor physiology and metabolism can be undertaken prior to organ procurement which can alter recipient outcomes; but ethical issues, relating to consent from donor families, make large randomised controlled trials comparing potential organ donor treatments and subsequent organ function difficult to perform(500).

The process of donor 'brain death' is detrimental to the human pancreas(501, 502) and careful maintenance of donor physiology is required to optimize organ quality(503).

Indeed studies have demonstrated accelerated acute rejection episodes in other solid organs procured from cadaveric donors which are attributed to abnormal donor physiology(504). Contreras *et al* studied islet isolation yields and functionality in relation to brain death in a rodent model. Induction of donor brain death was followed by a time dependent increase in expression of inflammatory cytokine mRNA (TNF $\alpha$ , IL-1 $\beta$  and IL-6) in the pancreas. Brain dead donor pancreata also had a lower perfusive insulin release, islet yield, viability and *in vitro* and *in vivo* islet function(505).

Outside of conventional ionotropic and vasoactive agents, pharmacological manipulation of human donor physiology has not been widely studied. However, Eckhoff *et al* achieved increased functional islet yields from experimental rodent donors treated with estradiol(506) after observing protection against pro-inflammatory cytokine mediated islet damage by *in vitro* estradiol exposure(507).

Glucose homeostasis of cadaveric donors is often disturbed prior to pancreas procurement. A state of insulin dependence occurs with hyperglycaemia and hyperinsulinaemia due to haemodynamic instability and abnormal plasma epinephrine levels at the time of brain death(508). Pancreatic allografts procured from such donors have reduced graft survival(509), and the islet yield from such donors is often reduced when hyperglycaemia is prolonged(510).

Optimising cardiovascular physiology is critical to optimal pancreas procurement from cadaveric donors(511). Where significantly reduced haemoglobin is present due to donor blood loss, whole blood or packed red cells are transfused and, if there is suspicion of thrombosis within the graft, heparinisation is carried out to improve circulation.

Heparinisation appears particularly applicable in NHBD where it is used to maintain graft microvascular architecture during *in situ* perfusion(512). Additionally it may have immunological advantages by decreasing anti-TNF $\alpha$  activity and monocyte and T-cell infiltration by reducing expression of graft MHC II(513, 514). In some cases vasodilator therapy is indicated to maximize graft perfusion. This uses either catecholamines(515), epoprostenol or nitroglycerine(516) which improves oxygen delivery without compromising splanchnic perfusion to the abdominal viscera.

The nutritional status of the multiorgan donor has been studied, most frequently relating to liver transplantation. Most have concentrated on modifying donor nutrition to allow maintenance of glycolysis, optimal adenosine triphosphate (ATP) levels and improved graft energy states. Support for this concept can be seen in the higher levels of ATP, energy charge and subsequent graft function when donor animals are treated with insulin and glucose to maintain glycolysis compared to non-treated or fasted donors(517).

Similarly levels of phosphorylated adenine nucleotides can be maintained during cold ischaemia where liver glycogen stores are replenished by donor intraportal infusion of insulin and hexoses(518). Nutritionally repleted liver grafts have shown improved *in vitro* function, such as bile production and cytosolic enzyme release, and graft sinusoidal epithelial morphology when subjected to isolated perfusion in both UW or Marshalls isotonic citrate solution (519). Clinical support for donor nutritional repletion was gleaned from evidence that peak post-operative AST levels demonstrated improved function of grafts from glucose infused donors than from non-treated controls(520). Some have argued conversely that donor malnutrition may contribute to improved function and survival after liver transplantation(521) and Sun *et al* demonstrated reduced liver injury and sinusoidal endothelial cell apoptosis where donor rats were fasted for 4 days prior to transplantation(522).

To date, Nishihara *et al* have performed the only experimental study of the effect of donor nutrition upon the VPT donor pancreas. Pancreas grafts from fasted donor rodents had a better tolerance to warm ischaemia than grafts from fasted donor animals, and a 41.7% pancreas graft survival rate was achieved when fasted donors were used in VPT compared to a 28.6% graft survival rate from fed donors(523, 524). The effect of donor nutrition upon islet isolation and transplantation is currently unknown. However, fasting augments insulin secretion whereas feeding causes degranulation of pancreatic exocrine enzymes. Such degranulation potentially alters the density of the exocrine fraction within purification gradients, and putatively results in increased contamination of the islet fraction.

### **3.2.3 Surgical organ procurement.**

Meticulous surgical technique during organ retrieval is critical to obtaining quality organs(525, 526). It is well acknowledged that suitably trained surgeons should perform pancreas procurement early in the retrieval process. Most procurement teams use low pressure UW splanchnic and portal perfusion, explanting the pancreas en bloc with the liver, or separately after liver retrieval and prior to the kidneys(527). An alternative technique, practiced in the UK, is systemic cold perfusion alone followed by the introduction of portal perfusion 'on the back table' after explantation.

Higher core pancreatic temperatures at the time of retrieval appear to correlate with a reduced islet yield and reduced *in vitro* assessment of islet function. Whilst maintenance of a core pancreatic temperature at or below 4°C during pancreas procurement seems to yield higher numbers of functional islets(528) and this is achieved by liberal ice packing into the lesser sac at the time of aortic cross clamping.

### **3.2.4 Pancreatic ischaemia.**

Inherent in the retrieval of organs is a period of ischaemia. A variable, but short, period of 'warm' ischaemia is encountered as blood flow ceases at the time of cross clamping. Organs are then subjected to 'cold' ischaemia during *in situ* organ cooling and pancreas explantation. Cold ischaemia ends when the graft is reperfused in the recipient. The sensitivity of tissues to ischaemia, and the injury which it causes, is species and organ specific and reflects the metabolic requirements of the tissues. Some organs tolerate periods of ischaemia and have the capacity to repair preservation induced injuries during a period of delayed graft function (DGF). It is generally well recognized that warm ischaemia and prolonged periods of cold ischaemia are detrimental to graft quality(529) and reducing ischaemic injury is associated with a lower incidence of DGF, acute

rejection and possibly less chronic graft dysfunction(530). This principle can be demonstrated by comparing living and cadaveric donor organs. Living donors generally experience very short periods of warm and cold ischaemia prior to transplantation, whilst cadaveric donors are subject to less controlled periods of ischaemia and organ preservation. The improved recipient survival following living donor transplantation may reflect these attributes(531), though this is compounded by the closer HLA tissue type matching which is usually achieved by using a live donor.

Slater *et al* performed the first studies of the ischaemic tolerance of the rodent pancreas and concluded that it resisted ischaemia more readily than other organs(532). Florack *et al* subsequently demonstrated ischaemic resistance in the rodent endocrine component compared to the exocrine(533). The canine donor pancreas was studied by disconnecting the pancreatic blood supply *in situ* with later graft harvesting and transplantation. The survival of dogs with grafts exposed to 1 hour of warm ischaemia was identical to that of the 0 hour ischemia controls (80%) whilst 2 hours of warm ischaemia was not tolerated and all animals died. The combination of 30 minutes of warm ischaemia and 24 hours of cold storage resulted in 80% recipient survival, whilst 1 hour warm ischaemia with 24 hours cold storage resulted in 60% recipient survival(534).

The quality of the human donor pancreas is similarly adversely affected by periods of warm ischaemia(535-538) and prolonged periods of cold ischaemia are associated with increasingly frequent surgical complications following pancreas transplantation(84, 539). Ischaemic injury increases the risk of graft pancreatitis, infection and vascular thrombosis(540); whilst the incidence of duodenal leaks increases with increasing cold ischaemia time and is attributed to the increased susceptibility of the duodenal patch to ischaemia(539, 541). Though VPT after 36 hours of cold ischaemia has been described, it is generally accepted that the success of VPT is limited by an 18-24 hour cold ischaemic tolerance of the human pancreas.

The gross histopathological ischaemic injury comprises vascular endothelial damage and activation, microcirculatory disturbances and leucocyte aggregation. Alterations in the production of vasoactive substances such as eicosanoids(542), platelet activating factor(543) and nitric oxide have been implicated in the process(544) and should be minimised to achieve optimal grafting of the transplanted pancreas(545).

Pancreatic ischaemia also contributes to the development of graft immunogenicity; an effect mediated by nitric oxide and neutrophil recruitment(546). Pressler *et al* showed that a prolonged period of 18 hours pancreatic static cold preservation led to a significant induction of ICAM-1 mRNA, enhancing leukocyte-adherence and extravasation

compared to a 2 hour preservation period(547). Whilst Toyama *et al* demonstrated a mixed population of T cell, B cells and macrophages, expressing a variety of MHC antigens, in islets isolated from both fresh and preserved pancreata. They proposed that elimination of immunocytes which accumulate during pancreas preservation as a means to prevent early islet graft loss(548).

IPTR data reinforces the impact of pancreatic ischaemia upon clinical graft function.

During 2000-2004, 5% of SPK, 7% of PAK and 11% of PTA grafts were stored for more than 24 hours and 40% of all grafts were stored for less than 12 hours. In PTA and PAK the duration of storage had no impact upon graft survival or technical failure rates.

However, in SPK longer preservation times were associated with reduced 1-year graft survival rates and higher technical failure rates.

Lakey *et al* studied the effect of pancreatic ischaemic time on the outcome of human islet isolation. Of 146 pancreata, 56 had been procured by a local retrieval team and had short cold ischaemia times. The remaining 90 had prolonged cold ischaemia times having been retrieved from distant centres. 'Successful' islet isolations were deemed to be those where >100,000 IEQ were present after purification, and was achieved in 83-86% of locally retrieved grafts with up to 8 hours cold ischaemia. Where cold ischaemia was between 8 to 16 hours or > 16 hours, successful isolation was achieved in 73% and 38% of cases respectively. Furthermore, islet *in vitro* viability, by glucose stimulated perfusion, was significantly reduced where cold storage time exceeded 16 hours(536, 549).

### ***3.3 Preservation of the donor pancreas.***

Pancreas preservation aims to maintain cell viability and achieve optimal post transplant graft function. It also allows transportation of organs over long distances, tissue and organ banking for long-term storage, and the potential for immunological manipulation of graft and recipient prior to transplantation. Furthermore, pancreas preservation allows transplantation in a semi elective timeframe rather than on an emergency basis.

The overwhelming biochemical injury that occurs during pancreatic ischaemia is loss of ATP from a lack of oxidative phosphorylation. Several strategies are used to maintain ATP during pancreas preservation. These centre on cooling to reduce cellular metabolism, prevention of ATP loss during the preservation period, and facilitating ATP recovery during reperfusion.

#### ***3.3.1 Organ cooling.***

The cornerstone of organ preservation is cooling. This reduces cellular metabolism to minimize ATP consumption, lowers the rate at which intracellular enzymes degrade the

cellular components and reduces generation of potentially harmful metabolites(550-552). Nevertheless cooling is potentially harmful to cells and the precise method by which it extends the duration of preservation is not known. Though it is slowed, a reduction in temperature does not arrest metabolism and even hypothermically stored organs have a viability limited by storage time. Moreover, freezing *per se* is lethal to cells so very low temperature preservation does not readily lend itself whole organ transplantation(553-555).

Therefore, organ cooling is a complex interplay between the effects of ischaemia and temperature reduction. Most enzymes show a 1.5 to 2.0 fold decrease in activity for each 10 °C drop in temperature and cooling from 37°C to 0°C reduces the metabolic rate by 12 to 13 fold to extent preservation times to around 12 hours. This was elegantly demonstrated by Calne *et al* who preserved renal function in the canine model by cold blood perfusion(556).

However, even during cooling, chemical and enzymatic reactions continue so the tissue still becomes irreversibly injured. A switch from aerobic metabolism to anaerobic glycolysis and glycogenolysis causes accumulation of lactate and hydrogen ions which is responsible for acidosis. Acidosis kills cells by inducing lysosomal instability, activating enzymes, disrupting binding of transition ions to carrier proteins, and altering mitochondrial properties.

Cooling further attenuates mitochondrial and cellular membrane changes. Membrane fluidity is reduced and they begin to solidify which induces changes in their intercalated enzymes. Phospholipids in the membranes attempt to establish a thermodynamically stable structure by the interaction between polar phospholipid heads and non-polar fatty acid tail groups. Re-establishing a normal membrane structure at reperfusion takes time and may explain some loss of organ viability during reperfusion. Similarly increased red cell rigidity is seen as calcium, previously bound to ATP, binds to the cell membrane. This causes red cell trapping in the microcirculation of reperfused organs.

Cooling also perpetuates cell swelling by inhibiting  $\text{Na}^+\text{-K}^+\text{-ATPase}$  pumps which control intracellular volume. Usually the impermeable nature of intracellular anions is counterbalanced by extrusion of chloride from the cell together with water. Underlying this is the active extrusion of sodium and influx of potassium in the ratio 3:2 resulting in a net loss of cations. Where this is reversed, due to denaturation of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , and cells are bathed in a fluid resembling the extracellular milieu, there is a net gain in sodium and water together with consequential cell swelling due to colloid osmotic pressure exerted by intracellular anionic proteins. It is these changes in intracellular

volume that can be opposed or manipulated by altering the composition of organ preservation solutions.

### **3.3.2 Preserving purine metabolites.**

During cold preservation cells have little capacity for adenosine nucleotide storage and production; though ATP generation does continue, albeit at a very reduced rate, through glucose-6 phosphate conversion to ribose-5 phosphate and subsequently to phosphoribosylpyrophosphate(557). During cold preservation the rate limiting step for ATP synthesis is the temperature sensitivity of adenine nucleoside translocase, a membrane bound enzyme that transports ADP into mitochondria. This enzyme displays a large increase in its required activation energy below 18 °C that reflects the membrane phase changes induced by cooling membrane lipids.

Therefore, during organ cooling continued metabolism of adenosine nucleotides occurs in the face of a reduced capacity for their generation. This produces purine metabolites such as adenosine, inosine and hypoxanthine which permeate the cell membrane and can be washed from the graft during storage and upon reperfusion tissue(558-560). Loss of these purines then limits the capacity of cells to regenerate ATP.

Preservation induced damage requires ATP for repair and, on reperfusion, a rapid return to ATP synthesis is required to allow the restoration of a normal intracellular milieu.

Therefore, in pancreas preservation, it is advantageous to limit loss of purine metabolites and optimize opportunities for ATP regeneration upon reperfusion. Indeed the viability of the organ, and prediction of reversibility of preservation injury, is dependent upon the cells ability to regenerate ATP following reperfusion(561-566) and for successful ATP replenishment to occur adenine precursors, adequate oxygen delivery and mitochondrial function must be preserved(567).

Tanioka *et al* demonstrated that regenerated ATP is used to restore the function of Na<sup>+</sup>-K<sup>+</sup>-ATPase and maintain membrane integrity of parenchymal cells and vascular endothelium in the donor pancreas. Where Na<sup>+</sup>-K<sup>+</sup>-ATPase was inhibited using ouabain there was an increase in nuclear trypan blue staining of vascular endothelial cells and a significant decrease in pancreatic perfusion(568). Several studies have subsequently demonstrated a correlation between pancreas graft viability and tissue levels of total adenine nucleotides (TAN) and ATP. Fujino *et al* used a canine pancreas autotransplantation model to show the effect of warm ischaemia upon tissue ATP content. Autografts subjected to 60 minutes warm ischaemia, with the addition of adenosine to EC solution, showed improved graft survival from 0 to 80% and allowed restoration of tissue ATP levels (1.90 +/- 0.54 vs 8.13 +/- 0.98 μmol/g dry wt). Four of five autografts

functioned immediately and maintained normoglycemia after transplantation(569). More recently a canine model of prolonged warm ischaemia (30 – 120 mins) followed by 24 hour UW cold storage and segmental pancreatic autotransplantation showed a similar good correlation between graft viability and tissue concentration of ATP and TAN(570). The efficacy of the two-layered method (TLM) at restoring graft ATP content has been shown by two studies. Fujino *et al* first demonstrated 100% graft survival, and no deterioration in tissue ATP content, when pancreata from either 72 hour fasted or fed dogs were subjected to 24 hour TLM preservation. Whilst Kuroda *et al* showed the tissue ATP concentrations of TLM preserved viable pancreas grafts were significantly higher than nonviable grafts (9.11 +/- 3.05 vs 5.22 +/- 1.02  $\mu\text{mol/g}$  dry wt). An ATP tissue concentration of 6.0  $\mu\text{mol/g}$  dry weight was determined as a threshold for graft viability and subsequent graft survival. Adopting this value achieved a sensitivity, specificity, positive predictive value, and negative predictive value of transplant success of 100%, 84.6%, 91.7%, and 94.3%, respectively(571).

### **3.3.3 Organ preservation solutions.**

Organ preservation solutions are designed to prolonged organ storage. They contain several generic components but are individually unique and lend themselves to optimal storage of certain tissues and organs over others. Lam *et al* illustrated this concept by demonstrating extended renal preservation times with a phosphate buffered solution, but very poor rates of graft survival when the same solution was used for 20 hour liver preservation(572). The heterogeneity of cell types in these tissues would logically suggest that this should be the case.

The constituents of preservation solutions oppose the effects of cooling and purine nucleotide loss; this is achieved by the inclusion of an 'impermeant' to prevent cell swelling, an ionic composition to oppose  $\text{Na}^+$  influx and supplementation with adenosine for ATP regeneration.

The impermeant included in preservation solutions are either uncharged saccharides (mannitol, sucrose, glucose, raffinose, trehalose) or impermeable anions (gluconate, glycerophosphate and lactobionate) in a concentration of 110-140mmol that oppose the osmotic effect of intracellular anions(566). Tri and disaccharides may be more effective at reducing cell oedema than monosaccharides which easily penetrate the cell membrane. Their inclusion in preservation solutions has been associated with better storage outcomes in pancreatic storage(573). Moreover there is evidence of a direct cytoprotective effect of saccharides over impermeable anions and it is proposed that saccharides in preservation

solutions may act as alternative substrates for cellular metabolism during preservation to maintain cellular ATP content and abrogate preservation injury(574-576).

Changing a solutions concentration and ratio of potassium and sodium can further reduce graft cellular oedema. Conventionally solutions have an 'intracellular' ionic composition; containing a high potassium and low sodium concentration to counter the osmotic driven shift of these cations(577). However the high potassium content has the potential to induce vasospasm and endothelial injury of the graft, and allow washout of high concentrations of potassium into the donor upon reperfusion. It is postulated that hyperkalaemic cardiac arrest, during *in situ* flushing of the donor and following reperfusion of the recipient, may be avoided by reversing the concentrations of sodium and potassium. Such 'extracellular' high sodium concentration solutions have also proven effective at reducing graft oedema(578-580).

Regeneration of ATP can be improved by the supplementation of either adenosine, or adenine and ribose, to preservation solutions(581-583) but this varies in efficacy.

Delmas-Beuvieux *et al* used <sup>31</sup>P nuclear magnetic resonance to demonstrate the absence of ATP in a heterogenous group of donor organs at the end of a 24 hour cold storage period regardless of preservation solution(584). Subsequently the ATP signal was seen to disappear by 8 hours where UW, histidine-tryptophan-ketoglutarate (HTK) and histidine buffered lactobionate solutions were used, but lost in only 3 hours when Krebs or EC solution were used for cold storage.(584, 585).

It appears that adenosine supplementation is particularly effective in whole pancreas preservation(569, 586) and improves the viability of the ischaemically damaged pancreas(565, 569). However, adenosine supplementation can have negative effects by promoting endothelial nitric oxide production(587) which potently induces an inflammatory response through release of endothelin and neutrophil recruitment(587). Hence high adenosine concentrations in the pancreas may exacerbate the post transplantation inflammatory response. Indeed, Pi *et al* observed a reduction in lipid peroxidation, neutrophil recruitment, thiobarbituric acid (TBA) reactive substance formation, myeloperoxidase (MPO) activity and tissue nitric oxide levels when the adenosine antagonist theophylline was added to UW solution, or adenosine was removed from UW solution(588).

Finally the addition of selective potassium channel opening drugs to preservation solutions has been shown to abrogate ischaemic injury in some organs(589). Such solutions may prove particularly applicable to pancreatic preservation as granular insulin storage, and the pulsatile control of insulin secretion, are disrupted by exposure to the

hyperglycaemic state in the organ donor. Administration of a  $\beta$  cell selective  $K^+$  ATP channel opener at the time of pancreas procurement could potentially induce a state of  $\beta$  cell rest, preserving insulin stores and pulsatile insulin secretion within the donor pancreas(590).

### ***3.4 Practical pancreas preservation methods.***

Static cold storage and continuous hypothermic perfusion have been used for preservation of the donor pancreas. Comparative studies, using various combinations of preservation solutions, show little difference in outcome up to 24 hours(591, 592). Meanwhile, the TLM means of preservation has shown itself particularly applicable to pancreas preservation and has improved pancreatic tolerance of warm and extended cold ischaemic periods.

#### ***3.4.1 Static cold storage for pancreas transplantation.***

Belzer(593) and Collins(594) first demonstrated extended renal cold ischaemic preservation times up to 30 hours with simple cold storage. This involved a flush with preservation solution to remove blood from the vasculature, followed by rapid cooling to around 0 to 4 °C by refrigeration. This method is simple and cost effective, requiring little specialist equipment; but results in considerable losses of purine metabolites. Baumgartner first reported successful static cold storage preservation of pancreas grafts for up to 24 hours in the original Collins solution(595); whilst Dreikorn proposed an improvement to the solution by omitting magnesium, which precipitated within the graft(596). This modified 'Euro-Collins' solution again proved to be effective in prolonging pancreas graft preservation(597). Serrou *et al* used a protein gel alternative to crystalloid solutions and demonstrated a survival advantage, compared to polysaccharide gel or Collins solution, in an 8 hour cold preserved canine pancreas transplant model(598). Florack confirmed that colloid solutions achieved efficient pancreatic preservation for 24 hours using a modified hyperosmolar silica gel filtered plasma (SGFP)(533). This solution was later modified by the addition of sucrose(599) and substitution of glucose with mannitol to produce the University of Minnesota solution specifically for pancreas preservation(600). Despite clinical success with this solution, the need for *de novo* preparation, batch to batch variation and infection risk from SGFP meant that, with the advent of new solutions, its clinical use was largely abandoned(601). Early clinical VPT used crystalloid and simple colloid solutions for short-term preservation. Of 617 preserved pancreata from 1983 to 1986 a higher graft survival and reduced technical failure rate was observed when grafts were stored for more than 6 but

less than 12 hours compared to those stored for less than 6 hours; but patient survival rates were unaffected by cold storage duration(602, 603). Hence for early VPT used pancreata be stored for no more than 6 to 10 hours(604) despite evidence that grafts were at increased risk of graft pancreatitis when intracellular preservation solutions were used for more than 6 hours(605).

In an effort to further extend pancreas preservation times, the effect of preservation fluid components upon pancreas storage were critically evaluated to define the optimal preservation solution. Oedema of pancreatic slices, stored in various saccharide containing solutions, decreased with the inclusion of saccharides and anions with ascending molecular mass(573). Consequently a preservation solution (University of Wisconsin solution) containing the high molecular mass saccharide raffinose and the anion lactobionate, was designed specifically for pancreatic preservation(573, 606). Following the introduction of UW solution the results of experimental and clinical pancreas preservation dramatically improved. UW preservation reduced pancreas graft oedema after reperfusion compared to EC solution(607) and extended canine pancreas preservation from 12-24 hours to 3 days compared to EC solution. It also functioned superiorly to more complex colloid solutions; achieving 50% graft survival after 96 hours UW preservation compared to 22% graft survival after SGFP preservation(608). It also appears that UW, for pancreatic preservation, confers a distinct bioenergetic advantage over other solutions. In a canine model of prolonged warm ischaemia (30 – 120 mins) followed by 24 hour UW cold storage and segmental pancreatic autotransplantation, a good correlation between graft viability and tissue concentration of ATP and TAN levels was observed(570).

Clinical series using UW pancreas preservation resulted in high rates of SPK graft viability. From 1987 to 1993, of 253 UW preserved kidney-pancreas grafts with a mean pancreas preservation time of 17 hours, immediate insulin independence was reported in 99% of recipients and an 86% pancreas 1 year graft survival rate was achieved(609-611). Following clinical studies by the Madison and Pittsburgh groups(612, 613), and a European multicentre study(614, 615), UW solution was advocated as the first choice solution for pancreas procurement and preservation via *in situ* donor aortic and portal perfusion and subsequent static cold storage though this is not widely practiced in the UK. This extended the mean pancreas preservation time to between 9 and 18 hours. The prospective multicentre study further concluded that cold ischemia time did not significantly influence pancreas graft survival when it exceeded 12 hours but that exceeding this risked a higher recipient complication rate(614).

Experimental studies have continued to critically evaluate each component of UW solution and alter its composition to further improve pancreas preservation. Ploeg *et al* used a 48 hour cold storage and segmental pancreas autotransplant canine model to study the impermeant colloid component of UW. Conventional UW, containing hydroxyethyl starch (HES), yielded 90% recipient survival, whilst without HES 50% of recipients died from graft failure within the first week after transplantation(616). Conversely, Urushihara *et al* omitted HES and insulin from UW, and reversed the sodium/potassium ratio to produce a simple 'extracellular' solution. In a syngeneic rodent transplant model, graft preservation for 48 hours at 4°C with the simplified solution resulted in 100% graft function compared to 44% graft function with commercially available UW solution(617). This extracellular type UW solution appears as effective as conventional UW solution for up to 72 hours of pancreas preservation in animal autotransplantation models(618, 619). Despite widespread success in experimental and clinical pancreas preservation, UW cold storage does have disadvantages. It is chemically unstable, must be stored cold until use and has a short shelf life that increases its cost. Its high viscosity makes the initial flush into the pancreas difficult and its distribution in the pancreas is unpredictable(620). Moreover, when cooled, adenosine crystals can accumulate in UW solution which require filtration through a pore filter to remove(621).

A number of other preservation solutions, with similar compositions to UW, have been studied to determine their suitability for whole pancreas preservation.

Bretschneiders HTK solution was designed for cardiac preservation. Histidine was included to retard acidosis, tryptophan to reduce membrane injury and ketoglutarate to provide a substrate for metabolism. Hesse *et al* first used HTK solution in pancreas transplantation with a segmental porcine pancreas autotransplantation model. When compared to UW storage, no difference in graft function was observed after 24 hours of preservation; after 48 hours preservation, 26% of HTK stored grafts and 9% of UW stored grafts achieved functional normoglycaemia in recipients; after 72 hours preservation all grafts stored in both HTK and UW failed to render the recipients normoglycemic(622). Leonhardt *et al* used a one-line reperfusion system at constant arterial pressure to compare *in vitro* pancreas function after 24 hours 4°C storage in HTK, UW and EC solutions. In this system the highest rate of arteriovenous flow was seen in pancreata preserved in HTK solution, followed by UW and EC solutions (5.7 +/- 0.91 ml/min vs 7.4 +/- 0.81 ml/min vs 3.0 +/- 0.26 ml/min). This mirrored the distribution of lowest lactate concentration in reperfusates (64.0 +/- 7.2  $\mu$ mol/50 ml vs 114.2 +/- 1.7  $\mu$ mol/50 ml vs 148.0 +/- 28.6  $\mu$ mol/50 ml) and amylase content in venous effluent

(189 +/- 72.6 U/ml vs 188 +/- 39.4 U/ml vs 416 +/- 71.7 U/ml). They proposed that *in vitro* post ischaemic pancreas quality and function was not jeopardized by HTK preservation compared to UW solution(623).

Recently a porcine model of NHBD, using a 15 minutes of warm ischaemia followed by 16 hours of cold ischaemic static preservation, has shown HTK to be as effective as UW for pancreas preservation. There was less biochemical evidence of pancreatitis within the grafts and similar values of intravenous glucose tolerance testing amongst autografted recipients(624). Clinical experience with small numbers of human whole pancreas allografts confirms similar efficacy of HTK and UW solutions. Grafts preserved for a mean cold ischaemia time of 11 +/-3 hours showed comparable post transplant function when fasting blood glucose, peak amylase and serial amylase levels were compared, along with 100% graft and patient survival in each group(625).

Celsior is a low viscosity, low sodium UW like solution originally developed for heart-lung preservation(626-628). A randomized clinical trial comparing Celsior with conventional UW solution found comparable rates of 1 year graft and patient survival (98% and 96% respectively), and comparable graft function assessed by mean serum levels of glucose, amylase, and lipase(629)

#### **3.4.2 Static cold storage for islet transplantation.**

Two principles are particularly important for static cold storage of the pancreas prior to islet isolation. First, an adequate vascular flush and possibly a ductal flush with preservation solution before cold storage are thought to be critical to the success of islet isolation. Ductal flush or infusion increases islet yield and viability by presumably preserving ductal architecture(630) and maintaining the integrity of the small pancreatic ducts to allow full distension by collagenase(631). White *et al* confirmed that porcine islet yields were increased when collagenase was delivered by intraductal infusion in organ preservation solution rather than HBSS at the time of retrieval prior to cold storage(220). Secondly, a static cold preservation time of at least 2 hours allows a degree of endogenous pancreatic enzyme activation(632) and it is thought that this has a permissive effect upon pancreas digestion(633) which improves islet yield.

Three main preservation solutions (UW solution, EC solution and hyperosmolar citrate (HOC)) have been evaluated in clinical islet cell transplantation. UW solution remains the clinical standard in the majority of centre as, when cold ischaemia is greater than 6 hours(634), UW solution has an advantage over EC(635, 636).

Prolonged cold ischaemic times are deleterious to islet yields. Yields are particularly reduced when the cold ischaemic time has exceeded 12 hours (536, 637) with very poor

yields after 16 hours of cold ischaemia(636). As part of the Edmonton protocol, the total time for pancreas preservation and islet isolation combined was less than 13 hours (mean 5 hours). Hence it seems that transplanting freshly isolated islets in a short timeframe confers the optimal chance of graft survival; though some authors advocate a period of cell culture before transplantation to allow post isolation recovery of the most viable islet mass.

As previously discussed, extended periods of static cold storage are complicated by ongoing cellular metabolism and loss of purine metabolites. It is questionable whether it will be possible to prolong static cold storage preservation times beyond that already achieved with UW solution(638). Longer preservation times may require more complex solutions with constituents able to reduce the effect of preservation induced injury.

#### ***3.4.3 Mechanical perfusive preservation for pancreas transplantation.***

Perfusion methods of organ preservation have been used for over 25 years. They rely on a mechanical means of achieving perfusate flow to and from the preserved organ; the rationale being dynamic substrate delivery and metabolite removal from the tissue. The perfusates studied have usually been conventional organ preservation fluids, and have been delivered using both continuous and pulsatile flow dynamics. However, recent improvements in technology now allow a closer mimicking of *in situ* perfusion by using hypo and normothermic blood perfusion in the *ex vivo* situation. Where appropriate substrates are supplied, most tissues can continue to synthesise ATP during hypothermic perfusion at physiological, even suprphysiological levels; and this is the advantage of continuous perfusion over static cold storage. However, perfusive preservation is complex and expensive requiring specialist equipment. It damages the vascular endothelium that is vital for subsequent repair and restoration of normal graft perfusion and function. This allows adherence of platelets and fibrin deposition during graft reperfusion. The pancreas, being a 'low flow' organ, has particularly delicate endothelium which is intolerant of barotraumas, shear stresses and injuries induced by high pressure perfusive preservation. However, such injury is less of a problem during islet transplantation where the graft is neovascularised gradually from recipient tissues. Reducing the perfusion pressure, or increasing the perfusate osmolality or viscosity, can overcome graft oedema during perfusion but these parameters have yet to be optimized to improve outcome. Therefore, for clinical whole pancreas transplantation, static cold storage is currently preferred over continuous pulsatile perfusion.

Experimental studies have used several machines and perfusates to attempt to improve the results of perfusive preservation. The Belzer kidney perfusion machine was first used

to preserve dog pancreata for 24 hours using a perfusate of cryoprecipitated plasma (CPP) at 50-60mmHg perfusion pressure at 6-10°C. Allografts from these organs survived for a mean 11 days(639) and islets within these grafts were recognizable and functioning, though the acinar tissue was largely replaced with fibrous tissue(640). A reduced perfusion pressure (5-30mmHg) allowed preservation of dog pancreaticoduodenal segments with CPP for 24 hours though allografts from these segments survived for only 4 days(641). The addition of human serum albumin, dextrose and methylprednisolone to the perfusate improved survival to 18.3 days(642) and Brynner *et al* used a human serum albumin perfusate at 50 mmHg to preserve dog pancreata for 24 hours and observed minimal amounts of organ oedema(643). Perfusive preservation of the canine pancreas for 24 and 48 hours, using SGFP at 11mmHg, resulted in 70% and 0% recipient survival in an autograft model(595); and significant loss of canine segmental pancreas grafts (30% and 40% respectively) compared to no graft loss amongst SGFP cold stored grafts(592).

Perfusive preservation has not been widely studied for pancreas preservation before islet isolation. In the only detailed published study, Leiser *et al* compared the isolation outcomes from human pancreata (n=4) preserved for a mean 13 hours using low flow machine pulsatile perfusion with non perfused pancreata stratified for cold ischaemia time. Islet yields from perfused pancreata and those cold stored for <8 hours and >8 hours were 3435 (+/-1951), 5134 (+/-2700) and 2640 (+/-1000) IEQ/g pancreas respectively. Mean islet viability after perfusion was 86% (vs 74% and 74% for the <8 hour and >8 hour groups) and insulin secretion index was 6.4 (vs 1.9 and 1.8 for the <8 hour and >8 hour groups). This implies that low-flow machine pulsatile perfusion of pancreata with prolonged cold ischemia time can result in islet yield, viability and function comparable to shorter periods of static cold storage(644).

#### **3.4.4 Two layered method of preservation.**

TLM pancreatic preservation uses the concept of normobaric oxygenation and was adapted from Fisher *et al* who described retrograde oxygen persufflation to rescue ischaemically damaged kidneys(645). The technique uses a cold conventional organ preservation solution and a perfluorochemical oxygen carrier solution, the pancreas being suspended between the two immiscible layers. Pefluorochemicals (PFCs) are hydrocarbons that have a very high capacity for binding dissolved oxygen but a negligible oxygen-binding constant. This allows them to release oxygen more effectively than hemoglobin into tissues with low oxygen tensions. In TLM preservation, PFC is oxygenated with a continuous pressure of 10 to 12 mmHg and a flow rate of 50 to 100cc

per minute. Pancreata are then oxygenated via the PFC whilst the organ preservation solution prevents graft oedema, provides substrates for metabolism and opposes the loss of purine metabolites (Figure 3-1)(646, 647).

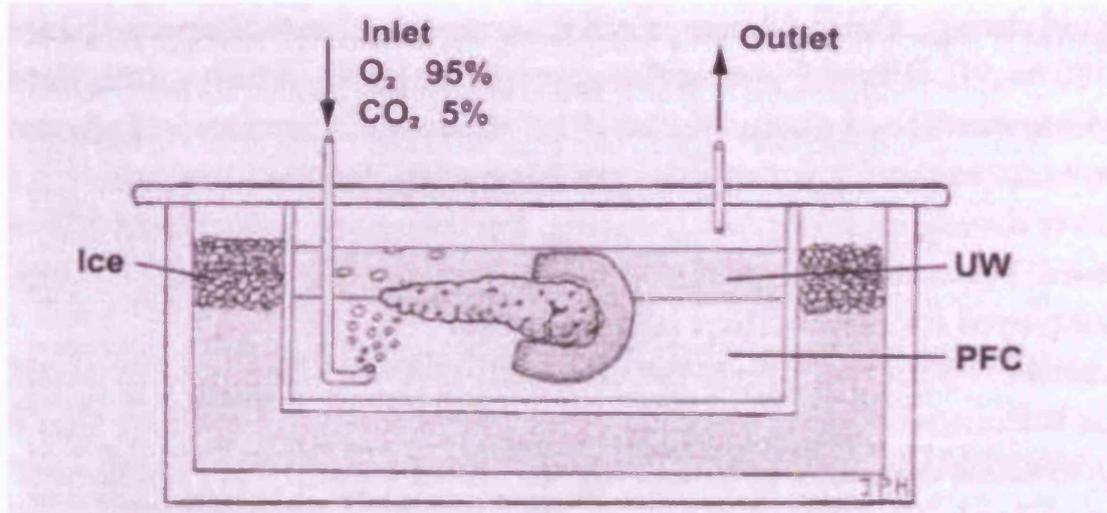


Figure 3-1. Schematic illustration of TLM.

Early experiments with the TLM using EC solution showed that whole canine pancreata preserved for 72 hours maintained normoglycaemia for 5 days after transplantation; and that their histological architecture was maintained up to 4 weeks after autotransplantation(648, 649). TLM was later modified to use UW solution(647) and canine pancreata were successfully preserved for 96 hours of cold ischaemia, and successfully resuscitated after short periods of warm ischaemia by this method(650). In the first clinical application of TLM for VPT, 10 grafts stored in TLM were compared with 44 grafts stored in static UW cold storage for mean cold ischaemic times of 16.5 and 18.1 hours respectively. At reperfusion none of the TLM grafts were oedematous, whilst 10 of the UW stored grafts had eodema. Nine of the TLM grafts allowed insulin independence immediately post-transplant, whilst only 31 of the UW stored grafts achieved this. Furthermore none of the TLM preserved grafts suffered acute rejection. It was concluded that TLM preservation was at least equivalent ,and may be superior, to UW static cold storage in both morphologic and functional assessment of the transplanted whole pancreas(651).

Tanioka *et al* first applied TLM to pancreata prior to islet isolation. They demonstrated improved isolation yields and *in vivo* viability of islets in a canine autotransplantation model compared to conventional static cold storage in UW(652). Subsequently TLM has proved successful in achieving high numbers of isolated porcine islets after prolonged storage(224) and improved the results of islet isolation in the primate model(653). A large study of non human primate islet isolations demonstrated that use of the TLM with

trypsin inhibition during the isolation phase correlated with high pre and post purification yields, and good viability and function of islets(654).

Hiraoka *et al* first demonstrated the efficacy of TLM for extending the preservation times of human pancreata without adversely affecting islet yield(655). Short duration TLM then demonstrated a significant improvement in islet yield from pancreata previously subjected to a mean of 11.8 hours UW static cold storage. This potentially increased opportunities for islet transplantation from human pancreases after prolonged cold ischemic periods; a group previously thought unsuitable for islet isolation(656). Subsequently both short (mean 11.3 hours) and long (mean 22.6 hours) periods TLM preservation following periods of cold storage have resulted in increased islet yield compared to periods of short and long UW cold storage alone(657). Recently islets isolated from pancreata with a very extended (24 hours) period of TLM preservation successfully reversed diabetes in nude mice and were also associated with a low yield of non viable ductal cells(658).

Hering *et al* have successfully used TLM preservation in a series of clinical single donor human islet transplants in which insulin independence was attained(269). However it is unclear as to whether this success was entirely attributable to TLM preservation or to islet cell culture and the immunosuppressive protocol used in this series. However, Tsujimura *et al* have since confirmed significantly improved islet recovery, *in vitro* islet function and ATP content in human isolated islets after short term TLM resuscitation of ischaemically damaged human pancreata(659, 660). A clinical study of marginal donor human islet isolations (age>50 years) showed those subjected to TLM preservation yielded an average of twice the number of islet equivalents than the control group subjected to UW cold storage alone. Subsequently 8 of 15 organs from the TLM group were used for clinical islet transplantation, whilst only 2 of 18 control pancreata were transplanted(661).

A retrospective analysis of human islet isolations over two years placed the limit of pancreatic cold storage at around 10 hours. However, TLM, allowed the majority of islet isolations to be successful after 10 to 16 hours of TLM storage, confirming the prolonged storage time which is possible with the TLM(662). TLM has advantages over UW cold storage even for short duration (<8 hours) cold ischaemia times after local pancreas procurement. After TLM 72% of isolation yielded >300,000 IEQ and were deemed suitable for clinical transplantation, compared to only 27% where UW cold storage alone was used(663). Therefore, it was largely accepted that, for clinical islet isolation, TLM preservation results in significantly higher yields of islets with comparable viability,

survival rate after culture, insulin content, and stimulation indices when compared to static cold stored pancreata(664-666).

However, a recent randomized controlled trial compared islet isolations from UW static cold stored and TLM preserved pancreata. No significant differences in tissue ATP content, pre- and post-purification islet yield or *in vitro* insulin secretory function were observed and each group functioned equivalently as a transplanted islet mass, allowing a significant decrease in insulin requirement amongst islet allograft recipients(667).

Similarly a retrospective study of 200 human islet isolations showed no difference in isolation outcome or post transplant function in PAK recipients when TLM and UW cold storage were compared(668). Consequently the clinical benefit of the routine use of TLM prior to islet isolation has now been questioned

As previously discussed, TLM putatively improves pancreatic ischaemic tolerance and 'resuscitates' the graft by efficient regeneration of tissue ATP levels. This maintains the function of cellular metabolism, preventing preservation injury caused by  $\text{Na}^+ - \text{K}^+$  -ATPase inhibition and cell swelling (*vide supra* 3.3.2). However a recent study of TLM, using a diffusion-reaction model to precisely measure oxygen distribution, showed poor external tissue penetration to a depth of only 1mm and oxygenation of only a small volume of pancreatic tissue. Hence the precise mechanisms by which ATP is replenished during TLM are more complex than merely reoxygenating the graft alone(669).

Kuroda *et al* studied the ATP content of TLM preserved whole pancreata and the mechanism by which ATP content was restored. They demonstrated the efficacy of 24 hours of TLM at 4°C for the preservation of canine pancreata subjected to warm ischaemia. In an autotransplant model, grafts preserved for 24 hours TLM and then subjected to 120 or 150 minutes of rewarming had 100% and 33% graft survival rates respectively; whereas grafts having 24 hours of UW static cold storage, or no preservation, all failed to function. Furthermore tissue concentrations of ATP reached supraphysiological levels (8.23 +/- 0.72 vs. 4.44 +/- 0.49  $\mu\text{mol/g}$  dry weight) and remained significantly higher when compared to grafts stored by the UW static cold storage method for all durations of warm ischaemia(670). The regenerated ATP formed during TLM arises from the restoration of oxidative phosphorylation which allows *de novo* ATP production from exogenous substrates such as glucose and adenosine, and endogenous graft adenine nucleotides. Substituting the glucose within EC solution for mannitol caused little change in tissue ATP concentrations in EC/PFC two layer preserved pancreata (7.91 +/- 1.21 vs 7.59 +/- 0.97  $\mu\text{mol/g}$  dry weight); but the addition of 2,4 dinitrophenol, an uncoupler of oxidative phosphorylation, significantly reduced the

ATP concentration (0.61 +/- 0.07 vs. 7.91 +/- 1.21  $\mu$ mol/g dry weight). Similarly, supplementation of adenosine to EC solution resulted in a significantly increased tissue ATP content (7.23 +/- 2.17 vs. 1.90 +/- 0.53  $\mu$ mol/g dry weight) suggesting that this exogenous substrate improves ATP reconstitution. Further [2-3 H] adenosine incorporation studies confirmed that during TLM, exogenous adenosine was used in ATP production that could not be reproduced by replacing adenosine with other exogenous nucleosides (671, 672).

Matsumoto performed detailed studies of the effect of TLM on vascular endothelial cells, graft reperfusion and ATP tissue levels. Pancreata subjected to 90 min of warm ischaemia alone were not viable (0/5). However, 5 hour TLM preservation made it possible to recover the pancreas (5/5), whilst 8 hour preservation was not successful (0/3). ATP tissue levels, assessed by HPLC, after 5 and 8 hours preservation were 9.40 +/- 2.09 and 7.37 +/- 1.06 micromol/g dry weight, respectively. By trypan blue exclusion staining, the viability of vascular endothelium was reduced in grafts preserved using 8 hours TLM compared with 5 hours TLM (37.6 +/- 11.6% vs 5.0 +/- 3.0%). By H<sub>2</sub> clearance technique, pancreatic perfusion was 57.1 +/- 4.4 ml/ min/100 g and 28.5 +/- 7.5 ml/min/100 g after 5 and 8 hours of TLM preservation respectively. Furthermore, the beneficial effects of TLM upon endothelial viability, microcirculation and reperfusion were further enhanced by the addition of the thromboxane A<sub>2</sub> synthesis inhibitor OKY046 which allowed extension of the TLM preservation period(673).

Iwanaga et al used HPLC analysis of ATP and light and electron microscopy to study the morphology of TLM preserved canine pancreata. Though significantly higher levels of ATP were found in TLM preserved grafts, light microscopy demonstrated no marked morphological abnormalities. However, EM demonstrated significantly increased mitochondrial swelling and rough endoplasmic reticulum vacuolization in the acinar cells of UW preserved grafts compared with those undergoing TLM preservation. This confirmed that intact cellular and intracellular organelle membranes is likely to contribute to the maintained graft ATP levels in TLM preserved islets(674).

Efforts continue to rationalize and improve TLM. Miyamoto *et al* demonstrated an increased islet yield from porcine pancreata preserved using a modified two-layer preservation method. This used PFC and University of Kyoto solution, a novel extracellular type preservation solution containing the impermeant trehalose (ET-K)(675). Recently the cumbersome equipment required for TLM has been modified to allow pre-oxygenation of PFC sufficient to maintain oxygen concentrations for 24 hours. This allows pancreata to be transported after TLM without oxygen insufflation in custom-

made containers (HugBox). However oxygen persufflation is unlikely to be omitted completely from the technique as pre-oxygenated PFC storage alone appears insufficient to resuscitate pancreata exposed to short duration warm ischaemia(676). Further simplifying the technique to a one layer method using PFC as the single preservation solution may be feasible. Porcine islet isolations following one layer preservation showed islet yield, purity, viability, ATP content and mitochondrial viability comparable to unstored pancreata. Moreover, improved *in vitro* stimulation indices were obtained following one layer preservation compared to TLM, and *in vivo* functionality of islets from one and two layer storage methods were comparable(677).

### **3.5 Conclusion.**

The procurement of a high quality pancreas, for either VPT or islet transplantation, commences with the identification and optimization of a suitable organ donor. Meticulous surgical technique with low pressure *in situ* cold perfusion with UW allows optimal explantation. Pancreatic warm ischaemia is detrimental to post transplant function of VPT and islets and should be minimized. In VPT no method has yet proven superior to static cold storage in UW solution for up to 18 hours. However, TLM preservation prior to islet cell transplantation has definite advantages over other preservation methods. It allows extension of cold ischaemic tolerance and has the capacity to 'resuscitate' the graft following short periods of warm ischaemia. This is achieved by maintenance of oxidative phosphorylation and efficient regeneration of ATP from endogenous and exogenous substrates.

Despite the huge advances in pancreas preservation technology, cellular injury and death still occur which has a detrimental effect upon VPT graft function, islet yield and function. It would be advantageous if an assessment of cellular death could be made throughout the procurement and preservation process. The efficacy of pancreas preservation and a possible prediction of graft viability could then be inferred.

## ***Chapter 4. Apoptosis in Pancreas and Islet Cell Transplantation.***

### ***4.1 Introduction.***

Cells die by necrosis or apoptosis, and both are observed during pancreas procurement, preservation, islet isolation and after transplantation.

Necrosis is an irreversible commitment to cell death characterized by cell swelling, organelle disruption and lysis that excites a local inflammatory reaction and triggers the immune response. Apoptosis, in contrast, follows sequential changes resulting in the dismantling of the cell without membrane rupture, little consequent inflammatory reaction or immune response, and disposal by neighbouring or phagocytic cells.

Moreover it is a potentially reversible process until mitochondrial bioenergetic changes commit the cell to death(678). It is relevant to study apoptosis, rather than necrosis as a marker of pancreatic preservation injury, as cells at an early stage of apoptosis may be salvaged. Preservation methods causing low numbers of apoptotic cells are likely to have low rates of cell necrosis and consequently good graft function. Conversely high numbers of apoptotic cells infer increased cell injury which is then likely to progress to secondary necrosis. Both primary and secondary necrosis then cause poor graft function and possibly augment graft immunogenicity(679) (680).

### ***4.2 Morphology of apoptosis.***

Apoptosis causes distinct morphological changes in cells (Table 4-1).

<b><u>NECROTIC DEATH</u></b>	<b><u>APOPTOTIC DEATH</u></b>
<b>INCREASED CELL SIZE</b>	<b>CELL SHRINKAGE</b>
<b>ORGANELLE DISORGANISATION</b>	<b>CHROMATIN CONDENSATION</b>
<b>MITOCHONDRIAL SWELLING AND LIPID ACCUMULATION</b>	<b>SWELLING OF ENDOPLASMIC RETICULUM, PRESERVATION OF ORGANELLE ARCHITECTURE</b>
<b>SHRINKING INNER MITOCHONDRIAL ENVELOPE</b>	<b>CELL MEMBRANE BLEBBING</b>
<b>CHROMATIN FLOCCULATION AND DISPERSAL</b>	<b>SHEDDING OF APOPTOTIC BODIES</b>
<b>CELL MEMBRANE RUPTURE AND CONTENTS RELEASED</b>	
<b>RECRUITMENT OF MONOCYTES AND MACROPHAGES TO THE SITE OF CELL DEATH</b>	

**Table 4-1. Morphology of necrotic and apoptotic cell death.**

The nuclear changes of apoptosis are complex and involve both the chromatin and nuclear skeleton. The nuclear envelope remains intact but nuclear pores are redistributed concurrent with breakdown of the nuclear lamellae(681). Chromatin collapses and condenses, then marginates to form dense nodules at the inner nuclear envelope. Eventually the nucleus fragments and distributes unevenly amongst the generated apoptotic bodies(682). DNA degradation occurs by digestion of linker regions between nucleosomes producing characteristic DNA 'laddering' patterns of varying base pair lengths(683-688).

Apoptosis alters the cell membrane causing cells to become isolated from their neighbours and adopt a more spherical shape. Cell membrane blebs(689) become fused with the outer cisternae of the endoplasmic reticulum, orientating phosphatidyl-serine residues from their intracellular position to the external surface of the cell and marking it for future phagocytosis(690). Eventually the cell membrane coalesces forming small apoptotic bodies from the cellular contents that may persist for hours, though actual cell death is normally rapid (Figure 4-1)(689).

Histologically apoptotic cells can appear transient and in low numbers as the time from apoptosis induction to phagocytosis may be as little as 1 hour. Additionally cells enter the apoptotic cycle at differing times, proceed at different rates and are usually rapidly phagocytosed to remove them prior to membrane rupture, inflammation and consequent secondary necrosis(691). Failure to phagocytose an apoptotic cell results in secondary necrosis and cell lysis. In tissues with high numbers of apoptotic cells, phagocytic capacity may be overwhelmed resulting in the appearance of a secondary necrotic rather than apoptotic phenotype. Indeed though necrosis and apoptosis are viewed as separate forms of cell death, they share many early signaling pathways and a continuum exists whereby morphological necrosis is sometimes seen in cells whose initial commitment was towards death by apoptosis(692).

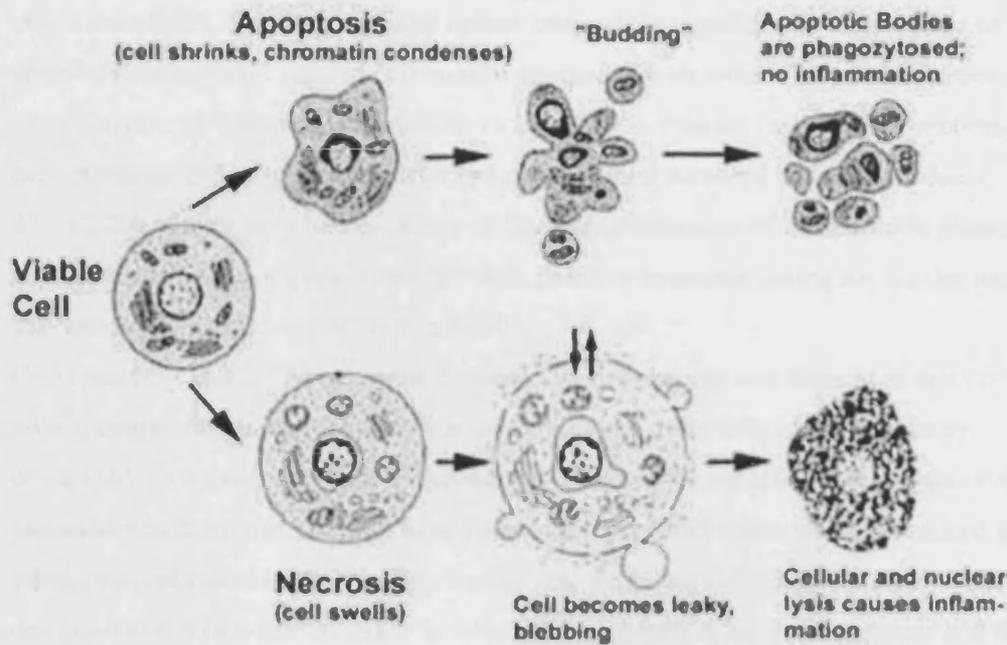


Figure 4-1. Morphology of apoptosis and necrosis.

#### 4.2.1 Apoptosis in pancreas preservation.

Apoptotic cell death is well documented during whole pancreas preservation and rates of apoptosis increase with the duration of pancreas preservation. The histological cellular injuries in the ischaemic pancreas closely mimic those seen in acute pancreatitis, most notably high rates of acinar cell apoptosis(693). Significantly increased rates of apoptosis are seen in reperfused rodent allografts after 18 hour UW cold storage compared to either unstored or 6 hour UW cold stored grafts(694) and high rates of acinar cell apoptosis are observed after reperfusion of experimental whole pancreas allografts(695) which correlate well with reduced pancreatic functional capillary density and increased leucocyte adhesion; both measures of microcirculatory damage within the pancreas(696). Furthermore, rates of acinar cell apoptosis varied, dependent upon procurement technique, in 6 hour cold preserved and reperfused pancreatic grafts (697) and using a short period of warm ischaemic reperfusion prior to 6 hours of cold preservation to 'precondition' the donor pancreas failed to prevent microcirculatory damage and increased the occurrence of apoptosis in a rodent syngeneic VPT model(698).

#### 4.2.2 Apoptosis during islet isolation and culture.

Apoptosis causes loss of islet mass during islet isolation. After experimental canine islet isolation a significant increase in cellular DNA fragmentation is observed coinciding with the appearance of pyknotic nuclei and apoptotic bodies upto 48 hours in cell culture; and rates of cellular apoptosis increased from 5% to 60% from the day of isolation to day 5 in

cell culture(699). Similarly isolated rodent islets show significantly higher rates of apoptosis following 7 days of cell culture compared with either 7 day preservation in UW or overnight cell culture (38% vs 27% vs 11%)(700). Paraskevas *et al* first reported islet cell apoptosis following human islet isolation. After a standard isolation protocol, 31.4±2.2% of islet cells had evidence of DNA fragmentation of an apoptotic phenotype and the distribution of cells coincided with positive immunostaining for insulin implying that apoptosis was occurring in  $\beta$  cells(701).

Until recently TNF $\alpha$ /TNF receptor mediated islet apoptosis was thought to arise only after transplantation and upregulation of TNF $\alpha$  expression in host inflammatory cells(336). However, significant increases in TNF $\alpha$  in the serum and pancreata of rodent experimental brain dead donors have been observed which coincide with reduced islet yield, reduced islet *in vitro* viability and *in vivo* function(505). Subsequently, Hanley *et al* demonstrated a two-fold increase in islet TNF $\alpha$  expression between days one and three in isolated islets, with TNF $\alpha$  secretion peaking at day one. Cell culture for up to 1 week with a TNF $\alpha$  inhibitor reduced TNF $\alpha$  secretion by 70.6%, the frequency of TNF $\alpha$  secreting islets by 64.4% and apoptotic levels by 26.4% in the first day after isolation. These findings implicate intraislet cytokine production prior to and during isolation as a factor contributing to islet apoptosis and loss of islet mass.

Berney *et al* used a murine model to compare rates of islet apoptosis following isolation with and without the presence of contaminant endotoxin. Following islet isolation using collagenase 17.2% of islet cells appeared apoptotic; this rate fell to only 7.1% when endotoxin free Liberase was used for isolation(702). Whilst Balamurugan *et al* recently showed that the duration of human islet exposure to exogenous enzymes during islet isolation correlated with rates of islet cell apoptosis(218).

Pancreatic warm and cold ischaemia during organ preservation also correlate with rates of isolated islet cell apoptosis and can be partially abrogated with organ preservation solutions(703). Additionally the preservation method preceding islet isolation affects rates of apoptosis. A recent study, using gene array analysis, found twelve fold increases in the expression of the antiapoptotic genes IAP and survivin, and reduced expression in proapoptotic genes (Bad, Bax and activated caspases) in islets isolated from TLM preserved pancreata(704).

Dependent upon the assay used, apoptosis can be detected as early as 3 hours after islet isolation. A study of four different apoptotic assays was undertaken by Cattran *et al* to determine their sensitivities at detecting early islet apoptosis. Following isolation, and islet exposure to IL-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$ , early apoptosis was first detected by a rise in

caspace 3 activity ( $0.8 \pm 0.3$  U/100 IEQ to  $1.4 \pm 0.45$  U/100 IEQ) and annexin V binding ( $21 \pm 5.8\%$  to  $27.5 \pm 8.1\%$ ) after 3 and 6 hours respectively. Whereas an increase in the numbers of TUNEL positive nuclei ( $1.5 \pm 0.5\%$  to  $17 \pm 5\%$ ) was observed after 24 hours with a peak at 48 hours ( $19 \pm 5\%$ ); and an increase in DNA fragmentation by gel electrophoresis was only apparent after 24 hours(705).

Methods to prevent isolation induced islet apoptosis have included intraductal administration of glutamine which reduced islet oxidative stress, increased islet yield and improved *in vivo* function of islets compared to the use of intraductal collagenase alone(706). Anti inflammatory agents such as lisofylline protect isolated islets from inflammatory cytokine induced apoptosis whilst maintaining glucose responsiveness and insulin secretion and allow a 30% reduction in islet mass required to achieve insulin independence and autoimmune recurrence in a murine transplant models(707, 708). Islet exposure to  $17\beta$ -estradiol shows a dose dependent increase in islet viability, reduced cytochrome c release and caspase 9 activation. This implies a degree of protection against pro-inflammatory cytokine induced islet apoptosis and, when transplanted into NOD-SCID mice, 50% of recipients of estradiol treated grafts achieved euglycaemia compared to only 8% of untreated graft recipients(507). Finally caspase inhibitors, such as the specific caspase 3 inhibitor Z-DEVD-FMK(709) and pancaspase inhibitor Z-VAD-FMK(710), have demonstrated dose dependent reduction in islet apoptosis during cell culture with successful reversal of diabetes in diabetic nude mouse transplant models.

#### **4.2.3 Apoptosis following islet transplantation.**

Apoptosis is a source of early islet graft loss and PNF(711). Davalli *et al* used a murine model to study the early fate of transplanted islets and reported a 6%  $\beta$  cell apoptosis rate attributed to hypoxia in the centrally located  $\beta$  cells of large islets(316). A recent experimental study, which mimicked the hypoxic and nutrient deficient conditions of the immediate post transplantation environment, concluded that 24 hours of hypoxia was sufficient to cause central islet necrosis with concomitant apoptotic features such as nuclear pyknosis and DNA fragmentation(712). Central islet apoptosis reduces both *in vitro* and *in vivo* islet function, and recent studies have proposed that small diameter islets suffer less central apoptosis resulting in an improved functioning islet mass after transplantation(713, 714).

Hypoxia is not the sole pro-apoptotic stimulus encountered by transplanted islets, and the hyperglycaemic state in the diabetic recipient contributes to the pro-apoptotic milieu.

Biarnes *et al* used a syngeneic mouse model to examine the effect of persisting

hyperglycaemia following transplantation of a borderline islet mass. Apoptosis was observed immediately following transplantation but also continued during chronic hyperglycaemia as an ongoing mechanism of islet graft damage(715). A further study showed apoptosis was present in 60% of transplanted islet tissue at 30 days even where insulin was used around the time of engraftment to abrogate recipient hyperglycaemia(715).

Therefore islets are exposed to many potential apoptogenic stimuli before and during transplantation such as IBMIR, hypoxia, hyperglycaemia, recruited lymphocytes, cytokines(716) and immunosuppressants(717, 718). Therefore the effects of pancreas preservation and islet isolation on islet apoptosis, viability and function is ideally studied *ex vivo*, before transplantation and their exposure to these other potentially apoptogenic stimuli.

#### ***4.3 Induction of apoptosis.***

Apoptosis was originally described by morphological characteristics but these occur in the later stages at a time when the cell has been committed to the apoptotic pathway for some time. Prior to altered morphology are phases whereby pro and anti apoptotic stimuli act on pathways of gene expression and protein synthesis. To enter apoptosis the cell must be 'induced' by one of three main pathways; either 'extrinsic' ligand-receptor interaction, direct 'intrinsic' signalling mediated via the mitochondrion (Figure 4-2) or by loss of cell-cell and cell-extracellular matrix (ECM) interaction, which is of particular relevance after islet isolation.

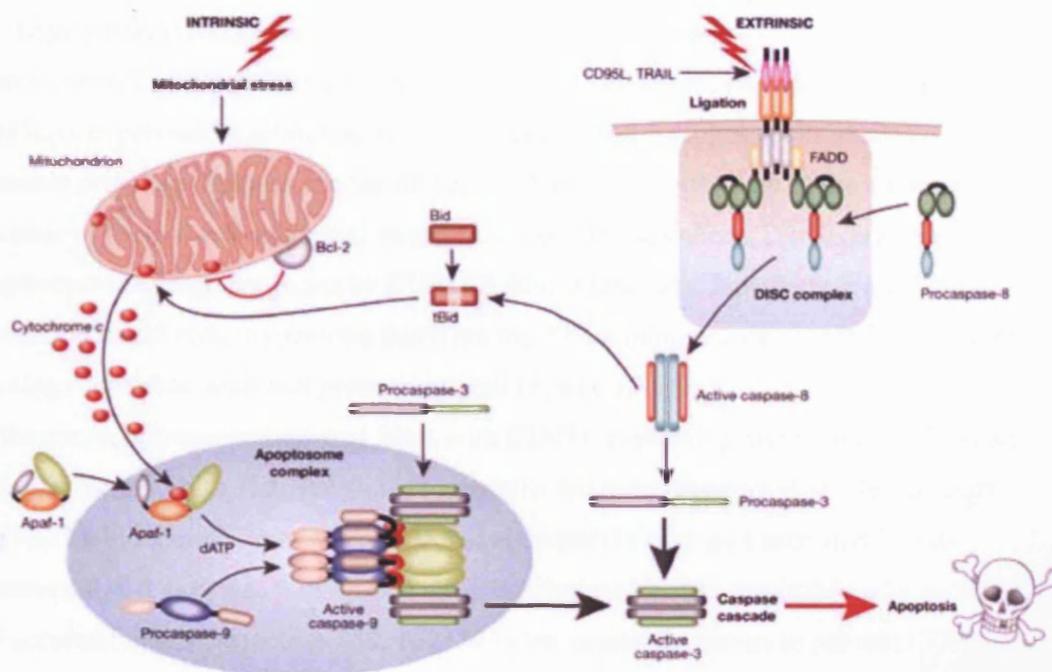


Figure 4-2. Extrinsic and intrinsic apoptotic induction pathways(719).

### 4.3.1 'Extrinsic' induction of islet apoptosis .

#### 4.3.1.1 Death receptor-ligand interaction.

Apoptogenic stimuli originating outside of the cell interact with cell surface death receptors to transduce signals through the cell membrane(720, 721). The majority of death receptors are members of the of type 1 tumour necrosis factor receptor family (TNF-R1)(721-724) which contain 4 extracellular cysteine rich domains for ligand interaction(725) and a death domain at the intracellular C terminus(726). Receptor ligation provokes both pro and anti apoptotic actions via downstream protein interactions with the death domain.

The ligands of the TNF receptors are trimeric transmembrane proteins with extracellular cell surface domains(722, 727, 728). Many have the capacity to interact with multiple TNF-R1 receptors(729), decoy receptors, and other TNFR ligands(727) to regulate a large number of cellular processes other than apoptosis (Figure 4-2).

The CD95/CD95L (Fas/FasL) ligand- death receptor pathway is of particular relevance in diabetes and islet transplantation (724, 730-736).

CD95/CD95L is a recognized pathway to apoptotic  $\beta$  cell destruction in autoimmune insulinitis prior to the onset of diabetes (*vide supra* 1.5)(15, 737-739) and in inflammatory

cytokine mediated islet apoptosis(730). In the pro-inflammatory milieu encountered during pancreas preservation, islet isolation and transplantation, cytokine induced islet expression of CD95 provides a mechanism for islet cell apoptosis and loss of  $\beta$  cell mass. CD95L is expressed by immunocytes(740-742) and non-lymphoid cells at sites of 'immune privilege' such as the Sertoli cells of the testis, epithelium of the anterior chamber of the eye and subretinal space(743-746). At such sites CD95 expressing lymphocytes undergo apoptosis by CD95/CD95L interaction. Putatively graft CD95L expression could induce apoptotic death amongst host immunocytes conferring 'immune privilege' upon the graft and preventing graft rejection(744).

Furthermore, cotransplantation of islets with CD95L expressing myoblasts(747-749) and testicular Sertoli cells(750, 751) has successfully extended the period of islet allograft survival by inducing immune privilege and apoptosis of anti-islet activated T cells.

However, direct expression of CD95L on islets themselves has resulted in islet apoptosis and accelerated graft rejection(750, 752). A more successful means to prevent CD95 mediated islet apoptosis may be to engineer the cells to endogenously express anti-CD95 molecules which specifically cleave CD95 mRNA(753).

Experimental studies using alternative ligands and 'decoy receptors' to prevent pro-apoptotic signaling by TNF receptor pathways have showed some promise. Wu *et al* recently demonstrated that the soluble TNF decoy receptor TR6 could prevent FasL and cytokine mediated islet apoptosis and islet graft PNF(754). Whilst Machen *et al* successfully prolonged islet graft survival using a adenovirally transfected soluble TNF-R1 immunoglobulin-Fc fusion transgene which prevented ligation of the TNF receptor(755).

#### 4.3.1.2 Non death receptor-ligand interaction.

Non-TNF receptor pro-apoptotic pathways are activated by the preservation and isolation induced rise in inflammatory cytokines, reactive oxygen species and nitric oxide; and are largely mediated through activation of nuclear factor kappa beta (NF- $\kappa$ B)(756, 757).

IL-1 $\beta$ , for example, is implicated in apoptotic islet death during the development of diabetes and in islet transplantation(758). IL-1 $\beta$  levels increase significantly following islet cell transplantation(336), and following whole pancreas ischaemia-reperfusion injury(759). Experimental models have demonstrated that inhibition of IL-1 $\beta$  release may prevent islet injury, that circulating anti-IL-1 $\beta$  antibodies prevents type 1 diabetes(760) and that preventing IL-1 $\beta$  receptor binding results in islet protection against IL-1 $\beta$

induced nitric oxide formation, impaired glucose-stimulated insulin production, and apoptosis(761).

IL-1 $\beta$  is secreted by activated macrophages, Kupffer cells and neutrophils, and binding of the IL-1 $\beta$  receptor indirectly results in NF- $\kappa$ B translocation from cytoplasm to the nucleus(762, 763). This ubiquitously expressed adaptor protein regulates a family of inducible genes(764, 765). At rest it is cytosolically sequestered to the inhibitory protein I $\kappa$ B $\alpha$ (766) but cytokine and stress induced phosphorylation of I $\kappa$ B $\alpha$  causes its degradation and subsequent release of NF $\kappa$ B which translocates to the nucleus(767).

NF $\kappa$ B is the major transcription factor responsible for *de novo* transcription of iNOS and elevated NO production(768), and is a key adaptor protein controlling cytokine induced beta cell dysfunction(769) and death(770, 771). There is evidence that NF- $\kappa$ B is activated in islets undergoing apoptosis(772, 773) and Giannoukakis *et al* demonstrated an upregulation of iNOS caused by NF- $\kappa$ B which contributed to islet apoptosis in a rodent model, whilst adenoviral gene transfer of the NF- $\kappa$ B inhibitor I $\kappa$ B resulted in reduced IL-1 $\beta$  mediated islet dysfunction and apoptosis(774). Similarly over expression of other NF- $\kappa$ B inhibiting genes, such as A20, have resulted in reduced islet apoptosis and successful reversal of diabetes using a marginal islet mass(775).

Abdelli *et al* recently demonstrated delayed accumulation of iNOSmRNA activity concomitant with a delayed increase in NF- $\kappa$ B DNA binding activity during islet isolation compared to exogenous administration of cytokines. This suggests that NF- $\kappa$ B mediated iNOS induction, arising from the isolation process, is unlikely to contribute to islet loss during or immediately following purification; but that iNOS induction, arising from cytokine exposure and the non-death receptor pathway, remains a potent mechanism for islet cell loss(776).

#### **4.3.2 'Intrinsic' mitochondrial pathway induction.**

Stressors such as hyperglycaemia(715) and hypoxia(777) can activate apoptotic pathways in isolated islets without the need for surface receptor interaction. This intracellular mitochondrial pathway requires phosphorylation of members of the mitogen activated protein kinases (MAPK) such as c-Jun NH<sub>2</sub>-terminal kinases (JNK)(778-780) and p38 kinase(781), which stimulate apoptosis(782). It is postulated that a balance exists between the pro-apoptotic JNK/p38 pathways and antiapoptotic extracellular regulated kinase (ERK) pathway in determining the fate of the cell(783). This balance is altered by the presence of extracellular stressors and apoptotic modulators which determines the fate of the cell(784).

#### **4.3.3 Cell detachment induction of anoikis.**

In the donor pancreas the islets of Langerhans interact with neighbouring pancreatic cells and their extracellular environment. Such cell interactions are required for survival and protection from apoptosis(785). The process of islet isolation disrupts such interactions causing apoptosis in islet cells and reducing the islet cell mass for transplantation(711).

When apoptosis occurs in response to a lack of cellular adhesion it is called 'anoikis'.

Though anoikis has morphological characteristics and effector mechanisms largely identical to apoptosis, the signaling mechanisms initiating the process arise uniquely from disruption of cellular adhesion(786, 787).

Therefore it is advantageous for the islet microenvironment to be maintained, or rapidly reconstituted, following isolation; and manipulation of islet cell interactions may reduce islet apoptosis and improve the outcome of islet isolation and transplantation(788). The requirement for trophic support from the surrounding microenvironment may explain why islets retaining a mantle of acinar tissue, after incomplete collagenase digestion, have good *ex vivo* survival and *in vitro* function(789). It also explains why transplantation of a relatively small number of unpurified islets achieves graft function and insulin independence(790) more readily than purified(791) or immunoisolated autologous islets(792, 793); as islet trophic support is maintained.

The ECM is comprised of interstitial matrix and basement membrane, and provides multiple cellular adherence and signaling pathways for proliferation, differentiation, migration and survival(786, 787). Enzymatic pancreas digestion readily destroys the interstitial matrix and can also disrupt the collagen IV, laminin and fibronectin islet basement membrane(788, 794). Disruption of both the islet cell-matrix(787, 795, 796) and islet cell-basement membrane relationships lead to induction of islet anoikis(797).

#### **4.4 Bcl-2 genes controlling apoptosis and anoikis.**

A number of genes, and their protein products, exert further control over apoptosis. The most extensively studied are the Bcl-2 genes consisting of pro and antiapoptotic members(798-800). Analogous to apoptosis, expression of Bcl-2 family genes and proteins also determines the sensitivity of cells to anoikis(801). Bcl-2 family gene expression is also regulated by cell interactions and adhesion(802-805), and through cytoskeletal protein signalling(806, 807).

The Bcl-2 sub family exert antiapoptotic effects and share sequence homology in three 'Bcl-2 Homology' regions (BH 1, 2 and 4)(808). Bax sub family members exert proapoptotic activity and share sequence homology only in the BH 3 region(809). Bcl-2 family proteins have the capacity to form homo and heterodimers(809-811) which

regulates their function. A 'rheostatic model' explains how such protein dimers control the fate of the cell. When Bcl-2 homodimerisation predominates cells develop an anti-apoptotic phenotype conferring protection against apoptosis, whilst Bax homodimerisation usually bestows apoptotic death upon the cell(809, 812, 813). Mismatching of Bax and Bcl2 expression forms heterodimers, and it is the ratio of Bax to Bcl-2 proteins in such complexes which dictates signalling for or against apoptosis(809, 814).

#### **4.4.1 Bcl-2**

Bcl-2 is one of the most prominently expressed antiapoptotic Bcl-2 sub family genes.(815, 816). Antiapoptotic activity is proportional to the level of Bcl-2 gene expression(817-819) which prevents apoptosis and necrosis(820-823) caused by several stressors and stimuli(824, 825).

Bcl-2 protein acts within the cytosol(826) and the mitochondrion(817, 827) to prevent the formation and opening of mitochondrial pores(828) and prevent the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) into the cytosol(829-835).

Gene and protein transduction provides a means to modify apoptotic gene and protein expression in pancreata during preservation and in islets following isolation. This can render them resistant to cytokine induced apoptosis but maintains insulin secretion(836, 837). However, viral vectors used in transfection can damage islet cells and interfere with biologic function. Such vectors are themselves capable of inducing islet apoptosis and reducing glucose-stimulated insulin secretion in a dose dependent manner during gene transfection studies(838-840).

Liu *et al* first demonstrated protection against cytokine stimulated apoptosis in insulinoma cells transfected to overexpress Bcl-2 (837); whilst groups led by Rabinovitch and Dupraz independently demonstrated resistance to cytokine and hypoxia induced apoptosis, and preservation of islet viability and function, in isolated pig, non human primate and human islets by adenovirally mediated transfer of Bcl-2(841, 842). Similarly detachment induced anoikis can be abolished by overexpression of the antiapoptotic proteins Bcl-2 and Bcl-xL(843-845).

Experimental studies of transgenic islets have shown Bcl-2 transfected non human primate islets to have similar *in vitro/vivo* function, and apoptotic protection, as mantle islets surrounded by ECM. It appears that preservation of the surrounding ECM may confer an anti-apoptotic signal equivalent to Bcl-2 over expression(846). A similar study,

using viral mediated transfection of several antiapoptotic genes into an insulin producing  $\beta$  cell derived cell line, showed different gene combinations conferring varying degrees of protection against apoptosis; the most potent of which offered apoptotic resistance equivalent to the use of a chemical caspase inhibitor(847).

In animal transplant models, Bcl-2 transfected macaque islets showed stable long-term protection against islet apoptosis, maintenance of islet function and a reduced islet mass requirement for stable euglycaemia with transplantation of into severe combined immunodeficient mice(848). Other studies have failed to prevent rejection of Bcl-2 transfected islet allografts alone, but have achieved prolonged graft survival using Bcl-2 transfection and anti -T cell co-stimulatory monoclonal antibodies(849).

#### **4.4.2 Bax**

Bax protein exists in the cytosol in inactive form and translocates to the mitochondria during apoptosis(819, 850, 851). It aids the formation of mitochondrial permeability transition pores(835, 852-854), mitochondrial release of cytochrome c and AIF(833, 855) and loss of the mitochondrial membrane potential( $\Delta\phi_M$ )(833, 856).

After detachment from ECM and changes in cell adhesion signalling(857, 858), identical pro-apoptotic changes are observed with a rapid translocation of Bax from cytosol to the outer mitochondrial membrane(859-861), membrane permeabilisation(862, 863), cytochrome c release(864) and effector caspase activation(865, 866). Overexpression of Bax induces apoptosis *in vivo*(867), whilst 'knockout' disruption of Bax results in cellular apoptotic resistance(868, 869).

Bax expression is known to be high in anoikitic isolated human islets and reducing Bax expression may preserve islet cell mass following isolation(870).

#### **4.5 The Bioenergetics of Apoptosis and Pancreas Preservation.**

Identical mitochondrial changes are observed during pancreatic preservation and early apoptosis. Concurrently ATP levels decline by hydrolysis(585), the electron transfer chain dissipates and oxidative phosphorylation uncouples(871, 872); whilst protein mitochondrial permeability transition (PT) pores open in the mitochondrial outer envelope(831, 832, 873-881) and  $\Delta\phi_M$  dissipates(872, 882-887). After mitochondrial permeabilisation downstream apoptotic pathways are activated that lead to the acquisition of the biochemical and morphological hallmarks of apoptosis(690, 830, 888-891).

#### **4.5.1 PT pore formation.**

Bcl-2 family pro and anti-apoptotic proteins are intimately associated with PT pore formation (*vide supra* 4.4); either by direct pore generation(861, 892) or by inducing formation of pores from mitochondrial protein voltage-dependent anion channels(893, 894).

PT pores allow release of the mitochondrial intermembrane fraction containing apoptogenic factors(895) like cytochrome c, procaspases (especially procaspase 2, 3 and 9), heat shock proteins, AIF and DNase into the cytosol(829, 831, 834). The significance of this is subject to wide interpretation. Some data suggest that intermembrane fraction release is an obligatory step for subsequent apoptosis(817, 832, 885, 895-900), some view it as an amplification step to accelerate the process(901), whilst others see it as a potentially reversible event in the apoptotic cascade(902, 903). Some even view the mitochondrial membrane changes as irrelevant after studies demonstrating a direct pathway to caspase activation without mitochondrial permeabilisation(904). Furthermore, in some cells, the bioenergetic and redox catastrophe of mitochondrial permeabilisation is sufficient to induce apoptosis regardless of the contribution from the intermembrane fraction(883, 885, 905).

#### **4.5.2 The intermembrane fraction.**

Release of intermembrane cytochrome c appears critical to the initiation of later apoptotic pathways via binding of procaspase 9, proteolysis and activation of the initiator procaspase 3(906-909). This step is closely regulated by endogenous inhibitors of apoptosis (IAP) which bind proteins in the pathway(910, 911) or degrade cytochrome c, so apoptosis cannot proceed until a critical threshold concentration of cytosolic cytochrome c is achieved.

Manipulation of IAP activity has been used experimentally to prevent islet apoptosis. Transgenic islet grafts expressing the IAP survivin achieved long-term engraftment and stable euglycaemia in a syngeneic murine transplant model(912); whilst human islets transduced with the endogenous X linked inhibitor of apoptosis (XIAP) showed resistance to apoptosis, functional recovery after *in vitro* hypoxia and a reduction in islet number required to reverse chemical diabetes in diabetic nude mice(913, 914). Recently islet treatment with a cell permeable pentapeptide apoptosis inhibitor has caused up and downregulation of antiapoptotic and proapoptotic protein expression respectively. This coincided with an increase in islet yield of 44% and *in vitro* insulin secretion by fourfold,

allowing successful reversal of streptozotocin induced diabetes by a third of the usual islet mass(915).

Other inducible enzymes in the intermembrane fraction, such as hemoxygenase-1 (HO-1), act as non specific inhibitors of apoptosis and are released during cytokine induced beta cell stress(916-918) and islet isolation(919). Overexpression of such genes can protect islets from inflammatory cytokine mediated apoptosis *in vitro*, and prolongs the survival time of islet allografts compared to untreated islets(920).

#### **4.5.3 ATP requirements.**

Cytochrome c mediates in caspase activation which is co-dependent upon activation of ATP binding proteins(907-909). This renders progression of apoptosis an 'active' energy consuming process dependent upon the availability of adequate ATP(829). When ATP is absent apoptosis cannot proceed and cells die by necrosis even though the early components of apoptosis may be present and active(832, 873, 881, 921-923).

Therefore the mode of cell death during pancreas preservation and islet isolation is a function of cellular levels of ATP(832, 873, 881, 921-924) and ATP repletion results in a return to apoptotic capability, and reduction in necrotic cell death(925). Pure hypoxic injury rapidly depletes ATP levels and results almost uniformly in cell necrosis(926-928); whereas pancreatic reperfusion, or resuscitation during preservation, potentially repletes ATP levels allowing a mixed morphology with variable rates of apoptosis and necrosis(929-934). Hence a rapid, reliable and reproducible method to assay ATP might allow the predominant mode of cell death, and efficacy of pancreas preservation, to be assessed prior to pancreas transplantation or islet isolation.

#### **4.5.4 Luciferin-luciferase measurement of ATP in apoptosis.**

Firefly luciferase can be used to measure the ATP content of isolated mitochondria and permeabilised whole cells. The luciferin-luciferase assay uses the phenomenon of bioluminescence whereby the enzyme luciferase catalyses the reaction between ATP and luciferin to generate light at wavelength 562nm with an intensity proportional to the concentration of substrates in the reaction mixture (Figure 4-3). Light intensity is measured using a luminometer generating an output in mV. ATP concentration can then be determined with reference to a standard curve.

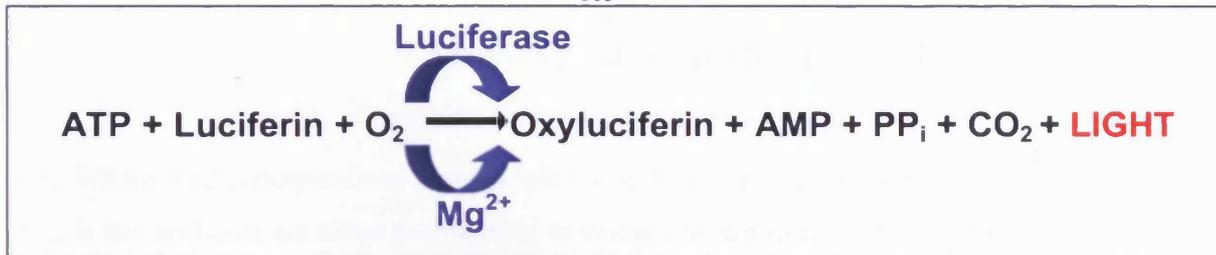


Figure 4-3 The luciferase reaction to generate bioluminescence.

The reaction can be used to differentiate between proliferation, apoptosis and necrosis in cells using a two-phase method. Firstly, ATP within the cells is measured directly by the bioluminescent reaction. Following a decay phase, ADP is measured by its conversion to ATP, using a reagent comprised of phosphoenolpyruvate (PEP) and pyruvate kinase (Figure 4-4), and repetition of the bioluminescent reaction.

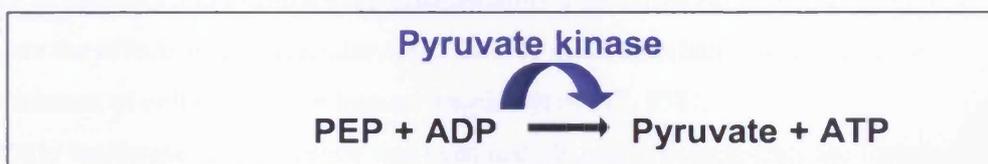


Figure 4-4. Conversion of ADP to ATP using ADP converting reagent.

The ratio of ADP/ATP (Equation 4-1) then distinguishes cellular proliferation, apoptosis or necrosis (Figure 4-5).

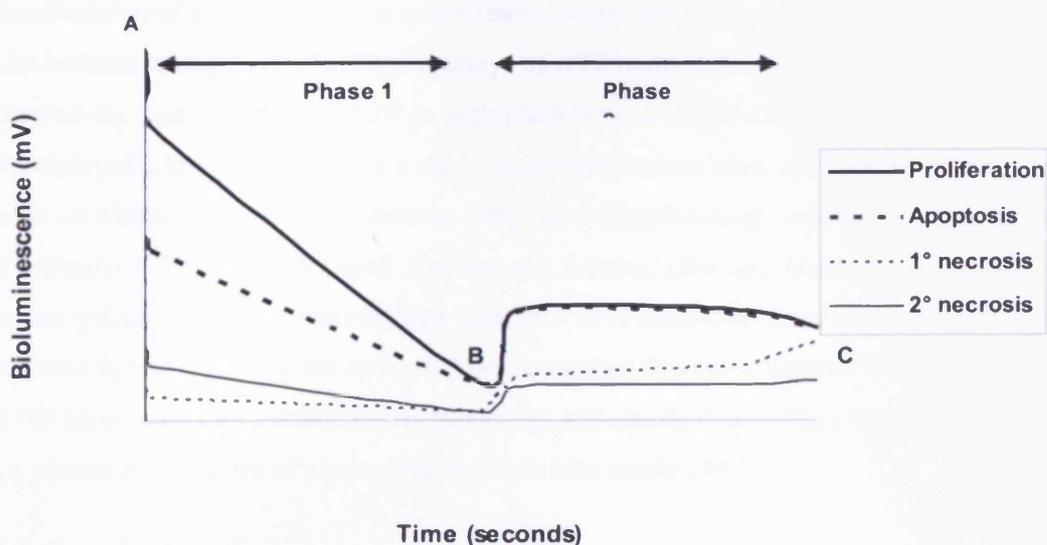


Figure 4-5. Modes of cell death and output from the ATP assay.

$$\text{ADP:ATP ratio} = (C-B)/A$$

**Equation 4-1. Calculation of ADP:ATP ratio.**

Where ATP concentrations are high relative to ADP concentrations, the ADP:ATP ratio is low and cells are either proliferating or viable and in a state of growth arrest.

Apoptosis, being an active ATP driven process, results in a reduced ATP concentration, relative to the ADP concentration, and a moderate increase in the ADP:ATP ratio. In primary necrosis a rapid loss of cellular ATP results in a very high, often reversed, ADP:ATP ratio; whereas secondary necrosis results in an intermediate fall in ATP and consequently high ADP:ATP ratio(935).

Luciferin – luciferase ATP bioluminescence was first used in human tissue to quantify ATP in platelets and plasma(936). Subsequently a modified assay, using ATPase to negate the effects of extracellular ATP, showed good correlation with fluorescent assessment of cell viability in human lymphocytes(937, 938).

Latterly luciferase luminescence was been used *in situ* to demonstrate the histological distribution of ATP in normal and malignant tissue(939) and the technique was used to study ATP content in rodent brain tissue sections following cold induced brain injury(940). Luciferase bioluminescence provides a highly sensitive quantification of adenosine nucleotides in human tissue allowing a detailed evaluation of its metabolic status comparable to standard spectrophotometric assays(941); and the technique is practical, reproducible and sensitive compared with other ATP assays(942).

Mundwiler *et al* previously made quantitative assessments of pancreas viability prior to islet isolation using firefly luciferase assays of ATP content(943) and a similar assay has allowed the quantification of ATP in multiple(944) and single islets(945). Brandhorst *et al* employed a luciferase assay in a study of isolated human islets and demonstrated a large variability of ATP concentration. They recommended clarification of the relevance of intraislet ATP content for graft function and survival after islet transplantation(946). Recently Goto *et al* demonstrated that islet ADP:ATP ratio correlated with transplant outcome in immunodeficient rodents. They suggested that the simplicity and sensitivity of the assay, using an established methodology and standard laboratory equipment, made it a promising measure of post-transplantation islet quality(947).

#### **4.6 Conclusion.**

Islet mass is lost during pancreas procurement, preservation and islet isolation by apoptosis and anoikis, and by primary and secondary necrosis. Pancreatic ischaemic hypoxia, cytokine, mechanical and enzymatic stressors act via receptor mediated and

mitochondrial pathways, and are closely regulated by pro and antiapoptotic proteins such as Bax and Bcl-2. Manipulation of apoptotic and anoikitic signaling can reduce isolation induced and post-transplantation islet apoptosis, and potentially preserve islet mass.

The mitochondrial changes induced by pancreas preservation are synonymous with those seen in apoptosis, and apoptotic pathways are likely activated during periods of pancreas preservation. A luciferase based ATP assay is an established means to indirectly assess apoptosis and necrosis by ADP:ATP ratio, and this ratio is predictive of post-transplant porcine islet function in a nude murine transplant model(947).

The remainder of this thesis studies the ADP:ATP ratio in pancreas and islet tissue subjected to various preservation protocols. Pre-clinical rodent and porcine models are studied, and finally results in a small number of human pancreata are presented. The ADP:ATP ratio is evaluated as a marker of pancreas and islet cell damage, and compared to PCR and immunohistochemical assays of Bax and Bcl-2 mRNA and protein expression respectively, apoptosis, and standard measures of islet yield, purity and *in vitro* function.

***Part II – Experimental Chapters.***

## ***Chapter 5. Development of Novel Methodology.***

### ***5.1 Introduction.***

This appendix summarises the development of novel assays and methods used during the experimental chapters. The rationale, methods, results and analysis of these preliminary experiments are described with the implications for the experimental work.

### ***5.2 Preliminary experiments using the luciferin assay.***

#### ***5.2.1 Rationale and aims.***

These experiments studied the basic kinetics and optimal conditions for conducting the ATP chemoluminescence assay. As ATP is ubiquitous within living tissues it was hypothesised that ATP concentration would be increased as more tissue was assayed. Experiments using increasing volumes of pancreas and digest tissue sections, and increasing islet numbers, were designed to test this basic hypothesis, and to optimise the amount of tissue required to achieve readings within limits of accuracy of the assay. A further experiment examined the effect of sampling different pancreatic sites to optimise the origin of pancreatic biopsy. Finally, to allow experimental pancreatic preservation, digestion and islet isolation to continue unimpeded it was deemed advantageous to perform ATP assay *after* completion of islet isolation; allowing the ATP assay to be performed upon stored samples of tissue from throughout the isolation. Hence the feasibility of pancreatic tissue storage to facilitate *en masse* assaying of stored tissue was studied.

#### ***5.2.2 Methods.***

Rodent pancreata were procured from three freshly culled animals and immediately 'snap frozen' in liquid nitrogen. Increasing numbers of serial 10µm frozen sections were assayed to determine ATP concentrations using increasing amounts of tissue. Confirmation of the effect of increasing tissue volume upon assayed ATP concentration was sought by increasing the thickness of single frozen sections used. To determine the effect of sampling from different areas of the pancreas biopsies from proximal, mid and distal pancreas were studied. The feasibility of performing the assay upon pancreatic digest tissue was investigated by assaying serial frozen sections of pancreatic digest frozen in flexible microwells, and confirmed by assaying single frozen sections cut at 10µm increments.

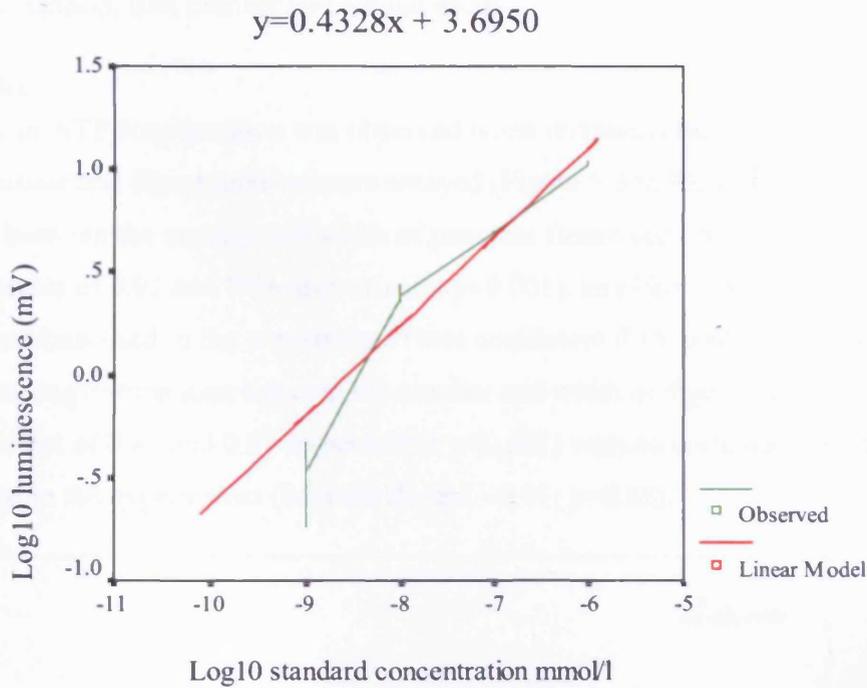
The influence of tissue storage conditions upon the ATP assay result was examined by storage of procured blocks at varying temperatures for differing lengths of time. Previous studies have demonstrated the unstable nature of ATP requiring rapid assays to prevent degradation of the molecule. The rationale was to obtain the optimal storage temperature and duration for frozen sections prior to ATP assay to allow completion of islet isolation without degradation of ATP during storage.

Finally increasing numbers of isolated rodent islets were exposed to the ATP assay before and after a period of overnight cell culture. This would confirm the required number of islets to obtain a chemoluminescent signal and allow the assay to proceed.

Bioluminescent reagents were supplied by BioWhittaker (Apoglow<sup>TM</sup>, Cambrex, Maine, USA). After allowing reagents to equilibrate to room temperature experiments were conducted in triplicate. To the Eppendorf containing the sample 100µl of RPMI (Sigma, UK) was added and vortexed for ten seconds. 100µl of nucleotide releasing reagent was added and the reaction allowed to proceed at room temperature for 5 minutes. 180µl was transferred from the Eppendorf into a white walled DNase treated luminometer tube, 20µl of ATP monitoring reagent (firefly luciferase, D-luciferin, 50mg BSA, 0.5 mmol magnesium acetate, 0.1 µmol inorganic pyrophosphate) was rapidly added and the luminometer (Wallac Series 1900, UK) cassette turned to allow a reading to be obtained in mV on a chart recorder (Wallac, UK). After the initial rapid peak of ATP luminescence had been recorded the reaction was left to decay for 10 minutes before a further baseline reading was obtained. 20µl of ADP converting reagent was then added to generate a second peak of ATP luminescence on the recorder. ADP:ATP ratios were calculated from the readings made using the equations previously described (*vide supra* 4.5.4).

A standard curve was constructed by performing the assay using ATP standard dilutions to permit the calculation of the absolute ATP content from the peak chemoluminescence. Standards were analysed in triplicate for the construction of each standard curve. Logarithmic transformation of these values appeared to show a linear correlation between log[standard concentration] and log[peak mV] of the form  $y=mx+c$ . Consistency between standard curves throughout the experiments is illustrated in the graph of cumulative standard curves from the first twelve calibrations of the luminometer (Figure 5-1).

Figure 5-1. Logarithmic transformation of ATP standard luminescence.



The equation of the slope of Figure 5-1 can be rearranged to allow the calculation of a value of ATP concentration thus:

**Equation 5-1.  $[ATP]=10^{(y-3.695)/0.4328}$**

For improved illustration figures use the log [ATP] value on the y axis to allow ATP concentrations, increasing by logarithmic progression, to be observed on the same axes.

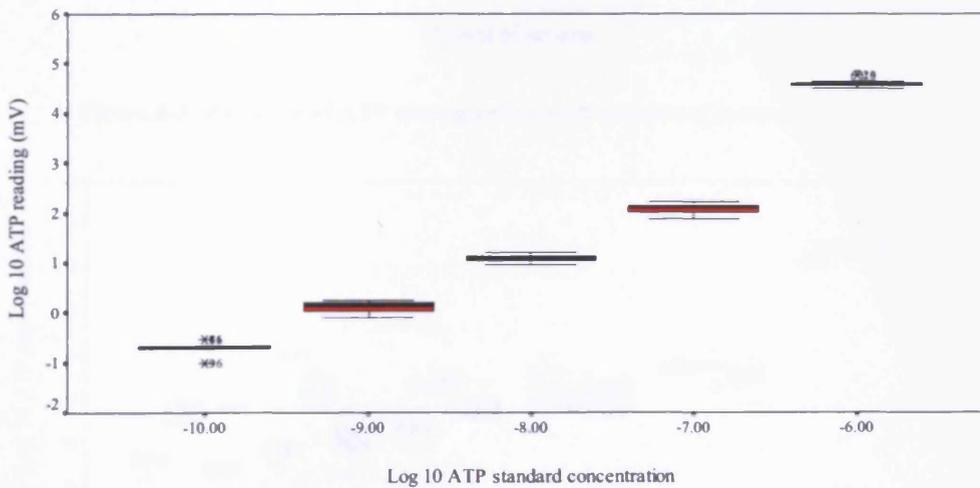


Figure 5-2. Cumulative results of luminometer ATP standard calibrations.

Multiple regression analysis was used to determine correlations between the number and width of sections assayed, and the origin of the biopsy in the pancreas. This attempts to construct a linear equation that predicts the value of a dependent variable (ATP

luminescence) from two or more independent variables (section number, width, biopsy site, storage method, islet number and animal used).

### 5.2.3 Results.

An increase in ATP concentration was observed when increasing numbers and widths of pancreatic tissue and digest sections were assayed (Figure 5-3 to Figure 5-6). A strong correlation between the number and width of pancreas frozen section was observed by beta coefficients of 0.91 and 0.93 respectively;  $p < 0.001$ ), an effect independent of the individual animals used in the experiments (beta coefficient 0.13;  $p = 0.87$ ). Similarly there were strong correlations between the number and width of digest sections assayed (beta coefficient of 0.83 and 0.87 respectively;  $p < 0.001$ ) with no correlation to individual animals used in the experiments (beta coefficient - 0.01;  $p = 0.88$ ).

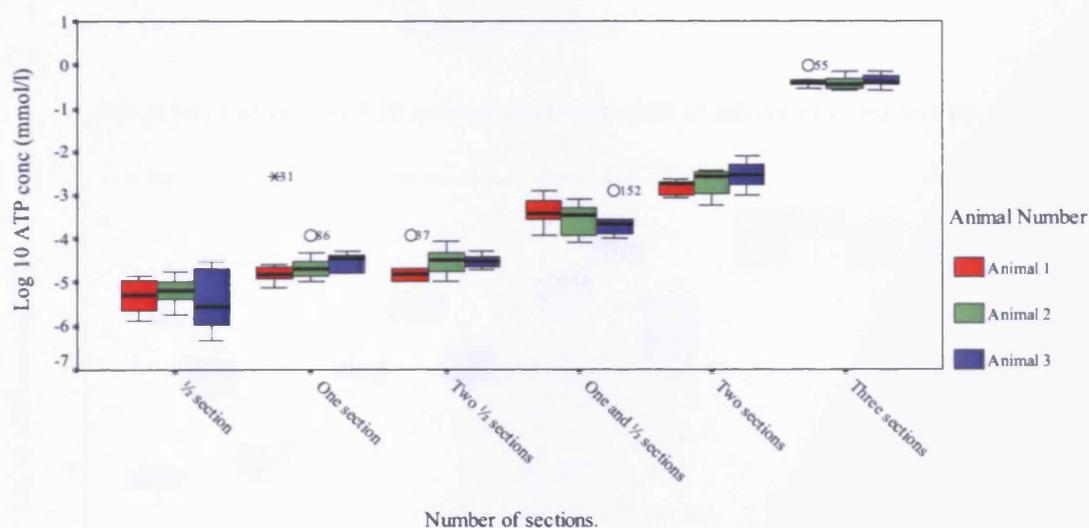


Figure 5-3. Variation of ATP concentration with number of assayed pancreas sections.

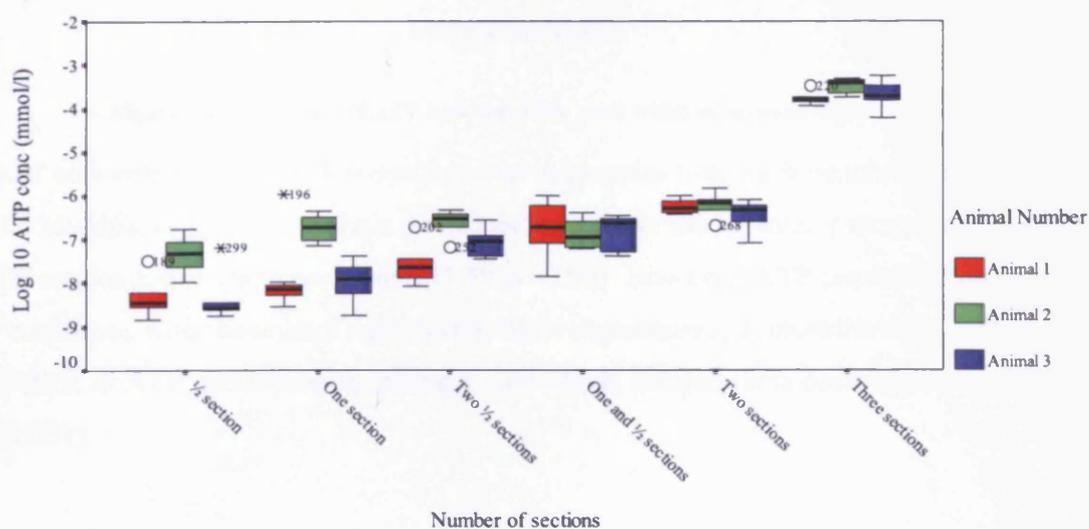


Figure 5-4. Variation of ATP concentration with number of assayed digest sections.

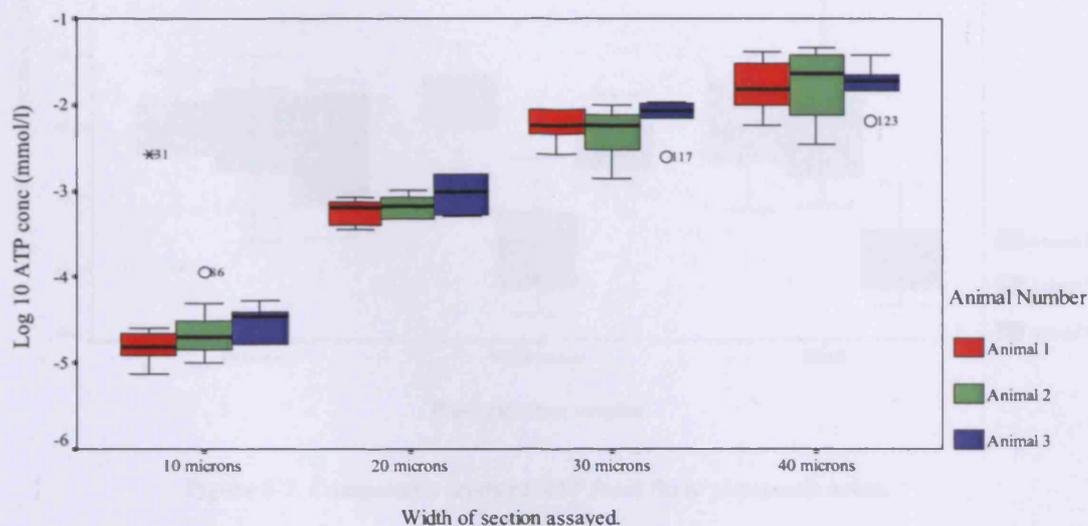


Figure 5-5. Variation of ATP concentration with width of assayed pancreas section.

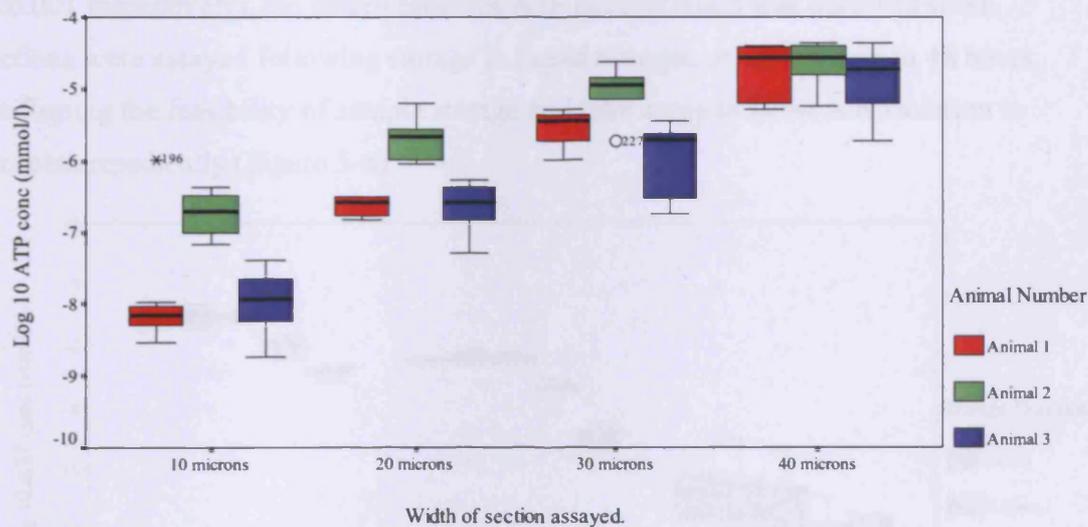


Figure 5-6. Variation of ATP concentration with width of assayed digest section.

Equal concentrations of ATP were observed in biopsies from each pancreatic area (Figure 5-7). Multiple regression analysis confirmed no correlation between pancreatic area and ATP concentration (beta coefficient -1.39;  $p=0.08$ ). However, ATP concentration could be correlated with the animal used during these experiments, demonstrating a degree of variation in ATP concentration amongst individuals assayed (beta coefficient -3.8;  $p<0.001$ ).

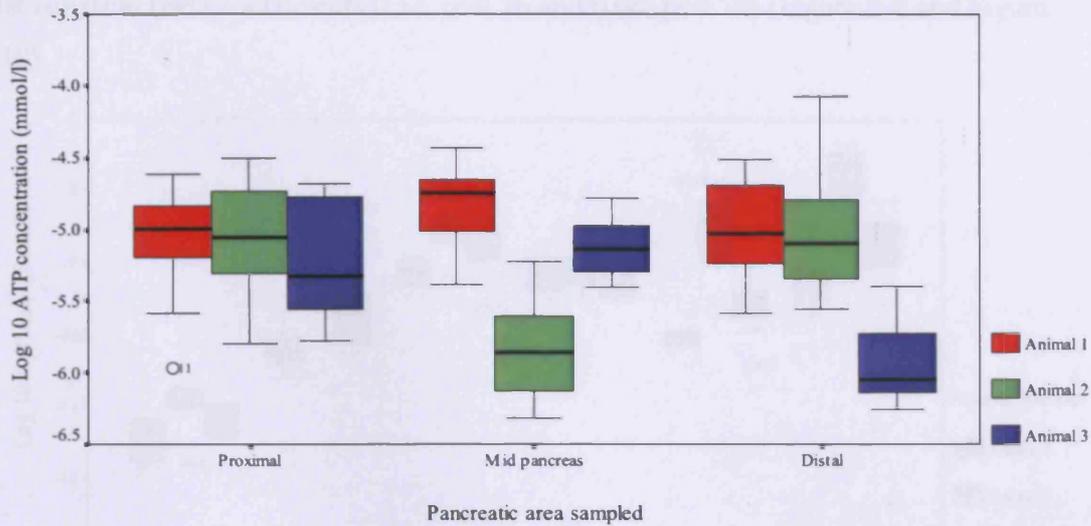


Figure 5-7. Comparable levels of ATP from three pancreatic areas.

Multiple regression analysis demonstrated negative correlations between the storage temperature and increasing duration of section storage (beta coefficients -0.88 and -0.22;  $p < 0.001$  respectively). No deterioration in ATP concentration was observed when sections were assayed following storage in liquid nitrogen or -80C for up to 48 hours, confirming the feasibility of sample storage and later assay to allow islet isolation to proceed expediently (Figure 5-8).

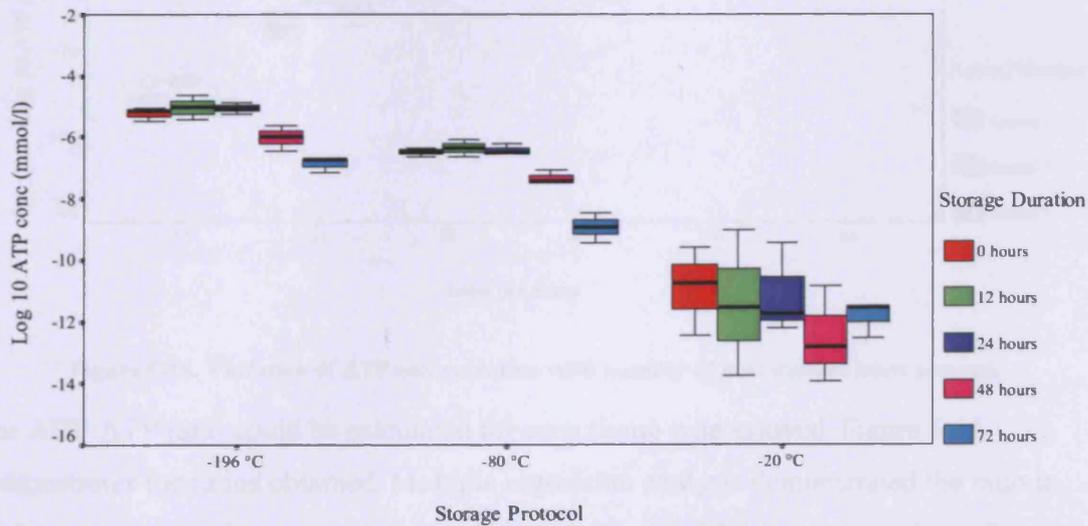


Figure 5-8. Variation of ATP concentration with section storage temperature and duration.

Multiple regression analysis demonstrated a significant correlation between the number of both pre-and post-culture islets assayed and ATP concentration (beta coefficients 0.63 and 0.95 respectively;  $p < 0.001$ ); again this was independent of the animal used for the

islet isolation (beta coefficients 0.11,  $p=0.26$  and 0.04,  $p=0.70$ ) (Figure 5-9 and Figure 5-10).

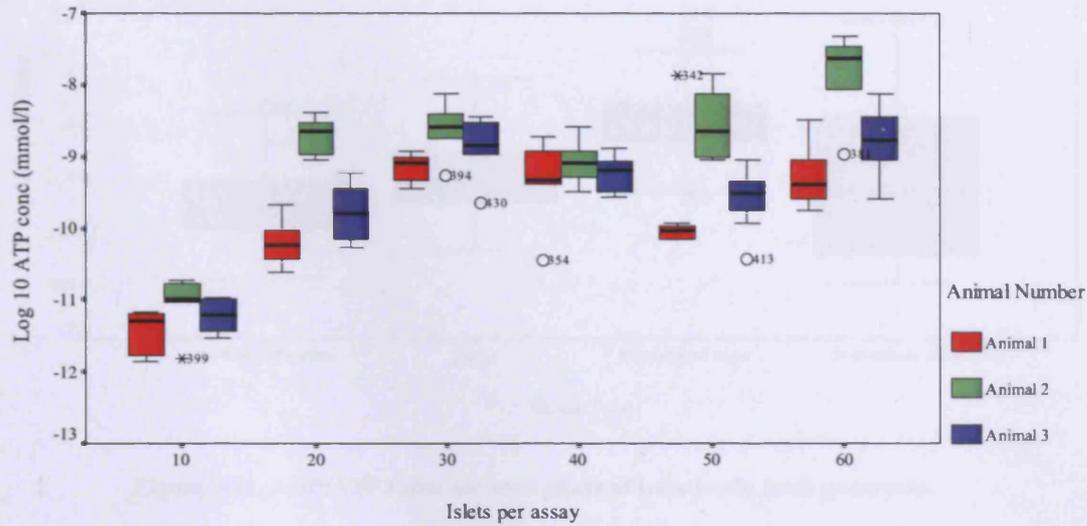


Figure 5-9. Variation of ATP concentration with number of pre-cultured islets assayed.

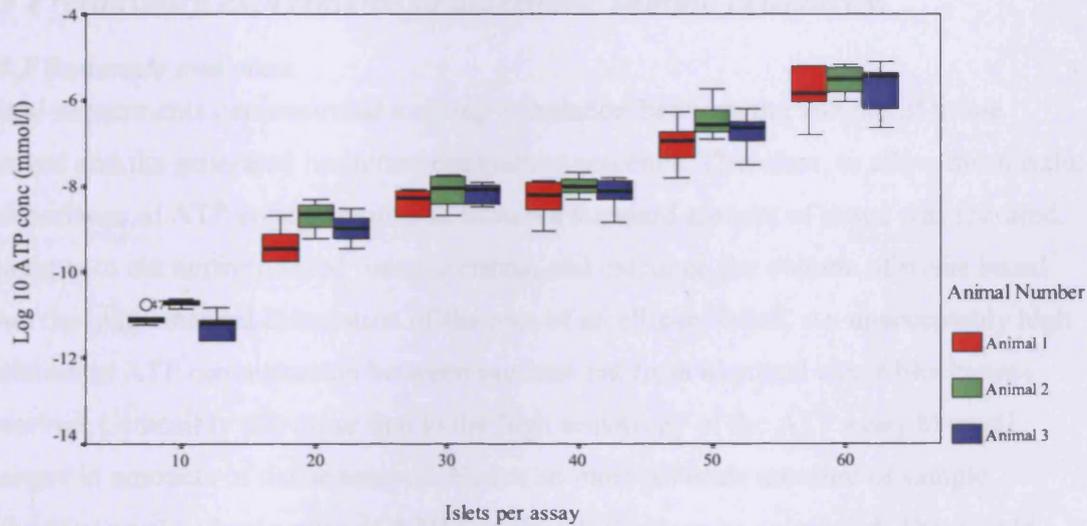


Figure 5-10. Variation of ATP concentration with number of post-culture islets assayed.

The ADP:ATP ratio could be calculated for each tissue type assayed. Figure 5-11 demonstrates the ratios obtained. Multiple regression analysis demonstrated the ratio to be dependent upon tissue type (beta coefficient 0.28;  $p<0.001$ ) but independent of number of sections assayed (beta coefficient 0.02;  $p=0.73$ ), section width (beta coefficient -0.06;  $p=0.48$ ), islet number (beta coefficient -0.09;  $p=0.16$ ) and animal used for isolation (beta coefficient 0.07;  $p=0.12$ ).

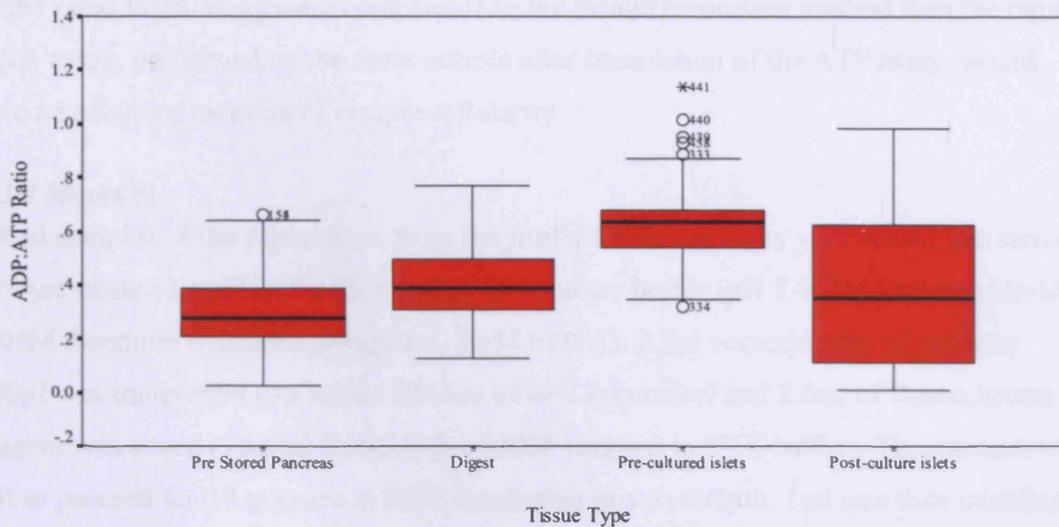


Figure 5-11. ADP:ATP ratios for each phase of isolation in fresh pancreata.

### 5.3 Preliminary experiments to determine sample cellularity.

#### 5.3.1 Rationale and aims.

Initial experiments demonstrated a strong correlation between the volume of tissue assayed and the generated luciferase chemoluminescence. Therefore, to allow meaningful comparisons of ATP concentrations in tissues a standard amount of tissue was required. Attempts to cut uniform sized tissue sections, and calculate the volume of tissue based upon the mathematical calculation of the area of an ellipse, failed. An unacceptably high variation in ATP concentration between sections cut from identical tissue blocks was observed. Ostensibly this arose due to the high sensitivity of the ATP assay to small changes in amounts of tissue assayed. Hence an more accurate measure of sample cellularity would allow a ratio of ATP:sample cellularity to be calculated. This would facilitate more meaningful comparison of ATP levels, whilst abrogating the need to standardize the amount of tissue sampled.

A conventional technique to precisely measure the cellularity of a tissue sample is to assay its DNA content. This requires DNA to be liberated from cells via mechanical or enzymatic cleavage of cell membranes. During the firefly luciferase assay cells are permeabilised using a cationic detergent to release adenine nucleotides. Hence the feasibility of performing a rapid DNA assay of sample cellularity upon the material previously used in the ATP assay was studied using frozen pancreatic sections, frozen digest sections and isolated islets. The results of these rapid DNA assays were then compared with a conventional DNA assay using sonication to release DNA. If the results

of the rapid DNA assay were comparable to the formal sonication method then the rapid DNA assay, performed on the same sample after completion of the ATP assay, would give an adequate measure of sample cellularity.

### **5.3.2 Methods.**

500 $\mu$ l samples of the supernatant from the firefly luciferase assay were added to a sterile DNase treated Eppendorf with 1.5ml of DNA assay buffer (pH 7.4; 2M sodium chloride, 0.05M disodium hydrogen phosphate, 2mM EDTA). After vortexing for 10 seconds 400 $\mu$ l was transferred to a sterile DNase treated Eppendorf and 2.6ml of fluorochrome reagent was added (1 $\mu$ g/ml Hoechst dye 33258 (Sigma) in NTE buffer). The reaction was left to proceed for 30 minutes at 30°C incubation in a waterbath. 1ml was then transferred to a DNase treated fluorometer cuvette and read on a fluorometer at wavelength of 350nm excitation and 455nm emission. A serial dilution of 50 $\mu$ g/ml calf thymus DNA (Sigma D1501) in DNA assay buffer was used to generate standards from which standard curve was plotted. Sample fluorescence readings in mV were then converted to DNA concentrations by comparison with the standard curve.

Conventional DNA assay methodology was performed on comparable samples. This used the method described above with the additional step of sonification of the samples at 14mHz for 3 periods of 20 seconds prior to the addition of DNA assay buffer.

Multiple regression analysis was used to determine correlations between the volume of tissue assayed and rapid and conventional DNA assay results.

### **5.3.3 Results.**

Using the rapid DNA assay an increase in DNA concentration was observed using increasing number and width of pancreatic (beta coefficient of 0.71 and 0.88 respectively;  $p < 0.001$ ) and digest sections (beta coefficient of 0.74 and 0.93 respectively;  $p < 0.001$ ) (Figure 5-12 and Figure 5-13).

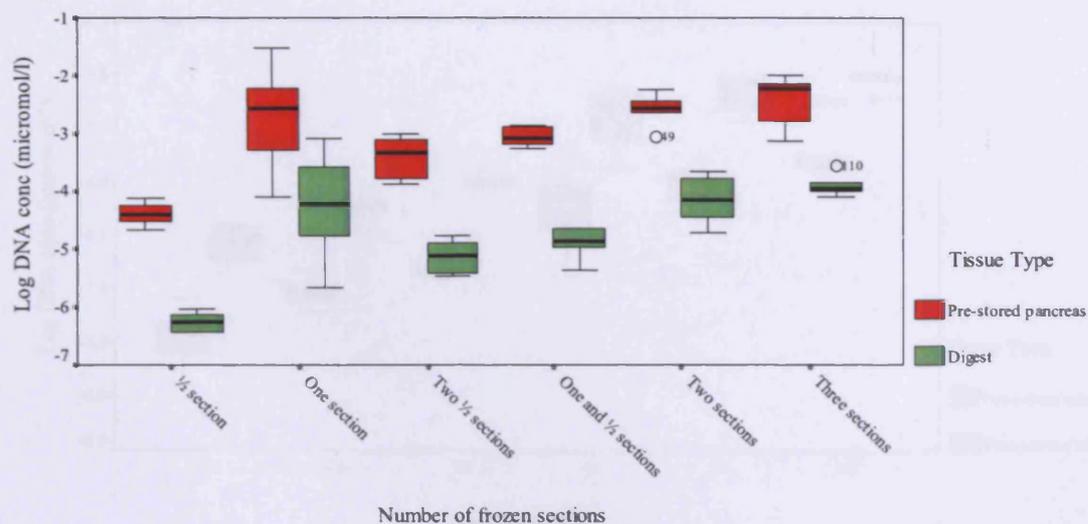


Figure 5-12. Variation in rapid DNA concentration with increasing number of sections assayed.

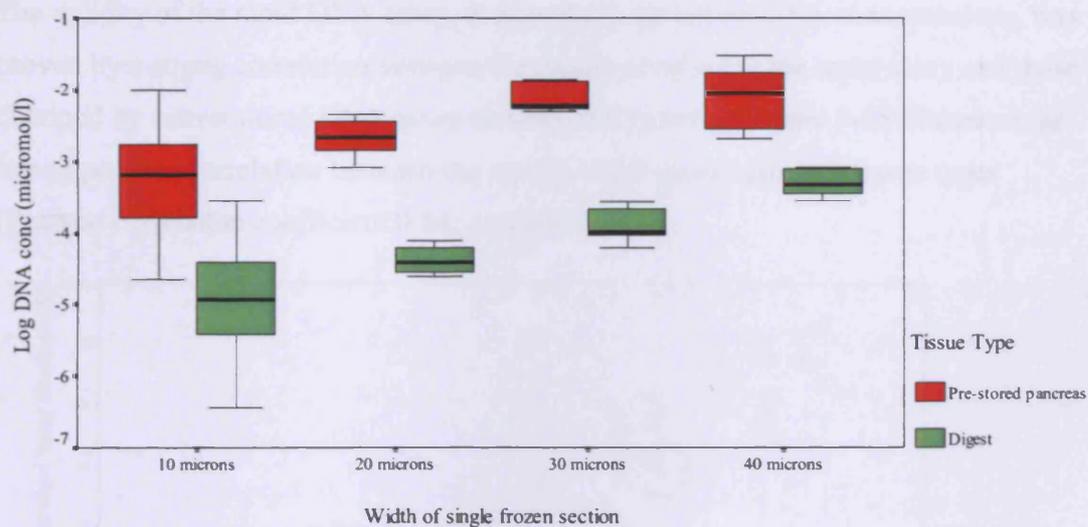


Figure 5-13. Variation in rapid DNA concentration with increasing width of section assayed.

Confirmation of the effect of increased tissue volume upon rapid DNA assay result was obtained using increasing numbers of isolated islets (Figure 5-14). A strong correlation was observed between rapid DNA assay result and number of islets used for both pre and post-culture islets (beta coefficient of 0.97 and 0.92 respectively;  $p < 0.001$ ).

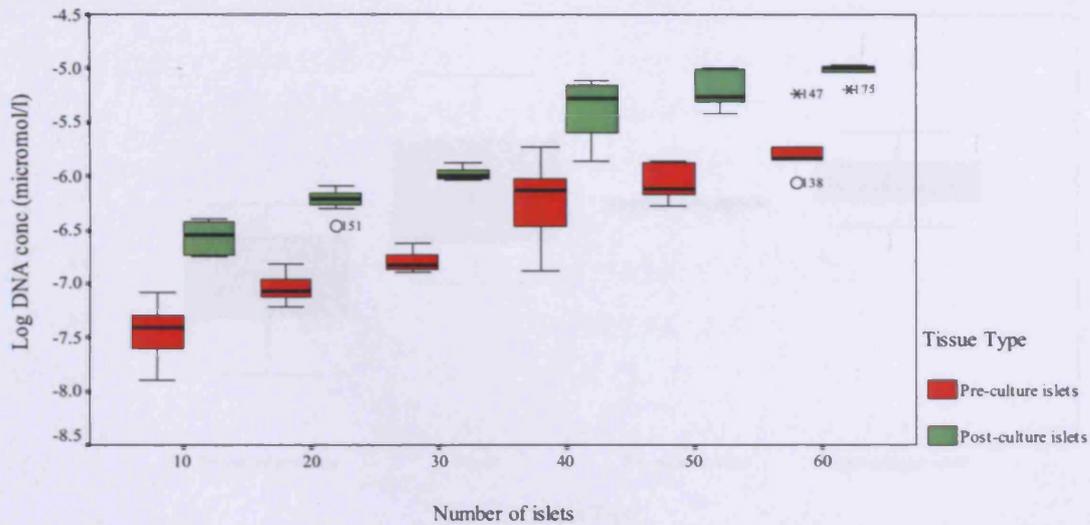


Figure 5-14. Variation in rapid DNA concentration with increasing number of islets assayed.

The validity of the rapid DNA assay, to accurately determine DNA concentrations, was proven by a strong correlation between the results obtained in the rapid assay and those obtained by conventional DNA assay on sonicated material. Figure 5-15 illustrates the strong positive correlation between the results of the assays using all tissue types (Pearson correlation coefficient 0.84;  $p < 0.001$ ).

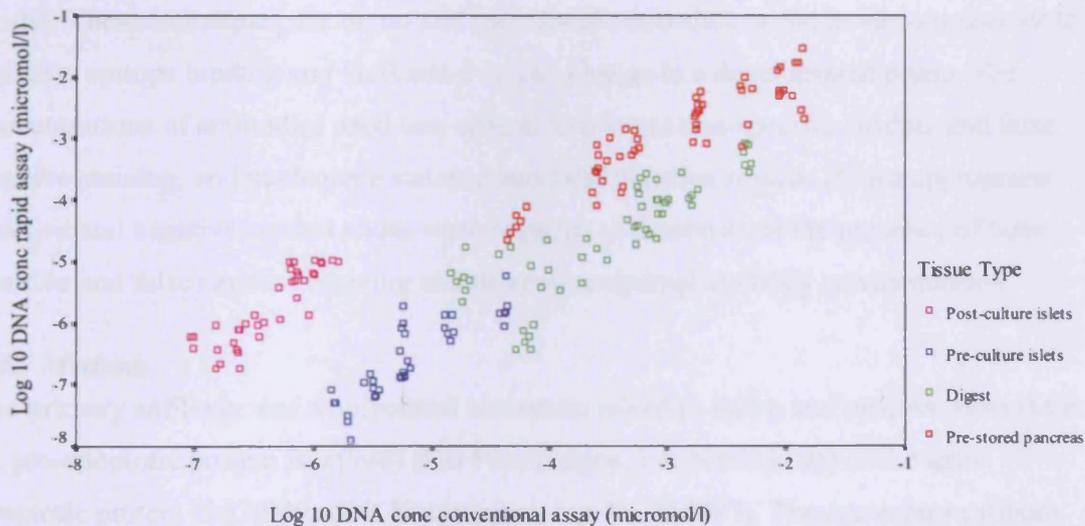


Figure 5-15. Scatter plot demonstrating correlation between rapid and conventional DNA assays.

Hence an ATP:DNA ratio could be calculated for each tissue type assayed. Figure 5-16 demonstrates the ratios obtained. Multiple regression analysis demonstrated the ratio to be dependent upon tissue type (beta coefficient 0.614;  $p < 0.001$ ) but independent of number of sections assayed (beta coefficient -0.04;  $p = 0.76$ ), section width (beta coefficient -0.02;  $p = 0.90$ ) and islet number (beta coefficient 0.15;  $p = 0.38$ ).

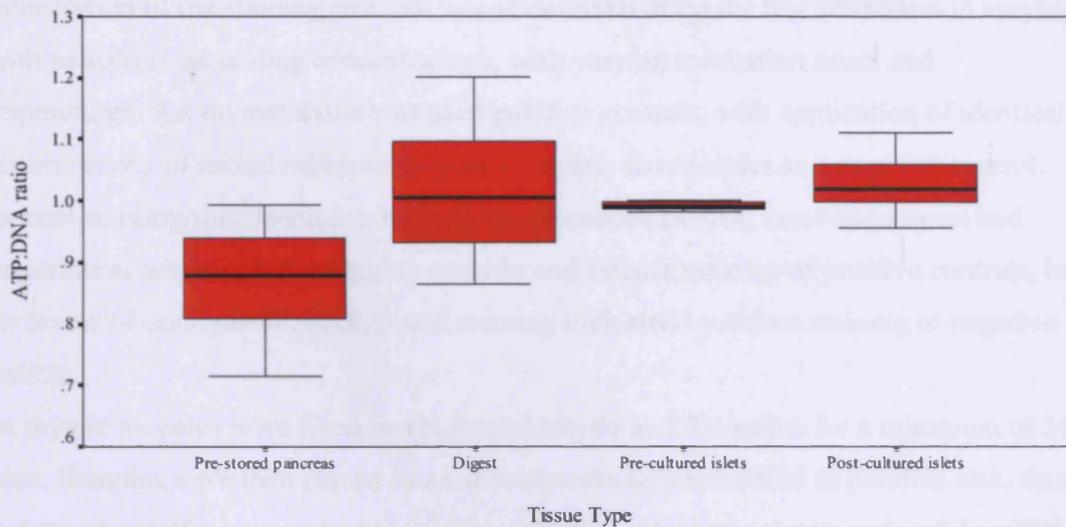


Figure 5-16. ATP:DNA ratios for each phase of isolation in fresh pancreata.

## 5.4 Preliminary experiments on immunohistochemistry of apoptotic proteins.

### 5.4.1 Rationale and aims.

These experiments optimised the immunohistochemical techniques to allow the semi-quantitative analysis of the pro and anti-apoptotic proteins Bax and Bcl-2 in the rodent model. These techniques use mono and polyclonal antibodies raised in various species to generate epitope binding and facilitate a colour change in a development phase. The concentrations of antibodies used was critical to prevent non-specific binding and false positive staining, and inadequate staining and false negative results. Hence appropriate positive and negative control slides were required to demonstrate the presence of false positive and false negative staining and determine optimal antibody concentrations.

### 5.4.2 Methods.

The primary antibody was a polyclonal antiserum raised in rabbit and reactive against the rat pro-apoptotic protein Bax(948) (DB Pharmingen, Lot No.554106) or the anti-apoptotic protein Bcl2(949) (DB Pharmingen, Lot No.554087). The secondary antibody was a biotinylated anti-rabbit polyclonal antiserum raised in goat (Stressgen Biotechnologies, Victoria, Canada. Lot No. SAB-303). The tertiary phase used an avidin/streptavidin complex reactive against biotin and biotinylated alkaline phosphatase. The final development phase utilized the colour change of fast red from colorless to red when acting as a substrate for alkaline phosphatase. Thus the Bax or Bcl2 epitopes are indirectly linked via a complex to alkaline phosphatase in an 'indirect sandwich' immunohistochemical assay (*vide infra* Figure 6-11).

Optimisation of the staining protocol was undertaken using the test antibodies in varying combinations in ascending concentrations, with varying incubation times and temperatures. Rat thymic tissue was used positive controls, with application of identical concentrations of mixed rabbit antibodies to thymic tissue slides as a negative control. Optimal staining was deemed to be at antibody concentrations, incubation times and temperatures which achieved highly specific and intense staining of positive controls, but low levels of non-specific background staining indicated by absent staining of negative controls.

Rat thymic biopsies were fixed in 4% formaldehyde in 0.9% saline for a minimum of 24 hours. Samples were then placed in a tissue cassette and embedded in paraffin wax. 4µm sections of paraffin wax embedded tissue were cut using a microtome and serial sections were floated in a waterbath to allow mounting on silane coated slides. Slides were dried at 37°C for 24 hours. Slides were de-waxed by serial immersions in xylene (2x 3 minutes), 99% industrial methylated spirit (IMS) (2x 3 minutes), 95% IMS (2x 3 minutes) and ultra-pure water (2x 3 minutes).

The process of fixation and paraffin blocking may obscure the antigens of interest and produce false negative staining of sections. Hence antigen retrieval was used to facilitate staining intensity where antigen expression may have been impaired by tissue processing. This required determination of optimal citrate buffer pH, temperature and duration of heating for a given concentration of primary and secondary antibody. For the antibody concentrations used during these experiments, antigen presentation by 850W microwave heating in 10mM citrate buffer at pH6 for 20 minutes followed by 40 minutes of room temperature cooling produced optimal retrieval conditions.

Slides were then washed in TRIS buffered saline (pH7.65, TRIS 20mM, NaCl 137mM, HCl 1M) and, prior to the addition of primary antibodies, were blocked with application of 50µl of a 1:20 dilution of normal goat serum in blocking solution (3% Bovine serum albumin, 0.1% Triton x-100, TBS) for 10 minutes to prevent non-specific binding of the primary antibody.

NGS was then poured from the slides and 100µl of primary rabbit anti-rat Bax polyclonal antiserum added to each (DB Pharmingen, Lot No.554106) for Bax IHC. For Bcl-2 IHC this was replaced by 100µl of a rabbit anti-rat Bcl-2 polyclonal antiserum (DB Pharmingen, Lot No.554087). Slides were placed in a darkened covered slide tray for a period of incubation at 4°C. The primary antibody solution was poured from each slide and 100µl of secondary biotinylated goat anti-rabbit polyclonal antiserum added to each (Stressgen Biotechnologies, Victoria, Canada. Lot No. SAB-303). Slides were covered

and incubated at 4°C for 1 hour. The secondary antibody was poured from each slide and the slides racked and washed in TBS for 5 minutes. 100µl of the tertiary phase avidin/streptavidin complex and biotin/alkaline phosphatase linkers was applied to each slide and the covered slides left to incubate. Slides were then racked in a Hellendahl and washed in ultrapure water on a rocking platform for 5 minutes. The wash was then poured away and replaced with veronal acetate buffer (VAB) (pH 9.2; 0.39%w/v sodium acetate trihydrate, 0.59%w/v sodium barbitone, 0.58%w/v sodium chloride) for a further 5 minutes. Slides were then transferred to a Hellendahl of filtered red developer solution (0.05% w/v naphthol ASBI phosphate in 500µl dimethylfemanide, 0.05%w/v fast red, 0.02% levamisole) to allow the final colour change to develop. Slides were washed in ultra pure water for 5 minutes, counterstained with haematoxylin for 5 minutes and rewashed before mounting using an aqueous mountant and cover slips.

### 5.4.3 Results.

#### 5.4.3.1 Bax immunohistochemistry.

Figure 5-17 to Figure 5-19 illustrate the most optimal staining protocol identified for rodent Bax staining. The optimal specificity and intensity of positive control staining was achieved by overnight incubation of slides at 4°C using a 1:100 dilution of primary antibody following 20 minutes of 850W microwaving for antigen presentation.

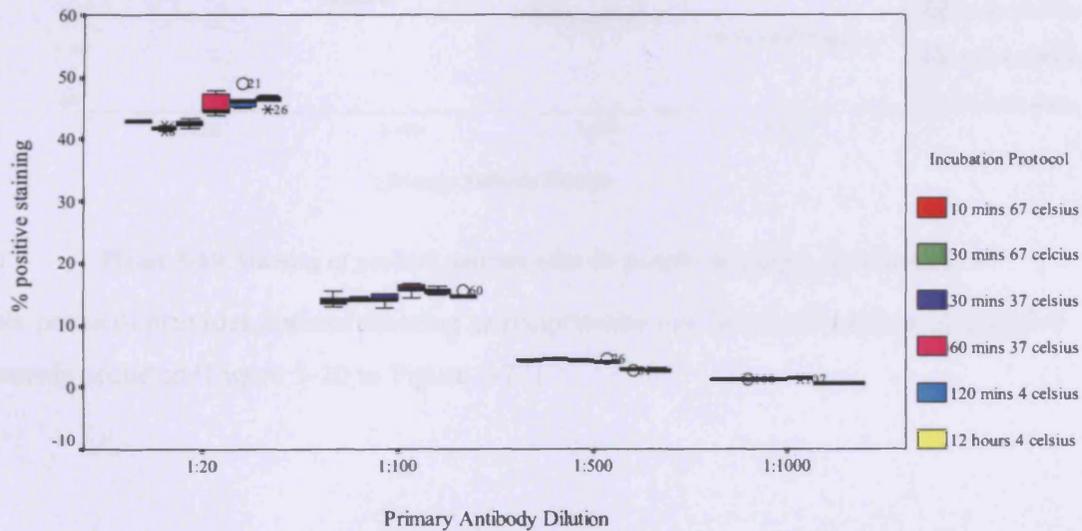


Figure 5-17. Staining of positive controls after 10 minutes of antigen presentation.

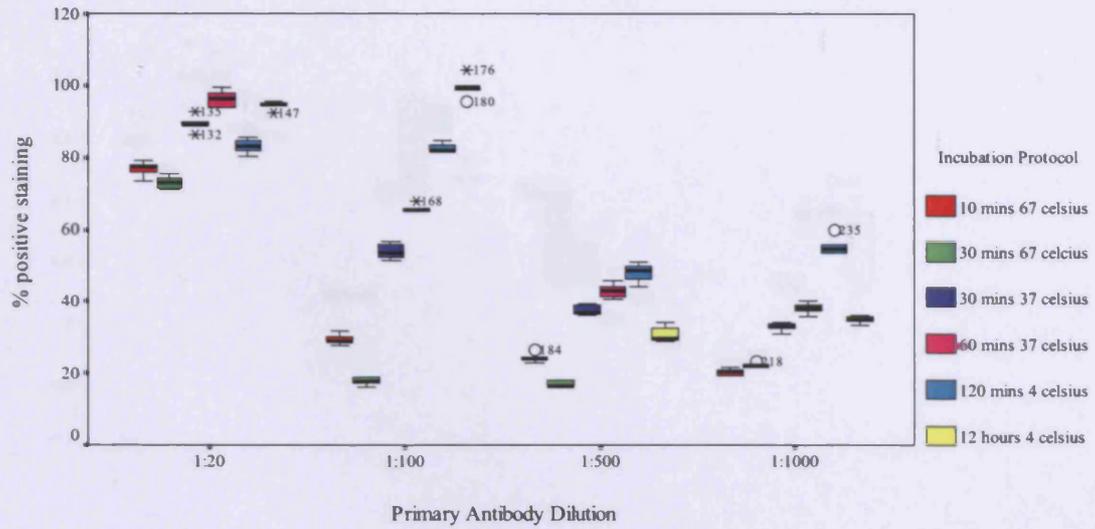


Figure 5-18. Staining of positive controls after 20 minutes of antigen presentation.

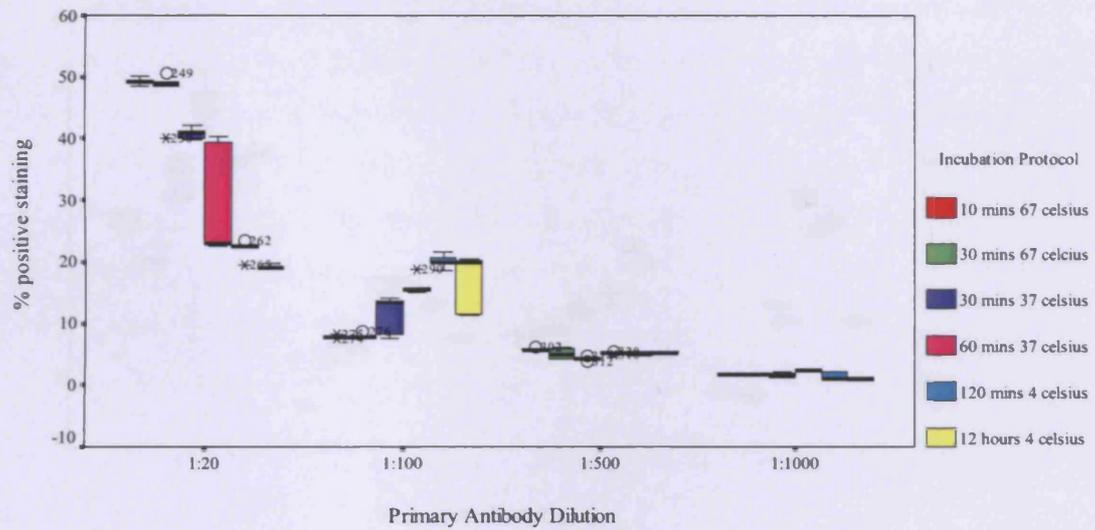


Figure 5-19. Staining of positive controls after 30 minutes of antigen presentation.

This protocol provides optimal staining as reciprocally low levels of staining of negative controls occurred (Figure 5-20 to Figure 5-22)

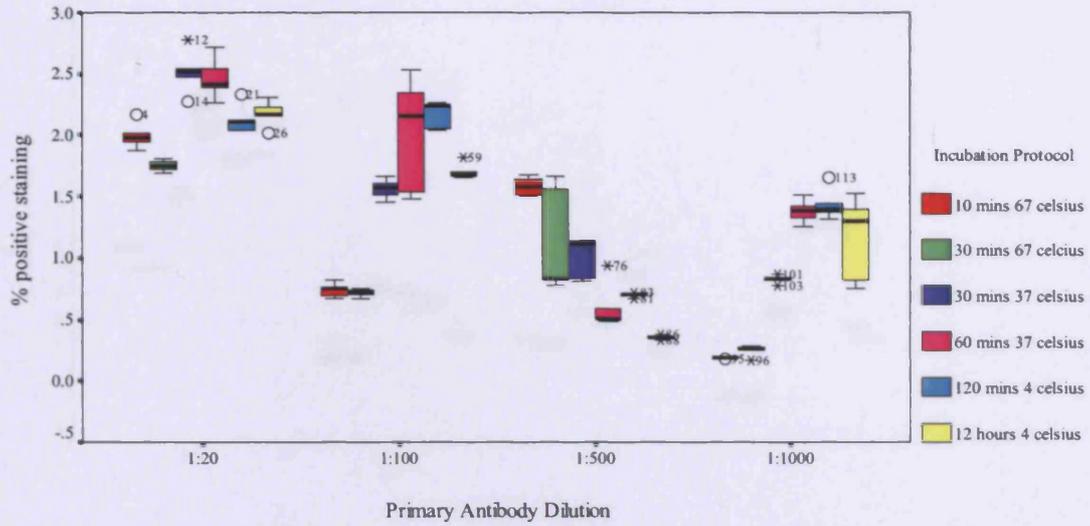


Figure 5-20. Staining of negative controls after 10 minutes of antigen presentation.

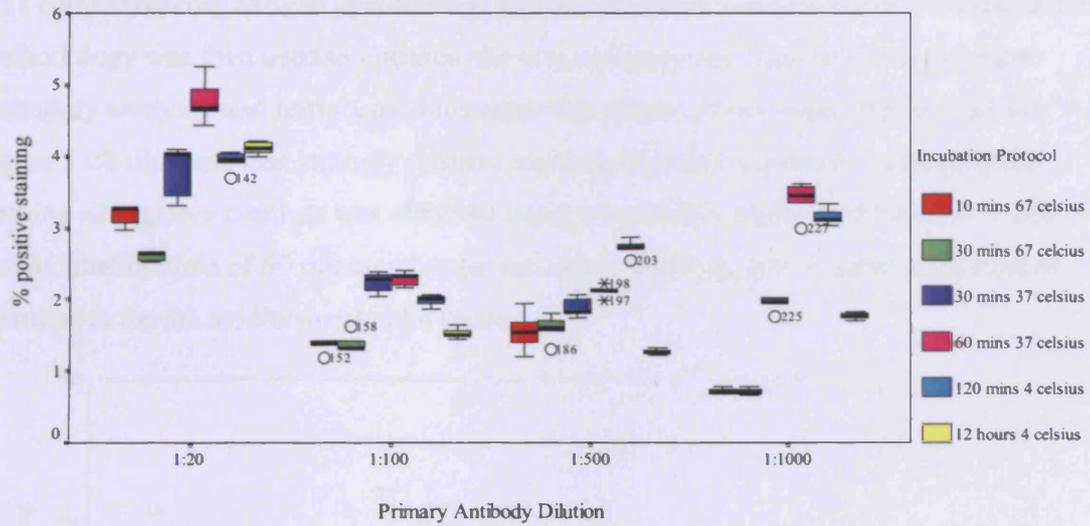


Figure 5-21. Staining of negative controls after 20 minutes of antigen presentation.

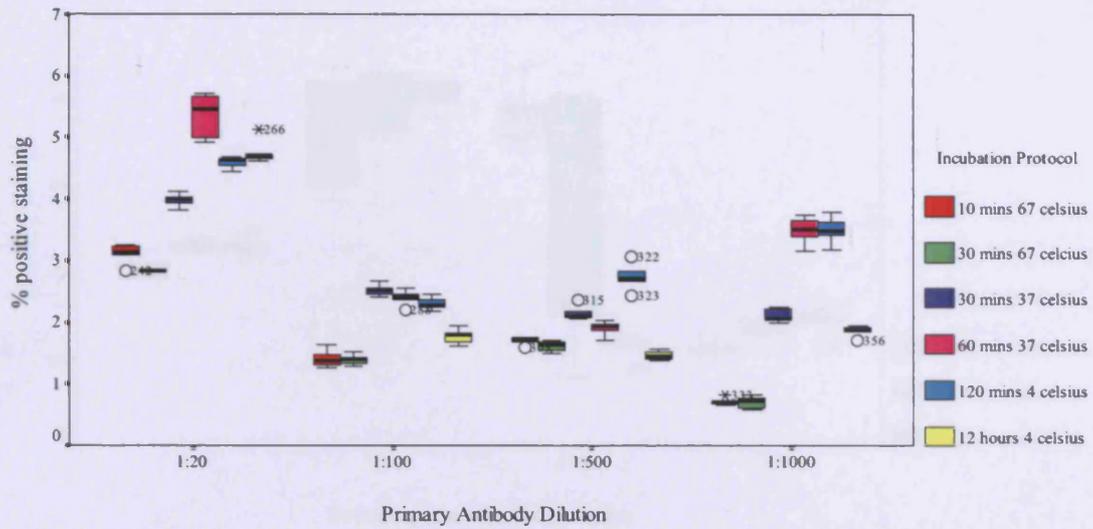


Figure 5-22. Staining of negative controls after 30 minutes of antigen presentation.

This combination of antigen presentation, primary antibody concentration, and incubation methodology was then used to optimise the concentration and incubation times of the secondary antibody and tertiary avidin/strepavidin phases of the assay. Figure 5-23 to Figure 5-28 illustrate that strongly positive staining of positive controls and negligible staining of negative controls was obtained using a secondary antibody dilution of 1:200 and incubation time of 60 minutes for the secondary antibody and an incubation time of 90 minutes for the avidin/strepavidin phase.

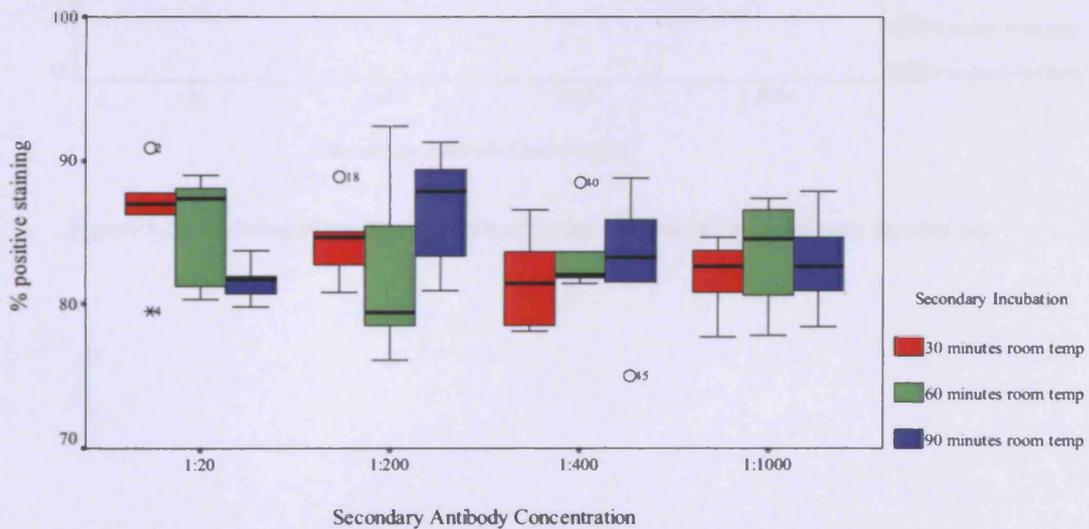


Figure 5-23. Staining of positive controls after 90 minutes tertiary antibody incubation.

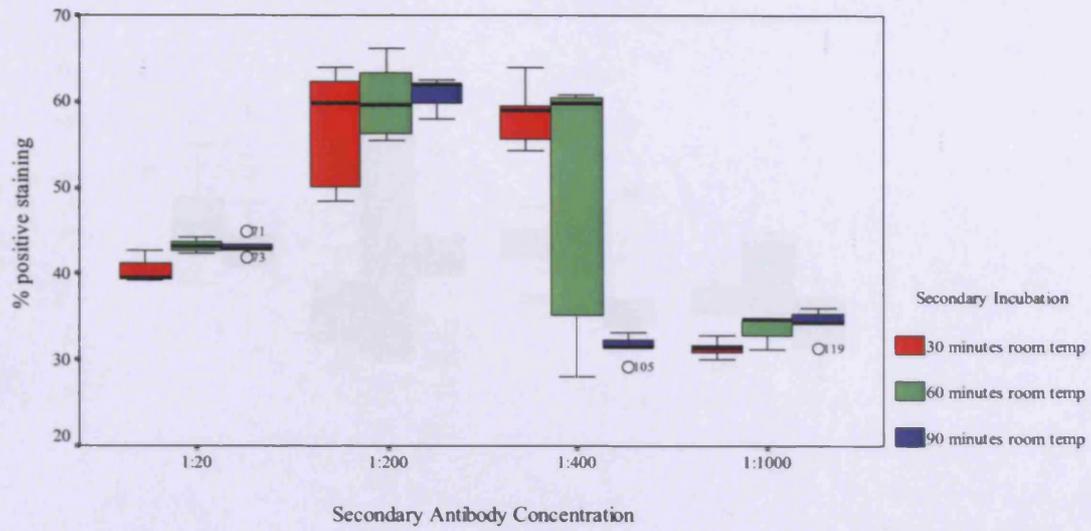


Figure 5-24. Staining of positive controls after 60 minutes tertiary antibody incubation.

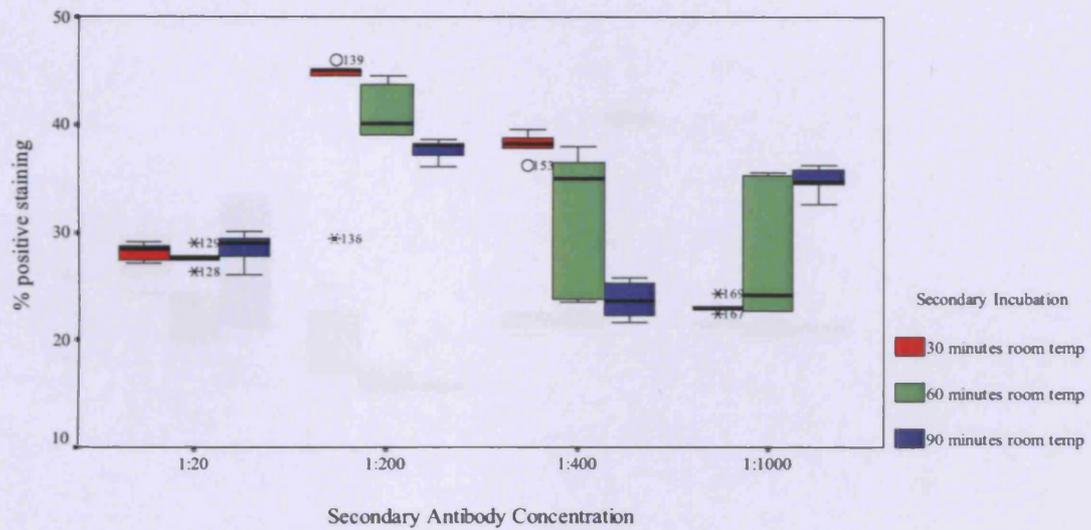


Figure 5-25. Staining of positive controls after 30 minutes tertiary antibody incubation.

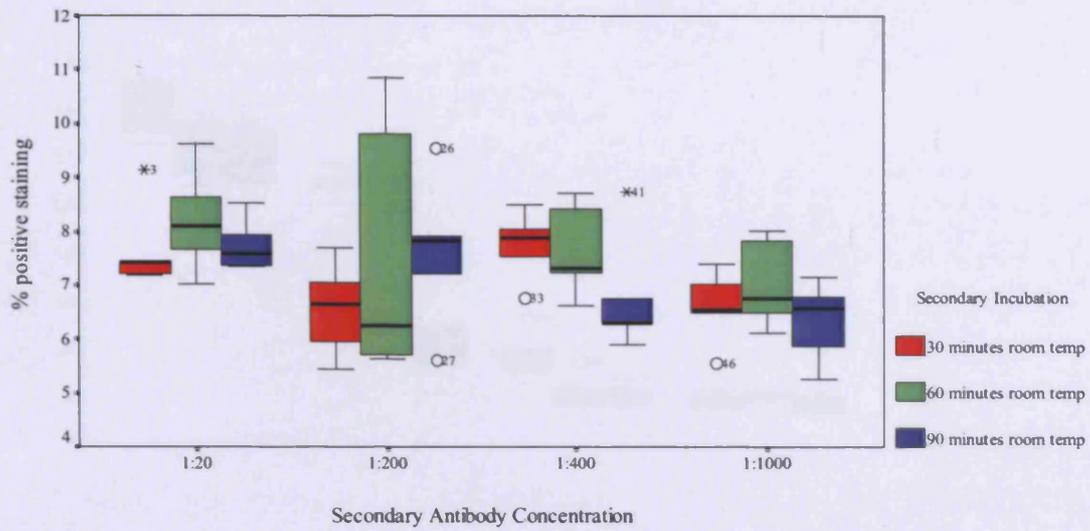


Figure 5-26. Staining of negative controls after 90 minutes tertiary antibody incubation.

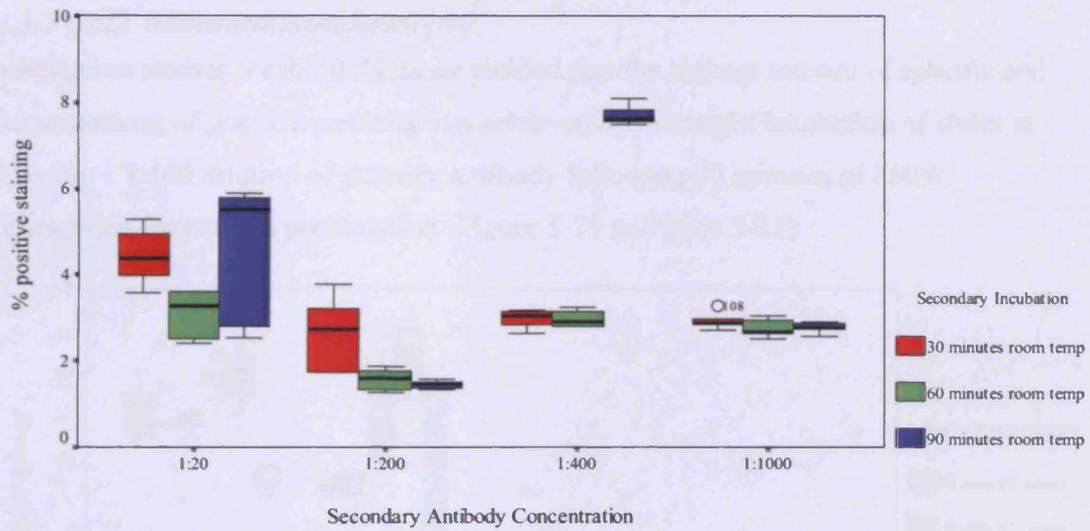


Figure 5-27. Staining of negative controls after 60 minutes tertiary antibody incubation.

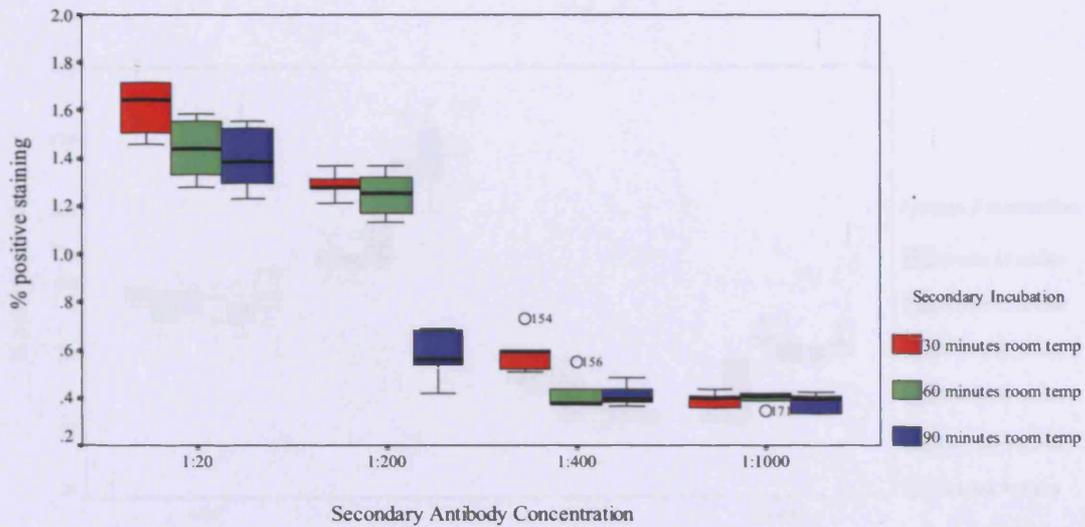


Figure 5-28. Staining of negative controls after 30 minutes tertiary antibody incubation.

#### 5.4.3.2 Bcl2 immunohistochemistry.

Optimisation studies for the Bcl2 assay yielded that the highest amount of specific and intense staining of positive controls was achieved by overnight incubation of slides at 4°C using a 1:100 dilution of primary antibody following 20 minutes of 850W microwaving for antigen presentation (Figure 5-29 to Figure 5-31)

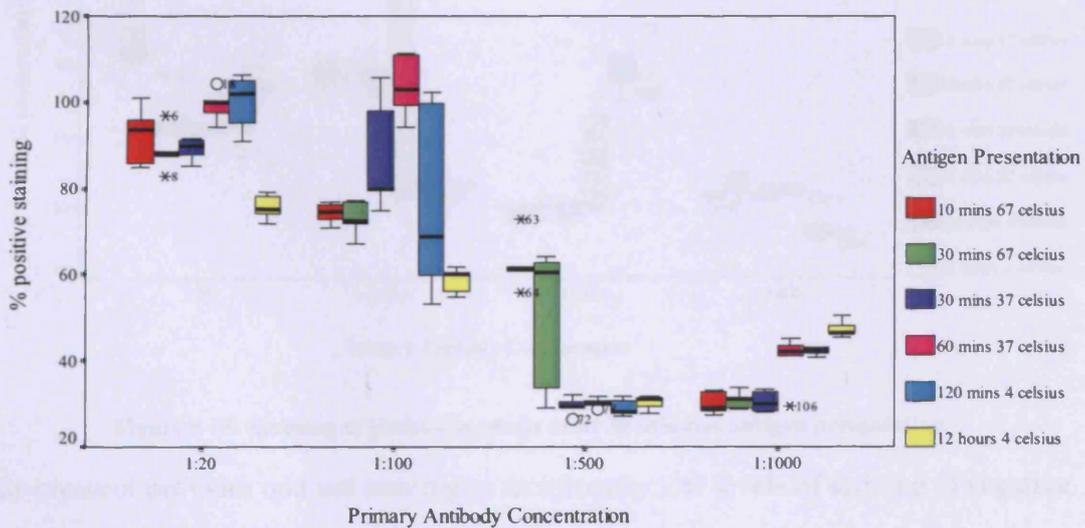


Figure 5-29. Staining of positive controls after 10 minutes antigen presentation.

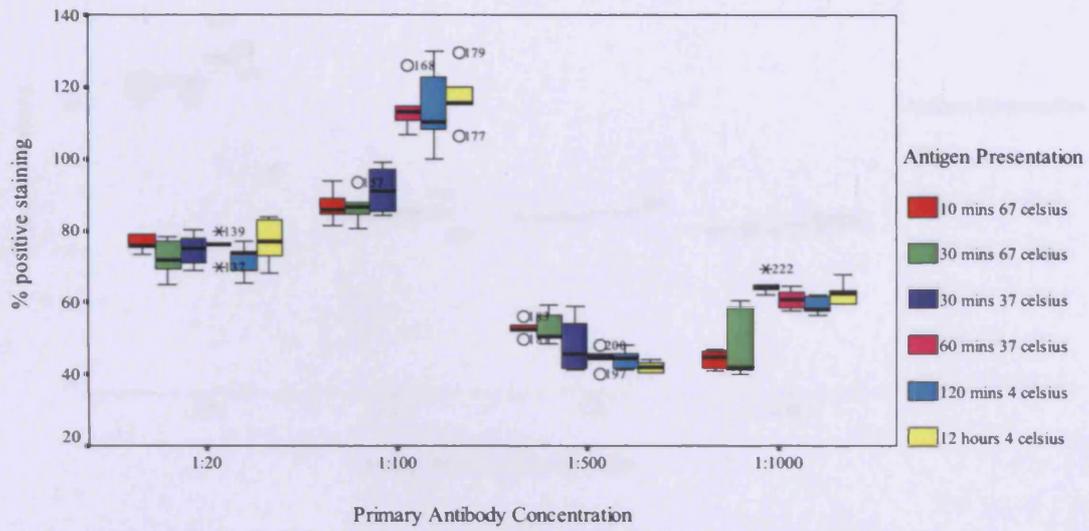


Figure 5-30. Staining of positive controls after 20 minutes antigen presentation.

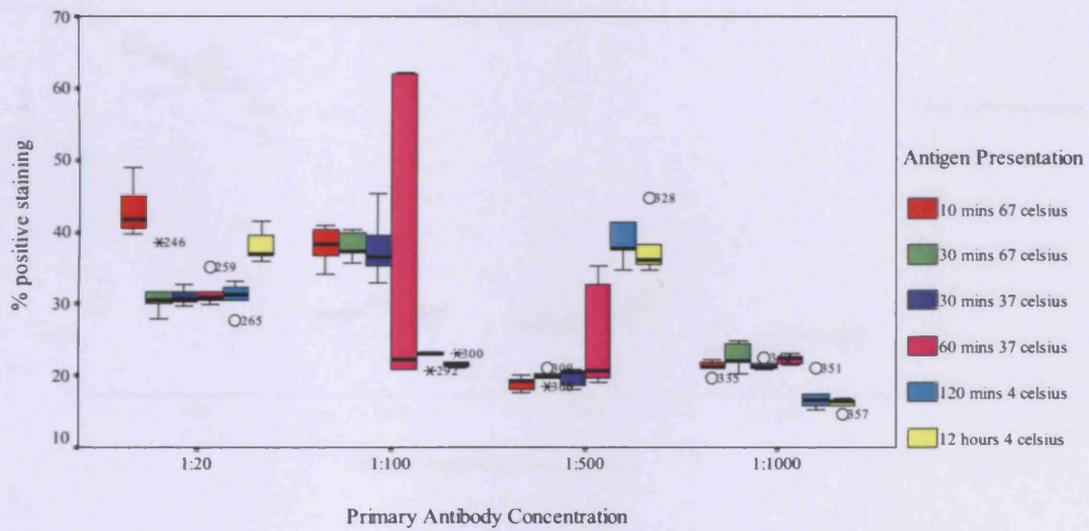


Figure 5-31. Staining of positive controls after 30 minutes antigen presentation.

This protocol provides optimal staining as reciprocally low levels of staining of negative controls occurred (Figure 5-32 to Figure 5-34).

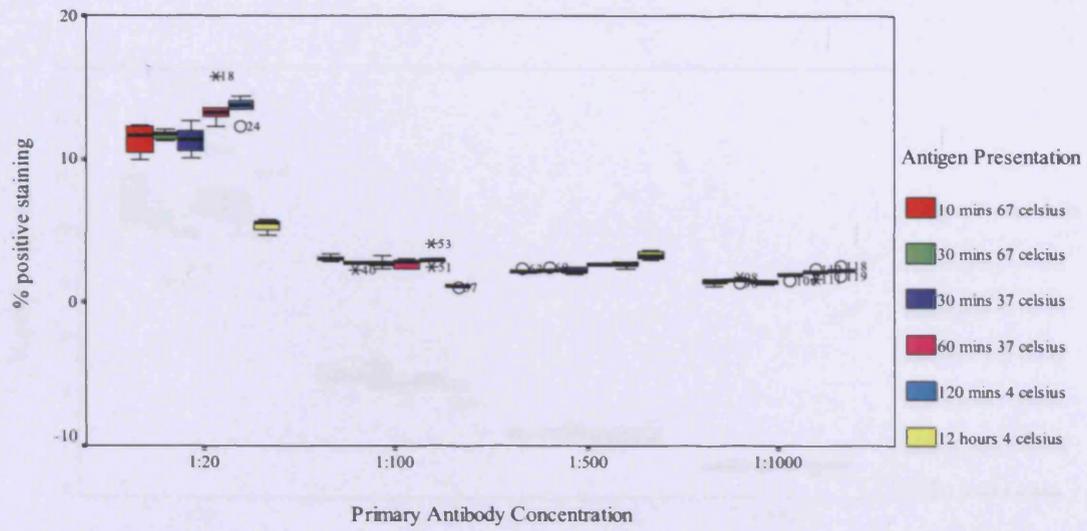


Figure 5-32. Staining of negative controls after 10 minutes antigen presentation.

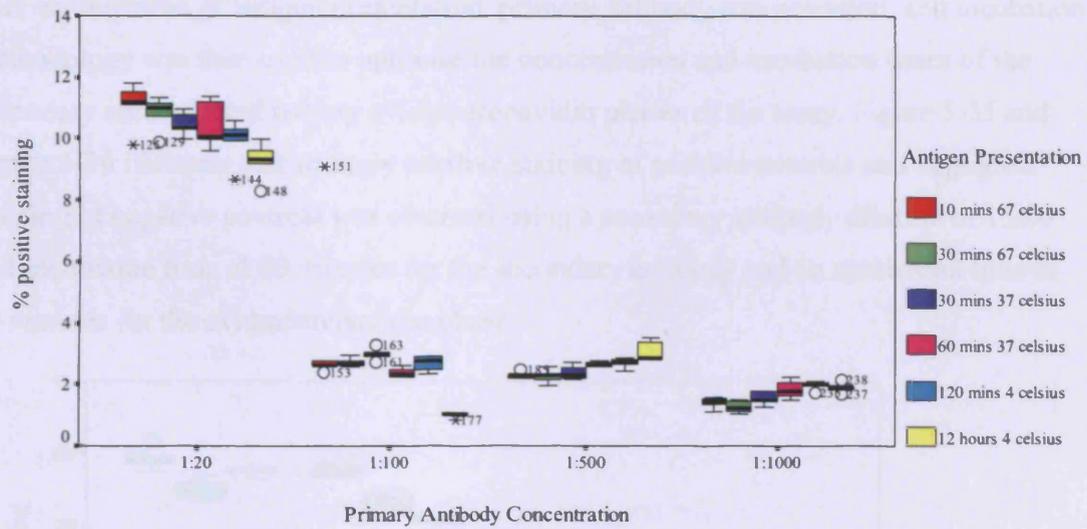


Figure 5-33. Staining of negative controls after 20 minutes antigen presentation.

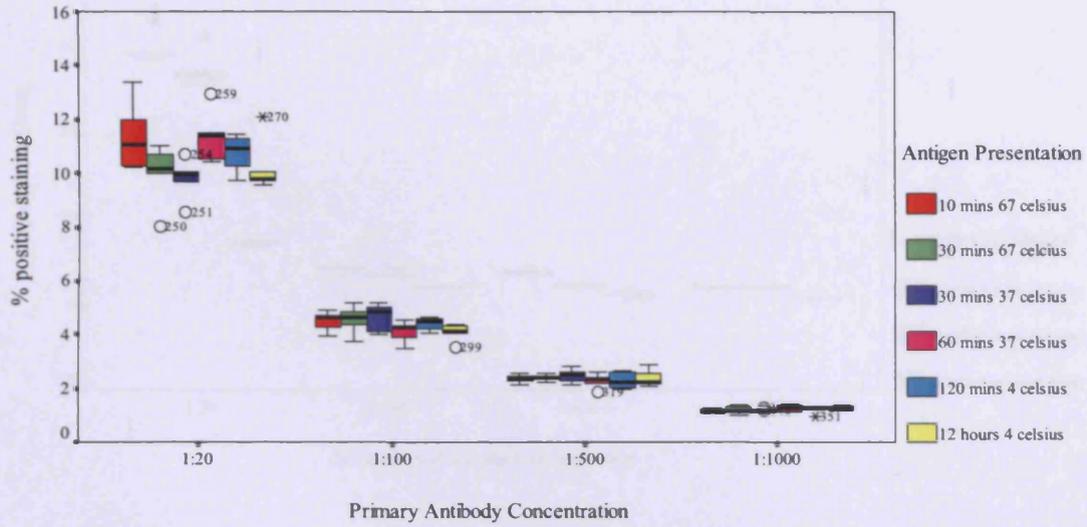


Figure 5-34. Staining of negative controls after 30 minutes antigen presentation.

This combination of antigen presentation, primary antibody concentration, and incubation methodology was then used to optimise the concentration and incubation times of the secondary antibody and tertiary avidin/streptavidin phases of the assay. Figure 5-35 and Figure 5-36 illustrate that strongly positive staining of positive controls and negligible staining of negative controls was obtained using a secondary antibody dilution of 1:200 and incubation time of 60 minutes for the secondary antibody and an incubation time of 90 minutes for the avidin/streptavidin phase.

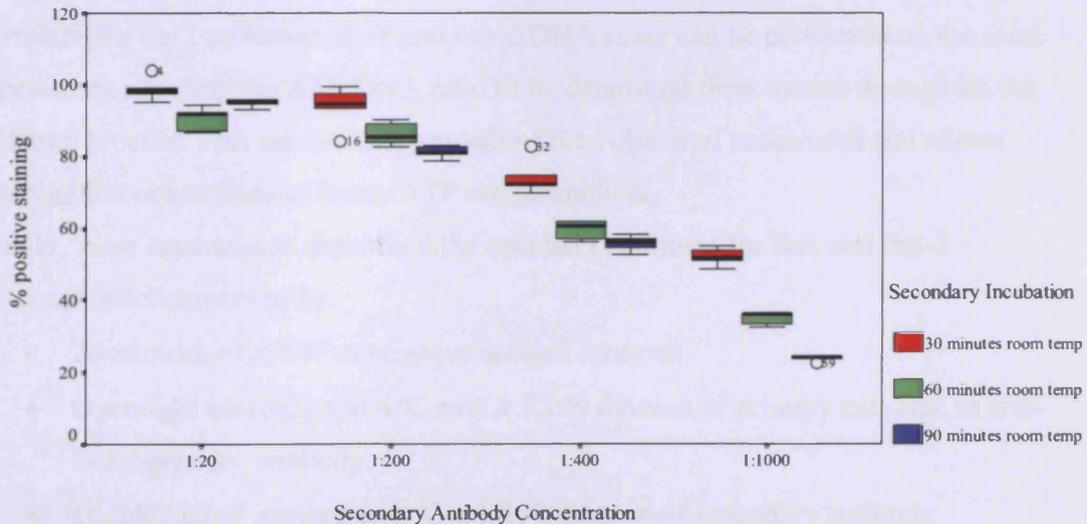


Figure 5-35. Staining of positive controls after 60 minutes tertiary antibody incubation.

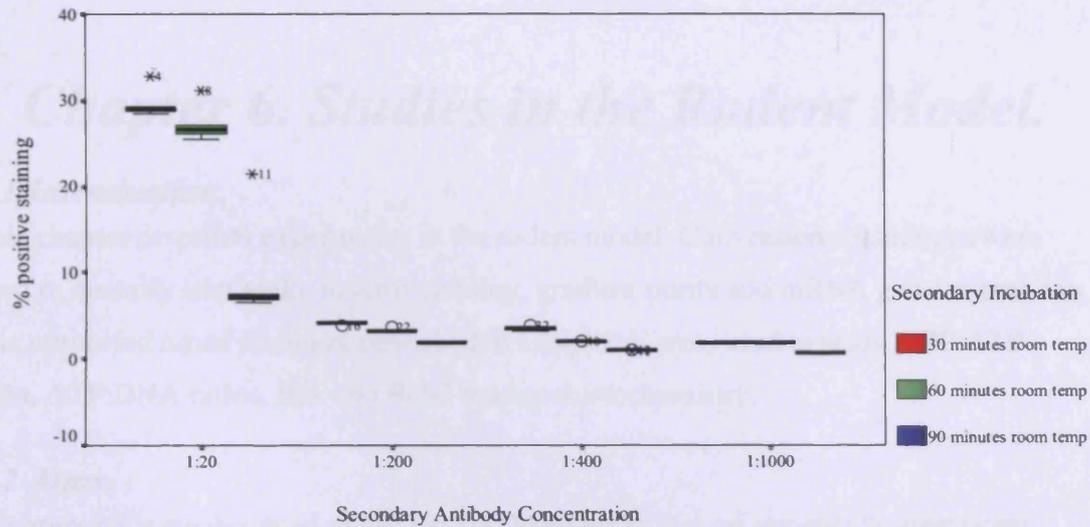


Figure 5-36. Staining of negative controls after 60 minutes tertiary antibody incubation.

### 5.5 Conclusion.

These preliminary experiments confirmed the feasibility of the Luciferase assay to determine the ADP:ATP ratio from tissues throughout the isolation process. However, the ATP chemoluminescent signal was dependent upon the volume of tissue assayed, the temperature and duration of tissue storage and individual variation in tissue ATP content. Adequate ATP signal could be obtained using single 10 $\mu$ m sections of pancreas or digest stored for up to 48 hours in liquid nitrogen, and from aliquots of ten or more islets upto 24 hours in cell culture.

Furthermore, the Luciferase assay and rapid DNA assay can be performed on the same supernatant allowing the ATP:DNA ratio to be determined from tissues throughout the isolation process. This ratio is independent of the volume of tissue used and allows meaningful comparisons of tissue ATP concentrations.

Finally, these experiments determined the optimal conditions for Bax and Bcl-2 immunohistochemistry to be:

- 20 minutes of 850W microwave antigen retrieval.
- Overnight incubation at 4°C with a 1:100 dilution of primary anti-Bax or anti-Bcl-2 primary antibody.
- 60 minutes of incubation with a 1:200 dilution of secondary antibody.
- 90 minutes for the avidin/streptavidin phase.

These optimised methods were then used throughout the remaining experiments using rodent, porcine and human pancreata.

## ***Chapter 6. Studies in the Rodent Model.***

### ***6.1 Introduction.***

This chapter describes experiments in the rodent model. Conventional techniques were used to quantify islet yield, *in vitro* viability, gradient purity and mRNA gene expression. The optimised novel methods, described in Chapter 5, were used to study ADP:ATP ratio, ATP:DNA ratios, Bax and Bcl-2 immunohistochemistry.

### ***6.2 Aims.***

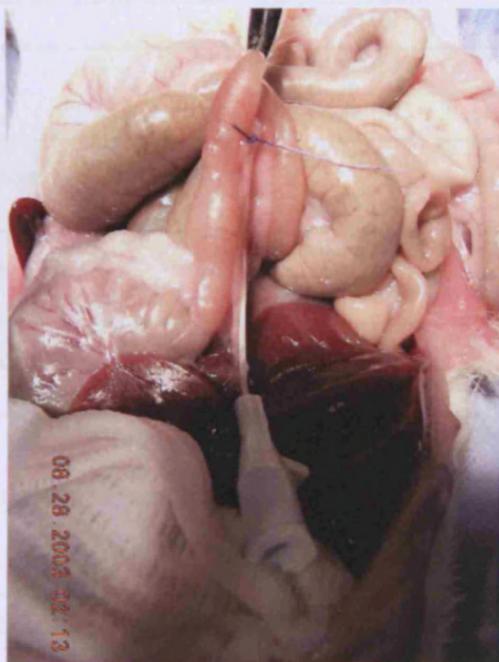
Experiments were designed to test the null hypotheses that no statistically significant difference in islet yield, gradient purity, viability, Bax and Bcl-2 gene expression and ADP:ATP or ATP:DNA ratio would be observed between the preservation methods studied.

### ***6.3 Methods.***

#### ***6.3.1 Rodent Pancreas Retrieval.***

Donor animals were 200-300g male inbred Wistar rats (Division of Biomedical Sciences, University of Leicester, U.K) housed in standard conditions and receiving a standard dietary regimen. Culling was by HMHO Schedule 1 approved cervical dislocation. Pancreas retrieval was performed expediently as follows. Following supine positioning on a cork board the abdomen was shaved and cleansed using 0.1% ethanol. A midline laparotomy allowed exposure of the abdominal viscera and pressure upon the inferior costal margin caused liver eversion cranially to expose the underlying biliary tract, pancreas and small bowel. The pancreas was dissected from its position within a circumferential duodenal loop and a tissue biopsy obtained and embedded in Tissuetek within flexible microwells prior to snap freezing in liquid nitrogen. A 26G×19mm cannula (Abbocath®-T, Abbott, Sligo, Rep. of Ireland) was positioned into the confluence of biliary and pancreatic ducts and this secured by a <sup>4</sup>/<sub>0</sub> silk suture (Mersilk®, Johnson and Johnson International) (Figure 6-1). For immediate islet isolation a haemostat was applied to the distal pancreatic duct to allow controlled distension of the pancreas and *in situ* distension was via introduction of 10ml of 2mg/ml collagenase solution (20mg collagenase(Serva Electrophoresis GmbH, Heidelberg, Germany; Cat. No 17456; Lot No 15022) in 10ml Eagles minimal essential medium (MEM)(Gibco)) over 20 seconds. The distended pancreas was excised from the circumferential duodenal loop

and placed into a Universal container on ice for immediate transport to the isolation laboratory.



**Figure 6-1. Rodent dissection demonstrating cannulation of the pancreatic duct.**

Where islet isolation was to be delayed until after a period of preservation a section of small bowel including the junction of common bile duct into bowel was isolated between  $4/0$  silk ligatures allowing the pancreas to be excised *en bloc* by division of the small bowel between the ligatures. The cannulated duct was then flushed with 0.5ml phosphate buffered saline to maintain duct patency and the pancreas was transferred into its allocated preservation method.

Prior to discarding the cadaver a biopsy was taken from the thymus lying in the anterior mediastinum. This was fixed in 4% formalin to be used as a positive control during subsequent immunohistochemistry (IHC).

### **6.3.2 Experimental Groups.**

Pancreata were randomised to experimental preservation protocols using combinations of warm and cold ischaemia and preservation solutions to mimic the retrieval and transportation times achieved in clinical human islet isolation.(Table 6-1).

PRESERVATION METHOD	COLD ISCHAEMIA DURATION
IMMEDIATE ISOLATION	0 HOURS (8)
UW COLD STORAGE ALONE	4 HOURS (8)
	8 HOURS (8)
ET-K COLD STORAGE ALONE	4 HOURS(8)
	8 HOURS (8)
UW TWO LAYER METHOD	8 HOURS AND 1 HOUR TLM (8)
	8 HOURS AND 4 HOURS TLM (8)
ET-K TWO LAYER METHOD	8 HOURS AND 1 HOUR TLM (8)
	8 HOURS AND 4 HOURS TLM (8)
UW COLD STORAGE WITH WARM ISCHAEMIA	30 MINS WARM ISCHAEMIA AND 4 HOURS COLD STORAGE (8)
	30 MINS WARM ISCHAEMIA AND 4 HOURS COLD STORAGE (8)

Table 6-1. Experimental protocols for the rodent studies. ()=n per group.

6.3.2.1 Static Cold Storage.

Pancreata subjected to periods of static cold storage were placed in sterile Universal containers (Sterilin, UK) in melting ice (4°C ) with 15mls of the respective preservation solution (Figure 6-2).



Figure 6-2. UW and ET-K solutions compared during rodent experiments.

After the allocated period of static cold storage the preservation solution was decanted and two small biopsies were taken; one snap frozen in Tissuetek in a flexible microwell and stored in liquid nitrogen for later ATP and DNA assays, the other fixed in 4%



After the allocated period of TLM preservation the pancreas was retrieved into a sterile Universal container and two small biopsies were taken; one snap frozen in Tissuetek in a flexible microwell and stored in liquid nitrogen for later ATP and DNA assays, the other fixed in 4% formalin for IHC. The pancreas was then distended with collagenase via the indwelling cannula as previously described and the isolation phase was begun. The perfluorodecalin was re-used by filtration through a 14 $\mu$ m filter (Millipore, UK) and autoclaving at 121°C for 40 minutes.

### ***6.3.3 Rodent Islet Isolation.***

In a Class II microbiological cabinet the pancreas was transferred to a Universal containing 10ml MEM in a waterbath at 38°C for 18 minutes to allow collagenase enzymatic digestion to proceed. Digestion was halted by decanting the supernatant from the Universal and the addition of 10 ml of MEM (4°C). The Universal was then vigorously shaken for 1 minute to facilitate mechanical pancreatic digestion. The pancreatic digest was poured through a sterile 500 $\mu$ m nylon mesh into a sterile 50 ml conical bottomed tube (Costar Corning, UK) to remove undigested tissue and the Universal and mesh washed into the conical tube with MEM supplemented with 5% bovine serum albumin (BSA) (Advanced Protein Products). The collected suspension was washed twice by centrifugation (270g, 1 minute, 4°C) and resuspension of the tissue pellet in MEM with 5%BSA. After the final washing the supernatant was aspirated leaving the digest pellet for purification, and a Drummond displacement pipette was used to extract a 200 $\mu$ l biopsy of digest into a flexible microwell. This was snap frozen and stored under liquid nitrogen for later assays.

### ***6.3.4 Density Gradient Purification of Rodent Islets.***

A 7ml discontinuous density gradient was used for digest purification and islet separation. This was comprised of three densities of BSA at 1.086, 1.079 and 1.056 g/cm<sup>3</sup>. BSA solutions were prepared by dilution of stock high density 1.1g/cm<sup>3</sup> BSA (Advanced Protein Products, U.K) with sterile distilled water and MEM, and densities were confirmed using a calibrated densitometer (Paar Scientific Ltd, UK). The digest pellet was resuspended in 4ml of 1.086g/cm<sup>3</sup> BSA by gentle pipetting and this was sufficient to provide the lower fraction in two 10ml sterile centrifugation tubes (Costar Corning, UK). 2ml of the resuspended digest was pipetted into the base of each tube and onto this 2ml of 1.079g/cm<sup>3</sup> BSA was layered with care not to mix the layers. A third

layer of  $1.056\text{g/cm}^3$  BSA was applied and finally 1ml of MEM to complete the gradient (Figure 6-5).



**Figure 6-5. Discontinuous BSA density gradients for rodent islet purification.**

Gradient tubes were centrifuged at  $270g$   $4^{\circ}\text{C}$  for 15 minutes to allow the digest components to migrate to their isodense points on the gradient. After centrifugation the islet fraction was extracted from the interface of the  $1.079\text{g/cm}^3$  and  $1.056\text{g/cm}^3$  layers by aspiration at this level using a 2ml pipette tip. Islets were transferred to a 50ml conical tube and washed of BSA by 2 successive centrifugations ( $270g, 4^{\circ}\text{C}$  for 5 minutes) and resuspension in 50ml MEM.

### **6.3.5 Islet Culture Purification.**

After isolation the islet pellet was resuspended in 10ml of RPMI culture medium (Gibco, UK) supplemented with 10%v/v fetal calf serum, 1%v/v penicillin, 1%v/v streptomycin, 2%v/v HEPES and 1%v/v pyruvate, and transferred to a large Petri dish. A Drummond pipette was used to extract a 200 $\mu\text{l}$  biopsy of the islet fraction which was streaked onto a small Petri dish. This was then examined under a light microscope  $\times 100$  and ten aliquots of 10 islets were handpicked from the biopsy using a drawn out glass micro-pipette. Islets were transferred to sterile Eppendorfs, snap frozen and stored under liquid nitrogen for later assays. The remaining islets were then placed in a cell culture incubator ( $37^{\circ}\text{C}$ , 5% $\text{CO}_2$ ) for overnight culture purification. After 14 hours of incubation islets were again biopsied using the method above, and the post-culture islets were used in the remaining quality control assessments (Figure 6-6).

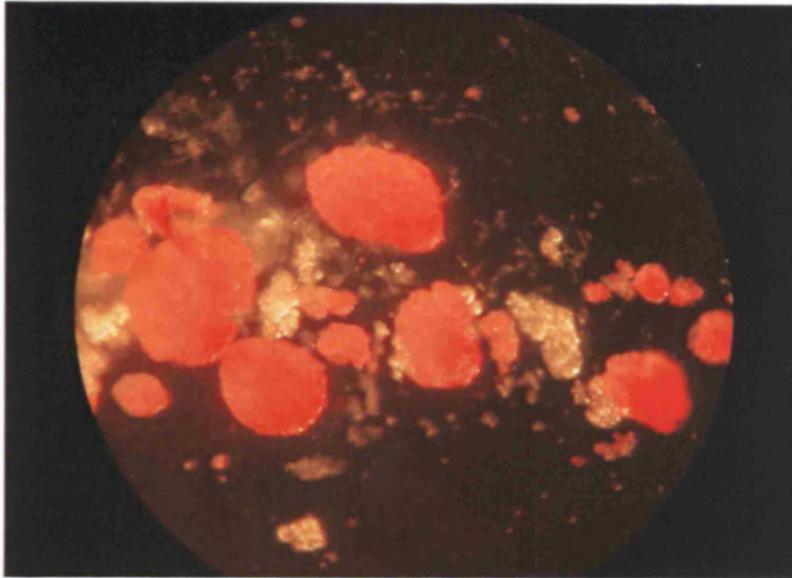


Figure 6-6. Rodent islets stained with dithizone - insulin granules within  $\beta$  cells staining red.

### 6.3.6 Islet yields.

Islets were removed from cell culture and five 100 $\mu$ l aliquots of culture medium streaked across a Petri dish using a Drummond pipette. To the streaks 20 $\mu$ l of 0.006%w/v dithizone (pH 7.8) was added with a Gilson pipette. Streaks were examined under a light microscope  $\times$  100 with a graticule (Cell Finder, Holland). Islets with diameters of greater than 50 $\mu$ m were counted in groups of 50 $\mu$ m ascending sizes. Islet counts were adjusted to IEQs and multiplied by the dilution factor 20 to generate a total IEQ per pancreas yield according to islet size.

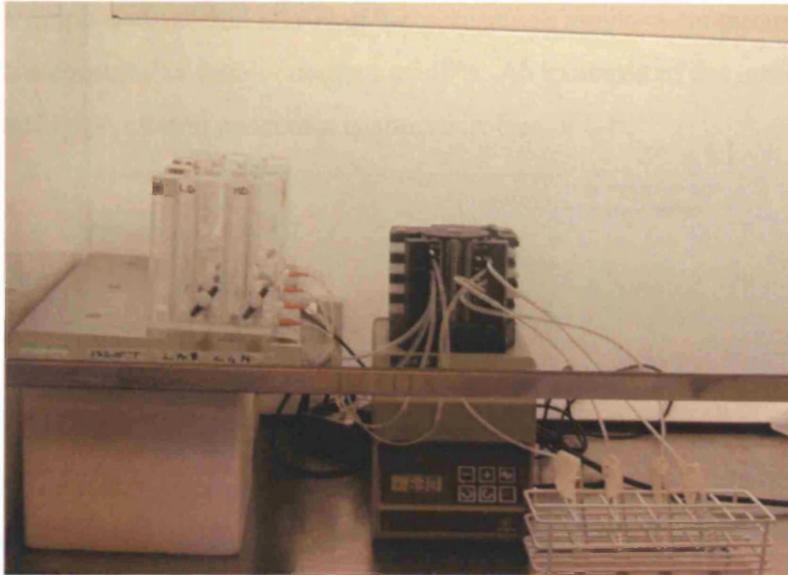
### 6.3.7 Gradient Purity.

An assessment of islet fraction purity was made by establishing the percentage of exocrine contamination in each 1ml gradient fraction, and comparisons made at the level of 60% insulin content. This is deemed to correspond to an acceptable islet yield from a continuous BSA minigradient.

#### 6.3.7.1 Continuous density gradient preparation.

Continuous linear mini-gradients were constructed after the pancreas had been processed to the digest stage. 5.2ml aliquots of high density (1.103g/cm<sup>3</sup>) and low density (1.075g/cm<sup>3</sup>) BSA were prepared by dilution of stock BSA (Advanced Protein Products, UK) with MEM and checked with a calibrated densitometer (Paar Scientific Ltd, UK). Each aliquot was added to a side of a two chamber gradient maker. A 1ml sample of digest was added to the bottom of a clear 12ml tube (Costar Corning, UK) and the

continuous density gradient layered onto this by running a roller pump and mixing the BSA densities with a magnetic flea in the base of the gradient maker (Figure 6-7).



**Figure 6-7. Apparatus for BSA continuous mini-gradients and measurement of gradient purity.**

Gradients were centrifuged for 5 minutes (500g, 22°C) to allow digest components to occupy their isodense points. Eleven 1ml aliquots were aspirated down each gradient, washed twice in MEM and resuspended in 2ml MEM in Eppendorfs. These were sonicated at 14MHz for three periods of 30 seconds (Soniprep150, MSE, UK). 1ml was then withdrawn and frozen in 1ml Zeigler reagent (90% v/v ethanol, 10%v/v 1M orthophosphoric acid, 5% v/v distilled water) at -20°C for insulin assay. The remaining 1ml was frozen at -20°C for amylase assay.

#### 6.3.7.2 *Amylase assay.*

Frozen gradient samples were thawed in a waterbath at 37°C. 200µl of the sample was mixed with 4 ml distilled water in a 10 ml tube (Costar Corning, UK) and returned to the water bath. To each tube a tablet of Phadebas reagent (Pharmacia Diagnostics, Sweden) was added and vortexed. This contained a water soluble cross-linked starch polymer which was hydrolysed by  $\alpha$ - amylase to generate a water soluble blue dye. Tubes were left for 15 minutes after which the reaction was ceased by the addition of 1ml 0.5M sodium hydroxide. After centrifugation at 1500g for 5 minutes the clear supernatant was aspirated into spectrophotometer cuvettes and the absorbance measured at 620nm wavelength against a blank cuvette containing ultra pure water. Amylase concentration was calculated from a series of standard absorbance curves. From the 11 samples the

cumulative percentage amylase concentration was calculated. A cumulative percentage insulin and amylase concentration was established for each 1ml portion of the continuous gradient, and a graph constructed allowing the percentage amylase contamination which coincided with a cumulative insulin content of 60%. An example of the cumulative results of in freshly processed pancreata is shown in Figure 6-8.

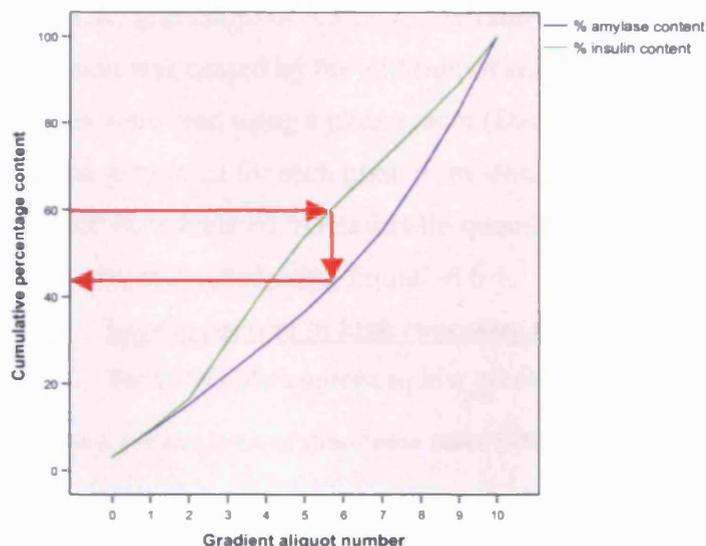


Figure 6-8. Cumulative insulin and amylase content in a freshly processed pancreas mini-gradient.

### 6.3.8 *In vitro* islet function.

Duplicate static incubations were performed after aliquots of 20 islets were handpicked from culture medium using a drawn out glass micro-pipette. Islets were placed onto 4µm mesh inserts (Costar Corning, UK) in a 12 well plate (Corning Costar, UK) containing 2ml per well of low concentration glucose solution (1.7mM; 66%v/v Geys balanced salt solution (GBSS), 33%v/v MEM, 0.05%w/v BSA). After 1 hour the mesh inserts were transferred to another 12 well plate containing low concentration glucose for 30 minutes, then to a plate containing high concentration glucose solution (25mM; 66%v/v GBSS, 33%v/v MEM, 0.05%w/v BSA, 0.3% w/v glucose) for 30 minutes and finally to low concentration glucose solution for 30 minutes. As the islets were moved between solutions, 1ml aliquots were aspirated from the wells and frozen at -20°C for insulin assay.

A solid phase two site enzyme linked immunosorbant assay (ELISA) kit was used to measure rodent insulin content. This used two monoclonal antibodies directed against different epitopes of rat insulin to generate a colour change.

The kit was supplied with standard insulin concentrations, and 25µl aliquots of these were added in duplicate to the first 16 wells of the 96 well assay plate. 25µl aliquots of the test samples were added in duplicate to the remaining wells. To each well 50µl of peroxidase conjugated anti-insulin antibodies were added, and the plate incubated for 2 hours. The plate was then washed in ultrapure water using a plate washer (Denley Wellwash, Denley, UK) and 200µl of 3,3',5,5' – tetramethylbenzidine was added. After 15 minutes the reaction was ceased by the addition of sulphuric acid to generate the end colour change. Plates were read using a plate reader (Denley, UK) at 450nm wavelength. A standard curve was generated for each plate from which the insulin content of the remaining wells could be calculated. After insulin quantification of the static supernatants, the SI was calculated using Equation 6-1.

**Insulin content in high concentration glucose**

**Mean insulin content in low glucose solution**

**Equation 6-1. Calculation of stimulation index following static incubations.**

***6.3.9 Apoptotic gene mRNA expression.***

RT-PCR methodology previously described for the measurement of mRNA in single human renal glomeruli(950) was used in this study. As an adjunct to this real time PCR analysis was performed to assess the concordance between these methods of mRNA quantification.

***6.3.9.1 Messenger RNA extraction***

Aliquots of 5 islets were handpicked from culture using a drawn out glass micro-pipette, immersed into 100µl of lysis binding buffer (Dyna, Bromborough, UK) and stored in sterile Eppendorfs at -80°C prior to mRNA extraction.

Prior to extraction samples were incubated with 50µg/ml proteinase K (Roche,Lewes, UK) for 1 hour at 37°C. After this 10µl oligo-dT paramagnetic Dynabeads<sup>®</sup> (Dyna, UK). were added and the reaction left to proceed at room temperature for 10 minutes. A magnet was applied to the sample Eppendorf which allowed the Dynabeads to bind tightly to mRNA whilst the supernatant was removed. The Dynabead bound mRNA was washed twice in wash buffer containing 1% lithium dodecyl sulphate (LiDS) and three times in buffer without LiDS (Dyna, UK) before being resuspended with the beads in 10µl DEPC treated water.

### 6.3.9.2 Reverse transcription.

Complementary DNA (cDNA) was synthesised from mRNA using avian myeloblastosis virus reverse transcriptase (RT) (Promega, Southampton, UK) and the oligo-dT of the Dynabeads<sup>®</sup> as a primer. 8µl of captured mRNA was incubated for 1 h at 42°C using a thermocycler in a final reaction volume of 25µl (5U reverse transcriptase, 1U RT buffer, 1mM dNTPs (Roche) and 25U RNAsin (Promega). A negative control comprising 2µl of captured mRNA without RT enzyme was included for each extraction.

### 6.3.9.3 Polymerase chain reaction.

PCR amplification of the endogenous gene  $\beta$ -actin was used to confirm the presence of cDNA and to assess the purity of the extracted mRNA. A negative and positive control was included for each reaction, containing no template and a previously amplified PCR product (dilution 1:50,000) respectively. The PCR reaction was performed in a 50µl reaction volume under oil and contained 1µl of template cDNA, 10 pmol  $\beta$ -actin forward and reverse primer (designed in-house, 5'forward AGATTACTGCCCTGGCTCCTA, 3' reverse CCACCAATCCACACAGAGTACTTG), 1U of JumpStart Taq polymerase (Sigma), 1U AJ buffer (100mM Tris pH 8.8, 100mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10mM ultrapure dNTPs (Roche), 100mM MgCl<sub>2</sub>, 2mg/ml ultrapure BSA, 100µM EDTA pH 8 and  $\beta$ -mercaptoethanol). Temperature cycling was performed using a thermocycler (Figure 6-9) as follows: 1 cycle of 95°C for 1 minute (template denaturation); 40 cycles of 95°C for 1 minute, 59°C for 30 seconds (primer annealing) and 72°C for 30 seconds (primer extension).

For PCR amplification of Bax cDNA the reaction mixture included 10 pmol Bax forward and reverse primers (Product No B8304, Sigma, 5'forward -GTT TCA TCC AGG ATC GAG CAG-, 3' reverse 5'-CAT CTT CTT CCA GAT GGT GA-); for amplification of Bcl-2 cDNA the mixture included 10 pmol Bcl-2 forward and reverse primers (Product No B9179, Sigma, 5'forward -CCT GTG GAT GAC TGA GTA CC-, 3' reverse 5'-GAG ACA GCC AGG AGA AAT CA-).



Figure 6-9. Thermocycler used for RT-PCR.



Figure 6-10. Gel electrophoresis used for PCR product analysis.

#### 6.3.9.4 Agarose gel electrophoresis.

PCR products were separated by electrophoresis on an agarose gel (3%w/v) and stained with ethidium bromide. To prepare the gel, agarose was heated in a microwave oven in TAE buffer (40mM Tris-acetate, 1mM EDTA). Ethidium bromide (0.5 $\mu$ g/ml) was added to the dissolved gel and the mixture poured into a gel cast tank and allowed to set for 1hr at room temperature (Figure 6-10). PCR product samples were diluted 5:1 with gel loading solution (0.25%w/v bromophenol blue, 50%v/v glycerol, 50%v/v TAE buffer) and were loaded into the gel alongside a 100 base pair control ladder. Gel electrophoresis performed using TAE running buffer at 150V for 30minutes. Gels were visualised under UV light and images captured using a gel documentation system. Electrophoretic banding of a 127bp PCR product was consistent with Bcl-2 expression, whilst banding of a 487bp PCR product was consistent with Bax expression.

#### 6.3.9.5 Real-time PCR.

Pre-optimised rat primers and probes were purchased from Applied Biosystems using their pre-developed assay range and assay-by-design service (Table 6-2). Constitutively expressed  $\beta$ -actin was used as an endogenous control to correct for variations in the concentrations of extracted mRNA. The PCR reaction was performed in a 50 $\mu$ l reaction volume and contained 1 $\mu$ l of template cDNA, 90nM forward and reverse primer, 1U of JumpStart Taq polymerase, 1U AJ buffer and 75nM ROX reference dye (Molecular Probes, Cambridge, UK). PCR was performed on a quantitative PCR instrument (Stratagene MX4000) using the thermal cycle: 1 cycle of 95 $^{\circ}$ C for 1 minute (template denaturation); 40 cycles of 95 $^{\circ}$ C for 30 seconds, 60 $^{\circ}$ C for 1 minute (primer annealing) and 72 $^{\circ}$ C for 30 seconds (primer extension). Quantitative measurements were recorded

by the instrument, which calculated a Ct value from an amplification plot. This was the cycle number at which the 'zero' threshold was crossed and an exponential increase in PCR product was detected. The amount of product in the original sample was indirectly proportional to the Ct value i.e. where high levels of expression were present in the original sample fewer PCR cycles were required to attain the threshold. A value of  $1/C_t$  was calculated for each gene product under test and expressed as a ratio over the expression of the endogenous control.

**Table 6-2. Primer and probe sequences used for real time PCR analysis.**

GENE	5' (FORWARD PRIMER)	3' (REVERSE PRIMER)	TAQMAN MGB PROBE (FAM DYE-LABELLED)
RAT B-ACTIN	AGATTACTGCCCTGGC TCCTA	CCACCAATCCACACAGAGTACT TG	ACCATGAAGATCAAGATC AT
RAT BAX	TGGCAGCTGACATGTT TGC	CCAGCCCATGATGGTTCTGATC	ACAGGGCCTTGAGCACC
RAT BCL-2	FOR THIS PRE-DEVELOPED ASSAY, THE START POINT IS 773 AND THE STOP POINT IS 876 WITHIN THE REFSEQ SEQUENCE NM_016993 (PRIMER SEQUENCES NOT AVAILABLE).		

### **6.3.10 Apoptotic protein immunohistochemistry.**

#### **6.3.10.1 Sample preparation and generic methods.**

Formalin fixed biopsies were placed in a tissue cassette and embedded in paraffin wax. Nine 4µm serial sections were cut from each block using a microtome and floated in a waterbath to allow mounting on silane coated slides. Two 4µm sections were cut from the thymic biopsy of each rat studied to act as positive controls. Slides were dried at 37°C for 24 hours before staining as described in Table 6-3

SECTION NUMBER	STAINING PROTOCOL
1	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE
2	1 <sup>ST</sup> BAX IHC
3	1 <sup>ST</sup> BCL-2 IHC
4	BAX NEGATIVE CONTROL
5	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE
6	2 <sup>ND</sup> BAX IHC
7	2 <sup>ND</sup> BCL-2 IHC
8	BCL-2 NEGATIVE CONTROL
9	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE

**Table 6-3. Serial section staining protocol for Bax and Bcl-2 immunohistochemistry.**

Slides were de-waxed by serial immersions in xylene (2x 3 minutes), 99% industrial methylated spirit (IMS) (2x 3 minutes), 95% IMS (2x 3 minutes) and ultra-pure water (2x 3 minutes). Antigen retrieval was by microwave (850W) heating in 10mM 1%w/v citrate buffer (pH 6) for 20 minutes followed by 40 minutes of room temperature cooling.

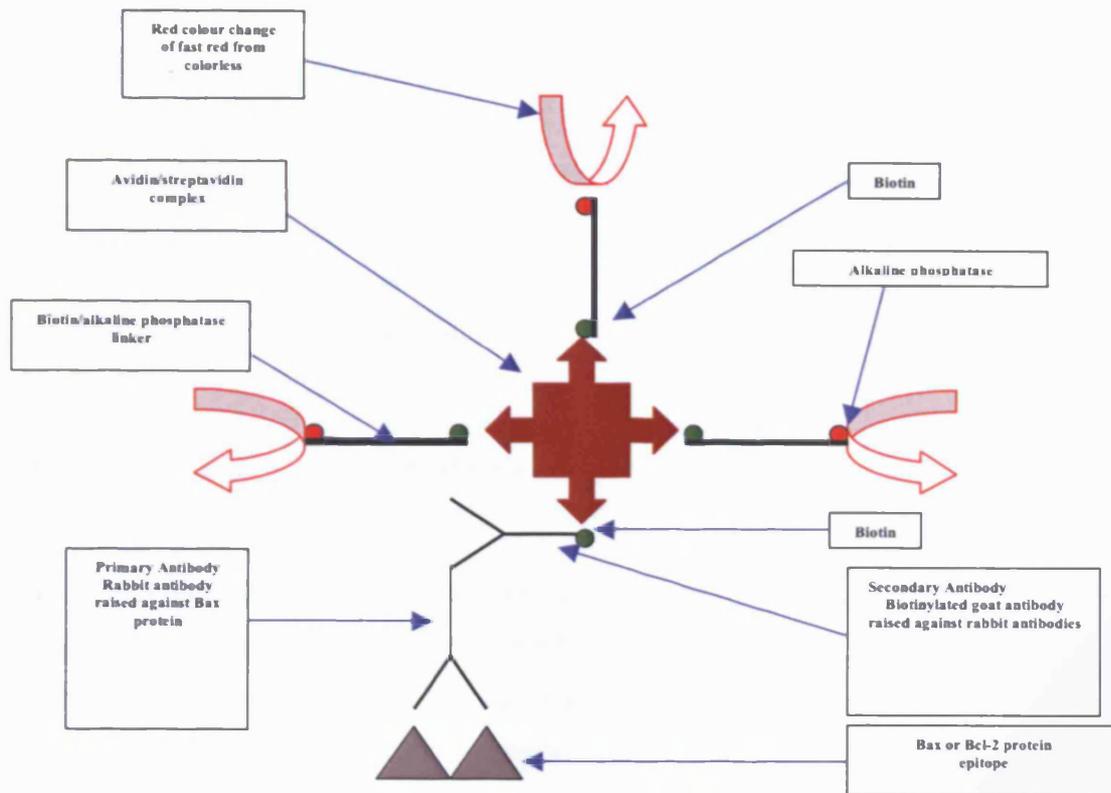
#### 6.3.10.2 Bax and Bcl-2 immunohistochemistry.

Bax and Bcl-2 immunohistochemistry was undertaken using the methods and antibody concentrations which were optimized in preliminary experiments (*vide supra* 5.4.2). De-waxed slides were washed in TRIS buffered saline (TBS)(pH 7.65; TRIS 20mM, NaCl 137mM, HCl 1M) and blocked with 50µl of a 1:20 dilution of normal goat serum (NGS) in blocking solution (3%w/v Bovine serum albumin, 0.1% w/v Triton x-100, 100ml TBS) for 10 minutes. NGS was then poured from the slides and 100µl of a 1:100 dilution of primary rabbit anti-rat Bax polyclonal antiserum added to each (DB Pharmingen, Lot No.554106) for Bax IHC. For Bcl-2 IHC this was replaced by 100µl of a 1:100 dilution of primary rabbit anti-rat Bcl-2 polyclonal antiserum (DB Pharmingen, Lot No.554087). Slides were placed in a darkened covered slide tray for overnight incubation at 4°C. The primary antibody solution was poured from each slide and 100µl of a 1:200 dilution of secondary biotinylated goat anti-rabbit polyclonal antiserum added to each (Stressgen Biotechnologies, Victoria, Canada. Lot No. SAB-303). Slides were covered and incubated at 4°C for 1hour. The secondary antibody was poured from each slide and the

sides racked and washed in TBS for 5 minutes. 100µl of the tertiary phase avidin/streptavidin complex and biotin/alkaline phosphatase linkers was applied to each slide and the covered slides left at room temperature for 90 minutes. Slides were then racked in a Hellendahl and washed in ultrapure water on a rocking platform for 5 minutes. The wash was then poured away and replaced with veronal acetate buffer (VAB) (pH 9.2; 0.39%w/v sodium acetate trihydrate, 0.59%w/v sodium barbitone, 0.58%w/v sodium chloride) for a further 5 minutes. Slides were then transferred to a Hellendahl of filtered red developer solution (0.05% w/v naphthol ASBI phosphate in 500µl dimethylfemanide, 0.05%w/v fast red, 0.02% levamisole) for 60 minutes to allow the final colour change to develop. Slides were washed in ultra pure water for 5 minutes, counterstained with haematoxylin for 5 minutes and rewashed before mounting using an aqueous mountant and cover slips.

#### *6.3.10.3 Preparation of controls.*

Positive controls comprised thymic tissue sections from the same animals from which the pancreata under study were procured. Thymic tissue was selected as the positive control due to the high rates of T cells apoptotic cell death during the process of T cell clonal deletion(951). The presence of staining in positive controls was desirable and confirms function of the indirect sandwich staining protocol used. Negative controls comprised pancreatic sections not exposed to the primary antibody. Instead 100µl of mixed rabbit antibodies (DB Pharmingen) were applied. The rest of the staining protocol for controls was identical to the studied slides. It was desirable to have no staining of negative controls to indicate that staining of study slides was entirely due to binding of Bax and Bcl-2 epitopes by the primary antibody. Where staining of the negative control occurred, positive staining of the test sections was quantified after first discarding the background staining seen in the negative control (Figure 6-11)



**Figure 6-11.** The indirect sandwich system of Bax and Bcl-2 immunohistochemistry.

#### 6.3.10.4 Insulin immunohistochemistry.

A three step indirect system was used for insulin immunohistochemistry. Endogenous peroxidase activity in dewaxed slides was blocked by immersion in 6% hydrogen peroxidase for 10 minutes. Slides were washed in TBS for 5 minutes and further blocked with 50 $\mu$ l of a 1:20 dilution of NGS in blocking solution (3%w/v Bovine serum albumin, 0.1% w/v Triton x-100, 100ml TBS) for 10 minutes. NGS was poured away and 100 $\mu$ l of a 1:50 dilution of the primary mouse anti-insulin antibody added to each (HB124 in house stock) for 30 minutes at room temperature. After washing in TBS 100 $\mu$ l of secondary biotinylated goat anti-mouse antibody was applied for 30 minutes and the sections rewashed in TBS. 100 $\mu$ l of the streptavidin and biotin/horseradish peroxidase detection complex (DAKO, UK) was applied for 30 minutes. Slides were then washed in TBS for 20 minutes and 100 $\mu$ l of the chromogen 3,3'-diaminobenzidine (DAKO, UK) applied to each to generate the brown immunoprecipitate. Slides were then counterstained using haematoxylin and mounted using aqueous mountant and cover slips.

#### **6.3.10.5 Bax and Bcl-2 semiquantification.**

Slides were randomised for assessment two blinded observers. Slides were assessed by light microscopy x 40 magnification (Nikon, Japan). Islets were identified by the position of positive insulin staining, and islet and acinar tissues separately assessed for the presence of dark red positive Bax and Bcl-2 staining. Ten microscope fields of both islet and acinar tissues were assessed for total cell number and number of positively stained cells. The proportion of positive staining was calculated as a percentage of the means of these values.

#### **6.3.11 Determination of ADP:ATP ratio.**

##### **6.3.11.1 Sample preparation.**

The optimal parameters for sample storage and methods for ATP:ADP analysis had been established during preliminary experiments (*vide supra* 5.2.2).

Frozen pancreas biopsies were removed from their freezing vials under liquid nitrogen storage and rapidly transferred into a cryostat (-20°C). All tissue handling was performed using metal forceps to prevent excessive warming of the samples. The flexible microwell encasing the biopsy was cut away using a scalpel blade (Swann Morton, UK) and the sample adhered in Tissuetek and mounted on cork by snap freezing in liquid nitrogen. Ten serial 10µm frozen sections were cut using the cryostat and transferred to individual pre-cooled sterile Eppendorfs. Sections were then stored temporarily under liquid nitrogen until required for ADP:ATP assay which proceeded expediently.

Samples of ten pre-and post-culture islets had previously been handpicked into Eppendorfs and stored under liquid nitrogen. Triplicate specimens from each pancreas at each phase of isolation were subjected to the assay.

##### **6.3.11.2 ADP:ATP Assay.**

Reagents were provided in an Apoglow™ kit (BioWhittaker, UK) and were allowed to warm to room temperature before commencing the assay. To the Eppendorf containing the sample 100µl of RPMI (Sigma, UK) was added and vortexed for ten seconds. 100µl of nucleotide releasing reagent was added and the reaction allowed to proceed at room temperature for 5 minutes. 180µl was transferred from the Eppendorf into a white walled DNase treated luminometer tube, 20µl of ATP monitoring reagent (firefly luciferase, D-luciferin, 50mg BSA, 0.5 mmol magnesium acetate, 0.1µmol inorganic pyrophosphate) was rapidly added and the luminometer (Wallac Series 1900, UK) cassette turned to

allow a reading to be obtained in mV on a chart recorder (Wallac, UK) (Figure 6-12 and Figure 6-13).



Figure 6-12. Luminometer cassette for ATP:ADP assay.

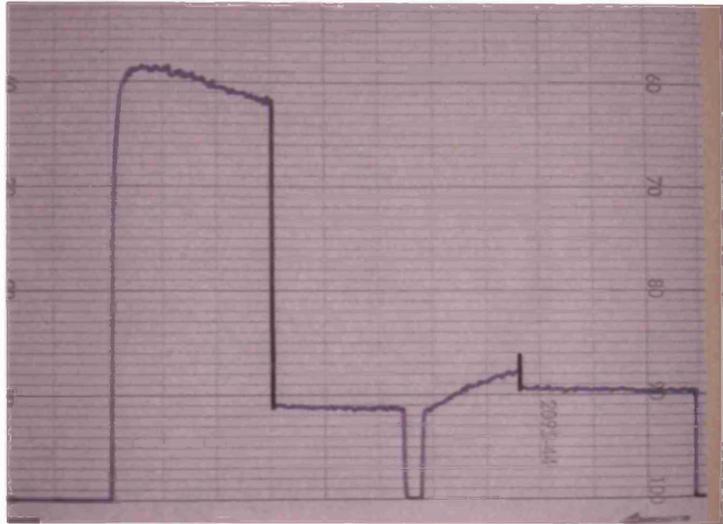


Figure 6-13. Chart recorder tracing of ATP:ADP luminescence.

After the initial rapid peak of ATP luminescence had been recorded the reaction was left to decay for 10 minutes before a further baseline reading was obtained. 20 $\mu$ l of ADP converting reagent was then added to generate a second peak of ATP luminescence on the recorder. ADP:ATP ratios were calculated from the readings made using the equations previously described. After completion of the assay 500 $\mu$ l of the supernatant was added to a sterile Eppendorf with 1.5ml of DNA assay buffer (pH 7.4; 2M sodium chloride, 0.05M disodium hydrogen phosphate, 2mM EDTA) and frozen for DNA assay (-20°C).

### 6.3.12 Determination of ATP:DNA ratio.

Samples of frozen supernatant from the firefly luciferase assay were thawed at room temperature for 30 minutes. After vortexing for 10 seconds 400 $\mu$ l was transferred to a sterile DNase treated Eppendorf and 2.6ml of fluorochrome reagent was added (1 $\mu$ g/ml Hoechst dye 33258 (Sigma) in NTE buffer). The reaction was left to proceed for 30 minutes at 30°C incubation in a waterbath. 1ml was then transferred to a DNase treated fluorometer cuvette and read on a fluorometer at wavelength of 350nm excitation and 455nm emission (Figure 6-14).

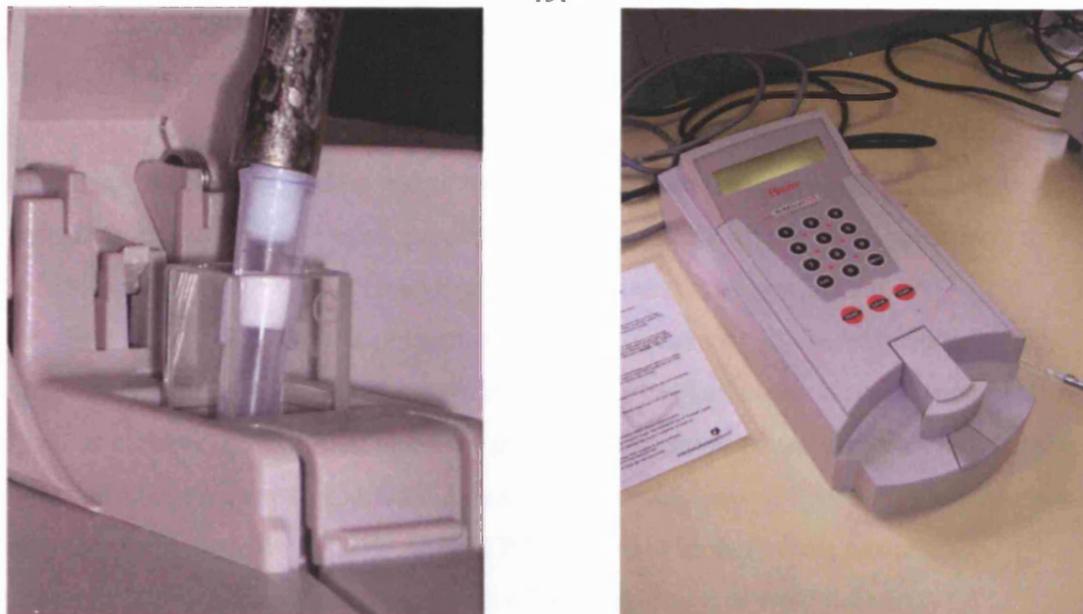


Figure 6-14. Luminometer used for DNA assay.

A serial dilution of 50 $\mu$ g/ml calf thymus DNA (Sigma D1501) in DNA assay buffer was used to generate standards from which standard curve was plotted. Sample fluorescence readings in mV were then converted to DNA concentrations by comparison with the standard curve.

The ATP concentration generated by the firefly luciferase assay was used to calculate the ATP:DNA ratio for each sample.

#### **6.4 Statistical Methodology.**

A one way analysis of variance (ANOVA) was used to compare mean islet yield, gradient contamination, stimulation indices, expressed levels of Bax, Bcl-2 and the endogenous control  $\beta$  actin. This test is used to compare three or more group means and 11 different preservation protocols were studied; rendering the preservation method a *between* subjects factor.

A two factor mixed ANOVA was used to compare the mean rates of Bax and Bcl-2 positive staining in islet and acinar tissues, mean ADP:ATP ratio and mean ATP:DNA ratio. This is because each pancreas was subjected to a different preservation protocol, but was also subjected to IHC, ATP bioluminescence and DNA assay at four different phases of islet isolation. Hence the preservation method is a *between* subjects factor and the phase of islet isolation is a *within* subjects factor upon which repeated measurements were made. Two factor ANOVA generates F statistics by comparing the variability *between* groups with the variability *within* groups.

ANOVA will determine if a statistically significant difference exists between *any* mean values but does not inform about precisely which mean values these are. Therefore, for all experiments a post hoc Tukey test was performed to allow a pairwise comparison of the group means. The Tukey test generates a table with means values that **do not differ significantly** occupying the same subset. Conversely, where the mean results occupy different subsets they are deemed to differ with statistical significance.

Finally a factor analysis was performed using the results of all the experiments. This technique seeks to establish if results in a series of variables can be explained by a smaller number of dominant latent variables. More potent latent variables have higher correlation coefficients (approaching 1.0) in factor analysis. Of particular interest was the correlation between ADP:ATP and ATP:DNA ratios in pancreatic tissue after preservation with established measures of islet yield and *in vitro* function.

## 6.5 Results.

### 6.5.1 Islet yields.

There were significant differences in mean islet yield between some preservation methods ( $F(10,87)= 23.3; p<0.001$ ). Figure 6-15 illustrates mean islet yield (total IEQ per pancreas) for each preservation method studied and the distribution of islet sizes obtained after islet isolation.

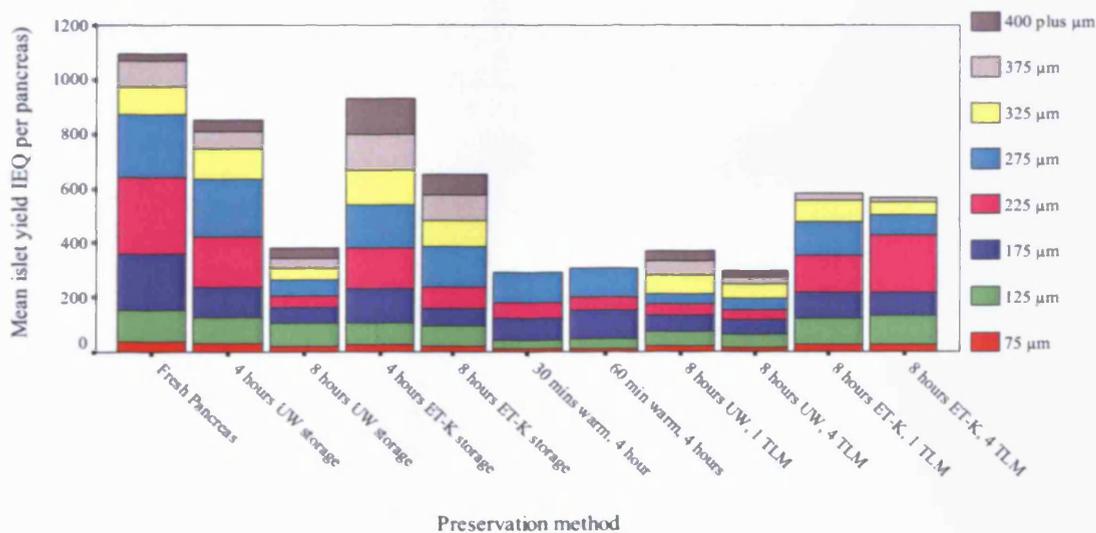


Figure 6-15. Stacked bar chart demonstrating islet yield and sizes from pancreata using different preservation methods.

Table 6-4 can be viewed as a league of increasing mean islet yields with a lack of subset overlap signifying a significant difference between preservation groups.

Preservation method	Subset					
	1	2	3	4	5	6
30 mins warm, 4 hours UW storage	292					
8 hours UW, 4 TLM	295	295				
60 min warm, 4 hours UW storage	309	309				
8 hours UW, 1 TLM	371	371	371			
8 hours UW storage	382	382	382	382		
8 hours ET-K, 4 TLM		566	566	566		
8 hours ET-K, 1 TLM			581	581	581	
8 hours ET-K storage				650	650	
4 hours UW storage					849	849
4 hours ET-K storage						931
Fresh Pancreas						1093

Table 6-4. Significant differences in islet IEQ yields from preservation groups.

6.5.2 Gradient purity.

There were significant differences in mean amylase contamination between preservation methods ( $F(10,99)= 15.1$ ;  $p<0.001$ ). Figure 6-16 illustrates the mean amylase contaminant concentration, at 60% cumulative insulin content, for each preservation method studied.

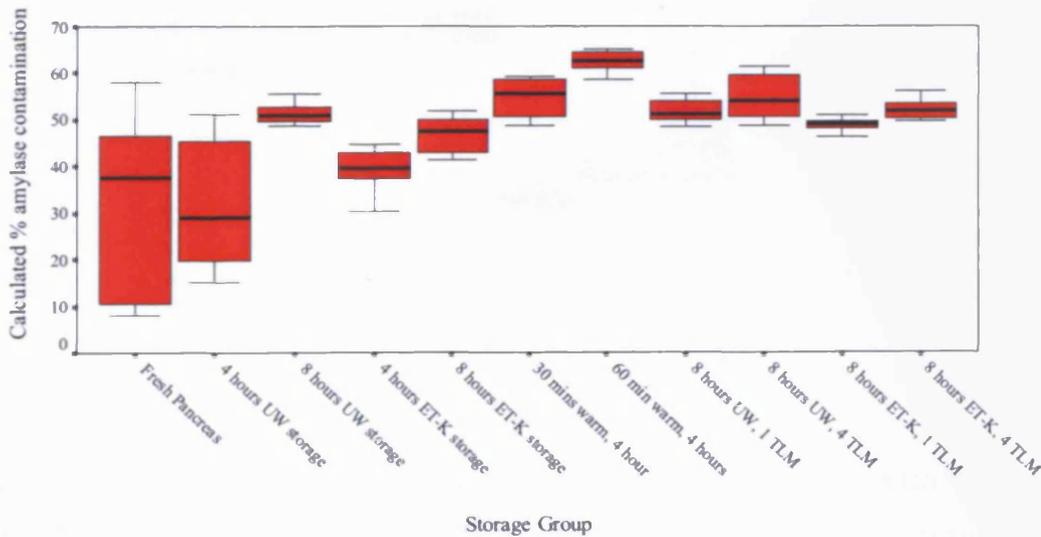


Figure 6-16. Calculated % amylase contamination at a 60% insulin content by different storage methods.

Table 6-5 is a league of increasing mean gradient contamination with a lack of subset overlap denoting a significant difference between preservation groups.

Storage Group	Subsets			
	1	2	3	4
4 hours UW storage	32.0			
Fresh Pancreas	33.6			
4 hours ET-K storage	39.3	39.3		
8 hours ET-K storage		47.1	47.1	
8 hours ET-K, 1 TLM		48.6	48.6	
8 hours UW storage			51.1	51.1
8 hours UW, 1 TLM			51.7	51.7
8 hours ET-K, 4 TLM			51.9	51.9
8 hours UW, 4 TLM			54.7	54.7
30 mins warm, 4 hours UW storage			54.8	54.8
60 min warm, 4 hours UW storage				62.3

Table 6-5. Significant differences in gradient contamination between preservation groups.

6.5.3 Islet in vitro function.

There were significant differences in mean SI between each preservation method when assessed by static incubation ( $F(10,99)= 29.8$ ;  $p<0.001$  (Figure 6-17).

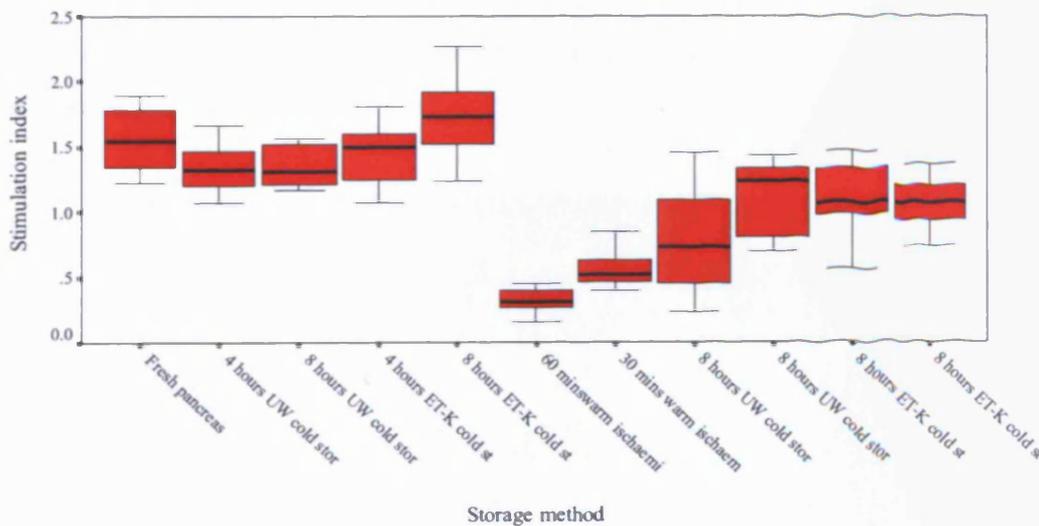


Figure 6-17. Islet stimulation indices by static incubation achieved after differing methods of preservation. Table 6-6 shows increasing mean SI from static incubations and a lack of subset overlap again denotes a significant difference between preservation methods.

Storage method	Subsets						
	1	2	3	4	5	6	7
60 mins warm ischaemia, 4 hours UW cold storage	.32						
30 mins warm ischaemia, 4 hours UW cold storage	.56	.56					
8 hours UW cold storage, 1 hour TLM		.74	.74				
8 hours ET-K cold storage, 4 hours TLM			1.05	1.05			
8 hours ET-K cold storage, 1 hour TLM			1.07	1.07			
8 hours UW cold storage, 4 hours TLM				1.12	1.12		
4 hours UW cold storage				1.33	1.33	1.33	
8 hours UW cold storage				1.34	1.34	1.34	
4 hours ET-K cold storage					1.45	1.45	1.45
Fresh pancreas						1.56	1.56
8 hours ET-K cold storage							1.73

Table 6-6. Differences in mean stimulation index assessed by static incubation.

### 6.5.4 Apoptotic gene mRNA expression.

#### 6.5.4.1 Reverse transcriptase polymerase chain reaction

Duplicate extractions and the use of a  $\beta$ -actin endogenous control demonstrated that pure mRNA could be consistently extracted from rodent islets. Figure 6-18 is a representative gel of  $\beta$ -actin amplification of cDNA synthesised from mRNA extracted from rat islets after 3 preservation methods.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

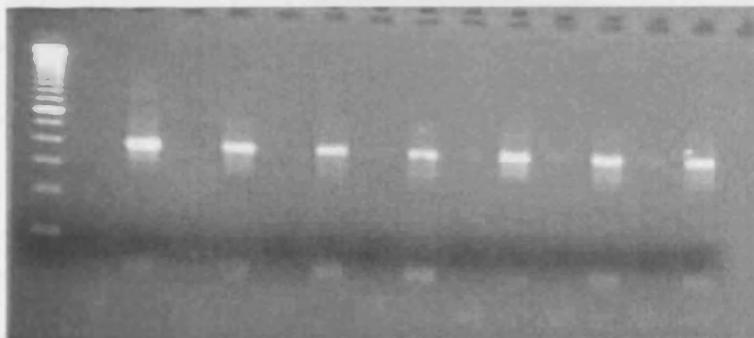


Figure 6-18. Example agarose gel of  $\beta$  actin amplification from 3 preservation groups.

(Lane 1: 100bp DNA ladder. Lane 2: no template control. Lane 3:  $\beta$ -actin positive control. Lane 4: 4 hours UW solution minus RT. Lane 5: 4 hours UW solution plus RT. Lane 6: 8 hours ETK solution minus RT. Lane 7: 8 hours ETK solution plus RT. Lane 8: 60 minutes warm ischemia minus RT. Lane 9: 60 minutes warm ischemia plus RT. Lane 10: 4 hours UW solution minus RT. Lane 11: 4 hours UW solution plus RT.

Lane 12: 8 hours ETK solution minus RT. Lane 13: 8 hours ETK solution plus RT. Lane 14: 60 minutes warm ischemia minus RT. Lane 15: 60 minutes warm ischemia plus RT.)

The consistent electrophoretic banding pattern between the groups represents reproducible amplification of the  $\beta$ -actin control cDNA in the positive control and test groups, and absence of  $\beta$ -actin amplification in the negative control groups.

However, during RT-PCR analysis of both Bax and Bcl-2 mRNA expression significant differences in the expression of the  $\beta$ -actin endogenous control was observed. This included positive  $\beta$ -actin signal being generated in a negative control sample containing no cDNA template.

#### 6.5.4.2 Real-time PCR.

$\beta$ -actin, Bax and Bcl-2 expression could be detected in isolated rat islet mRNA from all storage conditions using real time PCR. Figure 6-19 is a representative amplification plot illustrating the exponential increases in PCR product detection at different threshold counts.

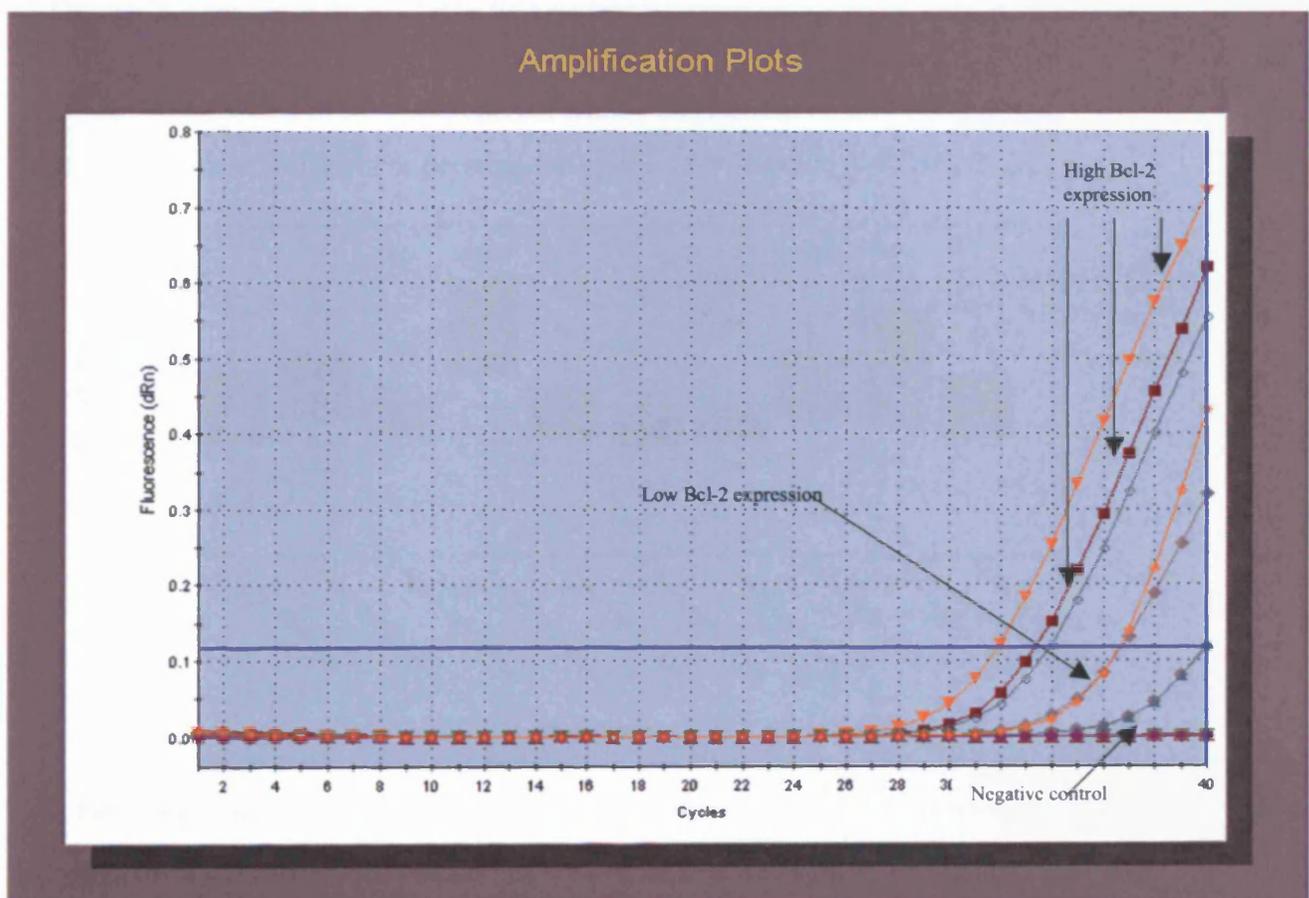


Figure 6-19. Representative real time PCR amplification plot of Bcl-2 expression.

Expression of Bax and Bcl-2 differed between groups, with a tendency to a reduced level of Bax, and increased level of Bcl-2 in TLM preserved groups. This equated to a reduced Bax/Bcl-2 ratio in these groups. (Figure 6-20).

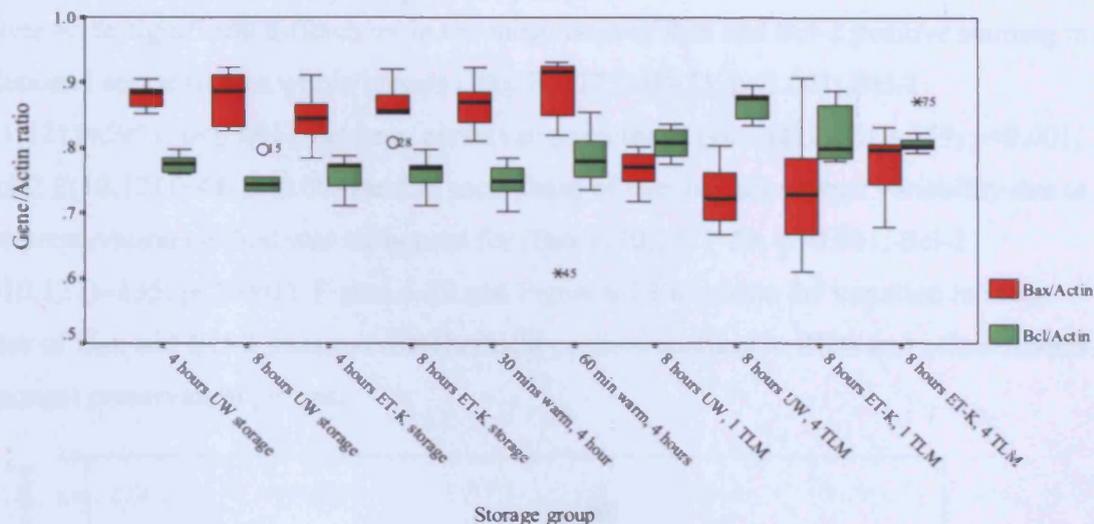


Figure 6-20. Variation in Bax and Bcl2 mRNA expression between storage groups assessed by real time PCR.

However comparison of the expression of  $\beta$  actin endogenous control by ANOVA showed significant differences between the groups ( $F(9,70)=2.5$ ;  $p=0.015$ ) (Figure 6-21).

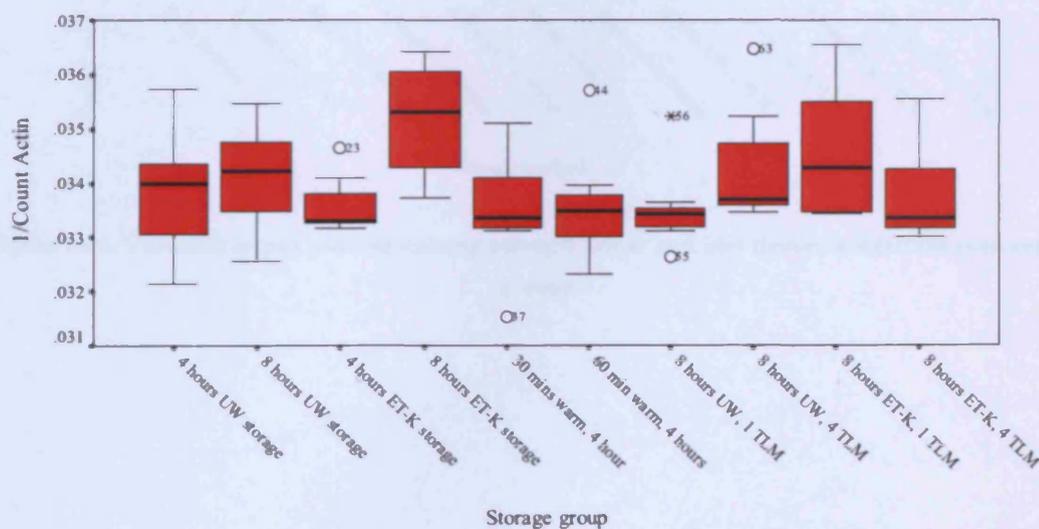


Figure 6-21. Variation in expression of the endogenous control beta actin between storage groups.

Though there appears to be a reduced Bax/Bcl-2 ratio in TLM preserved groups; it is impossible to draw any meaningful conclusions about the expression of Bax and Bcl-2

between the preservation groups due to significant differences in the expression of the  $\beta$ -actin endogenous control.

### 6.5.5 Immunohistochemistry of apoptotic proteins.

There were significant differences in the mean rates of Bax and Bcl-2 positive staining in islets and acinar tissues within groups (Bax  $F(1,121)=1973$ ;  $p<0.001$ ; Bcl-2  $F(1,121)=5953$ ;  $p<0.001$ ), between preservation methods (Bax  $F(10,121)=659$ ;  $p<0.001$ ; Bcl-2  $F(10,121)=44$ ;  $p<0.001$ ) and at each phase of islet isolation when variability due to the preservation method was accounted for (Bax  $F(10,121)=53$ ;  $p<0.001$ ; Bcl-2  $F(10,121)=135$ ;  $p<0.001$ ). Figure 6-22 and Figure 6-23 illustrate the variation in mean rates of Bax and Bcl-2 immunohistochemical positive staining in islets and acinar tissues amongst preservation groups.

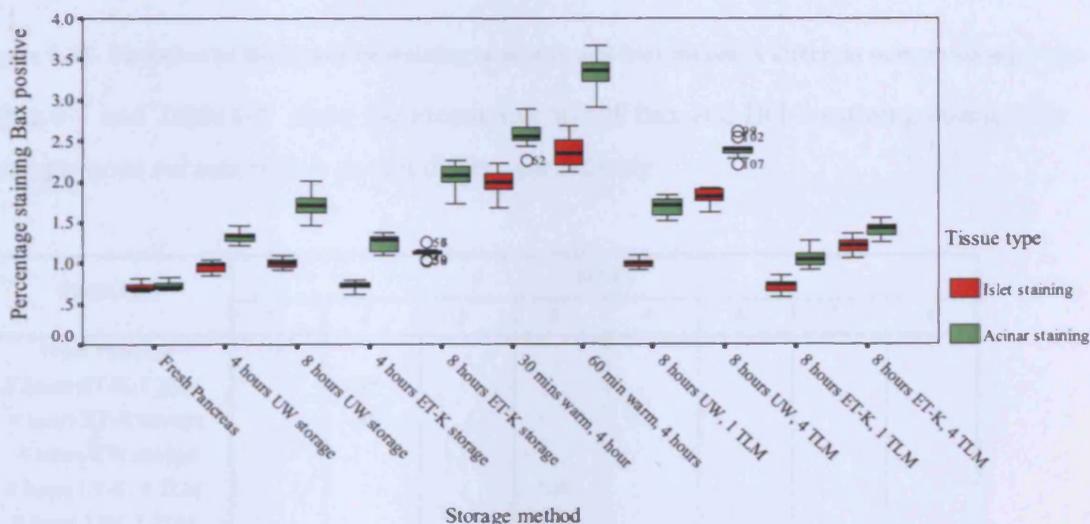


Figure 6-22. Variation in Bax positive staining amongst acinar and islet tissues in different preservation groups.

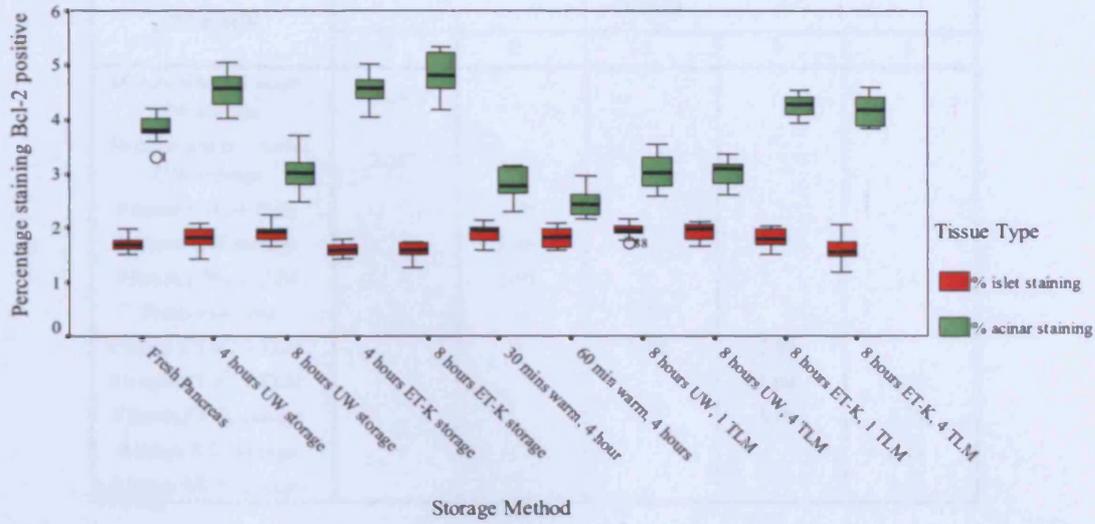


Figure 6-23. Variation in Bcl2 positive staining in acinar and islet tissues in different preservation groups.

Table 6-7 and Table 6-8 show the increasing rate of Bax and Bcl-2 staining arranged in homogeneous subsets which do not differ significantly.

STORAGE	Subset							
	1	2	3	4	5	6	7	8
Fresh Pancreas	.71							
8 hours ET-K, 1 TLM		.88						
4 hours ET-K storage		.99						
4 hours UW storage			1.14					
8 hours ET-K, 4 TLM				1.31				
8 hours UW, 1 TLM				1.34				
8 hours UW storage				1.36				
8 hours ET-K storage					1.60			
8 hours UW, 4 TLM						2.10		
30 mins warm, 4 hours UW storage							2.27	
60 min warm, 4 hours UW storage								2.86

Table 6-7. Significant differences in mean Bax immunohistochemistry staining across preservation groups.

STORAGE	Subset				
	1	2	3	4	5
60 min warm, 4 hours UW storage	2.14				
30 mins warm, 4 hours UW storage	2.35	2.35			
8 hours UW, 4 TLM		2.48			
8 hours UW storage		2.48			
8 hours UW, 1 TLM		2.50			
Fresh Pancreas			2.77		
8 hours ET-K, 4 TLM			2.89	2.89	
8 hours ET-K, 1 TLM				3.03	3.03
4 hours ET-K storage				3.09	3.09
4 hours UW storage					3.17
8 hours ET-K storage					3.21

**Table 6-8. Significant differences in Bcl-2 immunohistochemical staining across preservation groups.**

**6.5.6 ADP:ATP ratio.**

Significant differences in mean ADP:ATP ratios were observed at each phase of the isolation process ( $F(4,260)=262.3$ ;  $p<0.001$ ), between each preservation method ( $F(9,65)=146.7$ ;  $p<0.001$ ) and at each phase when variability due to the preservation method was accounted for ( $F(36,260)=19.0$ ;  $p<0.001$ ). Figure 6-24 to Figure 6-28 illustrate the ADP:ATP ratios obtained in the pre and post preservation pancreas, pancreatic digest and pre and post culture islets in the different groups.

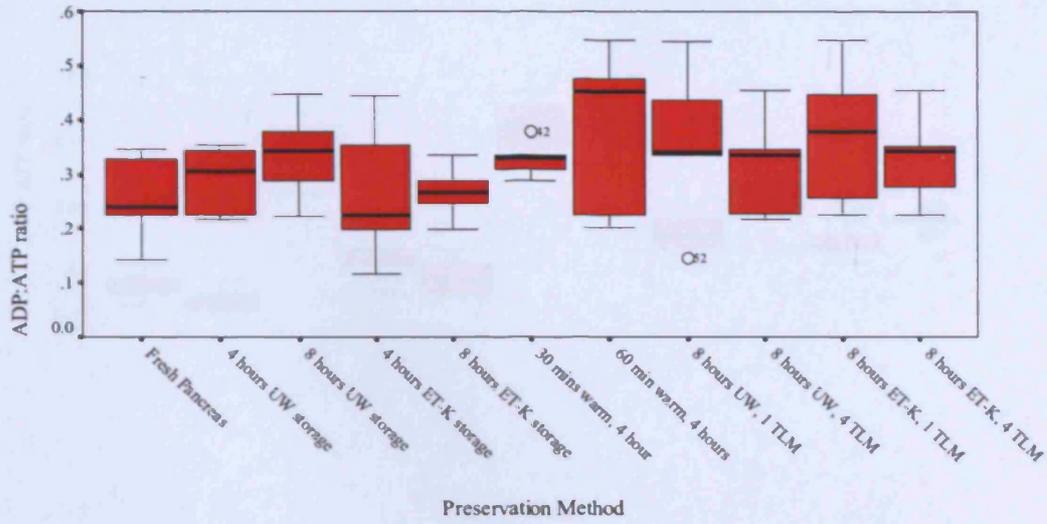


Figure 6-24. ADP:ATP ratios in the pre-stored pancreas across different preservation groups.

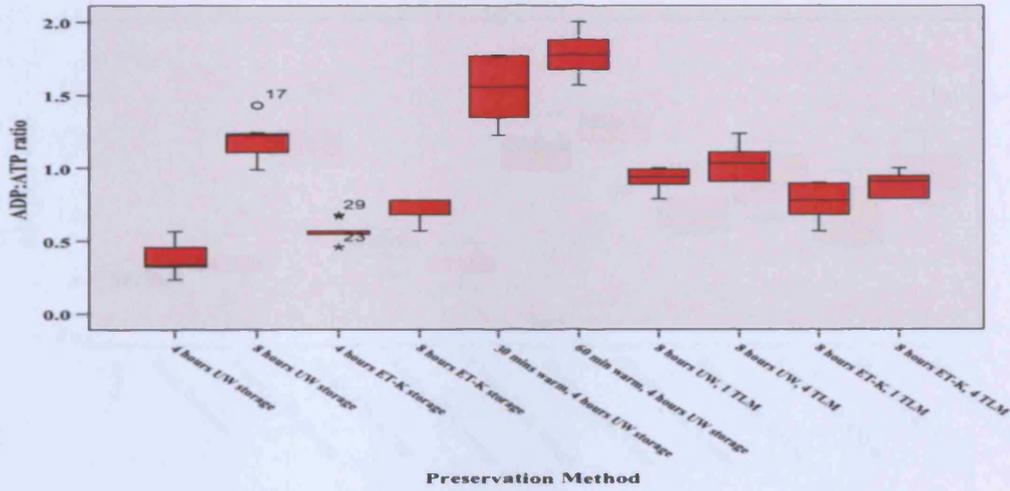


Figure 6-25. ADP:ATP ratios in the post-storage pancreas across different preservation groups.

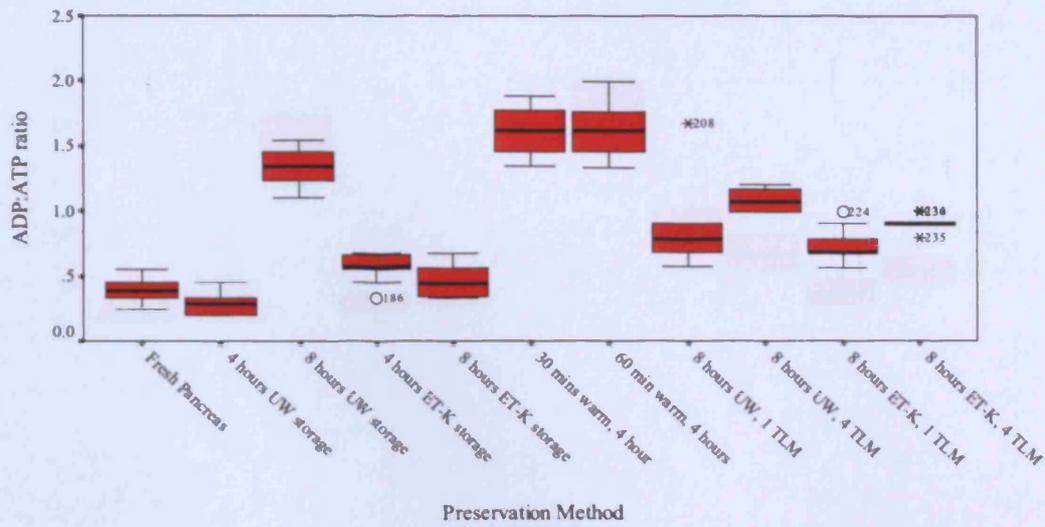


Figure 6-26. ADP:ATP ratios in the pancreatic digest across different preservation groups.

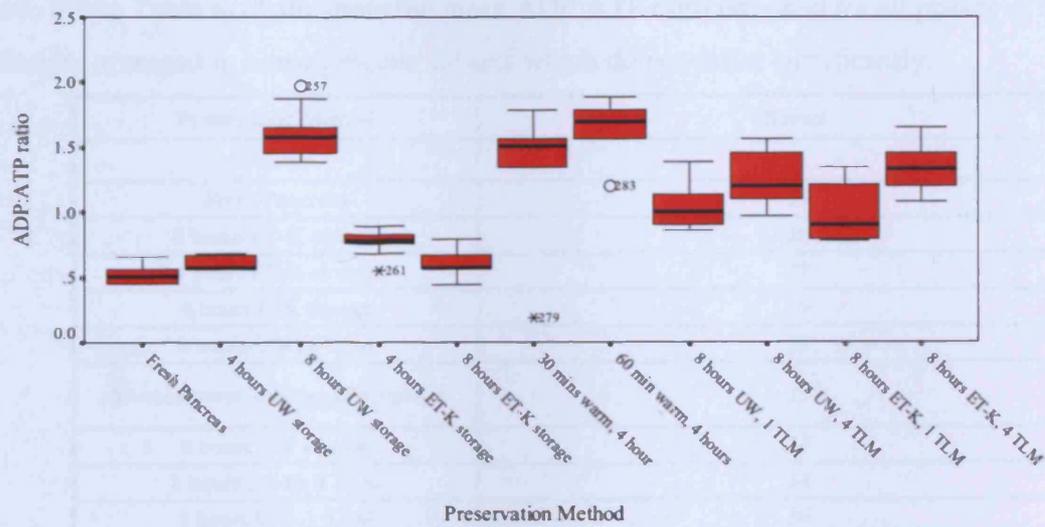


Figure 6-27. ADP:ATP ratios in pre-cultured islets across preservation groups.

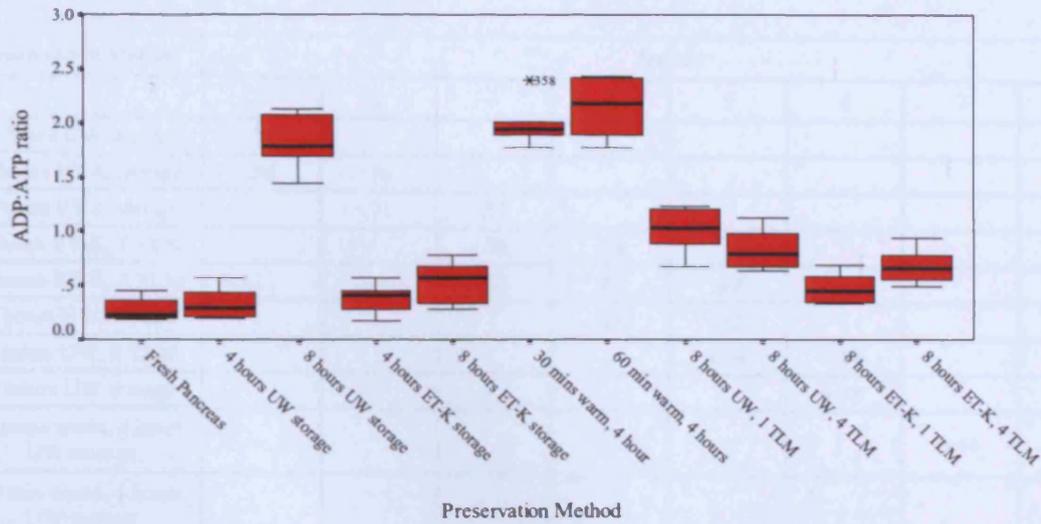


Figure 6-28. ADP:ATP ratios in post-culture islets across preservation groups.

Table 6-9 to Table 6-13 illustrate the mean ADP:ATP ratio obtained for all phases of islet isolation, arranged in homogeneous subsets which do not differ significantly.

Preservation Method	Subset
	1
Fresh Pancreas	.25
8 hours ET-K storage	.26
4 hours ET-K storage	.27
4 hours UW storage	.29
8 hours UW, 4 TLM	.32
30 mins warm, 4 hours UW storage	.33
8 hours UW storage	.33
8 hours ET-K, 4 TLM	.34
8 hours UW, 1 TLM	.36
8 hours ET-K, 1 TLM	.37
60 min warm, 4 hours UW storage	.39

Table 6-9. ADP:ATP ratios in pre-preservation pancreata of each group.

Preservation Method	Subset							
	1	2	3	4	5	6	7	8
4 hours UW storage	.37							
4 hours ET-K storage	.56	.56						
8 hours ET-K storage		.71	.71					
8 hours ET-K, 1 TLM			.78	.78				
8 hours ET-K, 4 TLM			.89	.89	.89			
8 hours UW, 1 TLM				.92	.92			
8 hours UW, 4 TLM					1.04	1.04		
8 hours UW storage						1.18		
30 mins warm, 4 hours UW storage							1.54	
60 min warm, 4 hours UW storage								1.78

**Table 6-10. Significant differences in post-preservation ADP:ATP ratios between preservation groups.**

Preservation Method	Subset						
	1	2	3	4	5	6	7
4 hours UW storage	.29						
Fresh Pancreas	.39						
8 hours ET-K storage	.45	.45					
4 hours ET-K storage	.57	.57					
8 hours ET-K, 1 TLM		.75	.75				
8 hours UW, 1 TLM			.90	.90			
8 hours ET-K, 4 TLM			.91	.91			
8 hours UW, 4 TLM				1.08	1.08		
8 hours UW storage					1.32	1.32	
30 mins warm, 4 hours UW storage						1.62	1.62
60 min warm, 4 hours UW storage							1.63

**Table 6-11. Significant differences in digest ADP:ATP ratios between preservation groups.**

Preservation Method	Subset			
	1	2	3	4
Fresh Pancreas	.53			
8 hours ET-K storage	.60			
4 hours UW storage	.60			
4 hours ET-K storage	.77	.77		
8 hours ET-K, 1 TLM		1.01	1.01	
8 hours UW, 1 TLM		1.05	1.05	
8 hours UW, 4 TLM			1.25	1.25
8 hours ET-K, 4 TLM			1.32	1.32
30 mins warm, 4 hours UW storage			1.32	1.32
8 hours UW storage				1.61
60 min warm, 4 hours UW storage				1.64

**Table 6-12. Significant differences in ADP:ATP ratios in pre-culture islets between preservation groups.**

Preservation Method	Subset				
	1	2	3	4	5
Fresh Pancreas	.28				
4 hours UW storage	.33				
4 hours ET-K storage	.38	.38			
8 hours ET-K, 1 TLM	.49	.49			
8 hours ET-K storage	.54	.54	.54		
8 hours ET-K, 4 TLM		.70	.70	.70	
8 hours UW, 4 TLM			.83	.83	
8 hours UW, 1 TLM				1.01	
8 hours UW storage					1.85
30 mins warm, 4 hours UW storage					1.99
60 min warm, 4 hours UW storage					2.15

**Table 6-13. Significant differences in ADP:ATP ratios of post-culture islets between preservation groups.**

**6.5.7 ATP:DNA ratio.**

Significant differences in mean ATP:DNA ratios were seen at each phase of the isolation process ( $F(4,260)=6181.1$ ;  $p<0.001$ ), between each preservation method ( $F(9,65)= 18.1$ ;  $p<0.001$ ) and at each phase when variability due to the preservation method was accounted for ( $F(36,260)=8.2$ ;  $p<0.001$ ). Figure 6-29 to 1-Figure 6-33 illustrate the ATP:DNA ratios obtained in the pre and post preservation pancreas, pancreatic digest and pre and post culture islets in the different groups.

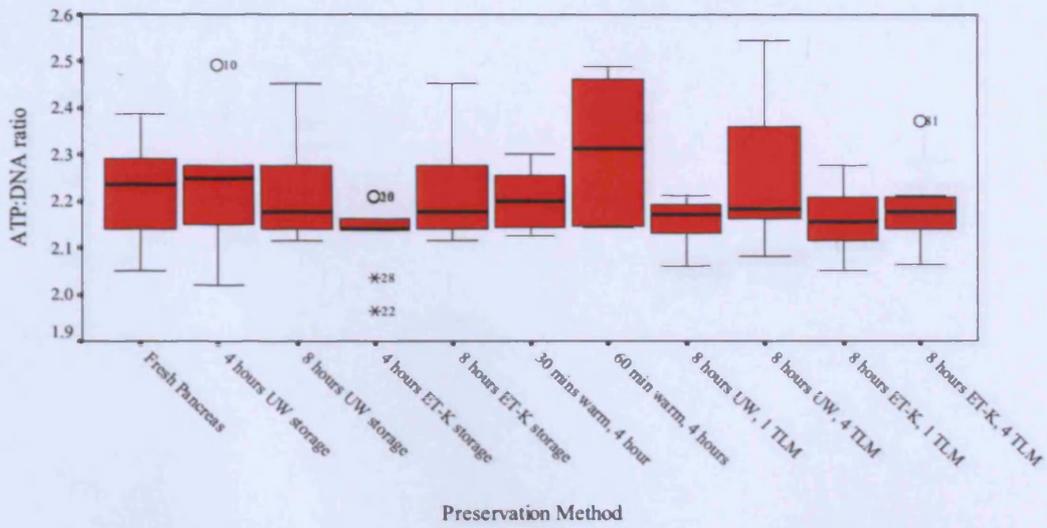


Figure 6-29. ATP:DNA ratios in pre-stored pancreata using different preservation methods.

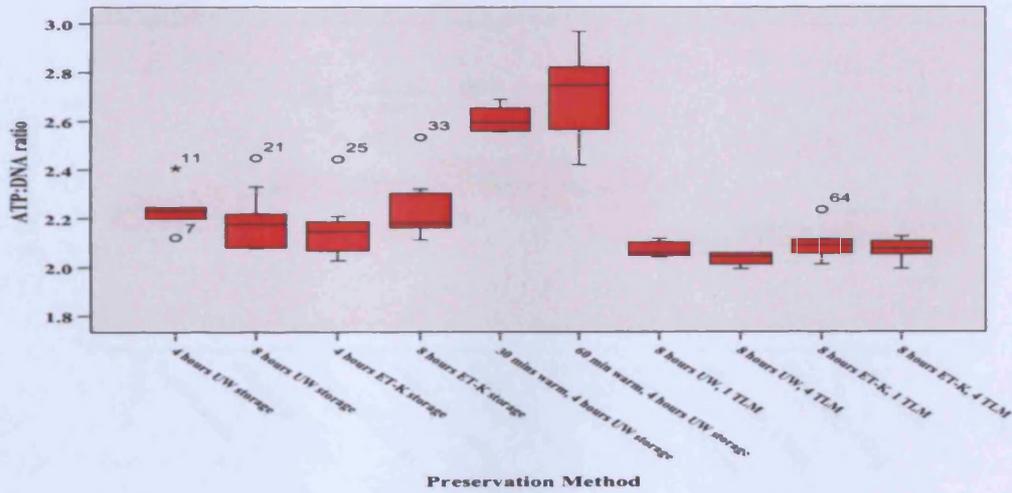


Figure 6-30. ATP:DNA ratios in post-storage pancreata using different preservation methods.

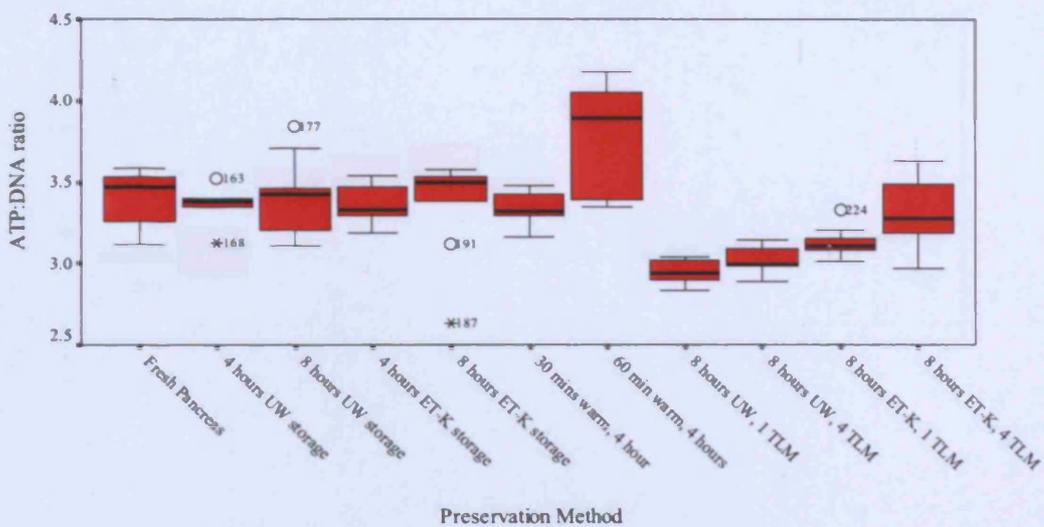


Figure 6-31. ATP:DNA ratios in pancreatic digest using different preservation methods.

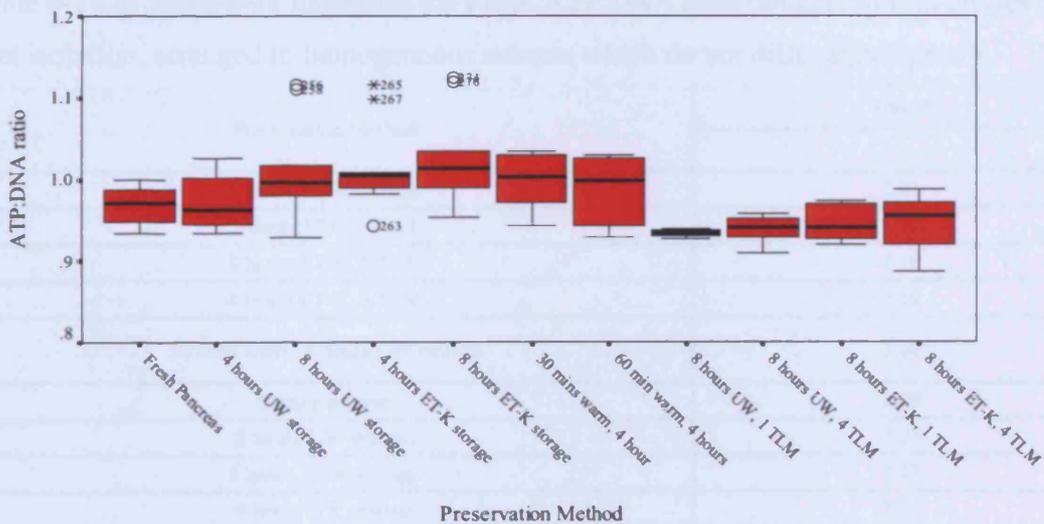


Figure 6-32. ATP:DNA ratios in pre-cultured islets using different preservation methods.

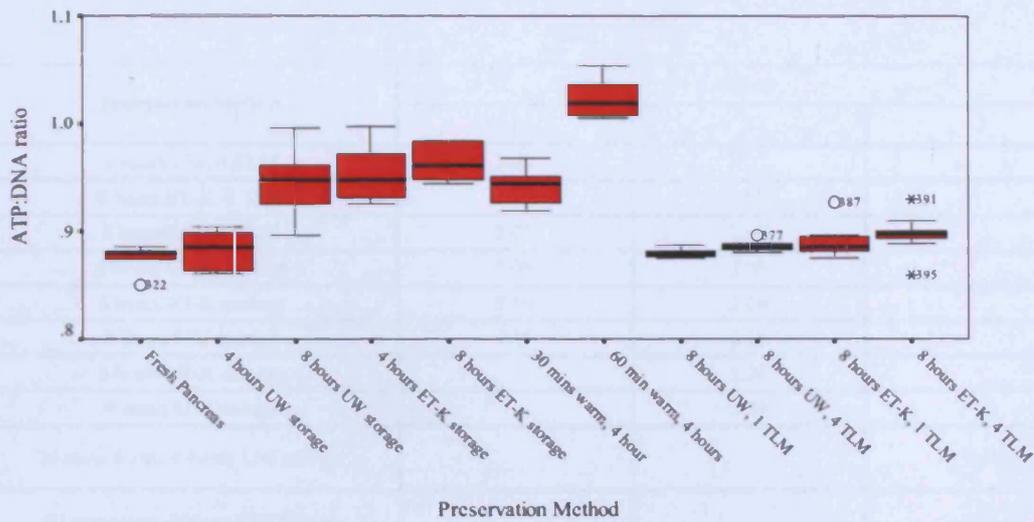


Figure 6-33. ATP:DNA ratios in post-culture islets using different preservation methods.

Table 6-14 to Table 6-18 illustrates the mean ATP:DNA ratio obtained for all phases of islet isolation, arranged in homogeneous subsets which do not differ significantly.

Preservation Method	Subset
	1
4 hours ET-K storage	2.13
8 hours UW, 1 TLM	2.16
8 hours ET-K, 1 TLM	2.16
8 hours ET-K, 4 TLM	2.19
30 mins warm, 4 hours UW storage	2.20
Fresh Pancreas	2.22
8 hours UW storage	2.23
8 hours ET-K storage	2.23
4 hours UW storage	2.24
8 hours UW, 4 TLM	2.25
60 min warm, 4 hours UW storage	2.31

Table 6-14. ATP:DNA ratios in pre-preservation pancreata of each group.

Preservation Method	Subset		
	1	2	3
8 hours UW, 4 TLM	2.03		
8 hours ET-K, 4 TLM	2.07	2.07	
8 hours UW, 1 TLM	2.07	2.07	
8 hours ET-K, 1 TLM	2.09	2.09	
4 hours ET-K storage	2.16	2.16	
8 hours UW storage	2.19	2.19	
8 hours ET-K storage		2.24	
4 hours UW storage		2.24	
30 mins warm, 4 hours UW storage			2.61
60 min warm, 4 hours UW storage			2.71

**Table 6-15. Significant differences in post-preservation ATP:DNA ratios between preservation groups.**

Preservation Method	Subsets			
	1	2	3	4
8 hours UW, 1 TLM	2.95			
8 hours UW, 4 TLM	3.01	3.01		
8 hours ET-K, 1 TLM	3.13	3.13	3.13	
8 hours ET-K, 4 TLM	3.28	3.28	3.28	
30 mins warm, 4 hours UW storage		3.33	3.33	
8 hours ET-K storage		3.35	3.35	
4 hours UW storage		3.36	3.36	
4 hours ET-K storage			3.37	
Fresh Pancreas			3.40	
8 hours UW storage			3.41	
60 min warm, 4 hours UW storage				3.79

**Table 6-16. Significant differences in digest ATP:DNA ratios between preservation groups.**

Preservation Method	Subsets		
	1	2	3
8 hours UW, 1 TLM	.93		
8 hours UW, 4 TLM	.94		
8 hours ET-K, 1 TLM	.95	.95	
8 hours ET-K, 4 TLM	.95	.95	
Fresh Pancreas	.97	.97	.97
4 hours UW storage	.97	.97	.97
60 min warm, 4 hours UW storage	.99	.99	.99
30 mins warm, 4 hours UW storage	1.00	1.00	1.00
8 hours UW storage		1.01	1.01
4 hours ET-K storage		1.01	1.01
8 hours ET-K storage			1.03

**Table 6-17. Significant differences in ATP:DNA ratios in pre-culture islets between preservation groups.**

Preservation Method	Subset		
	1	2	3
Fresh Pancreas	.87		
8 hours UW, 1 TLM	.88		
4 hours UW storage	.88		
8 hours UW, 4 TLM	.89		
8 hours ET-K, 1 TLM	.89		
8 hours ET-K, 4 TLM	.90		
30 mins warm, 4 hours UW storage		.94	
8 hours UW storage		.95	
4 hours ET-K storage		.95	
8 hours ET-K storage		.96	
60 min warm, 4 hours UW storage			1.02

**Table 6-18. Significant differences in ATP:DNA ratios of post-culture islets between preservation groups.**

### 6.5.8 Factor Analysis.

Table 6-19 and Table 6-20 show the results of factor analysis in the rodent experiments. The correlation matrix illustrates R values where positive correlations ( $R > 0.5$ ) and stronger correlations (R approaching 1.0) between experimental results can be seen.

	Post-stored pancreas ADP:ATP ratio	Digest ADP:ATP ratio	Pre-cultured islets ADP:ATP ratio	Post-cultured islets ADP:ATP ratio	Static stimulation index	Gradient amylase contamination at 60% insulin yield	Total IEQ yield per pancreas	Bax protein immuno in islets	Bax protein immuno in acinar	Bcl-2 protein immuno in acinar
Post-stored pancreas ADP:ATP ratio	1.000	.890	.719	.839	-.608	.726	-.666	.772	.796	-.785
Digest ADP:ATP ratio	.890	1.000	.730	.847	-.597	.675	-.675	.678	.649	-.769
Pre-cultured islets ADP:ATP ratio	.719	.730	1.000	.637	-.396	.597	-.542	.468	.443	-.667
Post-cultured islets ADP:ATP ratio	.839	.847	.637	1.000	-.488	.554	-.601	.614	.642	-.749
Static stimulation index	-.608	-.597	-.396	-.488	1.000	-.366	.399	-.502	-.380	.569
Gradient amylase contamination at 60% insulin yield	.726	.675	.597	.554	-.366	1.000	-.678	.582	.580	-.568
Total IEQ yield per pancreas	-.666	-.675	-.542	-.601	.399	-.678	1.000	-.557	-.541	.669
Bax protein immuno in islets	.772	.678	.468	.614	-.502	.582	-.557	1.000	.926	-.666
Bax protein immuno in acinar	.796	.649	.443	.642	-.380	.580	-.541	.926	1.000	-.654
Bcl-2 protein immuno in acinar	-.785	-.769	-.667	-.749	.569	-.568	.669	-.666	-.654	1.000

Table 6-19. Correlations of ADP:ATP ratio with other tests of islet function and viability.

	Post-stored pancreas ATP:DNA ratio	Digest ATP:DNA ratio	Pre-cultured islets ATP:DNA ratio	Post-cultured islets ATP:DNA ratio	Static stimulation index	Gradient amylase contamination at 60% insulin yield	Total IEQ yield per pancreas	Bax protein immuno in islets	Bax protein immuno in acinar	Bcl-2 protein immuno in acinar
Post-stored pancreas ATP:DNA ratio	1.000	.586	.299	.599	-.454	.257	-.211	.666	.672	-.397
Digest ATP:DNA ratio	.586	1.000	.565	.597	-.050	.049	.042	.350	.387	-.058
Pre-cultured islets ATP:DNA ratio	.299	.565	1.000	.446	.154	-.086	.054	.038	.123	.095
Post-cultured islets ATP:DNA ratio	.599	.597	.446	1.000	-.025	.278	-.082	.374	.481	-.147
Static stimulation index	-.454	-.050	.154	-.025	1.000	-.366	.399	-.502	-.380	.569
Gradient amylase contamination at 60% insulin yield	.257	.049	-.086	.278	-.366	1.000	-.678	.582	.580	-.568
Total IEQ yield per pancreas	-.211	.042	.054	-.082	.399	-.678	1.000	-.557	-.541	.669
Bax protein immuno in islets	.666	.350	.038	.374	-.502	.582	-.557	1.000	.926	-.666
Bax protein immuno in acinar	.672	.387	.123	.481	-.380	.580	-.541	.926	1.000	-.654
Bcl-2 protein immuno in acinar	-.397	-.058	.095	-.147	.569	-.568	.669	-.666	-.654	1.000

Table 6-20. Correlation of ATP:DNA ratios with other tests of islet function and viability.

## 6.6 Conclusion.

Highest islet yields were obtained from fresh pancreata and those preserved for 4 hours in either ET-K or UW solution. 8 hour ET-K cold storage and the addition of both short (1

hour) and long (4 hour) periods of TLM resulted in a higher yield than 8 hours of UW cold storage alone but this did not reach significance. Indeed 8 hour UW cold storage followed by short and long TLM exposure resulted in low islet yields comparable to pancreata subjected to 30 minutes and 60 minutes of warm ischaemia prior to UW cold storage.

Lowest levels of exocrine contamination were achieved in fresh pancreata and 4 hour ET-K and UW cold stored groups. Prolonged cold storage in ET-K solution, with or without short duration TLM, showed significantly increased contamination compared to fresh or 4 hour UW cold stored pancreata, but comparable contamination to short duration ET-K cold storage. Periods of warm ischemia prior to cold storage caused the highest gradient contamination.

*In vitro* function by static incubation was highest and comparable for islets isolated from unpreserved pancreata or those exposed to short or prolonged ET-K static cold storage ( $SI > 1.45$ ). The mean SI of islets from short and prolonged UW stored pancreata were comparable to short duration ET-K and unpreserved groups, but were significantly less than prolonged ET-K cold storage. Islets from all groups involving periods of TLM preservation had significantly reduced mean SI compared to unpreserved or prolonged ET-K stored groups.

High quality pure mRNA could be consistently extracted from rodent islets.

Unfortunately, RT-PCR analysis of both Bax and Bcl-2 mRNA expression showed significant differences in the expression of the  $\beta$ -actin endogenous control, and positive  $\beta$ -actin signal was generated in a negative control sample containing no cDNA template. Real-time PCR, as an alternative quantitative method of transcript analysis, appeared to show a reduced Bax/Bcl-2 ratio in TLM preserved groups. However, the significance of the disparate expression of Bax and Bcl-2 between the preservation groups must be interpreted cautiously due to significant differences in the expression of the  $\beta$ -actin endogenous control in these experiments.

The lowest rate of Bax immunostaining was in the unpreserved pancreas and the highest rates occurred in pancreata subjected to warm ischaemia. Short duration cold storage in ET-K solution resulted in significantly less Bax immunostaining, though this was reversed after 8 hours cold storage. TLM preservation using ET-K solution resulted in significantly reduced Bax immunostaining compared to equivalent durations using UW solution. Indeed Bax staining after 4 hours TLM in ET-K solution, and 1 hour TLM using UW solution, were equivalent.

Highest rates of Bcl-2 immunostaining were predominantly present in groups preserved in ET-K solution. Indeed, short and long duration ET-K cold storage and short duration TLM using ET-K solution, showed rates of Bcl-2 immunostaining comparable to short duration UW cold storage and significantly higher than unpreserved pancreata.

Rates of Bcl-2 staining were different between islet and acinar tissues. Groups with high mean rates of Bcl-2 immunostaining appeared to show increased Bcl-2 staining in acinar tissue whereas islet Bcl-2 staining remained consistent and comparable between all preservation groups.

The lowest mean ADP:ATP ratios were achieved in short duration cold stored pancreata and more prolonged cold storage in ET-K solution. The highest ADP:ATP ratios occurred in pancreata subjected to prolonged UW cold storage and when exposed to periods of warm ischaemia. ADP:ATP ratio in the pre-preservation biopsies in all groups were low and comparable. The increase in ADP:ATP ratio in warm ischaemic and 8 hour UW preserved pancreata was apparent in the post-storage biopsied tissue, and continued throughout sampling of the digest, pre-cultured and post-culture islets. Though the ADP:ATP ratio increased in all other groups throughout the isolation process, a decrease in ratio occurred following cell culture of isolated islets.

The highest ATP:DNA ratio occurred in the group exposed to 60 minutes of warm ischaemia; whilst the lowest ratios occurred in groups exposed to TLM preservation. Pre-preservation ATP:DNA ratios were comparable between all groups but began to rise during preservation resulting in significantly higher ratios in the warm ischaemia exposed pancreata. ATP:DNA ratios in pancreatic digest and pre-culture islets were lowest in TLM preserved groups.

Factor analysis showed strong correlation coefficients between high tissue ADP:ATP ratio and low islet yield (-0.67), low *in vitro* islet function by static incubation (-0.61) and high levels of gradient contamination (0.73). Additionally, high ADP:ATP ratios were strongly correlated with the immunostaining of the pro-apoptotic protein Bax in both islets (0.77) and acinar tissues (0.80), and a lack of immunostaining of the anti-apoptotic protein Bcl-2 in acinar tissues.

The ATP:DNA ratio in pancreatic tissue after preservation showed no correlations with standard measures of islet yield or *in vitro* function. Though there were positive correlations with the the percentage of Bax immunostaining in islets (0.67) and acinar tissues (0.67).

## ***Chapter 7. Studies in the Porcine Model.***

### ***7.1 Introduction.***

This chapter describes the experiments conducted in the porcine model. Methodology optimised during preliminary studies in the rodent, and used successfully in rodent experiments, were used with modifications where required for porcine pancreas and insulin.

### ***7.2 Aims.***

Experiments were performed to test the null hypotheses that no statistically significant difference in islet yield, gradient purity, *in vitro* function by SI, apoptotic index by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay and post-preservation pancreatic ADP:ATP or ATP:DNA ratio would be observed between the preservation methods studied.

### ***7.3 Methods.***

#### ***7.3.1 Porcine Pancreas Retrieval.***

Twenty porcine pancreata were procured from Landrace-Large White cross breed pigs aged 4 to 10 months (Sutton Bonnington, UK). Culling was by Home Office Schedule 1 approved electrocution and exsanguination. Pancreata were explanted expediently without *in situ* perfusion with care not to breach the pancreatic capsule or duodenum, and an aortic patch was maintained to facilitate later vascular perfusion. Pancreata were immersed in UW solution (DuPont, USA) at 4°C supplemented with 100u/ml penicillin G, 50 µl/ml gentamicin and 1µg/ml amphotericin B to reduce bacterial contamination(952) and transported to the isolation laboratory.

#### ***7.3.2 Experimental Groups.***

Pancreata were randomised to one of four preservation conditions. The combinations of cold ischaemia times, preservation solutions and methods were chosen to mimic the retrieval and transportation times achieved in clinical human islet isolation (Table 7-1).

<b>PRESERVATION METHOD</b>	<b>PANCREATA PER GROUP</b>
<b>IMMEDIATE ISLET ISOLATION WITHOUT PRESERVATION</b>	<b>N=5</b>
<b>4 HOURS UW STATIC COLD STORAGE</b>	<b>N=5</b>
<b>4 HOURS TLM PRESERVATION IN UW/PFC</b>	<b>N=5</b>
<b>4 HOURS MACHINE PERFUSION</b>	<b>N=5</b>

**Table 7-1. Preservation groups used in porcine studies.**

### **7.3.3 Static Cold Storage.**

Pancreata subjected to periods of static cold storage were placed in a Class II microbiological cabinet in a sterile bag containing UW solution in melting ice (4°C). After 0, 2 hours and 4 hours of static cold storage two 0.5cm<sup>3</sup> biopsies were taken; one snap frozen in Tissuetek in a flexible microwell and stored in liquid nitrogen for later ATP and DNA assays, the other fixed in 4% formalin.

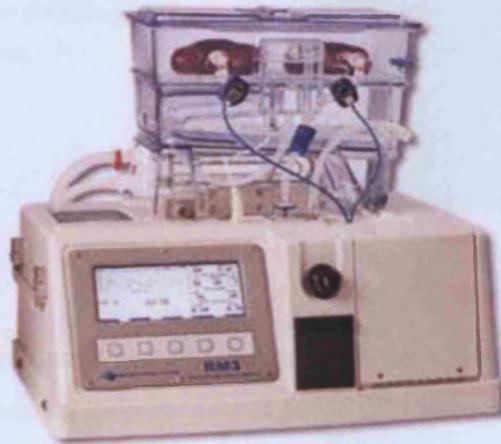
### **7.3.4 Two Layer Method (TLM).**

Where the pancreas was to be subjected to TLM preservation it was transferred to a 2 litre purpose built glass chamber (constructed in house). This comprised an oxygen inlet, with J shaped tube at the base of the chamber, and outlet. 500ml of perfluorodecalin solution and 1litre of UW solution was added to the chamber. Being immiscible, these formed two layers allowing the pancreas to float on the PFC whilst being bathed in the UW preservation solution. Oxygen (95%O<sub>2</sub>:5%CO<sub>2</sub>) was insufflated through the PFC layer via the inlet at 5 litre per minute, whilst the outlet allowed the release of carbon dioxide and excess oxygen. The whole apparatus was placed in an ice box to maintain a 4 °C temperature for the duration of TLM preservation.

After 0, 2 and 4 hours of TLM preservation two 0.5cm<sup>3</sup> biopsies were taken; one snap frozen in Tissuetek in a flexible microwell and stored in liquid nitrogen for later ATP and DNA assays, the other fixed in 4% formalin. Following each preservation the perfluorodecalin was re-used by filtration through a 14µm filter (Millipore, UK) and autoclaving at 121°C for 40 minutes, and the chamber was sterilized by autoclaving.

### **7.3.5 Pulsatile Machine Perfusion.**

Pancreata randomised to pulsatile machine perfusion were perfused via the arterial patch using the RM3 Waters machine (Figure 7-1). The aortic patch was cannulated into the superior mesenteric artery with a 4mm plastic cannula (Waters, USA) tied in position with  $3/0$  Vicryl™ ligatures (Ethicon®, Johnson & Johnson International, Belgium). The remainder of the small pancreatic vessels and distal splenic artery were ligated with  $3/0$  Vicryl™ ligatures to prevent leakage of the perfusate. UW solution was used to perfuse the cannulated pancreas with collection of effluent and recirculation via the in built counter current cooling system. The temperature of the system was monitored using a temperature probe throughout perfusion and varied with flow between 4 and 10°C. Similarly systolic perfusion pressure was monitored and maintained at 60mmHg which mimicked physiological mean arterial pressure. After 0, 2 and 4 hours of pulsatile perfusion two 0.5cm<sup>3</sup> biopsies were taken; one snap frozen in Tissuetek in a flexible microwell and stored in liquid nitrogen for later ATP and DNA assays, the other fixed in 4% formalin. Any leakage of perfusate from the pancreatic capsule after sampling was repaired using  $3/0$  Vicryl™ ligatures to maintain the perfusion pressure.



**Figure 7-1. The RM Waters renal perfusion machine used for perfusive preservation.**

### **7.3.6 Porcine Islet Isolation.**

Following the allocated preservation period a modified automated method was used for islet isolation(227). A digestion circuit was established containing Hanks balanced salt solution (HBSS) (Sigma, UK) supplemented with penicillin and streptomycin, polyvinyl chloride tubing (COBE Laboratories, UK), a roller pump (Watson Marlow, UK) at 80ml/min and a heat exchanger (COBE Laboratories, UK) at 4°C . pH 7.4 was maintained with aliquots of HEPES and sodium bicarbonate.

The pancreas was transferred to a cold Petri dish containing HOC at 4°C (Baxter, UK). Excess lymphoid, fat and connective tissue was excised and the pancreas weighed. The pancreatic duct was cannulated with a 14g cannula (Abbotcath, UK), secured with a  $2/0$  silk ligature, and distended by slow injection of 330ml Liberase<sup>®</sup> at 4°C (Roche, UK; 0.14%w/v in MEM) (Figure 7-2). The pancreas and Liberase<sup>®</sup> solution was placed in a stainless steel automated chamber divided by a 500µm mesh and containing seven 1cm<sup>3</sup> steel spheres to aid mechanical disruption (Figure 7-3).



**Figure 7-2. Human liberase used for porcine islet isolation.**



**Figure 7-3. Semi automated chamber for pancreatic digestion.**

The circuit was heated gradually to 37°C during which time the chamber was manually agitated two or three times per minute. Tissue exiting the chamber was sampled via a three way tap (5ml), stained with 0.006%w/v dithizone (pH 7.8) and examined under a light microscope (Nikon, Japan) for the presence of 'cleaved' islets devoid of surrounding acinar tissue. Once these were identified the circuit was opened, to allow collection of the digest into 250ml Falcon flasks containing 5ml newborn calf serum (Advanced Protein Products, UK), and the flow rate increased to 120ml/min with continuous manual agitation of the chamber until 4 litres of media had run through the circuit. Once empty the chamber was opened and the undigested pancreatic remnant was weighed. This was subtracted from the weight of the pre-digested tissue to give a total weight of digested pancreas.

Collected digest was stored at 4°C, centrifuged at 200g for 2 minutes, pooled and resuspended in UW solution at 4°C. The total digest was centrifuged at 200g for 2 minutes and the packed cell volume (PCV) documented. Four 100µl samples were resuspended in 1ml of 1.108g/cm<sup>3</sup> BSA (Advanced Protein Products, UK) for purification and analysis of gradient purity. After resuspension of the digest in 2.5 x PCV of UW solution, five 100µl samples were taken for islet yield quantification.

### **7.3.7 Islet Culture Purification.**

From the remaining three gradients sequential 1ml gradient fractions from 1 to 4ml, and 5 to 8ml were pooled into 50ml conical tubes, washed of BSA by 2 successive centrifugations (270g, 4°C for 5 minutes) and resuspended in 50ml MEM. After the final wash the tissue pellet was resuspended in 10ml of RPMI1640 culture medium (Gibco, UK) supplemented with 10%v/v fetal calf serum, 1%v/v penicillin, 1%v/v streptomycin, 2%v/v HEPES and 1%v/v pyruvate, and transferred to a large Petri dish. A Drummond pipette was used to extract a 200µl biopsy of the upper fractions which was streaked onto a small Petri dish for islet quantification. The remaining tissue was then placed in a cell culture incubator (37°C, 5%CO<sub>2</sub>) for overnight culture purification. After 14 hours of incubation islets were again biopsied using the method above and the remaining post-culture islets were used in the *in vitro* functional assessments.

### **7.3.8 Islet Yield and Cleavage.**

#### **7.3.8.1 Islet yield.**

Five 100µl aliquots of 2.5 times diluted digest were diluted in 0.9ml MEM. From these five further 100µl samples were streaked across a Petri dish using a Drummond pipette. To the streaks 20µl of 0.006%w/v dithizone (pH 7.8) was added with a Gilson pipette. Streaks were examined under a light microscope × 100 with a graticule (Cell Finder, Holland). Islets with diameters of greater than 50µm were counted in groups of 50µm ascending sizes. Islet counts were adjusted to IEQs and multiplied by the dilution factor 25 to generate a total IEQ count. This was divided by the weight of digested tissue to give a yield in IEQ per gram of pancreas.

#### **7.3.8.2 Cleavage index.**

During the determination of islet yield the morphology of each islet was noted. Where an islet was completely devoid of surrounding acinar tissue it was deemed to be 'cleaved'.

The number of cleaved islets was divided by the total number counted to calculate the cleavage index.

### **7.3.9 Gradient Purity.**

An assessment of islet fraction purity was made by establishing the percentage of exocrine contamination in each gradient fraction, and comparisons made at the level of 60% cumulative insulin content. This was deemed to correspond to an acceptable islet yield.

#### *7.3.9.1 Continuous density gradient purification.*

Continuous linear mini-gradients were constructed after the pancreas had been processed to the digest stage. 5.2ml aliquots of high density ( $1.103\text{g/cm}^3$ ) and low density ( $1.075\text{g/cm}^3$ ) BSA were prepared by dilution of stock BSA (Advanced Protein Products, UK) with MEM and checked with a calibrated densitometer (Paar Scientific Ltd, UK). Each aliquot was added to a side of a two chamber gradient maker. A 100 $\mu\text{l}$  sample of digest in 1ml of  $1.108\text{g/cm}^3$  BSA was added to the bottom of a clear 12ml tube (Costar Corning, UK) and the continuous density gradient layered onto this by running a roller pump and mixing the BSA densities with a magnetic flea in the base of the gradient maker. Gradients were centrifuged for 5 minutes (500g, 22°C) to allow digest components to occupy their isodense points.

Four gradients were prepared for each pancreas studied. From one gradient eleven 1ml aliquots were aspirated down each gradient, washed twice in MEM and resuspended in 2ml MEM in Eppendorfs. These were sonicated at 14MHz for three periods of 30 seconds (Soniprep150, MSE, UK). 1ml was then withdrawn and frozen in 1ml Zeigler reagent (90% v/v ethanol, 10%v/v 1M orthophosphoric acid, 5% v/v distilled water) at -20°C for insulin assay and the remaining 1ml was frozen at -20°C for amylase assay.

#### *7.3.9.2 Insulin assay.*

A solid phase two site ELISA kit was used to measure porcine insulin content. This used two monoclonal antibodies directed against different epitopes of pig insulin to generate a colour change.

The kit was supplied with standard insulin concentrations, and 25 $\mu\text{l}$  aliquots of these were added in duplicate to the first 16 wells of the 96 well assay plate. 25 $\mu\text{l}$  aliquots of the test samples were added in duplicate to the remaining wells. To each well 50 $\mu\text{l}$  of peroxidase conjugated anti-insulin antibodies were added, and the plate incubated for 2

hours. The plate was then washed in ultrapure water using a plate washer (Denley Wellwash, Denley, UK) and 200 $\mu$ l of 3,3',5,5' – tetramethylbenzidine was added. After 15 minutes the reaction was ceased by the addition of sulphuric acid to generate the end colour change. Plates were read using a plate reader (Denley, UK) at 450nm wavelength. A standard curve was generated for each plate from which the insulin content of the remaining wells could be calculated.

#### 7.3.9.3 Amylase assay.

Frozen gradient samples were thawed in a waterbath at 37°C. 200 $\mu$ l of the sample was mixed with 4 ml distilled water in a 10 ml tube (Costar Corning, UK) and returned to the water bath. To each tube a tablet of Phadebas reagent (Pharmacia Diagnostics, Sweden) was added and vortexed. This contained a water soluble cross-linked starch polymer which was hydrolysed by  $\alpha$ - amylase to generate a water soluble blue dye. Tubes were left for 15 minutes after which the reaction was ceased by the addition of 1ml 0.5M sodium hydroxide. After centrifugation at 1500g for 5 minutes the clear supernatant was aspirated into spectrophotometer cuvettes and the absorbance measured at 620nm wavelength against a blank cuvette containing ultra pure water. Amylase concentration was calculated from a series of standard absorbance curves. From the 11 samples the cumulative percentage amylase concentration was calculated.

From the results of the two assays a cumulative percentage insulin and amylase concentration was established for each 1ml portion of the continuous gradient, and a graph constructed to allow the percentage amylase accumulation to be found which coincided with a cumulative insulin content of 60% (*vide supra* 6.3.7).

#### 7.3.10 *In vitro* islet function.

Dynamic perfusion was used to study isolated islet function. Aliquots of low concentration (960ml) and high concentration (600ml) glucose solutions were prepared and placed in a waterbath at 37°C. An aliquot of 50 islets was handpicked from culture medium using a drawn out glass micro-pipette into a small chamber containing glass wool. The chamber was connected to the glucose solution reservoirs via a three way tap which allowed the islets to be bathed in a continuous flow of either glucose solution. A roller pump was used to perfuse the islets with low concentration glucose solution at a rate of 1ml per minute for 1 hour to reach a steady state. After perfusing the islets 1ml aliquots of effluent were collected from the circuit into sterile Eppendorfs and frozen at -20°C for insulin assay. Effluent collection began at 0,10 and 19 minutes after which the

bathing solution was switched to high concentration glucose. Further effluent samples were then collected at 2 minute intervals up to 29 minutes then at 10 minute intervals up to 79 minutes. After 79 minutes the circuit was switched back to low concentration glucose solution and the final samples collected at 10 minute intervals up to 140 minutes. After insulin quantification by ELISA, the SI was calculated using a plot of insulin concentration against elapsed time in SPSS for Windows v11 (Figure 7-4).

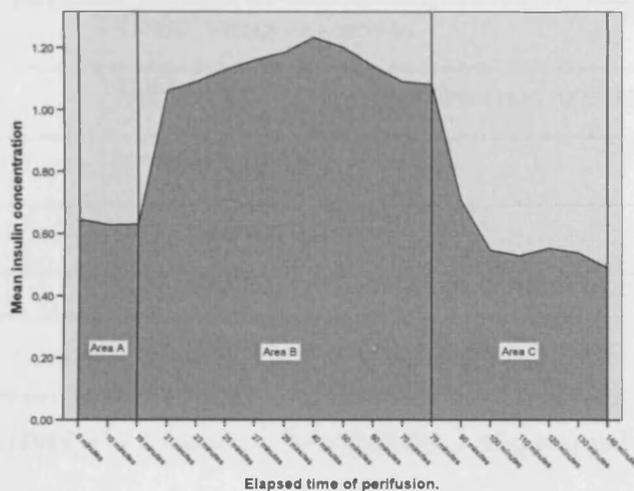


Figure 7-4. Example perfusion plot from porcine islets.

Calculation of the areas under the curve for periods 0-19 mins (Area A), 19-79 mins (Area B) and 79-140 mins (Area C) allowed calculation of the mean insulin secretion at low and high glucose concentrations. The SI was calculated by division of the mean high glucose secretion of insulin by the mean low glucose secretion of insulin (Equation 7-1).

$$\frac{(A/20)+(C/60)}{B/60}$$

$$B/60$$

Equation 7-1. Calculation of SI by perfusion.

### 7.3.11 Apoptotic index by TUNEL immunohistochemistry.

#### 7.3.11.1 Sample preparation.

Formalin fixed pancreatic biopsies were placed in a tissue cassette and embedded in paraffin wax. Seven 4µm serial sections were cut from each block using a microtome and floated in a waterbath to allow mounting on silane coated slides. Slides were dried at 37°C for 24 hours before staining as described in Table 7-2. Tissue from 5 out of 8

porcine pancreata in each group were available for IHC analysis as a storage problem had rendered 3 biopsies in each group unsuitable for analysis.

SECTION NUMBER	STAINING PROTOCOL
1	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE
2	1 <sup>ST</sup> TUNEL IHC
3	TUNEL NEGATIVE CONTROL
4	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE
5	2 <sup>ND</sup> TUNEL IHC
6	TUNEL NEGATIVE CONTROL
7	INSULIN IHC TO ALLOW IDENTIFICATION OF ISLET TISSUE

Table 7-2. Serial staining protocol for TUNEL assay.

Slides were de-waxed by serial immersions in xylene (2x 3 minutes), 99% industrial methylated spirit (IMS) (2x 3 minutes), 95% IMS (2x 3 minutes) and ultra-pure water (2x 3 minutes).

#### 7.3.11.2 TUNEL immunohistochemistry.

Slides were placed in a humidified darkened incubation box and 50µl of proteinase K (pH 7.6; 0.001%w/v proteinase K, TRIS 20mM, NaCl 137mM, HCl 1M) was applied to each. Slides were incubated for 1 hour at 37°C, removed, racked and washed in ultra pure water for 5 minutes. Slides were then immersed in TdT buffer (pH 7.0; 140mM sodium chloride, 1mM cobalt chloride) for 5 minutes at room temperature. TdT enzyme was added in the ratio 10u:50µl to a solution of digoxigenin labeled dUTP nucleotides (0.2%v/v in TdT buffer) to produce the labeling solution. A negative control comprised of digoxigenin labeled dUTP nucleotides alone. Slides were removed from TdT buffer and 50µl of labeling solution or negative control applied. Slides were incubated at 37°C for 2 hours. Slides were racked and washed twice in ultra pure water for 5 minutes then in blocking solution (3%w/v Bovine serum albumin, 0.1% w/v Triton x-100, 100ml TBS) for 10 minutes. 100µl of a 1:600 dilution of alkaline phosphatase conjugated anti-digoxigenin antibody in blocking solution was applied to each slide and the slides incubated at room temperature for 30 minutes. Slides were racked and washed twice in TBS for 10 minutes. Chromogen solution was prepared from a tablet of 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine and Nitro-Blue Tetrazolium Chloride

(BCIP/NBT) in sterile water. 100µl was added to each slide and left to develop for 15 minutes. Slides were washed in ultra pure water for 5 minutes, counterstained with haematoxylin for 5 minutes and rewashed before mounting using an aqueous mountant and cover slips.

During the assay TdT enzyme adds digoxigenin labeled dUTP nucleotides to the exposed 5' and 3' ends of DNA cleaved during apoptosis. Digoxigenin then binds the anti-digoxigenin antibody which itself is conjugated to alkaline phosphatase. Thus the exposed DNA ends are indirectly bound to alkaline phosphatase.

BCIP acts as a substrate for the enzyme alkaline phosphatase which phosphorylates BCIP. The product of this reaction is then oxidized NBT to give a dark blue color in cells with cleaved DNA

Figure 7-5).

#### *7.3.11.3 Insulin immunohistochemistry.*

A three step indirect system was used for insulin immunohistochemistry. Endogenous peroxidase activity in dewaxed slides was blocked by immersion in 6% hydrogen peroxidase for 10 minutes. Slides were washed in TBS for 5 minutes and further blocked with 50µl of a 1:20 dilution of NGS in blocking solution (3%w/v Bovine serum albumin, 0.1% w/v Triton x-100, 100ml TBS) for 10 minutes. NGS was poured away and 100µl of a 1:50 dilution of the primary mouse anti-insulin antibody added to each (HB124 in house stock) for 30 minutes at room temperature. After washing in TBS 100µl of secondary biotinylated goat anti-mouse antibody was applied for 30 minutes and the sections rewashed in TBS. 100µl of the streptavidin and biotin/horseradish peroxidase detection complex (DAKO, UK) was applied for 30 minutes. Slides were then washed in TBS for 20 minutes and 100µl of the chromogen 3,3'-diaminobenzidine (DAKO, UK) applied to each to generate the brown immunoprecipitate.

#### *7.3.11.4 TUNEL semiquantification.*

Each slide was randomised and assessed for staining by two observers. Slides were assessed by light microscopy x 40 magnification (Nikon, Japan). Islets were identified by the position of positive insulin staining, and islet and acinar tissues separately assessed for the presence of dark blue positive TUNEL staining. Ten microscope fields of both islet and acinar tissues were assessed for total cell number and number of positively



### 7.3.12.2 ADP:ATP Assay.

Reagents were provided in an Apoglow™ kit (BioWhittaker, UK) and were allowed to warm to room temperature before commencing the assay. To the Eppendorf containing the sample 100µl of RPMI (Sigma, UK) was added and vortexed for ten seconds. 100µl of nucleotide releasing reagent was added and the reaction allowed to proceed at room temperature for 5 minutes. 180µl was transferred from the Eppendorf into a white walled DNase treated luminometer tube, 20µl of ATP monitoring reagent (firefly luciferase, D-luciferin, 50mg BSA, 0.5 mmol magnesium acetate, 0.1µmol inorganic pyrophosphate) was rapidly added and the luminometer (Wallac Series 1900, UK) cassette turned to allow a reading to be obtained in mV on a chart recorder (Wallac, UK). After the initial rapid peak of ATP luminescence had been recorded the reaction was left to decay for 10 minutes before a further baseline reading was obtained. 20µl of ADP converting reagent was then added to generate a second peak of ATP luminescence on the recorder. ADP:ATP ratios were calculated from the readings made using the equations previously described. After completion of the assay 500µl of the supernatant was added to a sterile Eppendorf with 1.5ml of DNA assay buffer (pH 7.4; 2M sodium chloride, 0.05M disodium hydrogen phosphate, 2mM EDTA) and frozen for DNA assay (-20°C).

### 7.3.13 Determination of ATP:DNA ratio.

Samples of frozen supernatant from the firefly luciferase assay were thawed at room temperature for 30 minutes. After vortexing for 10 seconds 400µl was transferred to a sterile DNase treated Eppendorf and 2.6ml of fluorochrome reagent was added (1µg/ml Hoechst dye 33258 (Sigma) in NTE buffer). The reaction was left to proceed for 30 minutes at 30°C incubation in a waterbath. 1ml was then transferred to a DNase treated fluorometer cuvette and read on a fluorometer at wavelength of 350nm excitation and 455nm emission. A serial dilution of 50µg/ml calf thymus DNA (Sigma D1501) in DNA assay buffer was used to generate standards from which standard curve was plotted. Sample fluorescence readings in mV were then converted to DNA concentrations by comparison with the standard curve.

The ATP concentration generated by the firefly luciferase assay was used to calculate the ATP:DNA ratio for each sample.

### 7.4 Statistical Methodology.

A one way analysis of variance (ANOVA) was used to compare mean islet yield and cleavage, gradient contamination and stimulation indices. A two factor mixed ANOVA was used to compare the mean proportions of TUNEL positive staining in islet and acinar tissues, ADP:ATP and ATP:DNA ratios. Post hoc Tukey tests were performed for multiple pairwise comparisons of group means. Factor analysis was used to establish if islet yield and *in vitro* function correlated with any latent variables. The ADP:ATP ratio of post-preservation pancreatic tissue was specifically studied during factor analysis after it showed strong correlations with islet yield and *in vitro* function in the rodent experiments (*vide supra* 6.5.8).

### 7.5 Results.

#### 7.5.1 Islet yield and cleavage.

There were significant differences in mean islet yield ( $F(3,31)= 7.7; p=0.001$ ) but no difference in mean cleavage index ( $F(3,31)=1.5; p=0.23$ ) between preservation methods. Figure 7-6 and Figure 7-7 illustrate the mean islet yields per gram of pancreas and mean islet cleavage indices respectively for each preservation method.

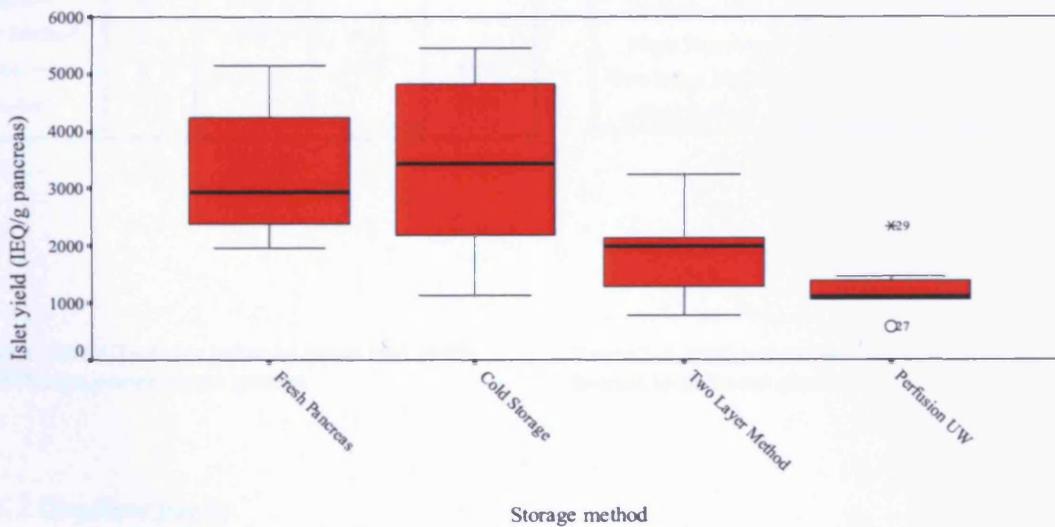


Figure 7-6. Mean islet yields after pancreas preservation by different methods.

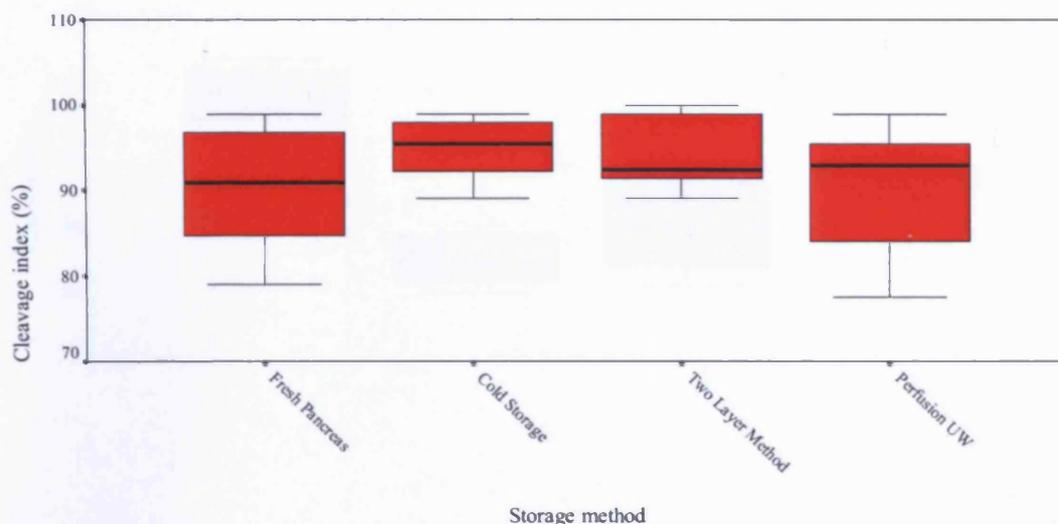


Figure 7-7. Mean islet cleavage indices after pancreatic preservation by different methods

Table 7-3 and Table 7-4 show increasing mean islet yields and cleavage indices between groups. A lack of subset overlap denotes a significant difference between preservation methods.

Storage method	N	Subsets		
		1	2	3
Perfusion UW	8	1250		
Two Layer Method	8	1858	1858	
Fresh pancreas	8		3279	3279
Cold Storage	8			3438

Storage method	Mean cleavage index (%)
Perfusion UW	90
Fresh Pancreas	90
Two Layer Method	94
Cold Storage	95

Table 7-3. Differences between mean islet yields in different preservation groups.

Table 7-4. Differences between islet cleavage indices in different preservation groups.

### 7.5.2 Gradient purity.

There were significant differences in gradient purity between the preservation methods used ( $F(3,31) = 42.0$ ;  $p < 0.001$ ). Figure 7-8 shows the mean % amylase contamination in each preservation group.

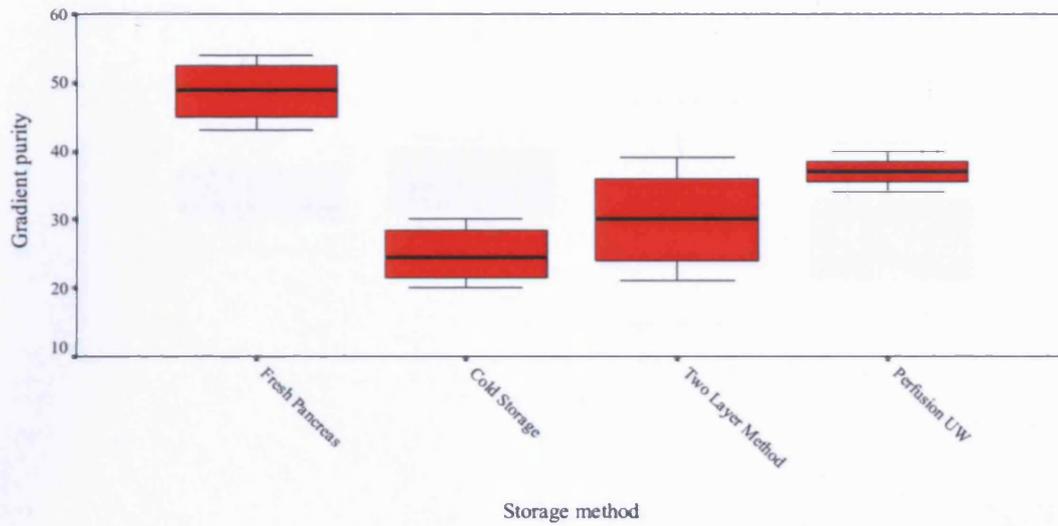


Figure 7-8. Minigradient contamination at 60% islet yield after different preservation methods.

Table 7-5 shows the comparable amylase contamination in cold stored and TLM preserved pancreata, with a significant increase in contamination in perfused and unpreserved pancreata.

Storage method	Subset		
	1	2	3
Cold Storage	24.9		
Two Layer Method	30.0		
Perfusion UW		37.0	
Fresh Pancreas			48.8

Table 7-5. Significantly increasing levels of gradient contamination between preservation groups.

### 7.5.3 *In vitro* islet function.

No significant differences in stimulation indices were observed between preservation methods ( $F(3,31) = 1.8$ ;  $p = 0.16$ ). Figure 7-9 illustrates the mean SI amongst the four preservation groups.

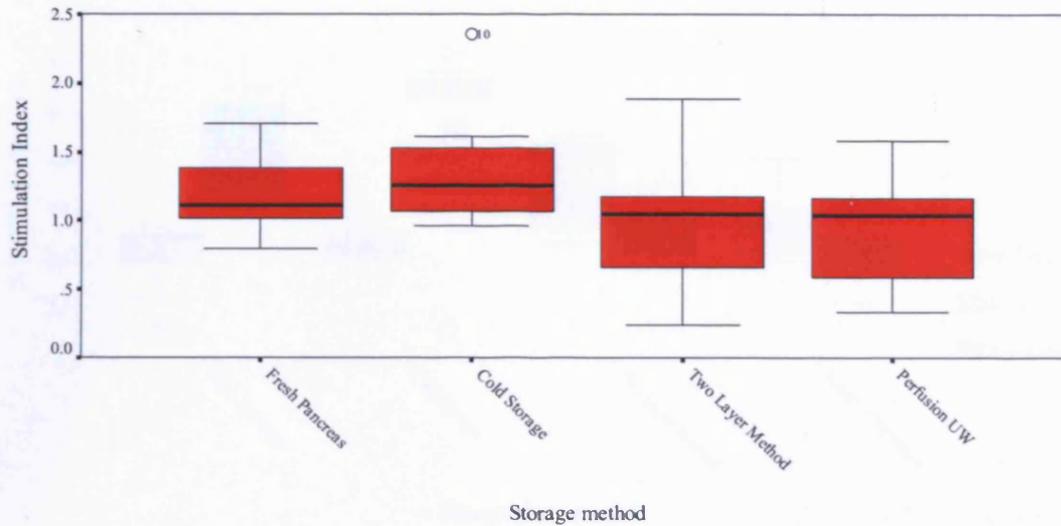


Figure 7-9. Perfusion stimulation indices after different methods of pancreas preservation.

Table 7-6 illustrates the ascending mean stimulation indices across the preservation groups.

Storage method	N
Perfusion UW	.94
Two Layer Method	.98
Fresh Pancreas	1.19
Cold Storage	1.38

Table 7-6. Increasing perfusion stimulation indices by preservation method.

#### 7.5.4 Assessment of Apoptotic Index by TUNEL Histochemistry.

There were significant differences in the mean proportion of TUNEL positive cells in islets and acinar tissues within groups ( $F(1,44)=86.9$ ;  $p<0.001$ ), between preservation methods ( $F(3,44)=12.8$ ;  $p<0.001$ ) and in each tissue type when variability due to the preservation method was accounted for ( $F(3,44)=94.7$ ;  $p<0.001$ )(Figure 7-10).

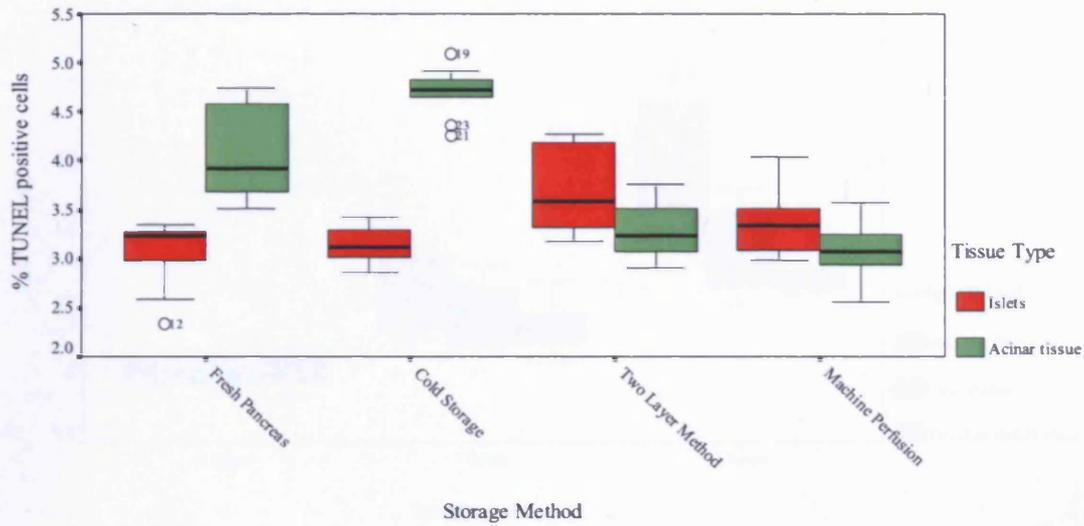


Figure 7-10. Variation in TUNEL staining in islets and acinar tissues after different preservation methods.

Table 7-7 shows the significantly reduced apoptotic index in the machine perfused group compared to freshly processed pancreata. These, in turn, had a significantly reduced apoptotic index compared with cold stored pancreata.

Storage Method	Subset		
	1	2	3
Machine Perfusion	3.2		
Two Layer Method	3.5	3.5	
Fresh Pancreas		3.6	
Cold Storage			3.9

Table 7-7. Differences in mean TUNEL apoptotic index between preservation methods.

**7.5.5 Pancreatic ADP:ATP ratio.**

There were significant differences in mean ADP:ATP between each preservation method ( $F(2,62)= 10.8$ ;  $p<0.001$ ), between each preservation duration ( $F(2,62)=84.9$ ;  $p<0.001$ ) and also when both these factors were accounted for ( $F(4,62)=4.0$ ;  $p=0.006$ ) (Figure 7-11).

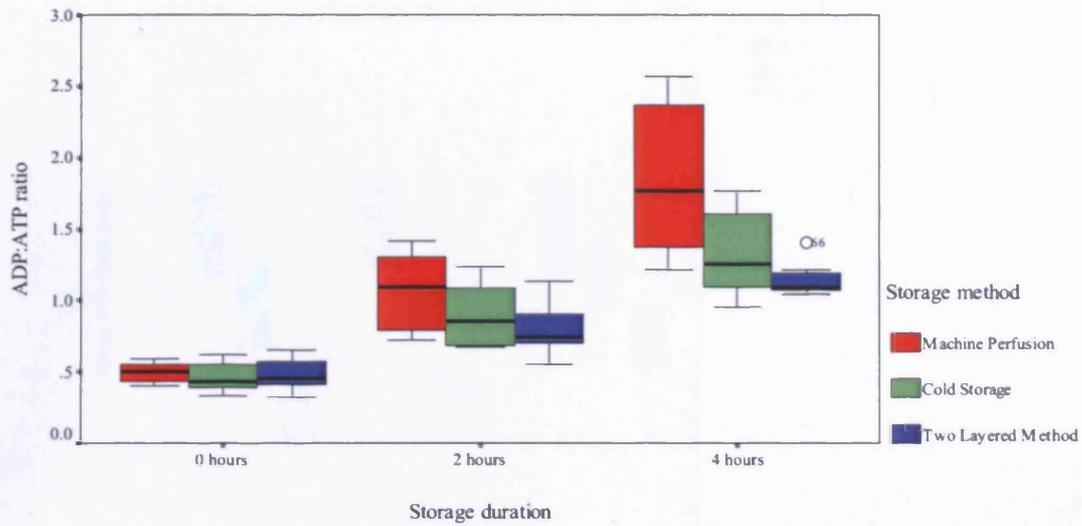


Figure 7-11. ADP:ATP ratios in the porcine pancreas preserved using different methods.

Table 7-8 and Table 7-9 show the significantly higher mean ADP:ATP ratio in the machine perfused group of pancreata compared to either TLM and cold stored groups, and the increase in mean ADP:ATP ratio with increasing duration of preservation.

Storage method	Subset		
	1	2	3
Fresh Pancreas	.73		
Two Layer Method	.99	.99	
Cold Storage		1.19	
Perfusion UW			1.78

Table 7-8. Mean porcine pancreas ADP:ATP ratios by preservation method.

Storage duration	Subset		
	1	2	3
0 hours	.48		
2 hours		.92	
4 hours			1.43

Table 7-9. Mean porcine ADP:ATP ratios with increasing duration of preservation.

### 7.5.6 Pancreatic ATP:DNA ratio.

There were significant differences in mean ATP:DNA ratio between each preservation method ( $F(2,62) = 3.3$ ;  $p < 0.043$ ) and between each preservation duration ( $F(2,62) = 55.5$ ;  $p < 0.001$ ) but not when both these factors were accounted for ( $F(4,62) = 1.4$ ;  $p = 0.234$ ).

Figure 7-12 shows the variation of mean ATP:DNA ratios in pancreatic tissue from each preservation group for all storage durations.

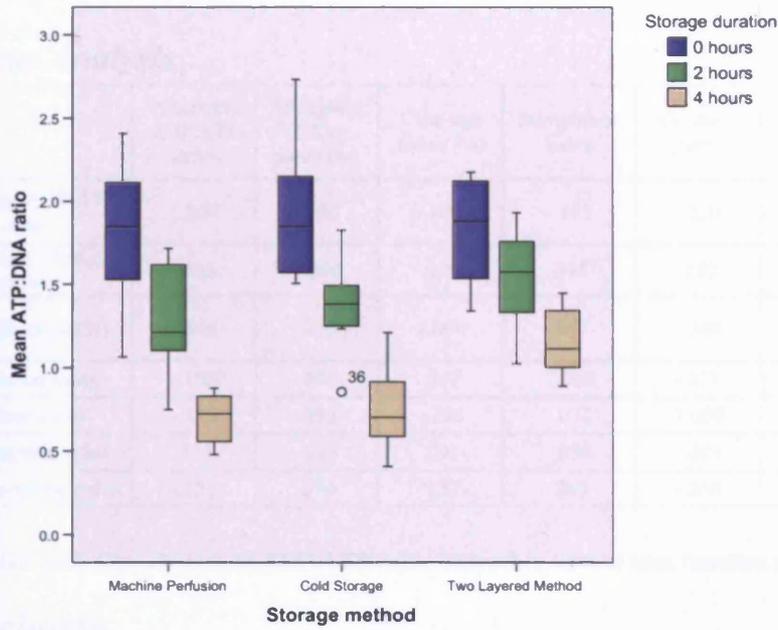


Figure 7-12. ATP:DNA ratios by different preservation methods.

Table 7-10 and Table 7-11 illustrate the comparable mean ATP:DNA ratios between the preservation methods and the significant reduction in mean ATP:DNA ratio as the duration of pancreas preservation is increased.

Storage method	
Machine Perfusion	1.30
Cold Storage	1.35
Two Layered Method	1.51

Storage duration	Subset		
	1	2	3
4 hours	.89		
2 hours		1.40	
0 hours			1.85

Table 7-10. Mean ATP:DNA ratios between different preservation groups.

Table 7-11. Mean ATP:DNA ratios with increasing duration of preservation preservation

### 7.6 Factor Analysis.

	Pancreatic ADP:ATP ratio	Islet yield (IEQ/g pancreas)	Cleavage index (%)	Stimulation Index	Gradient purity	Islet apoptotic index	Acinar apoptotic index
Pancreatic ADP:ATP ratio	1.000	-.505	-.006	-.183	-.220	.133	-.258
Islet yield (IEQ/g pancreas)	-.505	1.000	.016	.396	.092	-.221	.556
Cleavage index (%)	-.006	.016	1.000	.547	-.246	.091	.177
Stimulation Index	-.183	.396	.547	1.000	-.071	-.099	.385
Gradient purity	-.220	.092	-.246	-.071	1.000	-.203	-.268
Islet apoptotic index	.133	-.221	.091	-.099	-.203	1.000	-.275
Acinar apoptotic index	-.258	.556	.177	.385	-.268	-.275	1.000

Table 7-12. Correlations of ADP:ATP ratio with other tests of islet function and viability.

### 7.7 Conclusion.

Cold storage of the pancreas resulted in islet yields comparable to the fresh pancreas, and significantly higher yields than from TLM preserved or perfused pancreata. However, the cleavage index did not differ significantly between preservation groups.

Gradients from statically cold stored and TLM preserved pancreata showed significantly reduced levels of exocrine contamination compared to UW perfused and unperfused pancreata. Indeed, unperfused organs demonstrated the highest level of exocrine gradient contamination.

No significant difference in *in vitro* function was observed between preservation methods.

Machine perfused pancreata had a significantly reduced numbers of apoptotic cells compared to freshly processed pancreata; which, in turn, had a reduced apoptotic rate compared to cold stored pancreata.

Higher mean ADP:ATP ratios were found in machine perfused pancreata, compared to either TLM and cold stored groups, and the mean ADP:ATP ratio increased with the duration of preservation. High ADP:ATP ratio were correlated with a reduced islet yield (-0.51) but porcine pancreatic ADP:ATP ratio showed no correlation with other *in vitro* measures of gradient purity, islet function or apoptotic index.

## ***Chapter 8. Studies in the Human Donor Pancreas.***

### ***8.1 Introduction.***

This chapter describes experiments conducted using a small number of human donor pancreata. This work was conducted during the Diabetes UK led UK Islet Transplant Consortium (UKITC) attempt to replicate the Edmonton protocol in UK islet isolation and transplant centres. Working within the remit of this protocol prevented randomisation of pancreata into experimental groups and the use of different preservation methods. Nevertheless the ADP:ATP and ATP:DNA ratios, as a measure of pancreatic damage prior to human islet isolation, were studied alongside established measures of islet yield and function.

### ***8.2 Aims.***

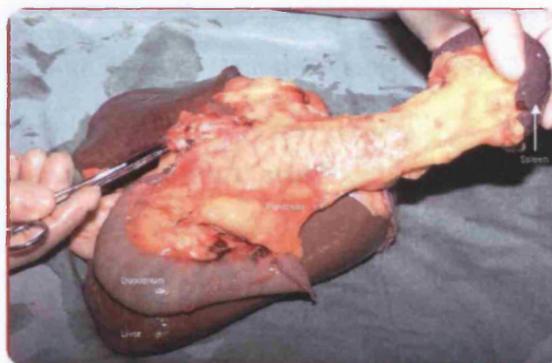
To correlate the ADP:ATP ratio in pancreatic tissue with the duration of pancreatic ischaemia, subsequent islet yield and *in vitro* function after human islet isolation.

### ***8.3 Methods.***

#### ***8.3.1 Human Donor Pancreas Procurement.***

As part of the Human Islet Transplant Programme eight human pancreata were procured from heart beating donors. Of these five were subjected to clinical islet isolation with the intention to perform clinical intraportal islet cell transplantation. Three were procured with donor family consent for research but were not processed for islet isolation as the isolation laboratory facilities were closed. These pancreata were subjected to further controlled periods of cold ischaemia to study the effect upon the ADP:ATP ratio. During multi-organ donor retrieval the pancreas was mobilised *in situ* by division of the splenic ligaments, Kocherisation of the duodenum and division of the gastrocolic omentum to enter the lesser sac prior to circulatory arrest. The splenic artery was identified close to its origin at the SMA and a 1/0 Vicryl™ (Ethicon®, Johnson & Johnson International, Belgium) ligature slung around the vessel but not tied. A 0.5cm<sup>3</sup> biopsy was taken with scissors from the pancreatic head and haemostasis secured with diathermy. Biopsy tissue was placed into a flexible microwell, snap frozen and stored under liquid nitrogen. After aortic cross clamping above the coeliac axis, and venting of

the inferior vena cava in the thorax, *in situ* cold perfusion was commenced via aortic and superior mesenteric arterial (SMA) cannulae. Hyperosmolar citrate solution was perfused via the aorta and UW solution via the SMA whilst the peritoneum and lesser sac were packed with sterile ice to maintain *in situ* cooling. After 1 litre of SMA perfusion the splenic artery ligature was tied to prevent over distension of the pancreas. After completion of *in situ* cold perfusion, and explantation of the liver, the first and third parts of the duodenum were divided using staplers (GIA 80mm, Autosuture, TycoHealthcare, UK). The pancreas was excised from the retroperitoneum from lateral to medial with sharp dissection and forward traction on the mobilised spleen (Figure 8-1).



**Figure 8-1. En bloc retrieval of the human donor pancreas.**

In a large kidney bowl of 4°C UW solution the main pancreatic duct entering the second part of the duodenum was identified, cannulated with a long 18g catheter (Abbotcath, UK) and secured with a 1/0 Vicryl™ ligature. A second 0.5cm<sup>3</sup> biopsy was taken from the pancreatic head and placed into a flexible microwell, snap frozen and stored under liquid nitrogen. The duodenum and spleen were then excised from the specimen and the pancreas placed in triple layer sterile plastic bags with 500ml of fresh 4°C UW solution. The pancreas was transported to the islet isolation laboratory on melting ice in a polystyrene human organ retrieval box with generic UK Transplant donor information forms.

### **8.3.2 Human Islet Isolation.**

Islet isolations were performed in the University Hospitals of Leicester Islet Isolation Laboratory. This facility was accredited by the Department of Health to perform clinical islet isolation and adheres to the Code of Practice for Tissue Banks. All procedures adhered to the laboratory Quality System as outlined in the Quality Manual(953).

### **8.3.3 Pancreas Distension.**

In a sterile Class II microbiological cabinet the pancreas was removed from its transport medium, weighed and disinfected by serial immersion into aqueous solutions of 10% povidone iodine (Betadine, Purdue, UK), cefuroxime (Zinacef, GlaxoSmithKline, UK), amphotericin B (Ambisome, Astellas Pharma, USA) and HBSS at 4°C. It was placed onto a cooled stainless steel tray (University of Alberta, Canada) with 100ml HOC 4°C and excess fat and lymphoid tissue was trimmed without disruption of the pancreatic capsule. This tissue was weighed prior to being discarded. Two 18G cannulae (Abbotcath, UK) were inserted retro- and antegrade after dissecting into the pancreatic duct and secured with 2/0 Vicryl™ ligatures (Ethicon®, Johnson & Johnson International, Belgium). A 0.5cm<sup>3</sup> biopsy was taken with scissors from the pancreatic head, placed into a flexible microwell, snap frozen and stored under liquid nitrogen.

The pancreas was distended with 250ml human Liberase solution (Roche, UK; 0.14%w/v in HBSS) by controlled perfusion with Watson-Marlow pumps on a bespoke stainless steel platform (University of Alberta, Canada). Temperature was monitored with Oakton thermometers and maintained at 4 to 7°C. Perfusion pressure was monitored through rigid PVC tubing using pressure transducers and maintained at 80mmHg for the first 5 minutes of distension. After this the pump rate was increased to generate 180mmHg perfusion pressure for a further 5 minutes. Leaks from the pancreatic capsule were reduced by application of haemostats to ensure uniform pancreatic distension.

### **8.3.4 Pancreas Digestion.**

The distended pancreas was divided into 8 equal sized portions to aid digestion and transferred, with the Liberase® solution, into a stainless steel automated chamber divided by a 500µm mesh and containing seven 1cm<sup>3</sup> steel spheres to aid mechanical disruption. The chamber was connected to a closed circuit of PVC tubing (Gambro, UK) comprising a Watson –Marlow pump, heat exchanger and bubble trap/air release valve (designed in house; manufactured by COBE Laboratories, UK). The circuit was primed with MEM (Cambrex, UK) and heated gradually to 37°C during which time the chamber was manually agitated two or three times per minute. Tissue exiting the chamber was sampled via a three way tap (5ml), stained with 0.006%w/v dithizone (pH 7.8) and examined under a light microscope (Nikon, Japan) for the presence of ‘cleaved’ islets devoid of surrounding acinar tissue. Once these were identified, or after a maximum closed circuit digestion period of 19 minutes, the circuit was opened to allow collection of the digest into 250ml centrifuge flasks (Jencons, UK) containing 15ml 20% human albumin

solution (Zenalb, Bio Products Laboratory, UK). Digest was collected at 120ml/min with continuous manual agitation of the chamber until 8 litres of media had run through the circuit. Once empty the chamber was opened and the undigested pancreatic remnant was weighed. This, and the mass of previously excised tissue, was subtracted from the weight of the pre-digested tissue to give a total weight of digested pancreas.



Figure 8-2. Pancreatic digest prior to COBE purification.

### **8.3.5 Islet Purification.**

Flasks containing digest were centrifuged at 200g for 2 minutes at 4°C and the supernatant decanted. Tissue pellets were resuspended in MEM and pooled by 3 serial centrifuge washings to obtain the digest in a single 250ml flask (Figure 8-2). A 100µl digest sample was taken with a Drummond pipette and mixed with 900µl MEM for islet quantification. The digest was left on melting ice for 15 minutes before a final centrifugation to determine the digest packed cell volume. Each 35 ml of PCV was resuspended in 100ml of UW solution it was left on melting ice for a further 45 minutes. Islets were purified using continuous density gradient separation on the COBE 2991 cell processor (COBE Laboratories, UK). Up to 35ml of resuspended digest PCV was used for each COBE gradient. The density gradient was constructed from 260ml high density (1.100g/cm<sup>3</sup>) Ficoll 400 ® (Biocoll, Gentaur, Belgium) and 140ml low density (1.077g/cm<sup>3</sup>) Ficoll 400 ®. Using a two sided gradient maker, Watson –Marlow pump and stirring magnetic flea, 130ml of high density solution was run into the COBE 2991 processor bag whilst running the COBE at 1000rpm. With a further 130ml of high density solution and 140ml of low density solution in either side of the gradient maker the

remainder of the linear density gradient was overlaid by running the pump and turning the flea on a magnetic stirring platform.

As the gradient maker was about to empty the 100ml aliquot of digest was added to the gradient and the COBE rate increased to 1800rpm. Finally 50ml of UW was added to complete the gradient. The hydraulics of the COBE were used to expel air from the bag and the COBE run for 4 – 6 minutes at 1800 rpm.

110ml was decanted from the top of the gradient followed by 12 fractions of 30ml collected into separate 50ml conical tubes. 100µl samples of each fraction were sampled with a Drummond pipette for islet quantification. The remainder of each fraction was washed in MEM and centrifuged at 600g for 2 minutes at 4°C, then twice more with centrifugation at 200g for 2 minutes at 4°C. The packed cell volume of each fraction was recorded and fractions were pooled based upon their islet content.

Where islet numbers were insufficient or the packed cell volume was too high for transplantation, pooled islet fractions were transferred to sterile T25 tissue culture flasks (Sarstedt, UK) containing a total volume of 40ml M199 transplant medium (12.5%v/v 20% human albumin solution; Cambrex, UK) for overnight culture at 37°C.

### ***8.3.6 Unprocessed pancreata.***

Three procured pancreata were not processed for islet transplantation. After arrival in the islet isolation laboratory pancreata were left in static cold storage in their transport UW media at 4°C. Biopsies were taken from the pancreatic head, mid pancreas and tail after 1 hour, 2 hours, 4 hours and 8 hours of cold ischaemia. Biopsies were placed in flexible microwells, snap frozen and stored under liquid nitrogen.

### ***8.3.7 Digest and fraction yields.***

A 100µl sample of digest was diluted in 0.9ml MEM. From this two further 100µl samples were streaked across a Petri dish using a Drummond pipette. To the streaks 20µl of 0.006%w/v dithizone (pH 7.8) was added with a Gilson pipette. Streaks were examined under a light microscope (Nikon, Japan) × 100 with a 100µm graticule (Cell Finder, Holland). Islets with diameters of greater than 50µm were counted in groups of 50µm ascending sizes. Islet counts were adjusted to IEQs and multiplied by the dilution factor 25000 to generate a total digest IEQ count. This was divided by the weight of digested tissue to give a yield in IEQ per gram of pancreas. The method described was also used for purified islets before and after cell culture purification, though the dilution

factor was adjusted to reflect the volume in which fractions were pooled and resuspended.

### **8.3.8 Islet *in vitro* function.**

#### *8.3.8.1 Static incubations.*

Duplicate static incubations were performed after aliquots of 20 islets were handpicked from culture medium using a drawn out glass micro-pipette. Islets were placed onto 4µm mesh inserts (Costar Corning, UK) in a 12 well plate (Corning Costar, UK) containing 2ml per well of low concentration glucose solution (1.7mM; 66%v/v GBSS, 33%v/v MEM, 0.05%w/v BSA). After 1 hour the mesh inserts were transferred to another 12 well plate containing low concentration glucose for 30 minutes, then to a plate containing high concentration glucose solution (25mM; 66%v/v GBSS, 33%v/v MEM, 0.05%w/v BSA, 0.3% w/v glucose) for 30 minutes and finally to low concentration glucose solution for 30 minutes. As the islets were moved between solutions, 1ml aliquots were aspirated from the wells and frozen at -20°C for insulin assay.

After insulin quantification the SI was calculated using Equation 6-1 (*vide supra* 6.3.8).

#### *8.3.8.2 Insulin assay.*

A solid phase two site ELISA kit was used to measure human insulin content. This used two monoclonal antibodies directed against different epitopes of human insulin to generate a colour change.

The kit was supplied with standard insulin concentrations, and 25µl aliquots of these were added in duplicate to the first 16 wells of the 96 well assay plate. 25µl aliquots of the test samples were added in duplicate to the remaining wells. To each well 50µl of peroxidase conjugated anti-insulin antibodies were added, and the plate incubated for 2 hours. The plate was then washed in ultrapure water using a plate washer (Denley Wellwash, Denley, UK) and 200µl of 3,3',5,5' – tetramethylbenzidine was added. After 15 minutes the reaction was ceased by the addition of sulphuric acid to generate the end colour change. Plates were read using a plate reader (Denley, UK) at 450nm wavelength. A standard curve was generated for each plate from which the insulin content of the remaining wells could be calculated.

### **8.3.9 Determination of ADP:ATP ratio.**

#### *8.3.9.1 Sample preparation.*

Frozen pancreas biopsies were removed from their freezing vials under liquid nitrogen storage and rapidly transferred into a cryostat (-20°C). All tissue handling was performed using metal forceps to prevent excessive warming of the samples. The flexible microwell encasing the biopsy was cut away using a scalpel blade (Swann Morton, UK) and the sample adhered in Tissuetek and mounted on cork by snap freezing in liquid nitrogen. Ten serial 10µm frozen sections were cut using the cryostat and transferred to individual pre-cooled sterile Eppendorfs. Sections were then stored temporarily under liquid nitrogen until required for ADP:ATP assay which proceeded expediently. Triplicate specimens from each pancreas were subjected to the assay.

#### *8.3.9.2 ADP:ATP Assay:*

Reagents were provided in an Apoglow™ kit (BioWhittaker, UK) and were allowed to warm to room temperature before commencing the assay. To the Eppendorf containing the sample 100µl of RPMI (Sigma, UK) was added and vortexed for ten seconds. 100µl of nucleotide releasing reagent was added and the reaction was allowed to proceed at room temperature for 5 minutes. 180µl was transferred from the Eppendorf into a white walled DNase treated luminometer tube, 20µl of ATP monitoring reagent (firefly luciferase, D-luciferin, 50mg BSA, 0.5 mmol magnesium acetate, 0.1µmol inorganic pyrophosphate) was rapidly added and the luminometer (Wallac Series 1900, UK) cassette turned to allow a reading to be obtained in mV on a chart recorder (Wallac, UK). After the initial rapid peak of ATP luminescence had been recorded the reaction was left to decay for 10 minutes before a further baseline reading was obtained. 20µl of ADP converting reagent was then added to generate a second peak of ATP luminescence on the recorder. ADP:ATP ratios were calculated from the readings made using the equations previously described. After completion of the assay 500µl of the supernatant was added to a sterile Eppendorf with 1.5ml of DNA assay buffer (pH 7.4; 2M sodium chloride, 0.05M disodium hydrogen phosphate, 2mM EDTA) and frozen for DNA assay (-20°C).

### 8.4 Statistical Methodology.

A one way ANOVA was used to compare mean islet yields in pancreatic digest, following COBE purification and after overnight cell culture; and for comparison of islet *in vitro* function. A two factor mixed ANOVA was used to compare ADP:ATP ratios from pancreata subjected to controlled periods of cold ischaemia.

### 8.5 Results.

#### 8.5.1 Donor Pancreas Characteristics.

Donor demographics, clinical parameters and ischaemia times were extracted from accompanying UK Transplant donor information forms and digest data from measurements made during the islet isolation. These are summarized in Table 8-1.

(\* indicates processed for islet isolation).

Pancreas Number	Donor age & sex	Donor BMI	Warm ischaemia time (mins)	Cold ischaemia time prior to arrival (mins)	Digested tissue weight (grams)	Closed circuit digestion time (mins)	Open circuit collection time (mins)	Digest PCV (ml)
1*	49 years, female	24 kg/m <sup>2</sup>	0	233	159	17	63	33
2*	34 years, female	26 kg/m <sup>2</sup>	3	247	179	14	55	28
3*	44 years, female	29 kg/m <sup>2</sup>	0	258	203	18	48	44 (2 COBE runs)
4*	55 years, female	25 kg/m <sup>2</sup>	6	279	116	14	61	30
5*	41 years, male	28 kg/m <sup>2</sup>	0	242	136	16	59	35
6	41 years, female	31 kg/m <sup>2</sup>	0	228	-	-		
7	47 years, female	28 kg/m <sup>2</sup>	5	158	-	-		
8	50 years, female	25 kg/m <sup>2</sup>	0	193	-	-		

Table 8-1. Human pancreas donor and digest data.

### 8.5.2 Digest and purified islet yields.

There were comparable digest ( $F(4,9)=0.4$ ;  $p=0.8$ ), post-purification ( $F(4,9)=0.53$ ;  $p=0.72$ ) and post-cell culture ( $F(4,9)=1.33$ ;  $p=0.38$ ) islet yields from all five processed human pancreata. Figure 8-3 to Figure 8-5 show the distribution of islet yield by size in pancreatic digest, after purification and cell culture.

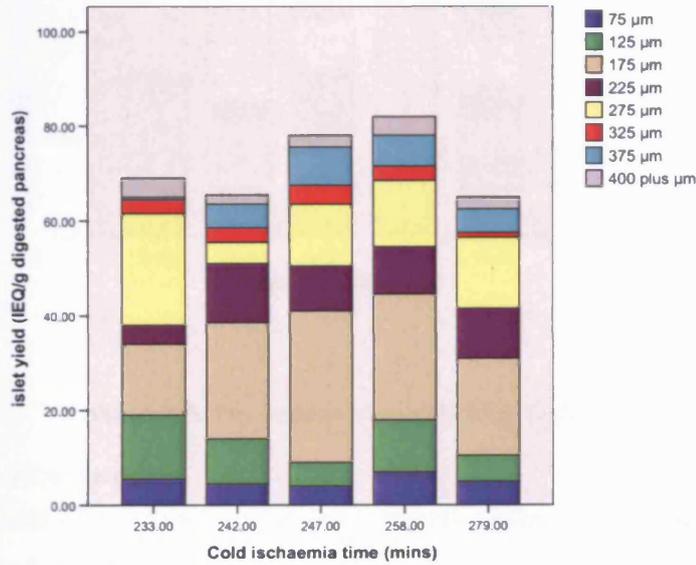


Figure 8-3. Digest yields from human pancreata.

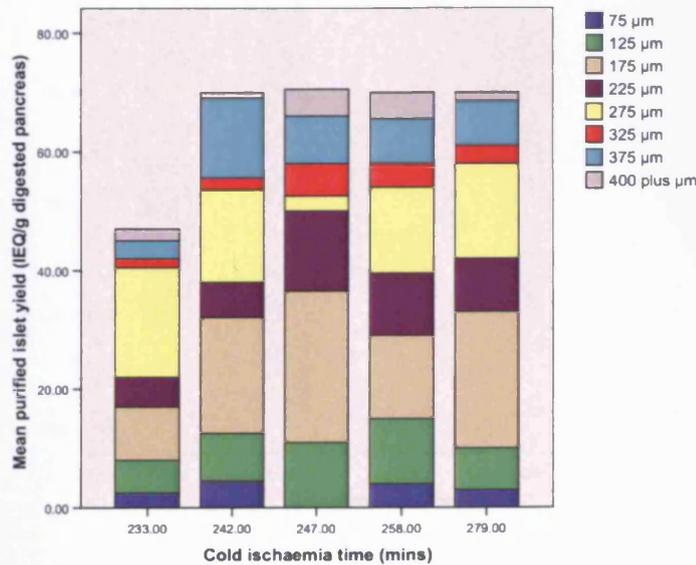


Figure 8-4. Purified islet yields from human pancreata.

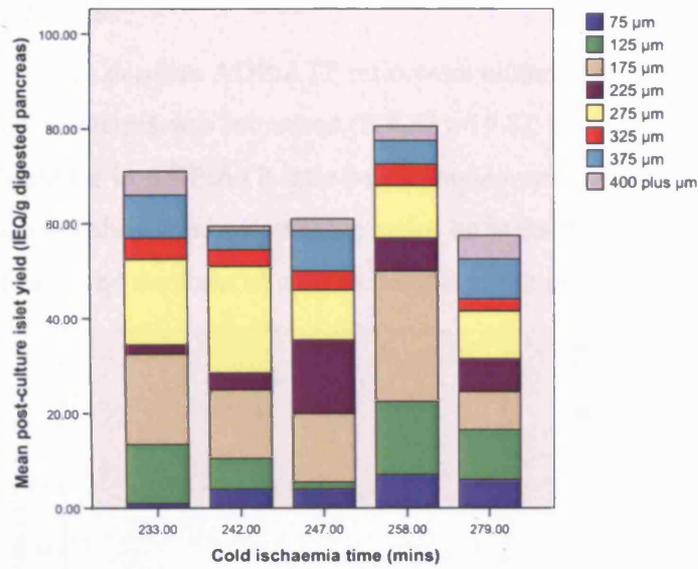


Figure 8-5. Post-culture islet yields from human pancreata.

### 8.5.3 Islet in vitro function.

Comparable islet stimulation indices were obtained from all pancreata ( $F(4,9)=0.25$ ;  $p=0.9$ ). Figure 8-6 shows the mean stimulation indices from islets isolated from the five pancreata subjected to islet isolation.

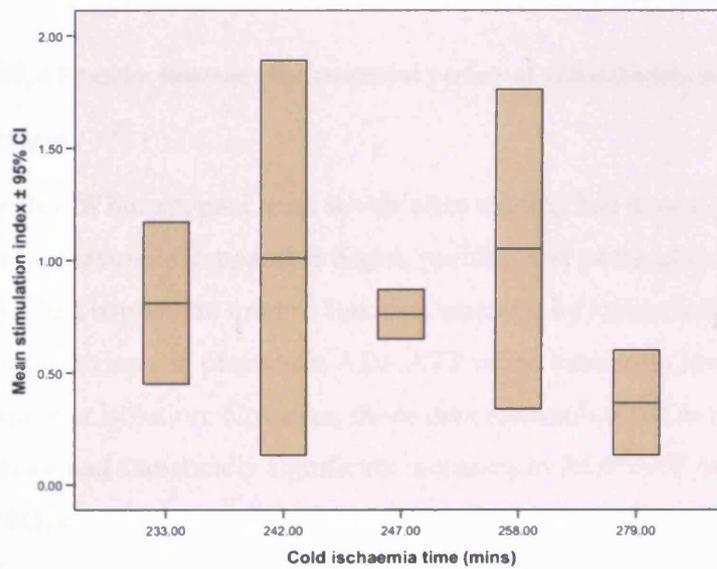


Figure 8-6. Islet stimulation indices from human pancreata.

### 8.5.4 ADP:ATP ratio.

Significant increases in mean ADP:ATP ratio were obtained as the duration of controlled pancreatic cold ischaemia was increased ( $F(4,32)=19.8$ ;  $p<0.001$ ). However, no significant difference in ADP:ATP ratio was seen *between* pancreata ( $F(28,32)=1.7$ ;  $p=0.07$ ). Figure 8-7 shows the comparable ratios between individual pancreata, but increasing ratios as the duration of cold ischaemia is increased.

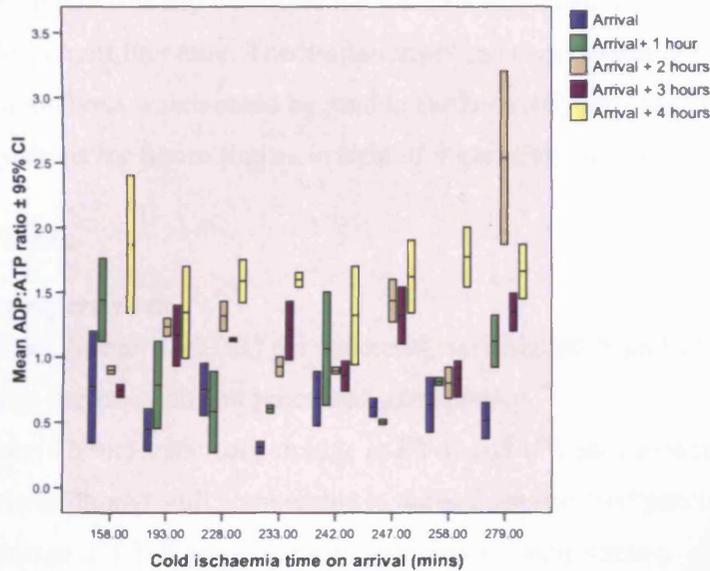


Figure 8-7. ADP:ATP ratios obtained after controlled periods of cold ischaemia in the human pancreas.

### 8.6 Conclusion.

The small number of human pancreata which were studied had comparable durations of warm and cold ischaemia. Comparable digest, purified and post-culture islet yields were obtained which had equivalent *in vitro* function assessed by static incubation.

No significant differences in pancreatic ADP:ATP ratios were seen between pancreata used for human islet isolation. However, those pancreata subjected to controlled periods of cold ischaemia had statistically significant increases in ADP:ATP ratio with increasing duration of storage.

## ***Chapter 9. Discussion, Limitations and Future Work.***

### ***9.1 Introduction.***

This chapter summarises and discusses the findings of the experimental work in the context of the current literature. The limitations of the experiments are discussed, together with methods which could be used to further verify the experimental findings. Finally suggestions for future studies in light of these experimental findings are made.

### ***9.2 Islet Yields.***

#### ***9.2.1 Rodent experiments.***

Total islet yields (mean 1093 IEQ per pancreas) were highest from fresh pancreata subjected to no preservation and processed immediately.

Short duration (4 hour) static cold storage in ET-K and UW solutions resulted in slightly lower islet yields, though still comparable to those from unstored pancreata. Prolonged (8 hour) cold storage in ET-K solution resulted in a yield comparable to short duration UW cold storage, but when UW solution was used for prolonged storage islet yield was significantly reduced. Notably pancreata exposed to short (1 hour) and long (4 hour) periods of TLM preservation showed no significant increase in islet yield over prolonged cold storage in either UW or ET-K solution.

The lowest yields were from pancreata exposed to warm ischaemia followed by short duration cold storage. Interestingly these yields were comparable with those obtained from 8 hour UW cold stored pancreata regardless of the duration of TLM used. However, the majority of methods involving ET-K cold storage resulted in yields significantly higher than pancreata exposed to warm ischaemia.

#### ***9.2.2 Porcine experiments.***

The highest islet yields were obtained from unpreserved and static cold stored porcine pancreata. Yields from TLM preserved pancreata compared to those obtained from unpreserved pancreas; whilst the yield from UW perfused organs was significantly less than both unpreserved and statically cold stored pancreata.

Though statically cold stored pancreata had the highest cleavage index, there was no significant difference in cleavage index between either group.

### **9.2.3 Human pancreas study.**

The human pancreata processed had comparable durations of warm and cold ischaemia (range 0 to 6 mins and 233 to 279 mins respectively) and had comparable islet yields in the digest, after COBE purification and after overnight islet cell culture.

### **9.2.4 Discussion.**

The best rodent islet yields, obtained from unpreserved pancreata, compare with previously published data(954).

There is little published literature on the use of trehalose containing ET-K solution in rodent pancreas preservation and its effect during static cold storage has not been previously established. Miyamoto *et al* reported increased islet yields from porcine pancreata exposed to a modified TLM preservation using PFC and Kyoto solution(675). Noguchi *et al* recently used a modified TLM, comprising PFC and a new Kyoto solution (M-Kyoto) containing the trypsin inhibitor ulinastatin. Porcine islet yields were significantly increased using this novel method, whilst trypsin activation was reduced and collagenase activity augmented(955). It is difficult to establish if the use of Kyoto solution alone, or its combination with TLM, contributed to the increased yields in these studies.

The low islet yields from TLM preserved rodent pancreata are contrary to the findings of previous studies. The study of canine pancreas TLM preservation by Tanioka *et al* demonstrated improved islet yields after only 3 hours of TLM(652); whilst Hiraoka *et al* achieved yields of upto 916 islets per pancreas after 24 hours of TLM preservation(655). It is possible that the 4 hours of TLM preservation used in these experiments was insufficient to allow resuscitation of rodent pancreata after a short duration cold ischaemic insult.

Reduced yields from warm ischaemically damaged pancreata are fairly well appreciated and consistent with results from the published literature. Tellez-Yudilevich *et al* first described the poor islet yield from rodent pancreata exposed to 30 minutes of warm ischaemia(956). This was also observed by Avila *et al* who reported a fall in the mean islet yield per pancreas from 503 to 247 islets after the same warm ischaemic period(957). Corlett *et al* demonstrated 45.7 and 52.5% rodent and canine islet yields after 45 minutes of warm ischaemia compared to freshly processed pancreata. These yields further decreased to 15.6 and 23.9% respectively after 90 minutes of warm ischaemia(958).

The porcine islet yields in this study compared with those of the published literature. Ching *et al* used a semi automated isolation method to achieve an islet yield of  $2398 \pm 143$  islets per gram of fresh pancreas but used a different animal strain and age, isoflurane anaesthesia, *in situ* UW arterial perfusion and collagenase P for pancreas digestion(959). Ricordi *et al* reported a wide range of islet yields (16 400 to 1 265 000 islets per pancreas) from large white porcine islet isolations, and found warm ischaemia time to be the limiting factor for successful isolation. After 18 to 20 minutes of warm ischaemia, exocrine cells were beginning to contaminate the gradients suggesting a degree of autolysis and exocrine cell swelling(960). Though not designed to investigate warm ischaemia, the porcine experiments reported in this thesis had an inherent period of pancreatic warm ischaemia (6.8 to 8.3 minutes) during explantation which may have been sufficient to allow early autolysis which could reduce islet yields.

No literature exists to corroborate the low islet yields from perfused porcine pancreata. Though Toledo-Pereya *et al* found machine perfusion to be 'a safe and reliable method for pancreas preservation', they used a canine model, a SGFP perfusate with over 24 hours of perfusion and report no data on overall islet yield(961). However, this study did report islet fragmentation following perfusive preservation which is consistent with the reduced mean islet diameters found in the perfused group in this thesis. Though not specifically studied in these experiments, it is possible that machine perfusion may exaggerate changes in islet and exocrine cell densities resulting in significant loss of islets into other gradient fractions and contamination of islet fractions by exocrine components. Islet yields from the small number of human pancreata were low compared to the published literature (mean digest count 71.9 IEQ/g, mean purified count 65.9 IEQ/g, mean post-culture count 65.5 IEQ/g). The most likely explanation for this is the short period of cold ischaemic storage (mean 252 minutes). Toso *et al* demonstrated an increased islet yield where cold ischaemia did not exceed 8 hours, but also showed reduced islet yields with cold ischaemia of less than 3 hours(962). Hence it would appear that a short period of cold ischaemic preservation is beneficial for human islet isolation allowing reversal of cellular oedema after organ retrieval and activation of endogenous proteases which has a permissive effect upon later collagenase digestion(962).

#### **9.2.5 Limitations.**

The discrepancies in islet yield, between the experiments herein and the published literature, may be explained by the methodology used. Variations in animal strains,

weight, age and collagenase batches are known to have effects upon islet yield, and make it difficult to make direct comparisons with published studies.

Notably the rodent experiments used intraductal flushing with PBS to maintain duct patency during preservation, and previous studies have advocated ductal flushing with HBSS and collagenase to optimize rodent islet yield(963). Preservation with intraductal collagenase, and the optimal flush solution, is likely to be dependent upon the animal model, duration and method of preservation. Whilst maintaining duct patency during preservation is desirable to allow good collagenase distribution through the pancreas; over exposure to collagenase can result in over-digestion and islet fragmentation, and UW flushing has an inhibitory effect upon the action of collagenase(964).

Timing of collagenase administration is similarly critical during porcine islet isolation. White *et al* reported significantly higher islet yields (mean 21 524 IEQ/g pancreas), than achieved in this thesis, by intraductal administration of collagenase in HBSS at the time of retrieval, followed by expedient islet isolation within 2 hours(220). Though this resulted in improved delivery of collagenase by preservation of the ductal system, this method is not applicable to porcine pancreata having a more prolonged period of preservation. Porcine islet fragility means that exposure to collagenase over prolonged periods causes reduced islet yields(965).

Further sources of error arise in islet sampling and counting to calculate islet yields. Despite examining repeated samples under a graticule, this method assumes a uniform distribution of islets of all sizes in the samples to calculate overall islet yield. In future automated methods of islet sorting and quantification may allow more accurate and reproducible methods of assessing yields(966).

Inherent limitations in the human studies were the small numbers reported and the failure to properly randomly allocate human pancreata to different periods and methods of ischaemic preservation. This was because the study was performed within constraints of the Diabetes UK methodology to reproduce the Edmonton protocol which did not allow for such randomization. In future, clarification of the results in the clinical setting using human pancreas and islets would require a robust randomised study with controlled periods of ischaemia and different preservation methods.

### **9.3 Gradient purity.**

#### **9.3.1 Rodent experiments.**

The exocrine contamination of BSA mini-gradients was lowest from unpreserved pancreata. This was comparable to the contamination of gradients from pancreata

subjected to short duration static cold storage in either UW or ET-K solution. Though prolonged (8 hour) cold storage in ET-K solution, with or without a short duration of TLM resuscitation increased gradient contamination, this remained statistically comparable to short duration ET-K storage. This was not the case for prolonged UW cold stored pancreata.

As expected, pancreata subjected to periods of warm ischaemia showed the highest gradient contamination (>50% mean amylase concentration). Notably all groups involving prolonged UW cold storage, with or without any period of TLM resuscitation, showed gradient contamination comparable to warm ischaemic pancreata (>51%); whereas prolonged ET-K storage alone or combined with 1 hour TLM showed significantly reduced gradient contamination.

### ***9.3.2 Porcine experiments.***

Gradients from statically cold stored and TLM preserved pancreata showed significantly reduced levels of exocrine contamination compared to both UW perfused and unpreserved pancreata. Unpreserved organs demonstrated the highest level of exocrine gradient contamination.

### ***9.3.3 Discussion.***

Following pancreatic ischaemia acinar tissue is very susceptible to cellular oedema. This reduces the acinar tissue density causing it to occupy a similar isodense position in the centrifuged gradient thus contaminating the islet fraction(954). The function of the impermeants in preservation solutions is to oppose the osmotic gradient generated by intracellular impervious anions. In this capacity it would seem that trehalose, contained in ET-K solution, may be superior to raffinose and lactobionate, in UW solution, for periods of static cold rat pancreas preservation from 4 to 8 hours. This potentially challenges the conventional use of UW solution as the optimal preservation solution for pancreatic *in situ* portal perfusion, static cold storage and storage of the pancreatic digest(246). The high rate of exocrine contamination arising in UW perfused porcine pancreata compared to UW cold stored organs may be explained by inadequate perfusion of the vasculature resulting in poor distribution of UW throughout the pancreas. UW is a highly viscose solution (34.7 centipoise (cp) at 37°C; 86.2 cp at 4°C)(967) making perfusion of the islet rich pancreatic tail, via superior mesenteric artery cannulation, difficult to achieve without the use of high perfusion pressures. The perfusion pressure (60mmHg)

used in this study represented a physiological mean blood pressure for pancreatic perfusion but may have been inadequate for UW perfusion of the whole of the pancreas.

#### **9.3.4 Limitations.**

The period of TLM used in the rodent experiments failed to significantly reduce gradient contamination. However, the current literature would suggest that TLM is an effective means to resuscitate the ischaemic pancreas and abrogate the cell swelling causing gradient contamination. It is possible that the period of TLM used in these studies was insufficient to allow adequate rodent pancreas resuscitation to be achieved.

As discussed, the perfusate and pressure parameters used in the porcine experiments are potentially inadequate for whole pancreas perfusion. Future experiments should seek to optimize such parameters by using less viscose perfusates or higher perfusion pressures, within the limits of inducing barotrauma to the ductal system and islets. The application of perfusive techniques to the human pancreas is complicated by variable vascular anatomy which differs from the porcine organ. Where perfusion via the SMA achieves perfusion of the splenic lobe in the pig; the tail and body of the human pancreas is supplied by the splenic artery with the head being supplied by small branches of the gastro-duodenal artery. Hence perfusion of two arteries is required in the human gland to ensure adequate delivery of perfusate to all pancreatic areas.

Calculations of gradient purity are based upon sampling gradient aliquots. Though repeated measures were used to minimize sampling errors, this assumes an equal distribution of islets and exocrine tissue within the aliquot studied. Additionally both the amylase assay and insulin ELISA, used to quantify the exocrine and endocrine components in each gradient, are prone to error. Rather than a direct assay, each provides an indirect calculation of reagent quantity via reference to a 'standard curve'. In performing such calculations the accuracy and reproducibility of such standards is assumed.

### **9.4 *In vitro* islet function.**

#### **9.4.1 Rodent experiments.**

*In vitro* function by static incubation was highest and comparable for islets isolated from unpreserved pancreata or those exposed to short or prolonged ET-K static cold storage (SI>1.45). The mean SI of islets from short and prolonged UW stored pancreata were comparable to short duration ET-K and unpreserved groups, but were significantly less

than prolonged ET-K cold storage. Islets from all groups involving periods of TLM preservation had significantly reduced mean SI compared to unpreserved or prolonged ET-K stored groups.

Interestingly the mean SI of islets from prolonged UW stored pancreata subjected to a long period of TLM resuscitation was significantly higher than those having a short TLM exposure. The latter group had a mean SI comparable to the worst functioning islets from groups exposed to warm ischaemia.

#### **9.4.2 Porcine experiments.**

All preservation groups produced islets with comparable stimulation indices when assessed by dynamic perfusion.

#### **9.4.3 Human pancreas study.**

Comparable SIs were obtained from all five pancreata when post-culture islets were tested by static glucose incubation.

#### **9.4.4 Discussion.**

In the rodent experiments, static incubation demonstrated that unpreserved and short cold stored pancreata generate the best functioning islets, and that warm ischaemia is deleterious to islet function. Nevertheless there is considerable variation in the SI's generated from each preservation group. Additionally the SI's calculated during these experiments are lower than other reported studies which vary from 4.5, after 24 hours of UW cold storage(954), to 12 after UW ductal flushing and 6 hours UW cold storage(630).

The SIs obtained from porcine perfusion experiments were low compared to those reported in current literature. An SI of less than 1 implies no response to changes in glucose concentrations, but the significance of an SI of greater than 1, and the threshold SI which correlates with *in vivo* islet function, has not been established. Though Holmes *et al* reported lower porcine islet SI compared to human islets(968) this study compared tissue culture media rather than methods of preservation and used disparate species. The low SI arising in the porcine experiments may reflect the susceptibility of porcine islets to ischaemia, islet fragmentation during preservation and presence of exocrine contamination in islet fractions.

#### **9.4.5 Limitations.**

The reasons for the SI variation between groups, and discrepancy between SI reported in the literature, may include the different rodent strain, preservation and isolation methods used in my experiments. Static and dynamic islet functional studies are also prone to inconsistencies between experiments with variation in islet size, individual islet viability, medium dissolved oxygen content and ambient temperature all contributing to errors in calculating the SI. Additionally the insulin ELISA is an indirect measure of insulin content reliant upon the accurate construction of a standard curve and calculation rather than direct measure of insulin content.

The problem of reproducing consistent results, especially using perfusion, is well documented in the literature. Bertuzzi et al studied the results of islet perfusions from 80 cadaveric human pancreata to demonstrate that preservation and isolation variables such as the concentration of collagenase used for isolation could be associated with subsequent islet SI(969). In most US centres perfusion has been superseded by simpler static glucose incubations for *in vitro* islet function assessment; whilst the majority of recent experimental studies have used bioassays of islet *in vivo* function by transplantation into diabetic nude rodents to determine reversal of hyperglycaemia. Although these give a valid measure of *in vivo* islet function they are impractical during the immediate peri-isolation period when there is a need to transplant the optimal islet mass expediently into the recipient.

### **9.5 Apoptotic gene expression.**

#### **9.5.1 Rodent experiments.**

The results of experiments involving both RT-PCR and realtime PCR were inconclusive. Pure mRNA was successfully extracted from islets using the paramagnetic Dynabead method. Gel electrophoresis of the amplified  $\beta$  actin endogenous control cDNA against a DNA ladder showed consistent amplification and electrophoretic banding in positive control and test wells, and a lack of expression in negative controls without template cDNA or RT. This demonstrates a lack of contamination in the extracted mRNA and amplified  $\beta$  actin cDNA.

Unfortunately significant differences in the expression of  $\beta$  actin were seen during amplification of both Bax and Bcl-2 PCR products. As  $\beta$  actin was functioning as an endogenous control, the amount of  $\beta$  actin PCR product expressed in each test well should be at a constant and consistent level. Where this occurred, any variation in Bax or

Bcl-2 expression between samples could be interpreted as being derived from differences in the amount of original Bax or Bcl-2 cDNA. However in several experiments the significant differences in  $\beta$  actin expression, including apparent  $\beta$  actin expression from a negative control containing no template cDNA, meant that no conclusion could be made regarding Bax or Bcl-2 expression.

Similarly Bax and Bcl-2 expression could be successfully detected in rodent islet mRNA extracted using the paramagnetic Dynabead method prior to real time PCR. There was an apparent reduction in the Bax:Bcl-2 ratio in islets from TLM preserved pancreata; arising from a reduced level of Bax, and increased level of Bcl-2 expression. However, scrutiny of the expression of the  $\beta$  actin endogenous control showed significant differences between groups, hence no conclusion can be made about the Bax:Bcl-2 ratios in any group after real time PCR analysis.

#### **9.5.2 Discussion.**

Positive amplification and significant variation from negative and endogenous controls during PCR is potentially caused by many factors. The slightest contamination of controls can result in amplification of contaminant cDNA so it is essential that good laboratory technique is followed precisely. Despite such protocols it is possible that controls in these experiments were contaminated.

PCR methodology requires strict parameters which, when adjusted, can result in 'false positive' gene amplification. In this case further experiments, using different cycle durations and temperatures for primer annealing and extension, different primer sequences or alternative endogenous control mRNA sequences like GAPDH or NADPH, may abrogate false positive results.

Future work might also include alternative PCR methodologies to increase the specificity of DNA amplification by reducing background due to non-specific amplification of DNA. For example, nested PCR uses two sets of primers in two successive PCR reactions. In the first reaction, a pair of primers generate DNA products which consist of the intended target and also non-specifically amplified DNA fragments. These products are then used in a second PCR reaction with a set of primers whose binding sites are different from, and located 3' of, each of the primers used in the first reaction. Touchdown PCR aims to reduce nonspecific background amplification by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees above the  $T_m$  of the primers used, while at the later cycles, it is a few degrees below the primer  $T_m$ . The higher temperatures give greater specificity for primer binding, and the lower temperatures

permit more efficient amplification from the specific products formed during the initial cycles. A further technique, hot-start PCR, could be used to reduce non-specific amplification during the initial set up stages of the PCR reaction. The technique involves heating the reaction components to the melting temperature (e.g., 95°C) before adding the polymerase (970) and specialized enzyme systems that inhibit polymerase's activity at ambient temperature. Such hybrid polymerases are only activated following the high-temperature activation step.

Three published studies have previously used PCR to assess mRNA expression of apoptotic genes in the preserved pancreas or isolated islets. Federici et al demonstrated the pro-apoptotic effect of human islet exposure to hyperglycaemia. RT-PCR confirmed increased expression of the pro-apoptotic genes Bad, Bid and Bik; whilst showing no change in the expression of anti-apoptotic Bcl-2 (971). An increase in Bax over Bcl-2 expression also occurs in human islets in response to isolation where real time PCR showed an islet ratio of Bax to Bcl-2 of 10.8:1 (870). Reverse transcriptase PCR was used by Shirouzu et al to study Bax and Bcl-2 ratios in isolated rodent islets in conventional cell culture and prolonged UW cold storage for 7 days. The ratio was lowest after overnight cell culture suggesting that apoptotic gene expression was highest immediately after isolation and after prolonged period of islet culture or cold storage (700). These studies prove that PCR analysis of apoptotic gene mRNA is possible and provides a quantitative means of determining apoptotic activity early after islet isolation and during islet culture.

## ***9.6 Apoptotic immunohistochemistry.***

### ***9.6.1 Rodent Bax and Bcl-2 immunohistochemistry.***

Immunostaining of the pro-apoptotic protein Bax was lowest in the unpreserved pancreas and showed similar distribution amongst islet and acinar tissue. Short duration ET-K cold storage and prolonged ET-K cold storage followed by short duration TLM showed the second lowest mean Bax immunostaining.

Prolonged ET-K cold storage with long (4 hour) TLM resuscitation showed comparable Bax immunostaining to groups having prolonged UW cold storage with or without 1 hour TLM resuscitation. These groups had levels of intra-islet Bax staining comparable to the first three groups but relatively increased levels of positive Bax staining in acinar tissue. The most frequent Bax staining was observed in groups exposed to warm ischaemia, prolonged cold storage in ET-K solution and prolonged UW cold storage followed by 4

hours of TLM. These groups showed increased levels of Bax staining in both islet and acinar tissues compared to all other groups.

Immunostaining of the anti-apoptotic protein Bcl-2 was highest in pancreata exposed to short duration cold storage in either preservation solution, or after prolonged ET-K cold storage combined with any duration of TLM resuscitation. These groups showed Bcl-2 immunostaining equal or more intense than that seen in the unpreserved pancreas.

Though the overall mean percentage Bcl-2 staining was highest in these groups, the distribution of staining is unequal with a disproportionately high amount of staining in acinar tissue. The intensity of Bcl-2 staining amongst islets themselves remained constant and comparable between all preservation groups.

Groups with the least Bcl-2 immunostaining included those exposed to warm ischaemia. Here there was a relative reduction in acinar Bcl-2 staining whilst islet staining remained comparable to other groups. This reduced the total mean number of Bcl-2 positive cells.

#### ***9.6.2 Porcine apoptotic index by TUNEL assay.***

The lowest apoptotic indices were found in pancreata subjected to machine perfusion and TLM. These were significantly reduced compared to the apoptotic index of cold stored pancreata. However the distribution of apoptotic cells may explain the differences between preservation groups, with relatively high numbers of apoptotic cells in the acinar tissues of unpreserved and cold stored pancreata. The numbers of apoptotic cells amongst islets themselves varied little between preservation groups.

#### ***9.6.3 Discussion.***

Published studies have confirmed increased Bax and reduced Bcl-2 staining in the beta cells of rat islets upto 1 month in cell culture(972) and that human pancreas before islet isolation stains positively for Bax but not Bcl-2 protein(870). Moreover, isolated islets contain high numbers of cells expressing Bax but low numbers expressing Bcl-2(870). Disparity between the intensity and distribution of apoptotic protein staining in pancreatic tissue was observed by Bateman et al. Their immunohistochemical study of human chronic pancreatitis showed a high rate of acinar cell apoptosis by TUNEL assay, concurrent with frequent staining of Bax and Bcl-2 proteins in acinar tissues compared to islets. The authors proposed that such differential rates of apoptosis might explain the loss of exocrine and preservation of islet function occurring after chronic pancreatitis(973). Given the inflammatory cytokine and autolytic mediation of

preservation injury in the pancreas, it would be reasonable to expect a similarly non uniform distribution of apoptotic cells and proteins after pancreas preservation.

#### **9.6.4 Limitations.**

IHC is a potentially unreliable method to study protein expression, which can be prone to a number of errors. One of the main difficulties was overcoming specific or non-specific background staining by optimising antigen retrieval, incubation methods and times, pre-treatment with blocking agents and optimising post-antibody wash buffers and wash times (Appendix A). In addition, the presence of robust positive and negative controls for staining were essential for determining specificity. Recognition of positive staining in tissue sections relied upon microscope field sampling and cell counting. This assumes a uniform distribution of staining across tissue sections but is prone to sampling and observer error despite corroboration using a second slide assessor. Slides were scored using a basic binary assessment of the presence or absence of staining. This may have been too simple and took no account of the precise distribution (nuclear vs cytoplasmic) or intensity of staining within each cell. Such factors are frequently incorporated into IHC scoring systems and the use of a more sophisticated method of scoring IHC slides could be used in future work. Automated methods of slide assessment, using computer based visual analysis software to perform cell counts and assessments of cell staining, might further improve IHC assessment by removing observer error.

### **9.7 Adenine nucleotide ratios.**

#### **9.7.1 Rodent experiments.**

Pancreatic tissue ADP:ATP and ATP:DNA ratios were comparable between all groups prior to pancreatic preservation. Therefore, it is likely that any differences in subsequent adenine nucleotide ratios arose as a result of the preservation method, subsequent islet isolation and period of cell culture.

The lowest ADP:ATP ratios, in pancreatic tissue and isolated islets, were achieved in short duration cold stored pancreata regardless of preservation solution and prolonged cold stored grafts using ET-K solution. Ratios in these groups were significantly reduced compared to any group where TLM resuscitation was used. Of TLM preserved pancreata, the group exposed to short duration TLM after ET-K cold storage had a significantly reduced ADP:ATP ratio compared to all others. Nevertheless all TLM preserved groups had significantly reduced ADP:ATP ratios compared to pancreata exposed to warm ischaemia or prolonged UW cold storage alone.

In groups with a low mean ADP:ATP ratio, the ratio at different stages of islet isolation was consistently low. Similarly when a high mean ADP:ATP ratio occurred this was observed in post-preservation pancreatic tissue, digest and islets before and after culture. However, groups exposed to TLM preservation showed a moderate increase in ratio at the post-preservation and digest stages, but a relatively high ADP:ATP ratio amongst pre-culture islets. This may explain the significant increase in mean ADP:ATP ratio compared to other methods of preservation. Following a period of cell culture, islets from TLM preserved groups had ADP:ATP ratios comparable with those from shorter duration cold preserved groups.

The ATP:DNA ratio gave a measure of ATP concentration relative to the cellularity of the assayed sample to allow comparison within and between groups. High ATP:DNA ratios suggest a high ATP concentration per cell, or a reduced sample cellularity or DNA concentration with normal or reduced ATP concentration.

The highest mean ATP:DNA ratios were found in groups exposed to warm ischaemia. This may be an artifact generated by cellular oedema during ischaemia and preservation, which reduces the DNA concentration in each cell, artificially increasing the calculated ATP:DNA ratio. High ratios were seen in post-preservation pancreatic biopsies exposed to warm ischaemia but these became comparable to other groups at the pre-culture islet stage of isolation. Indeed islets from pancreata exposed to 30 minutes warm ischaemia had similar ATP:DNA ratios to islets from all cold stored pancreata regardless of preservation solution.

The use of TLM appeared to significantly reduce the mean ATP:DNA ratio below that of the 30 minute warm ischaemic group. Of TLM preserved groups, those exposed to prolonged UW cold storage then short duration TLM resuscitation had a mean ATP:DNA ratio significantly lower than any of the static cold preserved groups.

#### ***9.7.2 Porcine experiments.***

Pancreatic tissue and digest ADP:ATP ratios were lowest amongst unpreserved and TLM preserved pancreata. Both these groups had significantly reduced mean ADP:ATP ratios compared to perfused pancreata.

For all preservation methods, increasing duration of preservation resulted in significant increases in the mean ADP:ATP ratio.

There were no significant differences in mean ATP:DNA ratios between preservation methods but a statistically significant reduction in ATP:DNA ratios occurred with increasing duration of preservation by all methods.

#### **9.7.3 Human pancreas study.**

No significant differences in pancreatic ADP:ATP ratios were seen between pancreata used for human islet isolation. Notably those pancreata subjected to controlled periods of cold ischaemia, but not used for islet isolation, had statistically significant increases in ADP:ATP ratio with increasing duration of cold ischaemia.

#### **9.7.4 Discussion.**

There are few published studies using the luciferin-luciferase method to determine adenine nucleotide content of either pancreas or islets.

Goto et al recently published the only study of ADP:ATP ratios in freshly isolated porcine and human islets. Porcine islets after 24 hours of cell culture had an ADP:ATP ratio of  $0.1 \pm 0.01$  which increased to  $0.24 \pm 0.01$  and  $0.39 \pm 0.02$  after freeze-thawing and heating respectively. Moreover the latter groups failed to restore euglycaemia in a streptozotocin induced athymic mouse transplant model. Porcine islet preparations which successfully cured diabetes in the model had significantly lower ADP:ATP ratios than unsuccessful preparations ( $0.08 \pm 0.02$  vs  $0.15 \pm 0.01$  respectively) and ADP:ATP ratio was significantly correlated to transplant outcome ( $\rho = -0.49$ ,  $p = 0.006$ ). Islets with ADP:ATP ratios of less than 0.10 consistently cured diabetes; whilst those with ratios greater than 0.10 cured diabetes in only 29.4% of cases(947).

According to an early study by Brandhorst et al, mean intracellular ATP content of freshly isolated human islets was  $130.4 \pm 53.4$  pg/ $\mu$ g of islet protein corresponding to  $20.7 \pm 6.3$  pg/IEQ. After  $5 \pm 1$  days of cell culture at 22 °C mean ATP content increased to  $265.5 \pm 113.3$  pg/ $\mu$ g islet protein or  $43.7 \pm 15.3$  pg/IEQ. These results suggest that islets in hypothermic cell culture retain the capacity to produce ATP but also showed a wide variation in ATP content between islets. This study used a different luciferase methodology combining liquid nitrogen tissue storage and islet sonification to release adenine nucleotides. ATP content was measured relative to islet protein content or islet size (IEQ). This, and the disparate species used, may explain the variation in ATP content between the published data and my experiments.

Ihm et al recently used ATP/DNA content to assess the impact of donor age upon isolated human islet function. ATP levels of  $115.7 \pm 17.7$  and  $75.7 \pm 6.6$  pmol/ $\mu$ g DNA were found

in young (<40 years) and older donors respectively. Concomitantly there were significantly higher levels of glucose stimulated insulin release, improved rates of insulin independence in a diabetic nude mouse bioassay and higher C peptide responses to IVGTT in clinical islet transplantation where young donors were used(974). This study used high pressure liquid chromatography to determine ATP content which may explain the differences in ATP:DNA content relative to my results. Though ATP:DNA ratio was strongly correlated with donor age and transplant outcome in this study, the feasibility of using such a complex and time consuming assay to predict transplant outcome is questionable. Moreover the experimental results contained herein would suggest that the ATP:DNA ratio is more prone to error than the direct calculation of ADP:ATP ratio. The latter being independent of tissue volume and less prone to error arising from cellular oedema during pancreas preservation.

#### ***9.7.5 Limitations.***

The method for ADP and ATP measurement is prone to error and spurious results. Samples had to be stored and analysed separately, with each assay taking 20 minutes to complete. Despite the use of meticulous technique the potential for sample deterioration during storage, contamination, batch to batch variation in reagent concentrations and variations in the ambient temperature from assay to assay could give rise to spurious results. In future it would be advantageous for the assay to be semi-automated to allow multiple repeated samples to be tested instantly and simultaneously with precise timing and volumes for the introduction of the various reagents. This would also allow simultaneous analysis of ATP standard concentrations to allow accurate calibration of the luminometer for each batch of assays. This might result in a more accurate measure of sample ATP concentration.

Similarly the rapid DNA assay required repeated calibration of the instrument and analysis of samples separately in the fluorometer. Despite the use of DNase treated equipment, the potential for sample contamination exists. Again a semi-automated method using a fluorometer capable of performing analysis of multiple samples and standard would allow a direct measure of sample DNA concentration.

### ***9.8 Factor Analysis.***

#### ***9.8.1 Rodent experiments.***

The ADP:ATP ratio in rodent pancreatic tissue after preservation may be of value in predicting the outcome of subsequent islet isolation. High ratios are strongly correlated

with low islet yield, low *in vitro* islet function, high levels of gradient contamination, strong immunostaining of the pro-apoptotic protein Bax in pancreatic tissue and lack of immunostaining of the anti-apoptotic protein Bcl-2 in acinar tissues.

The ATP:DNA ratio appears less valuable as a latent variable in predicting the success of islet isolation. This ratio showed no correlations with standard measures of islet yield or *in vitro* function. Despite positive correlations with the amount of Bax immunostaining in pancreatic tissue, this is not routinely used as measures of islet graft viability prior to transplantation.

### **9.8.2 Porcine experiments.**

The ADP:ATP ratio of porcine pancreatic tissue, after a period of preservation, may be of value in predicting subsequent yield during islet isolation. A high ADP:ATP ratio was correlated with a reduced islet yield (-0.51). Porcine ADP:ATP ratio did not show any correlation with other *in vitro* measures of gradient purity, islet function or apoptotic index.

### **9.8.3 Discussion.**

This thesis has demonstrated that the ADP:ATP ratio, determined by a rapid luciferin-luciferase based chemoluminescent assay, is a valid and reliable method to predict islet yield.

In the preserved rodent pancreas the ratio also predicts isolated islet purity and *in vitro* function, and higher ADP:ATP ratios are associated with increased expression of pro-apoptotic proteins.

The ADP:ATP ratio is superior to measures of ATP content which demand quantification of DNA content. This negates the effect of graft oedema and cell swelling during pancreas preservation which artificially increases the ATP:DNA ratio. Additionally the assay can be performed rapidly upon a small sample of tissue using standard simple laboratory equipment.

ADP:ATP ratios of isolated islets have been shown to be promising predictors of post-transplant function(947). However, this thesis shows that ADP:ATP ratios from preserved pancreatic tissue can be used to predict islet isolation outcomes. This has potential advantages for clinical islet transplantation to allow early assessment of the donor pancreas prior to the costly and time consuming process of islet isolation.

- 221 -

Performing the ADP:ATP assay on the preserved donor pancreas may potentially increase the number of islet isolations producing high numbers of good quality functioning islets for clinical transplantation.

## ***Bibliography.***

1. Dotta F, Mario UD. Antigenic determinants in type 1 diabetes mellitus. Review article. *Apmis* 1996;104(11):769-74.
2. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2000;23 Suppl 1:S4-19.
3. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 1998;21(9):1414-31.
4. Onkamo P, Vaananen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 1999;42(12):1395-403.
5. Molbak AG, Christau B, Marnar B, Borch-Johnsen K, Nerup J. Incidence of insulin-dependent diabetes mellitus in age groups over 30 years in Denmark. *Diabet Med* 1994;11(7):650-5.
6. Green A, Gale EA, Patterson CC. Incidence of childhood-onset insulin-dependent diabetes mellitus: the EURODIAB ACE Study. *Lancet* 1992;339(8798):905-9.
7. Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet* 2000;355(9207):873-6.
8. Rangasami JJ, Greenwood DC, McSparran B, Smail PJ, Patterson CC, Waugh NR. Rising incidence of type 1 diabetes in Scottish children, 1984-93. The Scottish Study Group for the Care of Young Diabetics. *Arch Dis Child* 1997;77(3):210-3.
9. Metcalfe MA, Baum JD. Incidence of insulin dependent diabetes in children aged under 15 years in the British Isles during 1988. *Bmj* 1991;302(6774):443-7.
10. Foulis AK. The pathology of the endocrine pancreas in type 1 (insulin-dependent) diabetes mellitus. *Apmis* 1996;104(3):161-7.
11. Imagawa A, Hanafusa T, Itoh N, Waguri M, Yamamoto K, Miyagawa J, et al. Immunological abnormalities in islets at diagnosis paralleled further deterioration of glycaemic control in patients with recent-onset Type I (insulin-dependent) diabetes mellitus. *Diabetologia* 1999;42(5):574-8.
12. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 1994;91(1):123-7.

13. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* 2002;346(22):1692-8.
14. Devendra D, Liu E, Eisenbarth GS. Type 1 diabetes: recent developments. *Bmj* 2004;328(7442):750-4.
15. Hui H, Dotta F, Di Mario U, Perfetti R. Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. *J Cell Physiol* 2004;200(2):177-200.
16. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 2004;350(7):664-71.
17. Redondo MJ, Rewers M, Yu L, Garg S, Pilcher CC, Elliott RB, et al. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *Bmj* 1999;318(7185):698-702.
18. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 2001;358(9277):221-9.
19. Todd JA, Farrall M. Panning for gold: genome-wide scanning for linkage in type 1 diabetes. *Hum Mol Genet* 1996;5 Spec No:1443-8.
20. Cavan D, Bain S, Barnett A. The genetics of type I (insulin dependent) diabetes mellitus. *J Med Genet* 1992;29(7):441-6.
21. Sutherland DE, Sibley R, Xu XZ, Michael A, Srikanta AM, Taub F, et al. Twin-to-twin pancreas transplantation: reversal and reenactment of the pathogenesis of type I diabetes. *Trans Assoc Am Physicians* 1984;97:80-7.
22. Roep BO. T-cell responses to autoantigens in IDDM. The search for the Holy Grail. *Diabetes* 1996;45(9):1147-56.
23. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 1994;331(21):1428-36.
24. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, et al. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. *PG - 816-22. Diabetologia* 1995;38(7):816-22.
25. Yu L, Robles DT, Abiru N, Kaur P, Rewers M, Kelemen K, et al. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci U S A* 2000;97(4):1701-6.

26. Barker JM, Goehrig SH, Barriga K, Hoffman M, Slover R, Eisenbarth GS, et al. Clinical characteristics of children diagnosed with type 1 diabetes through intensive screening and follow-up. *Diabetes Care* 2004;27(6):1399-404.
27. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996;45(7):926-33.
28. LaGasse JM, Brantley MS, Leech NJ, Rowe RE, Monks S, Palmer JP, et al. Successful prospective prediction of type 1 diabetes in schoolchildren through multiple defined autoantibodies: an 8-year follow-up of the Washington State Diabetes Prediction Study. *Diabetes Care* 2002;25(3):505-11.
29. Martin S, Wolf-Eichbaum D, Duinkerken G, Scherbaum WA, Kolb H, Noordzij JG, et al. Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. *N Engl J Med* 2001;345(14):1036-40.
30. Devendra D, Eisenbarth GS. 17. Immunologic endocrine disorders. *J Allergy Clin Immunol* 2003;111(2 Suppl):S624-36.
31. Arato A, Korner A, Veres G, Dezsofi A, Ujpal I, Madacsy L. Frequency of coeliac disease in Hungarian children with type 1 diabetes mellitus. *Eur J Pediatr* 2003;162(1):1-5.
32. Ashabani A, Abushofa U, Abusrewill S, Abdelazez M, Tuckova L, Tlaskalova-Hogenova H. The prevalence of coeliac disease in Libyan children with type 1 diabetes mellitus. *Diabetes Metab Res Rev* 2003;19(1):69-75.
33. Gale EA. A missing link in the hygiene hypothesis? *Diabetologia* 2002;45(4):588-94.
34. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347(12):911-20.
35. Hyoty H, Taylor KW. The role of viruses in human diabetes. *Diabetologia* 2002;45(10):1353-61.
36. Bodansky HJ, Staines A, Stephenson C, Haigh D, Cartwright R. Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population. *Bmj* 1992;304(6833):1020-2.
37. Lindberg B, Ahlfors K, Carlsson A, Ericsson UB, Landin-Olsson M, Lernmark A, et al. Previous exposure to measles, mumps, and rubella--but not vaccination during adolescence--correlates to the prevalence of pancreatic and thyroid autoantibodies. *Pediatrics* 1999;104(1):e12.

38. Ylipaasto P, Klingel K, Lindberg AM, Otonkoski T, Kandolf R, Hovi T, et al. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia* 2004;47(2):225-39.
39. Devendra D, Eisenbarth GS. Interferon alpha--a potential link in the pathogenesis of viral-induced type 1 diabetes and autoimmunity. *Clin Immunol* 2004;111(3):225-33.
40. Moriyama H, Wen L, Abiru N, Liu E, Yu L, Miao D, et al. Induction and acceleration of insulinitis/diabetes in mice with a viral mimic (polyinosinic-polycytidylic acid) and an insulin self-peptide. *Proc Natl Acad Sci U S A* 2002;99(8):5539-44.
41. Vaarala O. The gut immune system and type 1 diabetes. *Ann N Y Acad Sci* 2002;958:39-46.
42. Norris JM, Barriga K, Klingensmith G, Hoffman M, Eisenbarth GS, Erlich HA, et al. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *Jama* 2003;290(13):1713-20.
43. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *Jama* 2003;290(13):1721-8.
44. Norris JM, Beaty B, Klingensmith G, Yu L, Hoffman M, Chase HP, et al. Lack of association between early exposure to cow's milk protein and beta-cell autoimmunity. *Diabetes Autoimmunity Study in the Young (DAISY)*. *Jama* 1996;276(8):609-14.
45. Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. All-cause mortality rates in patients with type 1 diabetes mellitus compared with a non-diabetic population from the UK general practice research database, 1992-1999. *Diabetologia* 2006;49(4):660-6.
46. Nichols GA, Brown JB. Higher medical care costs accompany impaired fasting glucose. *Diabetes Care* 2005;28(9):2223-9.
47. Dixon S, Currie CJ, Peters JR. The cost of diabetes: time for a different approach? *Diabet Med* 2000;17(12):820-2.
48. Hogan P, Dall T, Nikolov P. Economic costs of diabetes in the US in 2002. *Diabetes Care* 2003;26(3):917-32.
49. McAlpine RR, Morris AD, Emslie-Smith A, James P, Evans JM. The annual incidence of diabetic complications in a population of patients with Type 1 and Type 2 diabetes. *Diabet Med* 2005;22(3):348-52.
50. Roberts SE, Goldacre MJ, Neil HA. Mortality in young people admitted to hospital for diabetes: database study. *Bmj* 2004;328(7442):741-2.

51. Cusick M, Meleth AD, Agron E, Fisher MR, Reed GF, Knatterud GL, et al. Associations of mortality and diabetes complications in patients with type 1 and type 2 diabetes: early treatment diabetic retinopathy study report no. 27. *Diabetes Care* 2005;28(3):617-25.
52. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* 1993;329(14):977-86.
53. Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. *Jama* 2002;287(19):2563-9.
54. Effects of insulin in relatives of patients with type 1 diabetes mellitus. *N Engl J Med* 2002;346(22):1685-91.
55. Gale EA, Bingley PJ, Emmett CL, Collier T. European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. *Lancet* 2004;363(9413):925-31.
56. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 2002;346(6):393-403.
57. Meyer L, Guerci B. Metformin and insulin in type 1 diabetes: the first step. *Diabetes Care* 2003;26(5):1655-6.
58. Gerstein HC, Yusuf S, Bosch J, Pogue J, Sheridan P, Dinccag N, et al. Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: a randomised controlled trial. *Lancet* 2006;368(9541):1096-105.
59. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, et al. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. *Diabetes Care* 2004;27(1):256-63.
60. Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 2001;344(18):1343-50.
61. Heneghan C, Thompson M, Perera R. Prevention of diabetes. *Bmj* 2006;333(7572):764-5.

62. Garg S, Rosenstock J, Silverman BL, Sun B, Konkoy CS, de la Pena A, et al. Efficacy and safety of preprandial human insulin inhalation powder versus injectable insulin in patients with type 1 diabetes. *Diabetologia* 2006;49(5):891-9.
63. Edgerton DS, Neal DW, Scott M, Bowen L, Wilson W, Hobbs CH, et al. Inhalation of insulin (Exubera) is associated with augmented disposal of portally infused glucose in dogs. *Diabetes* 2005;54(4):1164-70.
64. Quattrin T, Belanger A, Bohannon NJ, Schwartz SL. Efficacy and safety of inhaled insulin (Exubera) compared with subcutaneous insulin therapy in patients with type 1 diabetes: results of a 6-month, randomized, comparative trial. *Diabetes Care* 2004;27(11):2622-7.
65. Skyler JS, Weinstock RS, Raskin P, Yale JF, Barrett E, Gerich JE, et al. Use of inhaled insulin in a basal/bolus insulin regimen in type 1 diabetic subjects: a 6-month, randomized, comparative trial. *Diabetes Care* 2005;28(7):1630-5.
66. Rosenstock J, Cappelleri JC, Bolinder B, Gerber RA. Patient satisfaction and glycemic control after 1 year with inhaled insulin (Exubera) in patients with type 1 or type 2 diabetes. *Diabetes Care* 2004;27(6):1318-23.
67. Gerber RA, Cappelleri JC, Kourides IA, Gelfand RA. Treatment satisfaction with inhaled insulin in patients with type 1 diabetes: a randomized controlled trial. *Diabetes Care* 2001;24(9):1556-9.
68. Excellence. NfHaC. Inhaled insulin for the treatment of diabetes (types 1 and 2) - Technology Appraisal 113. In; 2006.
69. Morton-Eggleston E, Barrett EJ. Inhaled insulin. *Bmj* 2006;332(7549):1043-4.
70. Vajo Z, Duckworth WC. Genetically engineered insulin analogs: diabetes in the new millenium. *Pharmacol Rev* 2000;52(1):1-9.
71. Bolli GB, Owens DR. Insulin glargine. *Lancet* 2000;356(9228):443-5.
72. Peterson GE. Intermediate and long-acting insulins: a review of NPH insulin, insulin glargine and insulin detemir. *Curr Med Res Opin* 2006;22(12):2613-9.
73. Lepore M, Pampanelli S, Fanelli C, Porcellati F, Bartocci L, Di Vincenzo A, et al. Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting human insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro. *Diabetes* 2000;49(12):2142-8.
74. Rossetti P, Pampanelli S, Fanelli C, Porcellati F, Costa E, Torlone E, et al. Intensive replacement of basal insulin in patients with type 1 diabetes given rapid-acting insulin analog at mealtime: a 3-month comparison between administration of NPH insulin

four times daily and glargine insulin at dinner or bedtime. *Diabetes Care* 2003;26(5):1490-6.

75. Garg SK, Gottlieb PA, Hisatomi ME, D'Souza A, Walker AJ, Izuora KE, et al. Improved glycemic control without an increase in severe hypoglycemic episodes in intensively treated patients with type 1 diabetes receiving morning, evening, or split dose insulin glargine. *Diabetes Res Clin Pract* 2004;66(1):49-56.

76. Raskin P, Klaff L, Bergenstal R, Halle JP, Donley D, Mecca T. A 16-week comparison of the novel insulin analog insulin glargine (HOE 901) and NPH human insulin used with insulin lispro in patients with type 1 diabetes. *Diabetes Care* 2000;23(11):1666-71.

77. Rosenstock J, Park G, Zimmerman J. Basal insulin glargine (HOE 901) versus NPH insulin in patients with type 1 diabetes on multiple daily insulin regimens. U.S. Insulin Glargine (HOE 901) Type 1 Diabetes Investigator Group. *Diabetes Care* 2000;23(8):1137-42.

78. Murphy NP, Keane SM, Ong KK, Ford-Adams M, Edge JA, Acerini CL, et al. Randomized cross-over trial of insulin glargine plus lispro or NPH insulin plus regular human insulin in adolescents with type 1 diabetes on intensive insulin regimens. *Diabetes Care* 2003;26(3):799-804.

79. Chase HP, Dixon B, Pearson J, Fiallo-Scharer R, Walravens P, Klingensmith G, et al. Reduced hypoglycemic episodes and improved glycemic control in children with type 1 diabetes using insulin glargine and neutral protamine Hagedorn insulin. *J Pediatr* 2003;143(6):737-40.

80. Mohn A, Strang S, Wernicke-Panten K, Lang AM, Edge JA, Dunger DB. Nocturnal glucose control and free insulin levels in children with type 1 diabetes by use of the long-acting insulin HOE 901 as part of a three-injection regimen. *Diabetes Care* 2000;23(4):557-9.

81. Excellence. NIfHaC. Guidance on the use of long-acting insulin analogues for the treatment of diabetes - insulin glargine. Technology Appraisal Guidance No.53. In; 2002.

82. White SA, Nicholson ML, Hering BJ. Can islet cell transplantation treat diabetes? *Bmj* 2000;321(7262):651-2.

83. White SA, London NJ. Pancreas and islet transplantation. *Br J Surg* 1998;85(10):1313-5.

84. Humar A, Kandaswamy R, Granger D, Gruessner RW, Gruessner AC, Sutherland DE. Decreased surgical risks of pancreas transplantation in the modern era. *Ann Surg* 2000;231(2):269-75.
85. Stratta RJ. Mortality after vascularized pancreas transplantation. *Surgery* 1998;124(4):823-30.
86. White SA, James RF, Swift SM, Kimber RM, Nicholson ML. Human islet cell transplantation--future prospects. *Diabet Med* 2001;18(2):78-103.
87. Ryan EA, Lakey JR, Rajotte RV, Korbitt GS, Kin T, Imes S, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 2001;50(4):710-9.
88. Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000;343(4):230-8.
89. Weimar B, Rauber K, Brendel MD, Bretzel RG, Rau WS. Percutaneous transhepatic catheterization of the portal vein: A combined CT- and fluoroscopy-guided technique. *Cardiovasc Intervent Radiol* 1999;22(4):342-4.
90. Registry IPT. Annual Report. In. Minnesota, USA: University of Minnesota; 2004. p. Online Annual Report.
91. Sutherland DE, Gruessner RW, Dunn DL, Matas AJ, Humar A, Kandaswamy R, et al. Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann Surg* 2001;233(4):463-501.
92. Knoll GA, Nichol G. Dialysis, kidney transplantation, or pancreas transplantation for patients with diabetes mellitus and renal failure: a decision analysis of treatment options. *J Am Soc Nephrol* 2003;14(2):500-15.
93. Nath DS, Gruessner AC, Kandaswamy R, Gruessner RW, Sutherland DE, Humar A. Outcomes of pancreas transplants for patients with type 2 diabetes mellitus. *Clin Transplant* 2005;19(6):792-7.
94. Sutherland D, Gruessner A. Pancreas transplant results in the UNOS United States of America registry compared with non-USA data in the International Registry. Los Angeles: UCLA Tissue Typing Laboratory; 1995.
95. Douzdjian V, Abecassis MM, Cooper JL, Smith JL, Corry RJ. Incidence, management and significance of surgical complications after pancreatic transplantation. *Surg Gynecol Obstet* 1993;177(5):451-6.

96. Gruessner A, Sutherland D. Pancreas transplant outcomes for United States (US) and non-US cases. Los Angeles: UCLA Immunogenetics Center; 2003.
97. Sollinger HW, Odorico JS, Knechtle SJ, D'Alessandro AM, Kalayoglu M, Pirsch JD. Experience with 500 simultaneous pancreas-kidney transplants. *Ann Surg* 1998;228(3):284-96.
98. Kuo PC, Johnson LB, Schweitzer EJ, Bartlett ST. Simultaneous pancreas/kidney transplantation--a comparison of enteric and bladder drainage of exocrine pancreatic secretions. *Transplantation* 1997;63(2):238-43.
99. Bloom RD, Olivares M, Rehman L, Raja RM, Yang S, Badosa F. Long-term pancreas allograft outcome in simultaneous pancreas-kidney transplantation: a comparison of enteric and bladder drainage. *Transplantation* 1997;64(12):1689-95.
100. Stratta RJ, Gaber AO, Shokouh-Amiri MH, Grewal HP, Egidi MF, Kizilisik AT, et al. A 9-year experience with 126 pancreas transplants with portal enteric drainage. *Arch Surg* 2001;136(10):1141-9.
101. Stratta RJ, Shokouh-Amiri MH, Egidi MF, Grewal HP, Lo A, Kizilisik AT, et al. Long-term experience with simultaneous kidney-pancreas transplantation with portal-enteric drainage and tacrolimus/mycophenolate mofetil-based immunosuppression. *Clin Transplant* 2003;17 Suppl 9:69-77.
102. Stratta RJ, Gaber AO, Shokouh-Amiri MH, Reddy KS, Egidi MF, Grewal HP, et al. A prospective comparison of systemic-bladder versus portal-enteric drainage in vascularized pancreas transplantation. *Surgery* 2000;127(2):217-26.
103. Hakim NS, Gruessner AC, Papalouis BE, Troppmann C, Dunn DL, Sutherland DE, et al. Duodenal complications in bladder-drained pancreas transplantation. *Surgery* 1997;121(6):618-24.
104. Esterl RM, Stratta RJ, Taylor RJ, Radio SJ. Rejection with duodenal rupture after solitary pancreas transplantation: an unusual cause of severe hematuria. *Clin Transplant* 1995;9(3 Pt 1):155-9.
105. Dumas MD, Bude RO, Sonda PL, 3rd, Cohan RH, Merion RM. Urethral disruption with urinary extravasation: a delayed complication of pancreatic transplantation. *Radiology* 1996;201(3):761-5.
106. Del Pizzo JJ, Jacobs SC, Bartlett ST, Sklar GN. Urological complications of bladder-drained pancreatic allografts. *Br J Urol* 1998;81(4):543-7.
107. Gettman MT, Levy JB, Engen DE, Nehra A. Urological complications after kidney-pancreas transplantation. *J Urol* 1998;159(1):38-42; discussion 42-3.

108. Highshaw RA, Tunuguntla HS, Perez RV, Gandour-Edwards R, Evans CP. Initial report of bladder carcinoma following combined bladder-drained pancreas and kidney transplantation. *Clin Transplant* 2002;16(5):383-6.
109. Geetha D, Tong BC, Racusen L, Markowitz JS, Westra WH. Bladder carcinoma in a transplant recipient: evidence to implicate the BK human polyomavirus as a causal transforming agent. *Transplantation* 2002;73(12):1933-6.
110. Nymann T, Shokouh-Amiri MH, Elmer DS, Stratta RJ, Gaber AO. Diagnosis, management, and outcome of late duodenal complications in portal-enteric pancreas transplantation: case reports. *J Am Coll Surg* 1997;185(6):560-6.
111. Diem P, Abid M, Redmon JB, Sutherland DE, Robertson RP. Systemic venous drainage of pancreas allografts as independent cause of hyperinsulinemia in type I diabetic recipients. *Diabetes* 1990;39(5):534-40.
112. Katz H, Homan M, Velosa J, Robertson P, Rizza R. Effects of pancreas transplantation on postprandial glucose metabolism. *N Engl J Med* 1991;325(18):1278-83.
113. Blackman JD, Polonsky KS, Jaspan JB, Sturis J, Van Cauter E, Thistlethwaite JR. Insulin secretory profiles and C-peptide clearance kinetics at 6 months and 2 years after kidney-pancreas transplantation. *Diabetes* 1992;41(10):1346-54.
114. Nankivell BJ, Chapman JR, Bovington KJ, Spicer ST, O'Connell PJ, Allen RD. Clinical determinants of glucose homeostasis after pancreas transplantation. *Transplantation* 1996;61(12):1705-11.
115. Gaber AO, Shokouh-Amiri H, Grewal HP, Britt LG. A technique for portal pancreatic transplantation with enteric drainage. *Surg Gynecol Obstet* 1993;177(4):417-9.
116. Petruzzo P, Da Silva M, Feitosa LC, Dawahra M, Lefrancois N, Dubernard JM, et al. Simultaneous pancreas-kidney transplantation: portal versus systemic venous drainage of the pancreas allografts. *Clin Transplant* 2000;14(4 Pt 1):287-91.
117. Troppmann C, Gjertson DW, Cecka JM, McVicar JP, Perez RV. Impact of portal venous pancreas graft drainage on kidney graft outcome in simultaneous pancreas-kidney recipients reported to UNOS. *Am J Transplant* 2004;4(4):544-53.
118. Philosophe B, Farney AC, Schweitzer EJ, Colonna JO, Jarrell BE, Krishnamurthi V, et al. Superiority of portal venous drainage over systemic venous drainage in pancreas transplantation: a retrospective study. *Ann Surg* 2001;234(5):689-96.

119. Tang Y, Li YP, Li JS, Zhang ZD, Han FH, Hu WM, et al. Impact of portal versus systemic venous drainage on acute rejection of simultaneous pancreas-kidney transplantation in pig. *Transplantation* 2007;84(5):629-33.
120. Humar A, Khwaja K, Ramcharan T, Asolati M, Kandaswamy R, Gruessner RW, et al. Chronic rejection: the next major challenge for pancreas transplant recipients. *Transplantation* 2003;76(6):918-23.
121. Tyden G, Reinholt FP, Sundkvist G, Bolinder J. Recurrence of autoimmune diabetes mellitus in recipients of cadaveric pancreatic grafts. *N Engl J Med* 1996;335(12):860-3.
122. Sutherland DE, Goetz FC, Sibley RK. Recurrence of disease in pancreas transplants. *Diabetes* 1989;38 Suppl 1:85-7.
123. Esmatjes E, Rodriguez-Villar C, Ricart MJ, Casamitjana R, Martorell J, Sabater L, et al. Recurrence of immunological markers for type 1 (insulin-dependent) diabetes mellitus in immunosuppressed patients after pancreas transplantation. *Transplantation* 1998;66(1):128-31.
124. Petruzzo P, Andreelli F, McGregor B, Lefrancois N, Dawahra M, Feitosa LC, et al. Evidence of recurrent type I diabetes following HLA-mismatched pancreas transplantation. *Diabetes Metab* 2000;26(3):215-8.
125. Lohmann T, Klemm T, Geissler F, Uhlmann D, Ludwig S, Hauss J, et al. Islet cell-specific autoantibodies as potential markers for recurrence of autoimmune type 1 diabetes after simultaneous pancreas-kidney transplantation. *Transplant Proc* 2002;34(6):2249-50.
126. Drachenberg CB, Papadimitriou JC, Weir MR, Klassen DK, Hoehn-Saric E, Bartlett ST. Histologic findings in islets of whole pancreas allografts: lack of evidence for recurrent cell-mediated diabetes mellitus. *Transplantation* 1996;62(12):1770-2.
127. Drachenberg CB, Klassen DK, Weir MR, Wiland A, Fink JC, Bartlett ST, et al. Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation* 1999;68(3):396-402.
128. Dieterle CD, Schmauss S, Veitenhansl M, Gutt B, Illner WD, Land W, et al. Glucose metabolism after pancreas transplantation: cyclosporine versus tacrolimus. *Transplantation* 2004;77(10):1561-5.
129. Smith JL, Hunsicker LG, Yuh WT, Wright FH, Jr., Van Voorhis L, Corry RJ. Appearance of type II diabetes mellitus in type I diabetic recipients of pancreas allografts. *Transplantation* 1989;47(2):304-11.

130. Munn SR, Engen DE, Barr D, Carpenter HA, Perkins JD. Differential diagnosis of hypoamylasuria in pancreas allograft recipients with urinary exocrine drainage. *Transplantation* 1990;49(2):359-62.
131. Nakhleh RE, Benedetti E, Gruessner A, Troppmann C, Goswitz JJ, Sutherland DE, et al. Cystoscopic biopsies in pancreaticoduodenal transplantation. Are duodenal biopsies indicative of pancreas dysfunction? *Transplantation* 1995;60(6):541-6.
132. Gaber AO, Gaber LW, Shokouh-Amiri MH, Hathaway D. Percutaneous biopsy of pancreas transplants. *Transplantation* 1992;54(3):548-50.
133. Klassen DK, Weir MR, Cangro CB, Bartlett ST, Papadimitriou JC, Drachenberg CB. Pancreas allograft biopsy: safety of percutaneous biopsy-results of a large experience. *Transplantation* 2002;73(4):553-5.
134. Atwell TD, Gorman B, Larson TS, Charboneau JW, Ingalls Hanson BM, Stegall MD. Pancreas transplants: experience with 232 percutaneous US-guided biopsy procedures in 88 patients. *Radiology* 2004;231(3):845-9.
135. Lee BC, McGahan JP, Perez RV, Boone JM. The role of percutaneous biopsy in detection of pancreatic transplant rejection. *Clin Transplant* 2000;14(5):493-8.
136. Kayler LK, Merion RM, Rudich SM, Punch JD, Magee JC, Maraschio MA, et al. Evaluation of pancreatic allograft dysfunction by laparoscopic biopsy. *Transplantation* 2002;74(9):1287-9.
137. Drachenberg CB, Papadimitriou JC, Klassen DK, Racusen LC, Hoehn-Saric EW, Weir MR, et al. Evaluation of pancreas transplant needle biopsy: reproducibility and revision of histologic grading system. *Transplantation* 1997;63(11):1579-86.
138. Papadimitriou JC, Drachenberg CB, Wiland A, Klassen DK, Fink J, Weir MR, et al. Histologic grading of acute allograft rejection in pancreas needle biopsy: correlation to serum enzymes, glycemia, and response to immunosuppressive treatment. *Transplantation* 1998;66(12):1741-5.
139. Elmer DS, Hughes TA, Hathaway DK, Shokouh-Amiri MH, Gaber LW, Nymann T, et al. Use of glucose disappearance rates to monitor beta-cell function of pancreas allografts. *Transplant Proc* 1995;27(6):2985-6.
140. Battezzati A, Benedini S, Caldara R, Calori G, Secchi A, Pozza G, et al. Prediction of the long-term metabolic success of the pancreatic graft function. *Transplantation* 2001;71(11):1560-5.
141. Pfeffer F, Nauck MA, Drognitz O, Benz S, von Dobschuetz E, Hopt UT. Postoperative oral glucose tolerance and stimulated insulin secretion: a predictor of

endocrine graft function more than 10 years after pancreas-kidney transplantation.

Transplantation 2003;76(10):1427-31.

142. Hariharan S, McBride MA, Cherikh WS, Tolleris CB, Bresnahan BA, Johnson CP. Post-transplant renal function in the first year predicts long-term kidney transplant survival. *Kidney Int* 2002;62(1):311-8.

143. Tyden G, Bolinder J, Solders G, Brattstrom C, Tibell A, Groth CG. Improved survival in patients with insulin-dependent diabetes mellitus and end-stage diabetic nephropathy 10 years after combined pancreas and kidney transplantation.

Transplantation 1999;67(5):645-8.

144. Sudan D, Sudan R, Stratta R. Long-term outcome of simultaneous kidney-pancreas transplantation: analysis of 61 patients with more than 5 years follow-up.

Transplantation 2000;69(4):550-5.

145. Mohan P, Safi K, Little DM, Donohoe J, Conlon P, Walshe JJ, et al. Improved patient survival in recipients of simultaneous pancreas-kidney transplant compared with kidney transplant alone in patients with type 1 diabetes mellitus and end-stage renal disease. *Br J Surg* 2003;90(9):1137-41.

146. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. *N Engl J Med* 2003;349(24):2326-33.

147. Gruessner A, Sutherland D. Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004. *Clin Transpl* 2005;19:433.

148. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. Evolution and pathophysiology of renal-transplant glomerulosclerosis. *Transplantation* 2004;78(3):461-8.

149. Ojo AO, Meier-Kriesche HU, Hanson JA, Leichtman A, Magee JC, Cibrik D, et al. The impact of simultaneous pancreas-kidney transplantation on long-term patient survival. *Transplantation* 2001;71(1):82-90.

150. Reddy KS, Stablein D, Taranto S, Stratta RJ, Johnston TD, Waid TH, et al. Long-term survival following simultaneous kidney-pancreas transplantation versus kidney transplantation alone in patients with type 1 diabetes mellitus and renal failure. *Am J Kidney Dis* 2003;41(2):464-70.

151. Martinenghi S, Dell'Antonio G, Secchi A, Di Carlo V, Pozza G. Cancer arising after pancreas and/or kidney transplantation in a series of 99 diabetic patients. *Diabetes Care* 1997;20(3):272-5.
152. Roza AM, Johnson C, Juckett M, Eckels D, Adams M. Adenocarcinoma arising in a transplanted pancreas. *Transplantation* 2001;72(6):1156-7.
153. Buell JF, Gross TG, Hanaway MJ, Trofe J, Muthiak C, First MR, et al. Chemotherapy for posttransplant lymphoproliferative disorder: the Israel Penn International Transplant Tumor Registry experience. *Transplant Proc* 2005;37(2):956-7.
154. Tyden G, Tollemar J, Bolinder J. Combined pancreas and kidney transplantation improves survival in patients with end-stage diabetic nephropathy. *Clin Transplant* 2000;14(5):505-8.
155. Rayhill SC, D'Alessandro AM, Odorico JS, Knechtle SJ, Pirsch JD, Heisey DM, et al. Simultaneous pancreas-kidney transplantation and living related donor renal transplantation in patients with diabetes: is there a difference in survival? *Ann Surg* 2000;231(3):417-23.
156. Becker BN, Brazy PC, Becker YT, Odorico JS, Pintar TJ, Collins BH, et al. Simultaneous pancreas-kidney transplantation reduces excess mortality in type 1 diabetic patients with end-stage renal disease. *Kidney Int* 2000;57(5):2129-35.
157. Bunnapradist S, Cho YW, Cecka JM, Wilkinson A, Danovitch GM. Kidney allograft and patient survival in type I diabetic recipients of cadaveric kidney alone versus simultaneous pancreas kidney transplants: a multivariate analysis of the UNOS database. *J Am Soc Nephrol* 2003;14(1):208-13.
158. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions of diabetic nephropathy after pancreas transplantation. *N Engl J Med* 1998;339(2):69-75.
159. Farney AC, Borboroglu PG, Young C. Native renal function after pancreas transplantation alone. *JASN* 2002;13:188A.
160. Mazur MJ, Rea DJ, Griffin MD, Larson TS, Prieto M, Gloor JM, et al. Decline in native renal function early after bladder-drained pancreas transplantation alone. *Transplantation* 2004;77(6):844-9.
161. Hopt UT, Drognitz O. Pancreas organ transplantation. Short and long-term results in terms of diabetes control. *Langenbecks Arch Surg* 2000;385(6):379-89.
162. Zech JC, Trepsat D, Gain-Gueugnon M, Lefrancois N, Martin X, Dubernard JM. Ophthalmological follow-up of type 1 (insulin-dependent) diabetic patients after kidney and pancreas transplantation. *Diabetologia* 1991;34 Suppl 1:S89-91.

163. Pearce IA, Ilango B, Sells RA, Wong D. Stabilisation of diabetic retinopathy following simultaneous pancreas and kidney transplant. *Br J Ophthalmol* 2000;84(7):736-40.
164. Koznarova R, Saudek F, Sosna T, Adamec M, Jedinakova T, Boucek P, et al. Beneficial effect of pancreas and kidney transplantation on advanced diabetic retinopathy. *Cell Transplant* 2000;9(6):903-8.
165. Chow VC, Pai RP, Chapman JR, O'Connell PJ, Allen RD, Mitchell P, et al. Diabetic retinopathy after combined kidney-pancreas transplantation. *Clin Transplant* 1999;13(4):356-62.
166. Scheider A, Meyer-Schwickerath E, Nusser J, Land W, Landgraf R. Diabetic retinopathy and pancreas transplantation: a 3-year follow-up. *Diabetologia* 1991;34 Suppl 1:S95-9.
167. Recasens M, Ricart MJ, Valls-Sole J, Caballero A, Fernandez-Cruz L, Esmatjes E. Long-term follow-up of diabetic polyneuropathy after simultaneous pancreas and kidney transplantation in type 1 diabetic patients. *Transplant Proc* 2002;34(1):200-3.
168. Laftavi MR, Chapuis F, Vial C, Rahbar M, Lefrancois N, Feitosa LC, et al. Diabetic polyneuropathy outcome after successful pancreas transplantation: 1 to 9 year follow up. *Transplant Proc* 1995;27(1):1406-9.
169. Solders G, Tyden G, Persson A, Groth CG. Improvement of nerve conduction in diabetic neuropathy. A follow-up study 4 yr after combined pancreatic and renal transplantation. *Diabetes* 1992;41(8):946-51.
170. Muller-Felber W, Landgraf R, Scheuer R, Wagner S, Reimers CD, Nusser J, et al. Diabetic neuropathy 3 years after successful pancreas and kidney transplantation. *Diabetes* 1993;42(10):1482-6.
171. Navarro X, Sutherland DE, Kennedy WR. Long-term effects of pancreatic transplantation on diabetic neuropathy. *Ann Neurol* 1997;42(5):727-36.
172. Allen RD, Al-Harbi IS, Morris JG, Clouston PD, O'Connell PJ, Chapman JR, et al. Diabetic neuropathy after pancreas transplantation: determinants of recovery. *Transplantation* 1997;63(6):830-8.
173. Hurst GC, Somerville KT, Alloway RR, Gaber AO, Stratta RJ. Preliminary experience with midodrine in kidney/pancreas transplant patients with orthostatic hypotension. *Clin Transplant* 2000;14(1):42-7.

174. Gaber AO, Oxley D, Karas J, Cardoso S, Hathaway D, Shokouh-Amiri MH, et al. Changes in gastric emptying in recipients of successful combined pancreas-kidney transplants. *Dig Dis* 1991;9(6):437-43.
175. Hathaway DK, Abell T, Cardoso S, Hartwig MS, el Gebely S, Gaber AO. Improvement in autonomic and gastric function following pancreas-kidney versus kidney-alone transplantation and the correlation with quality of life. *Transplantation* 1994;57(6):816-22.
176. Gaber AO, Hathaway DK, Abell T, Cardoso S, Hartwig MS, el Gebely S. Improved autonomic and gastric function in pancreas-kidney vs kidney-alone transplantation contributes to quality of life. *Transplant Proc* 1994;26(2):515-6.
177. La Rocca E, Fiorina P, Astorri E, Rossetti C, Lucignani G, Fazio F, et al. Patient survival and cardiovascular events after kidney-pancreas transplantation: comparison with kidney transplantation alone in uremic IDDM patients. *Cell Transplant* 2000;9(6):929-32.
178. La Rocca E, Fiorina P, di Carlo V, Astorri E, Rossetti C, Lucignani G, et al. Cardiovascular outcomes after kidney-pancreas and kidney-alone transplantation. *Kidney Int* 2001;60(5):1964-71.
179. Secchi A, Caldara R, La Rocca E, Fiorina P, Di Carlo V. Cardiovascular disease and neoplasms after pancreas transplantation. *Lancet* 1998;352(9121):65; author reply 66.
180. Jukema JW, Smets YF, van der Pijl JW, Zwinderman AH, Vliegen HW, Ringers J, et al. Impact of simultaneous pancreas and kidney transplantation on progression of coronary atherosclerosis in patients with end-stage renal failure due to type 1 diabetes. *Diabetes Care* 2002;25(5):906-11.
181. Fiorina P, La Rocca E, Venturini M, Minicucci F, Fermo I, Paroni R, et al. Effects of kidney-pancreas transplantation on atherosclerotic risk factors and endothelial function in patients with uremia and type 1 diabetes. *Diabetes* 2001;50(3):496-501.
182. Manske CL, Wilson RF, Wang Y, Thomas W. Atherosclerotic vascular complications in diabetic transplant candidates. *Am J Kidney Dis* 1997;29(4):601-7.
183. Elliott MD, Kapoor A, Parker MA, Kaufman DB, Bonow RO, Gheorghiadu M. Improvement in hypertension in patients with diabetes mellitus after kidney/pancreas transplantation. *Circulation* 2001;104(5):563-9.
184. Fiorina P, La Rocca E, Astorri E, Lucignani G, Rossetti C, Fazio F, et al. Reversal of left ventricular diastolic dysfunction after kidney-pancreas transplantation in type 1 diabetic uremic patients. *Diabetes Care* 2000;23(12):1804-10.

185. Naf S, Jose Ricart M, Recasens M, Astudillo E, Fernandez-Cruz L, Esmatjes E. Macrovascular events after kidney-pancreas transplantation in type 1 diabetic patients. *Transplant Proc* 2003;35(5):2019-20.
186. Bruce DS, Newell KA, Josephson MA, Woodle ES, Piper JB, Millis JM, et al. Long-term outcome of kidney-pancreas transplant recipients with good graft function at one year. *Transplantation* 1996;62(4):451-6.
187. Kalker AJ, Pirsch JD, Heisey D, Sollinger HW, Belzer FO, Knechtle SJ, et al. Foot problems in the diabetic transplant recipient. *Clin Transplant* 1996;10(6 Pt 1):503-10.
188. Morrissey PE, Shaffer D, Monaco AP, Conway P, Madras PN. Peripheral vascular disease after kidney-pancreas transplantation in diabetic patients with end-stage renal disease. *Arch Surg* 1997;132(4):358-61; discussion 361-2.
189. Nankivell BJ, Lau SG, Chapman JR, O'Connell PJ, Fletcher JP, Allen RD. Progression of macrovascular disease after transplantation. *Transplantation* 2000;69(4):574-81.
190. La Rocca E, Minicucci F, Secchi A, Ciurlino D, Bonfatti D, Ferrari G, et al. Evolution of carotid vascular lesions in kidney-pancreas and kidney-alone transplanted insulin-dependent diabetic patients. *Transplant Proc* 1995;27(6):3072.
191. Registry CIT. Annual Report 2006. Bethesda, MD: National Institute of Diabetes & Digestive & Kidney Diseases; 2006.
192. Ryan EA, Lakey JR, Paty BW, Imes S, Korbitt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 2002;51(7):2148-57.
193. Hirshberg B, Rother KI, Digion BJ, 3rd, Lee J, Gaglia JL, Hines K, et al. Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience. *Diabetes Care* 2003;26(12):3288-95.
194. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 2006;355(13):1318-30.
195. Lehmann R, Weber M, Berthold P, Zullig R, Pfammatter T, Moritz W, et al. Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. *Am J Transplant* 2004;4(7):1117-23.

196. Brendel M. International Islet Transplant Registry Newsletter #9. Geissen: Center of Internal Medicine, University Hospital; 2001 June, 2001.
197. Wang XY, Sun J, Wang C, France M, Sheil AG. Effect of liver transplantation on islet allografts: up-regulation of Fas ligand and apoptosis of T lymphocytes are associated with islet graft tolerance. *Transplantation* 2001;71(1):102-11.
198. Carroll PB, Rilo HL, Alejandro R, Zeng Y, Khan R, Fontes P, et al. Long-term (> 3-year) insulin independence in a patient with pancreatic islet cell transplantation following upper abdominal exenteration and liver replacement for fibrolamellar hepatocellular carcinoma. *Transplantation* 1995;59(6):875-9.
199. Angelico MC, Alejandro R, Nery J, Webb M, Bottino R, Kong SS, et al. Transplantation of islets of Langerhans in patients with insulin-requiring diabetes mellitus undergoing orthotopic liver transplantation--the Miami experience. *J Mol Med* 1999;77(1):144-7.
200. Johnson PR, White SA, London NJ. Collagenase and human islet isolation. *Cell Transplant* 1996;5(4):437-52.
201. Gray DW, McShane P, Grant A, Morris PJ. A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* 1984;33(11):1055-61.
202. Gotoh M, Ohzato H, Porter J, Maki T, Monaco AP. Crucial role of pancreatic ductal collagenase injection for isolation of pancreatic islets. *Horm Metab Res Suppl* 1990;25:10-6.
203. Van Wart HE, Steinbrink DR. Complementary substrate specificities of class I and class II collagenases from *Clostridium histolyticum*. *Biochemistry* 1985;24(23):6520-6.
204. Barnett MJ, Zhai X, LeGatt DF, Cheng SB, Shapiro AM, Lakey JR. Quantitative assessment of collagenase blends for human islet isolation. *Transplantation* 2005;80(6):723-8.
205. Lakey JR, Smith ME, Fetterhoff TJ, Cavanagh TJ, Wright MJ, Dwulet FE. Sterile filtration of collagenase solution: effect of filter membrane composition on enzyme recovery. *Transplant Proc* 1998;30(2):347.
206. Vos-Scheperkeuter GH, van Suylichem PT, Vonk MW, Wolters GH, van Schilfhaarde R. Histochemical analysis of the role of class I and class II *Clostridium histolyticum* collagenase in the degradation of rat pancreatic extracellular matrix for islet isolation. *Cell Transplant* 1997;6(4):403-12.

207. Brandhorst D, Iken M, Brendel MD, Bretzel RG, Brandhorst H. Adaption of neutral protease activity for islet isolation from the long-term two-layer method-stored pig pancreas. *Transplant Proc* 2005;37(1):458-9.
208. Brandhorst H, Brendel MD, Eckhard M, Bretzel RG, Brandhorst D. Influence of neutral protease activity on human islet isolation outcome. *Transplant Proc* 2005;37(1):241-2.
209. Olack BJ, Swanson CJ, Howard TK, Mohanakumar T. Improved method for the isolation and purification of human islets of langerhans using Liberase enzyme blend. *Hum Immunol* 1999;60(12):1303-9.
210. Gill JF, Chambers LL, Baurley JL, Ellis BB, Cavanaugh TJ, Fetterhoff TJ, et al. Safety testing of Liberase, a purified enzyme blend for human islet isolation. *Transplant Proc* 1995;27(6):3276-7.
211. Linetsky E, Bottino R, Lehmann R, Alejandro R, Inverardi L, Ricordi C. Improved human islet isolation using a new enzyme blend, liberase. *Diabetes* 1997;46(7):1120-3.
212. Vargas F, Vives-Pi M, Somoza N, Armengol P, Alcalde L, Marti M, et al. Endotoxin contamination may be responsible for the unexplained failure of human pancreatic islet transplantation. *PG - 722-7. Transplantation* 1998;65(5):722-7.
213. Eckhardt T, Jahr H, Federlin K, Bretzel RG. Endotoxin impairs the engraftment of rat islets transplanted beneath the kidney capsule of C57BL/6-mice. *J Mol Med* 1999;77(1):123-5.
214. Vargas F, Julian JF, Llamazares JF, Garcia-Cuyas F, Jimenez M, Pujol-Borrell R, et al. Engraftment of islets obtained by collagenase and Liberase in diabetic rats: a comparative study. *Pancreas* 2001;23(4):406-13.
215. Jahr H, Hering B, Federlin K, Bretzel RG. Activation of human complement by collagenase and ficoll. *Exp Clin Endocrinol Diabetes* 1995;103(Suppl 2):27-9.
216. Stevens RB, Lokeh A, Ansite JD, Field MJ, Gores PF, Sutherland DE. Role of nitric oxide in the pathogenesis of early pancreatic islet dysfunction during rat and human intraportal islet transplantation. *Transplant Proc* 1994;26(2):692.
217. Stevens RB, Ansite JD, Mills CD, Lokeh A, Rossini TJ, Saxena M, et al. Nitric oxide mediates early dysfunction of rat and mouse islets after transplantation. *Transplantation* 1996;61(12):1740-9.

218. Balamurugan AN, He J, Guo F, Stolz DB, Bertera S, Geng X, et al. Harmful delayed effects of exogenous isolation enzymes on isolated human islets: relevance to clinical transplantation. *Am J Transplant* 2005;5(11):2671-81.
219. Bucher P, Mathe Z, Morel P, Bosco D, Andres A, Kurfuest M, et al. Assessment of a novel two-component enzyme preparation for human islet isolation and transplantation. *Transplantation* 2005;79(1):91-7.
220. White SA, Contractor HH, Hughes DP, Johnson PR, Clayton HA, Bell PR, et al. Influence of different collagenase solvents and timing of their delivery on porcine islet isolation. *Br J Surg* 1996;83(10):1350-5.
221. van Suylichem PT, Wolters GH, van Schilfgaarde R. Peri-insular presence of collagenase during islet isolation procedures. *J Surg Res* 1992;53(5):502-9.
222. Lakey JR, Warnock GL, Shapiro AM, Korbitt GS, Ao Z, Kneteman NM, et al. Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion. *Cell Transplant* 1999;8(3):285-92.
223. Socci C, Vignali A, Davalli AM, Maffi P, Magistretti P, Bertuzzi F, et al. Timing of collagenase injection influences the final islet yield in donors under 30 years of age. *Transplant Proc* 1992;24(6):2835-6.
224. Brandhorst D, Iken M, Tanioka Y, Brendel MD, Bretzel RG, Brandhorst H. Influence of collagenase loading on long-term preservation of pig pancreas by the two-layer method for subsequent islet isolation. *Transplantation* 2005;79(1):38-43.
225. Lu S, Jaeger C, Wolff-Vorbeck G, Khalaf AN, Kirste G. Increase of islet yields obtained from human pancreas donor following change of pH value in collagenase digestion medium. *Transplant Proc* 1995;27(6):3202-4.
226. Avila JG, Wang Y, Barbaro B, Gangemi A, Qi M, Kuechle J, et al. Improved outcomes in islet isolation and transplantation by the use of a novel hemoglobin-based O<sub>2</sub> carrier. *Am J Transplant* 2006;6(12):2861-70.
227. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes* 1989;38 Suppl 1:140-2.
228. Gray DW, Sudhakaran N, Titus TT, McShane P, Johnson P. Development of a novel digestion chamber for human and porcine islet isolation. *Transplant Proc* 2004;36(4):1135-8.
229. Dufrane D, Goebbels RM, Guiot Y, Squifflet JP, Henquin JC, Gianello P. A simple method using a polymethylpenten chamber for isolation of human pancreatic islets. *Pancreas* 2005;30(3):e51-9.

230. Arbet-Engels C, Darquy S, Capron F, Reach G. Isolation of islets of Langerhans from the rat and pig pancreas using a modified UW solution from organ storage to islet purification. *Diabetes Metab* 1993;19(6):590-6.
231. Mullen Y, Arita S, Kenmochi T, Une S, Smith CV. A two-step digestion process and LAP-I cold preservation solution for human islet isolation. *Ann Transplant* 1997;2(3):40-5.
232. Arias-Diaz J, Vara E, Garcia C, Torres-Melero J, Rodriguez JM, Balibrea JL. Pentoxifylline partially reverts the effect of tumor necrosis factor on human islets. *Transplant Proc* 1994;26(2):698-700.
233. Ichii H, Wang X, Messenger S, Alvarez A, Fraker C, Khan A, et al. Improved human islet isolation using nicotinamide. *Am J Transplant* 2006;6(9):2060-8.
234. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, et al. Insulin independence after living-donor distal pancreatectomy and islet allotransplantation. *Lancet* 2005;365(9471):1642-4.
235. Gores PF, Sutherland DE. Pancreatic islet transplantation: is purification necessary? *Am J Surg* 1993;166(5):538-42.
236. Froberg MK, Leone JP, Jessurun J, Sutherland DE. Fatal disseminated intravascular coagulation after autologous islet transplantation. *Hum Pathol* 1997;28(11):1295-8.
237. Wahoff DC, Papalois BE, Najarian JS, Kendall DM, Farney AC, Leone JP, et al. Autologous islet transplantation to prevent diabetes after pancreatic resection. *Ann Surg* 1995;222(4):562-75; discussion 575-9.
238. White SA, Robertson GS, Davies JE, Rees Y, London NJ, Dennison AR. Splenic infarction after total pancreatectomy and autologous islet transplantation into the spleen. *Pancreas* 1999;18(4):419-21.
239. Shapiro AM, Lakey JR, Rajotte RV, Warnock GL, Friedlich MS, Jewell LD, et al. Portal vein thrombosis after transplantation of partially purified pancreatic islets in a combined human liver/islet allograft. *Transplantation* 1995;59(7):1060-3.
240. Bennet W, Bjorkland A, Sundberg B, Brandhorst D, Brendel MD, Richards A, et al. Expression of complement regulatory proteins on islets of Langerhans: a comparison between human islets and islets isolated from normal and hDAF transgenic pigs. *Transplantation* 2001;72(2):312-9.
241. Secchi A, Socci C, Maffi P, Taglietti MV, Falqui L, Bertuzzi F, et al. Islet transplantation in IDDM patients. *Diabetologia* 1997;40(2):225-31.

242. Oberholzer J, Triponez F, Mage R, Anderegg E, Buhler L, Cretin N, et al. Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. *Transplantation* 2000;69(6):1115-23.
243. Lake SP, Bassett PD, Larkins A, Revell J, Walczak K, Chamberlain J, et al. Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator. *Diabetes* 1989;38 Suppl 1:143-5.
244. London NJ, Chadwick D, Johnson PR. Approaches to islet purification. In: Lanza P, Chick WL, editors. *Pancreatic islet transplantation*. Austin, Texas: Landes RG; 1995. p. 23-58.
245. Hesse UJ, Sutherland DE, Gores PF, Najarian JS. Experience with 3, 6, and 24 hours' hypothermic storage of the canine pancreas before islet cell preparation and transplantation. *Surgery* 1987;102(3):460-4.
246. Robertson GS, Chadwick D, Contractor H, Rose S, Chamberlain R, Clayton H, et al. Storage of human pancreatic digest in University of Wisconsin solution significantly improves subsequent islet purification. *Br J Surg* 1992;79(9):899-902.
247. Robertson GS, Chadwick DR, Davies J, Rose S, Contractor H, James RF, et al. The effectiveness of components of University of Wisconsin solution in improving human pancreatic islet purification. *Transplantation* 1994;57(3):346-54.
248. Chadwick DR, Robertson GS, Rose S, Contractor H, James RF, Bell PR, et al. Storage of porcine pancreatic digest prior to islet purification. The benefits of UW solution and the roles of its individual components. *Transplantation* 1993;56(2):288-93.
249. Robertson GS, Chadwick DR, Contractor H, James RF, Bell PR, London NJ. The use of continuous density gradients for the assessment of islet and exocrine tissue densities and islet purification. *Acta Diabetol* 1993;30(3):175-80.
250. London NJ, Toomey P, Contractor H, Thirdborough ST, James RF, Bell PR. The effect of osmolality and glucose concentration on the purity of human islet isolates. *Transplant Proc* 1992;24(3):1002.
251. Lake SP, Anderson J, Chamberlain J, Gardner SJ, Bell PR, James RF. Bovine serum albumin density gradient isolation of rat pancreatic islets. *Transplantation* 1987;43(6):805-8.
252. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE. The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation* 1973;16(6):686-9.

253. Olack B, Swanson C, McLear M, Longwith J, Scharp D, Lacy PE. Islet purification using Euro-Ficoll gradients. *Transplant Proc* 1991;23(1 Pt 1):774-6.
254. Hering BJ, Muench KP, Schelz J, Amelang D, Heitfeld M, Bretzel RG, et al. The evaluation of neutral density separation utilizing Ficoll-sodium diatrizoate and Nycodenz and centrifugal elutriation in the purification of bovine and canine islet preparations. *Horm Metab Res Suppl* 1990;25:57-63.
255. van Suylichem PT, Wolters GH, van Schilfgaarde R. The efficacy of density gradients for islet purification: a comparison of seven density gradients. *Transpl Int* 1990;3(3):156-61.
256. van der Burg MP, Graham JM. Iodixanol density gradient preparation in university of wisconsin solution for porcine islet purification. *ScientificWorldJournal* 2003;3:1154-9.
257. Huang GC, Zhao M, Jones P, Persaud S, Ramracheya R, Lobner K, et al. The development of new density gradient media for purifying human islets and islet-quality assessments. *Transplantation* 2004;77(1):143-5.
258. Ichii H, Pileggi A, Molano RD, Baidal DA, Khan A, Kuroda Y, et al. Rescue purification maximizes the use of human islet preparations for transplantation. *Am J Transplant* 2005;5(1):21-30.
259. Frutos MA, Ruiz P, Mansilla JJ. Pancreas donation for islet transplantation. *Transplant Proc* 2005;37(3):1560-1.
260. Kempf MC, Andres A, Morel P, Benhamou PY, Bayle F, Kessler L, et al. Logistics and transplant coordination activity in the GRAGIL Swiss-French multicenter network of islet transplantation. *Transplantation* 2005;79(9):1200-5.
261. Fraker C, Montelongo J, Szust J, Khan A, Ricordi C. The use of multiparametric monitoring during islet cell isolation and culture: a potential tool for in-process corrections of critical physiological factors. *Cell Transplant* 2004;13(5):497-502.
262. Jay TR, Paget MB, Heald KA, Downing R. Are organ preservation solutions useful for the storage of isolated human islets? *Transplant Proc* 2004;36(4):1130-2.
263. Papas KK, Avgoustiniatos ES, Tempelman LA, Weir GC, Colton CK, Pisanias A, et al. High-density culture of human islets on top of silicone rubber membranes. *Transplant Proc* 2005;37(8):3412-4.
264. Mendoza V, Klein D, Ichii H, Ribeiro MM, Ricordi C, Hankeln T, et al. Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction. *Transplant Proc* 2005;37(1):237-40.

265. Wideman RD, Yu IL, Webber TD, Verchere CB, Johnson JD, Cheung AT, et al. Improving function and survival of pancreatic islets by endogenous production of glucagon-like peptide 1 (GLP-1). *Proc Natl Acad Sci U S A* 2006;103(36):13468-73.
266. Todorov I, Omori K, Pascual M, Rawson J, Nair I, Valiente L, et al. Generation of human islets through expansion and differentiation of non-islet pancreatic cells discarded (pancreatic discard) after islet isolation. *Pancreas* 2006;32(2):130-8.
267. Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, Knoller S, Bar Y, Glandt M, et al. Expansion and redifferentiation of adult human pancreatic islet cells. *Biochem Biophys Res Commun* 2006;341(2):291-8.
268. Lipsett M, Aikin R, Castellarin M, Hanley S, Jamal AM, Laganriere S, et al. Islet neogenesis: a potential therapeutic tool in type 1 diabetes. *Int J Biochem Cell Biol* 2006;38(4):498-503.
269. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am J Transplant* 2004;4(3):390-401.
270. Froud T, Ricordi C, Baidal DA, Hafiz MM, Ponte G, Cure P, et al. Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. *Am J Transplant* 2005;5(8):2037-46.
271. Brazda E, Flautner L, Harsanyi L, Adeghate E, Donath T. In vitro amylase release of preserved pancreas: a simple test to assess the viability of pancreatic allograft during preservation in the pigs. *Acta Chir Hung* 1997;36(1-4):46-8.
272. London NJ, Contractor H, Lake SP, Aucott GC, Bell PR, James RF. A microfluorometric viability assay for isolated human and rat islets of Langerhans. *Diabetes Res* 1989;12(3):141-9.
273. Gray DW, Morris PJ. The use of fluorescein diacetate and ethidium bromide as a viability stain for isolated islets of Langerhans. *Stain Technol* 1987;62(6):373-81.
274. Barnett MJ, McGhee-Wilson D, Shapiro AM, Lakey JR. Variation in human islet viability based on different membrane integrity stains. *Cell Transplant* 2004;13(5):481-8.
275. Kumar P, Delfino V, McShane P, Gray DW, Morris PJ. Rapid assessment of islet cell viability by MTT assay after cold storage in different solutions. *Transplant Proc* 1994;26(2):814.
276. Merchant FA, Aggarwal SJ, Diller KR, Bartels KA, Bovik AC. Three-dimensional distribution of damaged cells in cryopreserved pancreatic islets as determined by laser scanning confocal microscopy. *J Microsc* 1993;169 ( Pt 3):329-38.

277. Amin MR, Rigel d'Lacey C, Occeleford CI, London NJ, James RF. Use of confocal laser scanning microscopy for assessing islet viability. *Transplant Proc* 1995;27(6):3351-2.
278. Ichii H, Inverardi L, Pileggi A, Molano RD, Cabrera O, Caicedo A, et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. *Am J Transplant* 2005;5(7):1635-45.
279. Dufrane D, D'Hoore W, Goebbels RM, Saliez A, Guiot Y, Gianello P. Parameters favouring successful adult pig islet isolations for xenotransplantation in pig-to-primate models. *Xenotransplantation* 2006;13(3):204-14.
280. Lacy PE, Walker MM, Fink CJ. Perfusion of isolated rat islets in vitro. Participation of the microtubular system in the biphasic release of insulin. *Diabetes* 1972;21(10):987-98.
281. Gerling IC, Kotb M, Fraga D, Sabek O, Gaber AO. No correlation between in vitro and in vivo function of human islets. *Transplant Proc* 1998;30(2):587-8.
282. Socci C, Davalli AM, Vignali A, Bertuzzi F, Maffi P, Zammarchi O, et al. Evidence of in vivo human islet graft function despite a weak response to in vitro perfusion. *Transplant Proc* 1992;24(6):3056-7.
283. Ricordi C, Scharp DW, Lacy PE. Reversal of diabetes in nude mice after transplantation of fresh and 7-day-cultured (24 degrees C) human pancreatic islets. *Transplantation* 1988;45(5):994-6.
284. Lake SP, Chamberlain J, Bassett PD, London NJ, Walczak K, Bell PR, et al. Successful reversal of diabetes in nude rats by transplantation of isolated adult human islets of Langerhans. *Diabetes* 1989;38(2):244-8.
285. London NJ, Thirdborough SM, Swift SM, Bell PR, James RF. The diabetic "human reconstituted" severe combined immunodeficient (SCID-hu) mouse: a model for isogeneic, allogeneic, and xenogeneic human islet transplantation. *Transplant Proc* 1991;23(1 Pt 1):749.
286. Sabek OM, Fraga DW, Minoru O, McClaren JL, Gaber AO. Assessment of human islet viability using various mouse models. *Transplant Proc* 2005;37(8):3415-6.
287. Papas KK, Colton CK, Nelson RA, Rozak PR, Avgoustiniatos ES, Scott WE, 3rd, et al. Human Islet Oxygen Consumption Rate and DNA Measurements Predict Diabetes Reversal in Nude Mice. *Am J Transplant* 2006.

288. Papas KK, Colton CK, Gounarides JS, Roos ES, Jarema MA, Shapiro MJ, et al. NMR spectroscopy in beta cell engineering and islet transplantation. *Ann N Y Acad Sci* 2001;944:96-119.
289. Armann B, Hanson MS, Hatch E, Steffen A, Fernandez LA. Quantification of basal and stimulated ROS levels as predictors of islet potency and function. *Am J Transplant* 2007;7(1):38-47.
290. Ryan EA, Paty BW, Senior PA, Lakey JR, Bigam D, Shapiro AM. Beta-score: an assessment of beta-cell function after islet transplantation. *Diabetes Care* 2005;28(2):343-7.
291. Ricordi C, Tzakis A, Alejandro R, Zeng YJ, Demetris AJ, Carroll P, et al. Detection of pancreatic islet tissue following islet allotransplantation in man. *Transplantation* 1991;52(6):1079-80.
292. Scopsi L, Andreola S, Socci C, Bertuzzi F, Di Carlo V, Pozza G, et al. Immunocytochemical detection and characterization of intrahepatic human pancreatic islets after combined liver-islet allotransplantation. *Cell Transplant* 1994;3(6):499-508.
293. Ritz-Laser B, Oberholzer J, Toso C, Brulhart MC, Zakrzewska K, Ris F, et al. Molecular detection of circulating beta-cells after islet transplantation. *Diabetes* 2002;51(3):557-61.
294. Han D, Xu X, Pastori RL, Ricordi C, Kenyon NS. Elevation of cytotoxic lymphocyte gene expression is predictive of islet allograft rejection in nonhuman primates. *Diabetes* 2002;51(3):562-6.
295. Han D, Xu X, Baidal D, Leith J, Ricordi C, Alejandro R, et al. Assessment of cytotoxic lymphocyte gene expression in the peripheral blood of human islet allograft recipients: elevation precedes clinical evidence of rejection. *Diabetes* 2004;53(9):2281-90.
296. Sindelar DK, Balcom JH, Chu CA, Neal DW, Cherrington AD. A comparison of the effects of selective increases in peripheral or portal insulin on hepatic glucose production in the conscious dog. *Diabetes* 1996;45(11):1594-604.
297. White SA, Koppiker NP, Burden AC, Davies MJ, Davies JE, Clayton HA, et al. Insulin deficiency and increased intact and 32/33 split proinsulin secretion following human islet autotransplantation. *Transplant Proc* 1998;30(2):627-8.
298. Hirshberg B, Mog S, Patterson N, Leconte J, Harlan DM. Histopathological study of intrahepatic islets transplanted in the nonhuman primate model using edmonton protocol immunosuppression. *J Clin Endocrinol Metab* 2002;87(12):5424-9.

299. Yin D, Ding JW, Shen J, Ma L, Hara M, Chong AS. Liver ischemia contributes to early islet failure following intraportal transplantation: benefits of liver ischemic-preconditioning. *Am J Transplant* 2006;6(1):60-8.
300. Luzi L, Hering BJ, Socci C, Raptis G, Battezzati A, Terruzzi I, et al. Metabolic effects of successful intraportal islet transplantation in insulin-dependent diabetes mellitus. *J Clin Invest* 1996;97(11):2611-8.
301. Pyzdrowski KL, Kendall DM, Halter JB, Nakhleh RE, Sutherland DE, Robertson RP. Preserved insulin secretion and insulin independence in recipients of islet autografts. *N Engl J Med* 1992;327(4):220-6.
302. Gupta V, Wahoff DC, Rooney DP, Poitout V, Sutherland DE, Kendall DM, et al. The defective glucagon response from transplanted intrahepatic pancreatic islets during hypoglycemia is transplantation site-determined. *Diabetes* 1997;46(1):28-33.
303. Kendall DM, Teuscher AU, Robertson RP. Defective glucagon secretion during sustained hypoglycemia following successful islet allo- and autotransplantation in humans. *Diabetes* 1997;46(1):23-7.
304. White SA, London NJ, Johnson PR, Davies JE, Pollard C, Contractor HH, et al. The risks of total pancreatectomy and splenic islet autotransplantation. *Cell Transplant* 2000;9(1):19-24.
305. Burke GW, Alejandro R, Cirocco R, Nery J, Mintz D, Miller J. Regional cytokine changes following OKT3 induction for islet cell transplantation in humans: early increase in portal and systemic levels of interleukin-6 and tumor necrosis factors. *Transplant Proc* 1992;24(3):962-4.
306. Xenos ES, Farney AC, Widmer MB, Casanova D, Stevens RB, Blazar BR, et al. Effect of tumor necrosis factor alpha and of the soluble tumor necrosis factor receptor on insulin secretion of isolated islets of Langerhans. *Transplant Proc* 1992;24(6):2863-4.
307. Shapiro AM, Gallant H, Hao E, Wong J, Rajotte R, Yatscoff R, et al. Portal vein immunosuppressant levels and islet graft toxicity. *Transplant Proc* 1998;30(2):641.
308. Stagner JI, Samols E. The induction of capillary bed development by endothelial cell growth factor before islet transplantation may prevent islet ischemia. *Transplant Proc* 1990;22(2):824-8.
309. Parr EL, Bowen KM, Lafferty KJ. Cellular changes in cultured mouse thyroid glands and islets of Langerhans. *Transplantation* 1980;30(2):135-41.

310. Vasir B, Aiello LP, Yoon KH, Quickel RR, Bonner-Weir S, Weir GC. Hypoxia induces vascular endothelial growth factor gene and protein expression in cultured rat islet cells. *Diabetes* 1998;47(12):1894-903.
311. Stagner J, Mokshagundam S, Wyler K, Samols E, Rilo H, Stagner M, et al. Beta-cell sparing in transplanted islets by vascular endothelial growth factor. *Transplant Proc* 2004;36(4):1178-80.
312. Narang AS, Sabek O, Gaber AO, Mahato RI. Co-expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improves human islet survival and function. *Pharm Res* 2006;23(9):1970-82.
313. Vajkoczy P, Olofsson AM, Lehr HA, Leiderer R, Hammersen F, Arfors KE, et al. Histogenesis and ultrastructure of pancreatic islet graft microvasculature. Evidence for graft revascularization by endothelial cells of host origin. *Am J Pathol* 1995;146(6):1397-405.
314. Vajkoczy P, Menger MD, Simpson E, Messmer K. Angiogenesis and vascularization of murine pancreatic islet isografts. *Transplantation* 1995;60(2):123-7.
315. Merchant FA, Diller KR, Aggarwal SJ, Bovik AC. Angiogenesis in cultured and cryopreserved pancreatic islet grafts. *Transplantation* 1997;63(11):1652-60.
316. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes* 1996;45(9):1161-7.
317. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 1993;42(1):12-21.
318. Sezai S, Sakurabayashi S, Yamamoto Y, Morita T, Hirano M, Oka H. Hepatic arterial and portal venous oxygen content and extraction in liver cirrhosis. *Liver* 1993;13(1):31-5.
319. Carlsson PO, Liss P, Andersson A, Jansson L. Measurements of oxygen tension in native and transplanted rat pancreatic islets. *Diabetes* 1998;47(7):1027-32.
320. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* 2001;50(3):489-95.
321. Carlsson PO, Palm F. Oxygen tension in isolated transplanted rat islets and in islets of rat whole-pancreas transplants. *Transpl Int* 2002;15(11):581-5.

322. Carlsson PO, Palm F, Andersson A, Liss P. Chronically decreased oxygen tension in rat pancreatic islets transplanted under the kidney capsule. *Transplantation* 2000;69(5):761-6.
323. Hughes SJ, Davies SE, Powis SH, Press M. Hyperoxia improves the survival of intraportally transplanted syngeneic pancreatic islets. *Transplantation* 2003;75(12):1954-9.
324. Juang JH, Hsu BR, Kuo CH, Uengt SW. Beneficial effects of hyperbaric oxygen therapy on islet transplantation. *Cell Transplant* 2002;11(2):95-101.
325. Linn T, Schmitz J, Hauck-Schmalenberger I, Lai Y, Bretzel RG, Brandhorst H, et al. Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. *Clin Exp Immunol* 2006;144(2):179-87.
326. Hara Y, Fujino M, Adachi K, Li XK. The reduction of hypoxia-induced and reoxygenation-induced apoptosis in rat islets by epigallocatechin gallate. *Transplant Proc* 2006;38(8):2722-5.
327. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 2000;190(3):255-66.
328. Ichikawa H, Flores S, Kvietys PR, Wolf RE, Yoshikawa T, Granger DN, et al. Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Circ Res* 1997;81(6):922-31.
329. Augstein P, Dunger A, Heinke P, Wachlin G, Berg S, Hehmke B, et al. Prevention of autoimmune diabetes in NOD mice by troglitazone is associated with modulation of ICAM-1 expression on pancreatic islet cells and IFN-gamma expression in splenic T cells. *Biochem Biophys Res Commun* 2003;304(2):378-84.
330. Chakrabarti D, Huang X, Beck J, Henrich J, McFarland N, James RF, et al. Control of islet intercellular adhesion molecule-1 expression by interferon-alpha and hypoxia. *Diabetes* 1996;45(10):1336-43.
331. Piemonti L, Leone BE, Nano R, Sacconi A, Monti P, Maffi P, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes* 2002;51(1):55-65.
332. Baker MS, Chen X, Rotramel A, Nelson J, Kaufman DB. Proinflammatory cytokines induce NF-kappaB-dependent/NO-independent chemokine gene expression in MIN6 beta cells. *J Surg Res* 2003;110(1):295-303.

333. Arai K, Sunamura M, Wada Y, Takahashi M, Kobari M, Kato K, et al. Preventing effect of anti-ICAM-1 and anti-LFA-1 monoclonal antibodies on murine islet allograft rejection. *Int J Pancreatol* 1999;26(1):23-31.
334. Katz SM, Bennett F, Stecker K, Clark JH, Pham T, Wang ME, et al. ICAM-1 antisense oligodeoxynucleotide improves islet allograft survival and function. *Cell Transplant* 2000;9(6):817-28.
335. Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur J Biochem* 1990;192(2):245-61.
336. Bottino R, Fernandez LA, Ricordi C, Lehmann R, Tsan MF, Oliver R, et al. Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. *Diabetes* 1998;47(3):316-23.
337. Gray DW, Sutton R, McShane P, Peters M, Morris PJ. Exocrine contamination impairs implantation of pancreatic islets transplanted beneath the kidney capsule. *J Surg Res* 1988;45(5):432-42.
338. Kolb H, Burkart V, Appels B, Hanenberg H, Kantwerk-Funke G, Kiesel U, et al. Essential contribution of macrophages to islet cell destruction in vivo and in vitro. *J Autoimmun* 1990;3 Suppl 1:117-20.
339. Schwizer RW, Leiter EH, Evans R. Macrophage-mediated cytotoxicity against cultured pancreatic islet cells. *Transplantation* 1984;37(6):539-44.
340. Heitmeier MR, Scarim AL, Corbett JA. Interferon-gamma increases the sensitivity of islets of Langerhans for inducible nitric-oxide synthase expression induced by interleukin 1. *J Biol Chem* 1997;272(21):13697-704.
341. Arnush M, Heitmeier MR, Scarim AL, Marino MH, Manning PT, Corbett JA. IL-1 produced and released endogenously within human islets inhibits beta cell function. *J Clin Invest* 1998;102(3):516-26.
342. Heitmeier MR, Scarim AL, Corbett JA. Prolonged STAT1 activation is associated with interferon-gamma priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. *J Biol Chem* 1999;274(41):29266-73.
343. Corbett JA, McDaniel ML. Intra-islet release of interleukin 1 inhibits beta cell function by inducing beta cell expression of inducible nitric oxide synthase. *J Exp Med* 1995;181(2):559-68.
344. Lacy PE, Finke EH. Activation of intra-islet lymphoid cells causes destruction of islet cells. *Am J Pathol* 1991;138(5):1183-90.

345. Yoshimura N, Kobayashi Y, Yamagishi H, Oka T. The role of tissue factor in ischemic reperfusion injury: (ii). The expression of tissue factor of the liver in hepatic injury. *Transplant Proc* 1998;30(7):3766-8.
346. Morrissey JH. Tissue factor: a key molecule in hemostatic and nonhemostatic systems. *Int J Hematol* 2004;79(2):103-8.
347. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 2002;360(9350):2039-45.
348. Sever CE, Demetris AJ, Zeng Y, Tzakis A, Fung JJ, Starzl TE, et al. Islet cell allotransplantation in diabetic patients. Histologic findings in four adults simultaneously receiving kidney or liver transplants. *Am J Pathol* 1992;140(5):1255-60.
349. Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, et al. Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes* 1999;48(10):1907-14.
350. Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. *Am J Transplant* 2006;6(2):305-12.
351. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 2002;51(6):1779-84.
352. Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Kallen R, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. *Diabetes* 2007;56(8):2008-15.
353. Moberg L, Olsson A, Berne C, Felldin M, Foss A, Kallen R, et al. Nicotinamide inhibits tissue factor expression in isolated human pancreatic islets: implications for clinical islet transplantation. *Transplantation* 2003;76(9):1285-8.
354. Goto M, Johansson U, Eich TM, Lundgren T, Engkvist M, Felldin M, et al. Key factors for human islet isolation and clinical transplantation. *Transplant Proc* 2005;37(2):1315-6.
355. Ansara MF, Saudek F, Newton M, Raynor AC, Cryer PE, Scharp DW. Pancreatic islet transplantation prevents defective glucose counter-regulation in diabetic dogs. *Transplant Proc* 1994;26(2):664-5.

356. Gray DW, Warnock GL, Sutton R, Peters M, McShane P, Morris PJ. Successful autotransplantation of isolated islets of Langerhans in the cynomolgus monkey. *Br J Surg* 1986;73(10):850-3.
357. Sutton R, Gray DW, Burnett M, McShane P, Turner RC, Morris PJ. Metabolic function of intraportal and intrasplenic islet autografts in cynomolgus monkeys. *Diabetes* 1989;38 Suppl 1:182-4.
358. Mellgren A, Schnell Landstrom AH, Petersson B, Andersson A. The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or spleen. *Diabetologia* 1986;29(9):670-2.
359. Kin T, Korbitt GS, Rajotte RV. Survival and metabolic function of syngeneic rat islet grafts transplanted in the omental pouch. *Am J Transplant* 2003;3(3):281-5.
360. Posselt AM, Naji A, Roark JH, Markmann JF, Barker CF. Intrathymic islet transplantation in the spontaneously diabetic BB rat. *Ann Surg* 1991;214(4):363-71; discussion 371-3.
361. Bobzien B, Yasunami Y, Majercik M, Lacy PE, Davie JM. Intratesticular transplants of islet xenografts (rat to mouse). *Diabetes* 1983;32(3):213-6.
362. Posselt AM, Barker CF, Tomaszewski JE, Markmann JF, Choti MA, Naji A. Induction of donor-specific unresponsiveness by intrathymic islet transplantation. *Science* 1990;249(4974):1293-5.
363. Arias-Diaz J, Vara E, Balibrea JL, Garcia C, Maranes A, Diaz A, et al. CT-guided fine-needle approach for intrathymic islet transplantation in a diabetic patient. *Pancreas* 1996;12(1):100-2.
364. Saito K, Iwama N, Takahashi T. Morphometrical analysis on topographical difference in size distribution, number and volume of islets in the human pancreas. *Tohoku J Exp Med* 1978;124(2):177-86.
365. Frey CF, Ho HS. Distal pancreatectomy in chronic pancreatitis. In: Trede M, Carter DC, editors. *Surgery of the pancreas*. London: Churchill and Livingstone; 1997. p. 347-357.
366. Gruessner RW, Kendall DM, Drangstveit MB, Gruessner AC, Sutherland DE. Simultaneous pancreas-kidney transplantation from live donors. *Ann Surg* 1997;226(4):471-80 discussion 480-2.
367. Matsumoto S, Tanaka K, Strong DM, Reems JA. Efficacy of human islet isolation from the tail section of the pancreas for the possibility of living donor islet transplantation. *Transplantation* 2004;78(6):839-43.

368. Webb MA, Illouz SC, Pollard CA, Musto PP, Berry D, Dennison AR. Long-term maintenance of graft function after islet autotransplantation of less than 1000 IEQ/kg. *Pancreas* 2006;33(4):433-4.
369. Clayton HA, Davies JE, Pollard CA, White SA, Musto PP, Dennison AR. Pancreatectomy with islet autotransplantation for the treatment of severe chronic pancreatitis: the first 40 patients at the leicester general hospital. *Transplantation* 2003;76(1):92-8.
370. Keymeulen B, Ling Z, Gorus FK, Delvaux G, Bouwens L, Gruppig A, et al. Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipients characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. *Diabetologia* 1998;41(4):452-9.
371. Keymeulen B, Gillard P, Mathieu C, Movahedi B, Maleux G, Delvaux G, et al. Correlation between beta cell mass and glycemic control in type 1 diabetic recipients of islet cell graft. *Proc Natl Acad Sci U S A* 2006;103(46):17444-9.
372. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. *Diabetes* 2005;54(7):2060-9.
373. Swift SM, Thirdborough SM, London NJ, Cahill M, James RF. Human islet preparations and the expression of MHC antigens. *Transplant Proc* 1992;24(3):1029.
374. Stock PG, Ascher NL, Chen S, Field J, Bach FH, Sutherland DE. Evidence for direct and indirect pathways in the generation of the alloimmune response against pancreatic islets. *Transplantation* 1991;52(4):704-9.
375. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today* 1994;15(1):32-8.
376. Hehmke B, Schroder D, Kloting I. Early appearance of complement-dependent antibody mediated cytotoxicity (C'AMC) to islet cells in serum of diabetes-prone BB/OK rats. *Diabetes Res* 1990;13(4):183-6.
377. Ozasa T, Newton MR, Dallman MJ, Shimizu S, Gray DW, Morris PJ. Cytokine gene expression in pancreatic islet grafts in the rat. *Transplantation* 1997;64(8):1152-9.
378. Diamond AS, Gill RG. An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J Immunol* 2000;165(1):247-55.
379. Arias J, Vara E, Gomez M, Garcia C, Moreno A, Balibrea JL. Effect of cytokines on "de novo" lipid synthesis and hormone secretion by isolated human islets. *Transplant Proc* 1992;24(6):2909-12.

380. Kawahara DJ, Kenney JS. Species differences in human and rat islet sensitivity to human cytokines. Monoclonal anti-interleukin-1 (IL-1) influences on direct and indirect IL-1-mediated islet effects. *Cytokine* 1991;3(2):117-24.
381. DeVault GA, Jr., Kohan DE, Nelson EW, Holman JM, Jr. The effects of oral pentoxifylline on the cytokine release syndrome during inductive OKT3. *Transplantation* 1994;57(4):532-40.
382. Benhamou PY, Moriscot C, Badet L, Halimi S. Strategies for graft immunomodulation in islet transplantation. *Diabetes Metab* 1998;24(3):215-24.
383. Scharp DW, Lacy PE, Santiago JV, McCullough CS, Weide LG, Boyle PJ, et al. Results of our first nine intraportal islet allografts in type 1, insulin-dependent diabetic patients. *Transplantation* 1991;51(1):76-85.
384. James RF, Lake SP, Chamberlain J, Thirdborough SM, Mistry N, Bassett PD, et al. Long-term survival of rat islet allografts after pretreatment with low dose gamma irradiation. *Transplant Proc* 1989;21(1 Pt 3):2725-6.
385. Stegall MD, Tezuka K, Oluwole SF, Engelstad K, Jing MX, Andrew J, et al. Interstitial class II-positive cell depletion by donor pretreatment with gamma irradiation. Evidence of differential immunogenicity between vascularized cardiac allografts and islets. *Transplantation* 1990;49(2):246-51.
386. Benhamou PY, Stein E, Mullen Y. Effect of ultraviolet-B irradiation on the function and immunogenicity of fetal porcine islets. *Transplant Proc* 1994;26(2):705.
387. Dellagiacoia G, Rossi M, Radin S, Vecchioni R. Isolated human islet transplanted into diabetic nude mice after ultraviolet B irradiation. *Transplant Proc* 1992;24(6):2853.
388. Goodman E, Fiedor P, Fein S, Hardy M, Athan E. Low-dose ultraviolet B pretreatment of human islets reduces graft immunogenicity through the induction of antigen-presenting cell apoptosis without adversely affecting insulin secretion. *Transplant Proc* 1995;27(1):1347-8.
389. Faustman D, Coe C. Prevention of xenograft rejection by masking donor HLA class I antigens. *Science* 1991;252(5013):1700-2.
390. Faustman D, Hauptfeld V, Lacy P, Davie J. Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to Ia determinants. *Proc Natl Acad Sci U S A* 1981;78(8):5156-9.

391. Faustman DL, Steinman RM, Gebel HM, Hauptfeld V, Davie JM, Lacy PE. Prevention of rejection of murine islet allografts by pretreatment with anti-dendritic cell antibody. *Proc Natl Acad Sci U S A* 1984;81(12):3864-8.
392. Merrell RC, Mahoney ME, Basadonna G, Cobb LF, Maeda M. Failure of canine islet allografts and autografts with cyclosporine. *Surgery* 1985;98(2):324-9.
393. Viviani GL, Borgoglio MG, Fontana I, Borgoglio A, Nocera A, Leprini A, et al. Azathioprine decreases insulin secretion in human islets. *Transplant Proc* 1989;21(1 Pt 3):2714-6.
394. Menger MD, Wolf B, Jager S, Walter P, Messmer K. The influence of prednisolone on revascularization of pancreatic islet grafts. *Transplant Proc* 1990;22(4):2042-3.
395. Robertson RP. Cyclosporin-induced inhibition of insulin secretion in isolated rat islets and HIT cells. *Diabetes* 1986;35(9):1016-9.
396. Nielsen JH, Mandrup-Poulsen T, Nerup J. Direct effects of cyclosporin A on human pancreatic beta-cells. *Diabetes* 1986;35(9):1049-52.
397. Morel P, Kaufman DB, Field MJ, Lloveras JK, Matas AJ, Sutherland DE. Detrimental effect of prednisone on canine islet autograft function. *Transplant Proc* 1992;24(3):1048-50.
398. Warnock GL, Kneteman NM, Ryan EA, Rabinovitch A, Rajotte RV. Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1992;35(1):89-95.
399. London NJ, James RFL, Robertson GM. Human islet transplantation: The Leicester Experience. In: Ricordi C, editor. *Pancreatic cell transplantation 1892-1992 one century of transplantation for diabetes*. Austin, Texas: Landes RG; 1992. p. 454-461.
400. Starzl TE, Todo S, Fung J, Demetris AJ, Venkataraman R, Jain A. FK 506 for liver, kidney, and pancreas transplantation. *Lancet* 1989;2(8670):1000-4.
401. Halloran PF. Sirolimus and cyclosporin for renal transplantation. *Lancet* 2000;356(9225):179-80.
402. Pirsch JD, Miller J, Deierhoi MH, Vincenti F, Filo RS. A comparison of tacrolimus (FK506) and cyclosporine for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Group. *Transplantation* 1997;63(7):977-83.

403. Tamura K, Fujimura T, Tsutsumi T, Nakamura K, Ogawa T, Atumaru C, et al. Transcriptional inhibition of insulin by FK506 and possible involvement of FK506 binding protein-12 in pancreatic beta-cell. *Transplantation* 1995;59(11):1606-13.
404. Xenos ES, Casanova D, Sutherland DE, Farney AC, Lloveras JJ, Gores PF. The in vivo and in vitro effect of 15-deoxyspergualin on pancreatic islet function. *Transplantation* 1993;56(1):144-7.
405. Kaufman DB, Gores PF, Field MJ, Farney AC, Gruber SA, Stephanian E, et al. Effect of 15-deoxyspergualin on immediate function and long-term survival of transplanted islets in murine recipients of a marginal islet mass. *Diabetes* 1994;43(6):778-83.
406. Gores PF, Najarian JS, Stephanian E, Lloveras JJ, Kelley SL, Sutherland DE. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-deoxyspergualin. *Lancet* 1993;341(8836):19-21.
407. Jindal RM, Soltys K, Yost F, Beer E, Tepper MA, Cho SI. Effect of deoxyspergualin on the endocrine function of the rat pancreas. *Transplantation* 1993;56(5):1275-8.
408. Hao L, Wang Y, Chan SM, Lafferty KJ. Effect of mycophenolate mofetil on islet allografting to chemically induced or spontaneously diabetic animals. *Transplant Proc* 1992;24(6):2843-4.
409. Kaplan B, Meier-Kriesche HU, Minnick P, Bastien MC, Sechaud R, Yeh CM, et al. Randomized calcineurin inhibitor cross over study to measure the pharmacokinetics of co-administered enteric-coated mycophenolate sodium. *Clin Transplant* 2005;19(4):551-8.
410. Guo Z, Chong AS, Shen J, Foster P, Sankary HN, McChesney L, et al. In vivo effects of leflunomide on normal pancreatic islet and syngeneic islet graft function. *Transplantation* 1997;63(5):716-21.
411. Rastellini C, Cicalese L, Leach R, Braun M, Fung JJ, Starzl TE, et al. Prolonged survival of islet allografts following combined therapy with tacrolimus and leflunomide. *Transplant Proc* 1999;31(1-2):646-7.
412. Yamasaki T, Inoue K, Hayashi H, Gu Y, Setoyama H, Ida J, et al. Effect of a new immunosuppressive agent, FTY720, on survival of islet allografts. *Cell Transplant* 1998;7(4):403-6.

413. Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med* 2006;12(3):301-3.
414. Kneteman NM, Lakey JR, Wagner T, Finegood D. The metabolic impact of rapamycin (sirolimus) in chronic canine islet graft recipients. *Transplantation* 1996;61(8):1206-10.
415. Kahan BD, Julian BA, Pescovitz MD, Vanrenterghem Y, Neylan J. Sirolimus reduces the incidence of acute rejection episodes despite lower cyclosporine doses in caucasian recipients of mismatched primary renal allografts: a phase II trial. Rapamune Study Group. *Transplantation* 1999;68(10):1526-32.
416. McAlister VC, Gao Z, Peltekian K, Domingues J, Mahalati K, MacDonald AS. Sirolimus-tacrolimus combination immunosuppression. *Lancet* 2000;355(9201):376-7.
417. Laugharne M, Cross S, Richards S, Dawson C, Ilchyshyn L, Saleem M, et al. Sirolimus toxicity and vascular endothelial growth factor release from islet and renal cell lines. *Transplantation* 2007;83(12):1635-8.
418. Eisenbarth GS, Stegall M. Islet and pancreatic transplantation--autoimmunity and alloimmunity. *N Engl J Med* 1996;335(12):888-90.
419. Stegall MD, Lafferty KJ, Kam I, Gill RG. Evidence of recurrent autoimmunity in human allogeneic islet transplantation. *Transplantation* 1996;61(8):1272-4.
420. Jaeger C, Hering BJ, Dyrberg T, Federlin K, Bretzel RG. Islet cell antibodies and glutamic acid decarboxylase antibodies in patients with insulin-dependent diabetes mellitus undergoing kidney and islet-after-kidney transplantation. *Transplantation* 1996;62(3):424-6.
421. Jaeger C, Brendel MD, Hering BJ, Eckhard M, Bretzel RG. Progressive islet graft failure occurs significantly earlier in autoantibody-positive than in autoantibody-negative IDDM recipients of intrahepatic islet allografts. *Diabetes* 1997;46(11):1907-10.
422. Kai N, Motojima K, Tsunoda T, Kanematsu T. Prevention of insulinitis and diabetes in nonobese diabetic mice by administration of FK506. *Transplantation* 1993;55(4):936-40.
423. Murase N, Lieberman I, Nalesnik MA, Mintz DH, Todo S, Drash AL, et al. Effect of FK 506 on spontaneous diabetes in BB rats. *Diabetes* 1990;39(12):1584-6.
424. Stiller CR, Dupre J, Gent M, Jenner MR, Keown PA, Laupacis A, et al. Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset. *Science* 1984;223(4643):1362-7.

425. Martel RR, Klicius J, Galet S. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can J Physiol Pharmacol* 1977;55(1):48-51.
426. Rabinovitch A, Suarez-Pinzon WL, Shapiro AM, Rajotte RV, Power R. Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 2002;51(3):638-45.
427. Yan H, Suzuki K, Li XK, Amemiya H, Suzuki S, Hiromitsu K. Immunosuppressive effect of FTY 720 on autoimmune diabetes models. *Transplant Proc* 1998;30(7):3436-7.
428. Stosic-Grujicic S, Dimitrijevic M, Bartlett R. Leflunomide protects mice from multiple low dose streptozotocin (MLD- SZ)-induced insulinitis and diabetes. *Clin Exp Immunol* 1999;117(1):44-50.
429. Soria B, Andreu E, Berna G, Fuentes E, Gil A, Leon-Quinto T, et al. Engineering pancreatic islets. *Pflugers Arch* 2000;440(1):1-18.
430. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC, et al. Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *Proc Natl Acad Sci U S A* 1980;77(6):3519-23.
431. Efrat S, Leiser M, Surana M, Tal M, Fusco-Demane D, Fleischer N. Murine insulinoma cell line with normal glucose-regulated insulin secretion. *Diabetes* 1993;42(6):901-7.
432. Knaack D, Fiore DM, Surana M, Leiser M, Laurance M, Fusco-DeMane D, et al. Clonal insulinoma cell line that stably maintains correct glucose responsiveness. *Diabetes* 1994;43(12):1413-7.
433. Roche E, Assimacopoulos-Jeannet F, Witters LA, Perruchoud B, Yaney G, Corkey B, et al. Induction by glucose of genes coding for glycolytic enzymes in a pancreatic beta-cell line (INS-1). *J Biol Chem* 1997;272(5):3091-8.
434. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, et al. Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A* 1988;85(23):9037-41.
435. Efrat S, Fejer G, Brownlee M, Horwitz MS. Prolonged survival of pancreatic islet allografts mediated by adenovirus immunoregulatory transgenes. *Proc Natl Acad Sci U S A* 1995;92(15):6947-51.
436. Efrat S, Fusco-DeMane D, Lemberg H, al Emran O, Wang X. Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. *Proc Natl Acad Sci U S A* 1995;92(8):3576-80.

437. Hughes SD, Johnson JH, Quaade C, Newgard CB. Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. *Proc Natl Acad Sci U S A* 1992;89(2):688-92.
438. Clark SA, Burnham BL, Chick WL. Modulation of glucose-induced insulin secretion from a rat clonal beta-cell line. *Endocrinology* 1990;127(6):2779-88.
439. Wang S, Beattie GM, Mally MI, Cirulli V, Itkin-Ansari P, Lopez AD, et al. Isolation and characterization of a cell line from the epithelial cells of the human fetal pancreas. *Cell Transplant* 1997;6(1):59-67.
440. Wang S, Beattie GM, Mally MI, Lopez AD, Hayek A, Levine F. Analysis of a human fetal pancreatic islet cell line. *Transplant Proc* 1997;29(4):2219.
441. Levine F, Wang S, Beattie GM, Mally MI, Cirulli V, Lopez AD, et al. Development of a cell line from the human fetal pancreas. *Transplant Proc* 1995;27(6):3410.
442. MacFarlane WM, Chapman JC, Shepherd RM, Hashmi MN, Kamimura N, Cosgrove KE, et al. Engineering a glucose-responsive human insulin-secreting cell line from islets of Langerhans isolated from a patient with persistent hyperinsulinemic hypoglycemia of infancy. *J Biol Chem* 1999;274(48):34059-66.
443. Scharfmann R. Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. *Diabetologia* 2000;43(9):1083-92.
444. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, et al. In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci U S A* 2000;97(14):7999-8004.
445. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 2000;49(2):157-62.
446. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med* 2000;6(3):278-82.
447. Ahlgren U, Pfaff SL, Jessell TM, Edlund T, Edlund H. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* 1997;385(6613):257-60.

448. Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest* 1996;98(1):216-24.
449. Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manzke O, et al. Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J Cell Biol* 1998;143(7):2045-56.
450. Otonkoski T, Beattie GM, Rubin JS, Lopez AD, Baird A, Hayek A. Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. *Diabetes* 1994;43(7):947-53.
451. Otonkoski T, Beattie GM, Mally MI, Ricordi C, Hayek A. Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J Clin Invest* 1993;92(3):1459-66.
452. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, et al. Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 1999;274(20):14112-21.
453. Gaur LK, Kennedy E, Nitta Y, Nepom GT, Nelson KA, Allen M, et al. Induction of donor-specific tolerance to islet allografts in nonhuman primates. *Ann N Y Acad Sci* 2002;958:194-8.
454. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;385(6619):810-3.
455. Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 1999;283(5401):534-7.
456. Todorov I, Nair I, Ferreri K, Rawson J, Kuroda A, Pascual M, et al. Multipotent progenitor cells isolated from adult human pancreatic tissue. *Transplant Proc* 2005;37(8):3420-1.
457. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet* 1994;344(8934):1402-4.
458. Auchincloss H, Jr., Sachs DH. Xenogeneic transplantation. *Annu Rev Immunol* 1998;16:433-70.

459. Eckstein V, Ulrichs K, Meincke G, Muller-Ruchholtz W. Natural xenophile antibodies from sera of type I diabetic patients differ strongly in their reactivity against various porcine pancreatic cells. *Transplant Proc* 1992;24(2):681-3.
460. Schaapherder AF, Daha MR, van der Woude FJ, Bruijn JA, Gooszen HG. IgM, IgG, and IgA antibodies in human sera directed against porcine islets of Langerhans. *Transplantation* 1993;56(6):1576-9.
461. Ross JR, Kirk AD, Ibrahim SE, Howell DN, Baldwin WM, 3rd, Sanfilippo FP. Characterization of human anti-porcine "natural antibodies" recovered from ex vivo perfused hearts--predominance of IgM and IgG2. *Transplantation* 1993;55(5):1144-50.
462. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 1989;38 Suppl 1:199-201.
463. Andersson A, Korsgren O, Jansson L. Intraportally transplanted pancreatic islets revascularized from hepatic arterial system. *Diabetes* 1989;38 Suppl 1:192-5.
464. Deng S, Buhler L, Lou J, Grau G, Redard M, Bubloz C, et al. Study of concordant xenografted islets of Langerhans rejection: humoral or cellular mechanism? *Transplant Proc* 1994;26(3):1184-5.
465. Moses RD, Auchincloss H, Jr. Mechanisms of cellular xenograft rejection. In: Cooper DK, Kemp E, Reemtsma K, editors. *Xenotransplantation: the transplantation of organs and tissues between species*. Berlin: Springer; 1991. p. 101-120.
466. Warrens AN, Dorling A, Lechler RI. The prospects for xenotransplantation. *Qjm* 1996;89(12):885-91.
467. Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. *J Immunol* 1995;155(11):5249-56.
468. Gotoh M, Kanai T, Dono K, Porter J, Maki T, Monaco AP. Gamma-irradiation as a tool to reduce immunogenicity of islet allo- and xenografts. *Horm Metab Res Suppl* 1990;25:89-96.
469. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* 1984;307(5947):168-70.
470. Li H, Ricordi C, Demetris AJ, Kaufman CL, Korbanic C, Hronakes ML, et al. Mixed xenogeneic chimerism (mouse+rat-->mouse) to induce donor-specific tolerance to sequential or simultaneous islet xenografts. *Transplantation* 1994;57(4):592-8.

471. Zeng YJ, Ricordi C, Tzakis A, Rilo HL, Carroll PB, Starzl TE, et al. Long-term survival of donor-specific pancreatic islet xenografts in fully xenogeneic chimeras (WF rat---B10 mouse). *Transplantation* 1992;53(2):277-83.
472. O'Shea GM, Sun AM. Encapsulation of rat islets of Langerhans prolongs xenograft survival in diabetic mice. *Diabetes* 1986;35(8):943-6.
473. Weber CJ, Zabinski S, Koschitzky T, Rajotte R, Wicker L, Peterson L, et al. Microencapsulated dog and rat islet xenografts into streptozotocin-diabetic and NOD mice. *Horm Metab Res Suppl* 1990;25:219-26.
474. Sun YL, Ma X, Zhou D, Vacek I, Sun AM. Porcine pancreatic islets: isolation, microencapsulation, and xenotransplantation. *Artif Organs* 1993;17(8):727-33.
475. Zhou D, Sun YL, Vacek I, Ma P, Sun AM. Normalization of diabetes in cynomolgus monkeys by xenotransplantation of microencapsulated porcine islets. *Transplant Proc* 1994;26(3):1091.
476. Weber CJ, Zabinski S, Koschitzky T, Wicker L, Rajotte R, D'Agati V, et al. The role of CD4+ helper T cells in the destruction of microencapsulated islet xenografts in nod mice. *Transplantation* 1990;49(2):396-404.
477. White DJ. Transplantation of organs between species. *Int Arch Allergy Immunol* 1992;98(1):1-5.
478. Langford GA, Yannoutsos N, Cozzi E, Lancaster R, Elsome K, Chen P, et al. Production of pigs transgenic for human decay accelerating factor. *Transplant Proc* 1994;26(3):1400-1.
479. Schmoekel M, Bhatti FN, Zaidi A, Cozzi E, Waterworth PD, Tolan MJ, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. *Transplantation* 1998;65(12):1570-7.
480. White DJ, Yannoutsos N. Production of pigs transgenic for human DAF to overcome complement-mediated hyperacute xenograft rejection in man. *Res Immunol* 1996;147(2):88-94.
481. White SA, Hughes DP, Contractor HH, Johnson PR, Clayton HA, Bell PR, et al. The intraductal administration of collagenase in University of Wisconsin solution at the time of harvesting significantly improves porcine islet isolation. *Transplant Proc* 1995;27(6):3366.
482. Chiche L, Adam R, Caillat-Zucman S, Castaing D, Bach JF, Bismuth H. Xenotransplantation: baboons as potential liver donors? Scientific and ethical issues. *Transplantation* 1993;55(6):1418-21.

483. Michaels MG, Simmons RL. Xenotransplant-associated zoonoses. Strategies for prevention. *Transplantation* 1994;57(1):1-7.
484. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med* 1997;3(3):282-6.
485. Martin U, Kiessig V, Blusch JH, Haverich A, von der Helm K, Herden T, et al. Expression of pig endogenous retrovirus by primary porcine endothelial cells and infection of human cells. *Lancet* 1998;352(9129):692-4.
486. Heneine W, Tibell A, Switzer WM, Sandstrom P, Rosales GV, Mathews A, et al. No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet* 1998;352(9129):695-9.
487. Fiedor P, Goodman ER, Sung RS, Czerwinski J, Rowinski W, Hardy MA. The effect of clinical and biochemical donor parameters on pancreatic islet isolation yield from cadaveric organ donors. *Ann Transplant* 1996;1(1):59-62.
488. Witkowski P, Liu Z, Cernea S, Guo Q, Poumian-Ruiz E, Herold K, et al. Validation of the scoring system for standardization of the pancreatic donor for islet isolation as used in a new islet isolation center. *Transplant Proc* 2006;38(9):3039-40.
489. O'Gorman D, Kin T, Murdoch T, Richer B, McGhee-Wilson D, Ryan E, et al. The standardization of pancreatic donors for islet isolation. *Transplant Proc* 2005;37(2):1309-10.
490. Brandhorst D, Hering BJ, Brandhorst H, Federlin K, Bretzel RG. Influence of donor data and organ procurement on human islet isolation. *Transplant Proc* 1994;26(2):592-3.
491. Lakey JR, Warnock GL, Rajotte RV, Ao Z, Suarez-Almazor ME, Shapiro AM, et al. Factors in cadaveric donors that affect recovery of human islets of Langerhans. *Transplant Proc* 1995;27(6):3265.
492. Brandhorst H, Brandhorst D, Hering BJ, Federlin K, Bretzel RG. Body mass index of pancreatic donors: a decisive factor for human islet isolation. *Exp Clin Endocrinol Diabetes* 1995;103 Suppl 2:23-26.
493. Nano R, Clissi B, Melzi R, Calori G, Maffi P, Antonioli B, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia* 2005;48(5):906-12.
494. Balamurugan AN, Chang Y, Bertera S, Sands A, Shankar V, Trucco M, et al. Suitability of human juvenile pancreatic islets for clinical use. *Diabetologia* 2006;49(8):1845-54.

495. Matsumoto I, Sawada T, Nakano M, Sakai T, Liu B, Ansite JD, et al. Improvement in islet yield from obese donors for human islet transplants. *Transplantation* 2004;78(6):880-5.
496. D'Alessandro AM, Odorico JS, Knechtle SJ, Becker YT, Hoffmann RM, Kalayoglu M, et al. Simultaneous pancreas-kidney (SPK) transplantation from controlled non-heart-beating donors (NHBDs). *Cell Transplant* 2000;9(6):889-93.
497. Sollinger HW, Ploeg RJ, Eckhoff DE, Stegall MD, Isaacs R, Pirsch JD, et al. Two hundred consecutive simultaneous pancreas-kidney transplants with bladder drainage. *Surgery* 1993;114(4):736-43; discussion 743-4.
498. Markmann JF, Deng S, Desai NM, Huang X, Velidedeoglu E, Frank A, et al. The use of non-heart-beating donors for isolated pancreatic islet transplantation. *Transplantation* 2003;75(9):1423-9.
499. Noguchi H, Iwanaga Y, Okitsu T, Nagata H, Yonekawa Y, Matsumoto S. Evaluation of islet transplantation from non-heart beating donors. *Am J Transplant* 2006;6(10):2476-82.
500. Powner DJ. Donor care before pancreatic tissue transplantation. *Prog Transplant* 2005;15(2):129-36; quiz 137.
501. Masson F, Thicoipe M, Gin H, de Mascarel A, Angibeau RM, Favarel-Garrigues JF, et al. The endocrine pancreas in brain-dead donors. A prospective study in 25 patients. *Transplantation* 1993;56(2):363-7.
502. Pratschke J, Wilhelm MJ, Kusaka M, Basker M, Cooper DK, Hancock WW, et al. Brain death and its influence on donor organ quality and outcome after transplantation. *Transplantation* 1999;67(3):343-8.
503. Salim A, Martin M, Brown C, Belzberg H, Rhee P, Demetriades D. Complications of brain death: frequency and impact on organ retrieval. *Am Surg* 2006;72(5):377-81.
504. Pratschke J, Wilhelm MJ, Kusaka M, Beato F, Milford EL, Hancock WW, et al. Accelerated rejection of renal allografts from brain-dead donors. *Ann Surg* 2000;232(2):263-71.
505. Contreras JL, Eckstein C, Smyth CA, Sellers MT, Vilatoba M, Bilbao G, et al. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes* 2003;52(12):2935-42.

506. Eckhoff DE, Eckstein C, Smyth CA, Vilatoba M, Bilbao G, Rahemtulla FG, et al. Enhanced isolated pancreatic islet recovery and functionality in rats by 17beta-estradiol treatment of brain death donors. *Surgery* 2004;136(2):336-45.
507. Contreras JL, Smyth CA, Bilbao G, Young CJ, Thompson JA, Eckhoff DE. 17beta-Estradiol protects isolated human pancreatic islets against proinflammatory cytokine-induced cell death: molecular mechanisms and islet functionality. *Transplantation* 2002;74(9):1252-9.
508. Yoshida H, Hiraide A, Iwai A, Kawaguchi N, Yoshioka T, Sugimoto T. Preservation of pancreatic endocrine function by hemodynamic stabilization following brain death. *Clin Transplant* 1997;11(5 Pt 1):420-5.
509. Gores PF, Gillingham KJ, Dunn DL, Moudry-Munns KC, Najarian JS, Sutherland DE. Donor hyperglycemia as a minor risk factor and immunologic variables as major risk factors for pancreas allograft loss in a multivariate analysis of a single institution's experience. *Ann Surg* 1992;215(3):217-30.
510. Watt PC, Mullen Y, Benhamou PY, Hober C, Nomura Y, Watanabe Y, et al. Donor factors affecting successful isolation of islets from the human pancreas. *Transplant Proc* 1994;26(2):594-5.
511. Sung RS, Fiedor PS, Goodman ER, Yaron I, Zakheim AR, Quadracci KM, et al. The influence of hemodynamic status in heart-beating cadaveric organ donors on subsequent pancreatic islet yield. *Transplant Proc* 1995;27(6):3250.
512. Richter S, Yamauchi J, Minor T, Menger MD, Vollmar B. Heparin and phentolamine combined, rather than heparin alone, improves hepatic microvascular procurement in a non-heart-beating donor rat-model. *Transpl Int* 2000;13(3):225-9.
513. Braun C, Schultz M, Fang L, Schaub M, Back WE, Herr D, et al. Treatment of chronic renal allograft rejection in rats with a low-molecular-weight heparin (reviparin). *Transplantation* 2001;72(2):209-15.
514. Shapira OM, Rene H, Lider O, Pfeffermann RA, Shemin RJ, Cohen IR. Prolongation of rat skin and cardiac allograft survival by low molecular weight heparin. *J Surg Res* 1999;85(1):83-7.
515. Kaisers U, Pappert D, Langrehr JM, Undi H, Neuhaus P, Rossaint R. Dopamine, dexamine and dobutamine in liver transplant recipients: a comparison of their effects on hemodynamics, oxygen transport and hepatic venous oxygen saturation. *Transpl Int* 1996;9(3):214-20.

516. Kaisers U, Neumann U, Kuhlen R, Sprenger M, Neuhaus P, Rossaint R. Nitroglycerin versus epoprostenol: effects on hemodynamics, oxygen delivery, and hepatic venous oxygenation after liver transplantation. *Liver Transpl Surg* 1996;2(6):455-60.
517. Morimoto Y, Kamiike W, Nishida T, Hatanaka N, Shimizu S, Huang TP, et al. Improvement of rat liver graft function by insulin administration to donor. *Gastroenterology* 1996;111(4):1071-80.
518. Cywes R, Greig PD, Morgan GR, Sanabria JR, Clavien PA, Harvey PR, et al. Rapid donor liver nutritional enhancement in a large animal model. *Hepatology* 1992;16(5):1271-9.
519. Morgan GR, Sanabria JR, Clavien PA, Phillips MJ, Edwards C, Harvey PR, et al. Correlation of donor nutritional status with sinusoidal lining cell viability and liver function in the rat. *Transplantation* 1991;51(6):1176-83.
520. Cywes R, Greig PD, Sanabria JR, Clavien PA, Levy GA, Harvey PR, et al. Effect of intraportal glucose infusion on hepatic glycogen content and degradation, and outcome of liver transplantation. *Ann Surg* 1992;216(3):235-46; discussion 246-7.
521. Sumimoto R, Southard JH, Belzer FO. Livers from fasted rats acquire resistance to warm and cold ischemia injury. *Transplantation* 1993;55(4):728-32.
522. Sun X, Kimura T, Kobayashi T, Noriki S, Imamura Y, Fukuda M, et al. Viability of liver grafts from fasted donor rats: relationship to sinusoidal endothelial cell apoptosis. *J Hepatobiliary Pancreat Surg* 2001;8(3):268-73.
523. Nishihara M, Sumimoto R, Asahara T, Fukuda Y, Southard JH, Dohi K. Effect of donor fasting on survival of pancreas and heart grafts after warm ischemia. *Hiroshima J Med Sci* 1996;45(3):93-7.
524. Nishihara V, Sumimoto R, Fukuda Y, Southard JH, Asahara T, Dohi K. Inhibition of warm ischemic injury to rat liver, pancreas, and heart grafts by controlling the nutritional status of both donor and recipient. *Surg Today* 1997;27(7):645-50.
525. Abri O, Kuhn F, Lippert H, Lohde E, Gutte N, Kraas E, et al. The effect of multiple-organ donation on the degree of preservation of isolated pancreas islets. *Transplant Proc* 1987;19(4 Suppl 4):99-102.
526. Raude H, Lohde E, Luck M, Kuhn F, Abri O, Kraas E. Influence of human donor operation and isolation techniques on the viability of islets of Langerhans. *Transplant Proc* 1991;23(5):2450.

527. Brockmann JG, Vaidya A, Reddy S, Friend PJ. Retrieval of abdominal organs for transplantation. *Br J Surg* 2006;93(2):133-46.
528. Lakey JR, Kneteman NM, Rajotte RV, Wu DC, Bigam D, Shapiro AM. Effect of core pancreas temperature during cadaveric procurement on human islet isolation and functional viability. *Transplantation* 2002;73(7):1106-10.
529. Mor E, Klintmalm GB, Gonwa TA, Solomon H, Holman MJ, Gibbs JF, et al. The use of marginal donors for liver transplantation. A retrospective study of 365 liver donors. *Transplantation* 1992;53(2):383-6.
530. Tilney NL, Guttman RD. Effects of initial ischemia/reperfusion injury on the transplanted kidney. *Transplantation* 1997;64(7):945-7.
531. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. *N Engl J Med* 1995;333(6):333-6.
532. Slater DN, Bardsley D, Mangnall Y, Smythe A, Fox M. Pancreatic ischaemia; sensitivity and reversibility of the changes. *Br J Exp Pathol* 1975;56(6):530-6.
533. Florack G, Sutherland DE, Heil J, Zweber B, Najarian JS. Long-term preservation of segmental pancreas autografts. *Surgery* 1982;92(2):260-9.
534. Florack G, Sutherland D, Dunning M, Zweber B, Najarian J. Function of segmental pancreas grafts subjected to warm ischaemia prior to hypothermic preservation. *Transplant Proc* 1984;16:111.
535. Benhamou PY, Watt PC, Mullen Y, Ingles S, Watanabe Y, Nomura Y, et al. Human islet isolation in 104 consecutive cases. Factors affecting isolation success. *Transplantation* 1994;57(12):1804-10.
536. Lakey JR, Rajotte RV, Warnock GL, Kneteman NM. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. *Transplantation* 1995;59(5):689-94.
537. Ketchum R, Nicolae M, Jahr H, Friedman AL, Naji A, Barker CF. Analysis of donor age and cold ischaemia as factors in cadaveric human islet isolation. *Transplant Proc* 1994;26:596-597.
538. Zeng Y, Torre MA, Karrison T, Thistlethwaite JR. The correlation between donor characteristics and the success of human islet isolation. *Transplantation* 1994;57(6):954-8.
539. Stratta RJ. Donor age, organ import, and cold ischemia: effect on early outcomes after simultaneous kidney-pancreas transplantation. *Transplant Proc* 1997;29(8):3291-2.

540. Kusterer K, Poschmann T, Friedemann A, Enghofer M, Zandler S, Usadel KH. Arterial constriction, ischemia-reperfusion, and leukocyte adherence in acute pancreatitis. *Am J Physiol* 1993;265(1 Pt 1):G165-71.
541. Humar A, Kandaswamy R, Drangstveit MB, Parr E, Gruessner AG, Sutherland DE. Prolonged preservation increases surgical complications after pancreas transplants. *Surgery* 2000;127(5):545-51.
542. Hotter G, Leon OS, Rosello-Catafau J, Lopez-Boado MA, Parellada PP, Henriques RD, et al. Tissular prostanoid release, phospholipase A2 activity, and lipid peroxidation in pancreas transplantation. *Transplantation* 1991;51(5):987-90.
543. Pi F, Hotter G, Closa D, Rosello-Catafau J, Bulbena O, Badosa F, et al. Effect of a platelet-activating factor antagonist and desferrioxamine administration on eicosanoid production in rat pancreas transplantation. *Transplantation* 1994;57(1):12-7.
544. Hotter G, Closa D, Pi F, Prats N, Fernandez-Cruz L, Bulbena O, et al. Nitric oxide and arachidonate metabolism in ischemia-reperfusion associated with pancreas transplantation. *Transplantation* 1995;59(3):417-21.
545. Lefer AM, Lefer DJ. Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. *Annu Rev Pharmacol Toxicol* 1993;33:71-90.
546. Hotter G, Pi F, Sanz C, Peralta C, Prats N, Gelpi E, et al. Endothelin mediated nitric oxide effects in ischemia--reperfusion associated with pancreas transplantation. *Dig Dis Sci* 1998;43(12):2627-33.
547. Preissler G, Massberg S, Enders G, Waldner H, Messmer K. [Effect of hypothermic preservation on ICAM-1 mRNA induction, leukocyte-endothelium interaction and microvascular perfusion after pancreas transplantation in the rat]. *Langenbecks Arch Chir Suppl Kongressbd* 1998;115(Suppl I):161-4.
548. Toyama H, Takada M, Tanaka T, Suzuki Y, Kuroda Y. Characterization of islet-infiltrating immunocytes after pancreas preservation by two-layer (UW/perfluorochemical) cold storage method. *Transplant Proc* 2003;35(4):1503-5.
549. Lakey JR, Warnock GL, Rajotte RV, Suarez-Alamazor ME, Ao Z, Shapiro AM, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation* 1996;61(7):1047-53.
550. Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. *Transplantation* 1988;45(4):673-6.
551. Blankensteijn JD, Terpstra OT. Liver preservation: the past and the future. *Hepatology* 1991;13(6):1235-50.

552. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992;53(5):957-78.
553. Rauen U, de Groot H. Cold-induced release of reactive oxygen species as a decisive mediator of hypothermia injury to cultured liver cells. *Free Radic Biol Med* 1998;24(7-8):1316-23.
554. Rauen U, Polzar B, Stephan H, Mannherz HG, de Groot H. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *Faseb J* 1999;13(1):155-68.
555. Rauen U, de Groot H. Mammalian cell injury induced by hypothermia- the emerging role for reactive oxygen species. *Biol Chem* 2002;383(3-4):477-88.
556. Calne RY, Pegg DE, Pryse-Davies J, Brown FL. Renal Preservation by Ice-Cooling: An Experimental Study Relating to Kidney Transplantation from Cadavers. *Br Med J* 1963;5358:651-5.
557. Lee RG, Clouse ME, Lanir A. Liver adenosine triphosphate and pH in fasted and well-fed mice after infusion of adenine nucleotide precursors. *Liver* 1988;8(6):337-43.
558. Collste H, Bergstrom J, Hultman E, Melin B. ATP in the cortex of canine kidneys undergoing hypothermic storage. *Life Sci* 1971;10:1204.
559. Cunarro JA, Johnson WA, Uehling DT, Updike SJ, Weiner MW. Metabolic consequences of low-temperature kidney preservation. *J Lab Clin Med* 1976;88(6):873-84.
560. Southard JH, Senzig KA, Hoffman RM, Belzer FO. Energy metabolism in kidneys stored by simple hypothermia. *Transplant Proc* 1977;9(3):1535-9.
561. Calman KC. The prediction of organ viability. I. An hypothesis. *Cryobiology* 1974;11(1):1-6.
562. Kamiike W, Watanabe F, Hashimoto T, Tagawa K, Ikeda Y, Nakao K, et al. Changes in cellular levels of ATP and its catabolites in ischemic rat liver. *J Biochem (Tokyo)* 1982;91(4):1349-56.
563. Yokota R, Fujiwara H, Miyamae M, Tanaka M, Yamasaki K, Itoh S, et al. Transient adenosine infusion before ischemia and reperfusion protects against metabolic damage in pig hearts. *Am J Physiol* 1995;268(3 Pt 2):H1149-57.
564. Kamiike W, Burdelski M, Steinhoff G, Ringe B, Lauchart W, Pichlmayr R. Adenine nucleotide metabolism and its relation to organ viability in human liver transplantation. *Transplantation* 1988;45(1):138-43.

565. Southard JH, van Gulik TM, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BL, et al. Important components of the UW solution. *Transplantation* 1990;49(2):251-7.
566. Southard JH, Belzer FO. Organ preservation. *Annu Rev Med* 1995;46:235-47.
567. Vajdova K, Smrekova R, Mislanova C, Kukan M, Lutterova M. Cold-preservation-induced sensitivity of rat hepatocyte function to rewarming injury and its prevention by short-term reperfusion. *Hepatology* 2000;32(2):289-96.
568. Tanioka Y, Kuroda Y, Kim Y, Matsumoto S, Suzuki Y, Ku Y, et al. The effect of ouabain (inhibitor of an ATP-dependent Na<sup>+</sup>/K<sup>+</sup> pump) on the pancreas graft during preservation by the two-layer method. *Transplantation* 1996;62(12):1730-4.
569. Fujino Y, Kuroda Y, Morita A, Tanioka Y, Ku Y, Saitoh Y. The effect of fasting and exogenous adenosine on ATP tissue concentration and viability of canine pancreas grafts during preservation by the two-layer method. *Transplantation* 1993;56(5):1083-6.
570. Yuan CH, Li GC, Zhang H, Cheng Y, Zhao N, Liu YF. Evaluation of the viability and energy metabolism of canine pancreas graft subjected to significant warm ischemia damage during preservation by UW solution cold storage method. *World J Gastroenterol* 2004;10(12):1785-8.
571. Kuroda Y, Morita A, Fujino Y, Tanioka Y, Ku Y, Saitoh Y. Restoration of pancreas graft function preserved by a two-layer (University of Wisconsin solution/perfluorochemical) cold storage method after significant warm ischemia. *Transplantation* 1993;55(1):227-8.
572. Lam FT, Mavor AI, Potts DJ, Giles GR. Improved 72-hour renal preservation with phosphate-buffered sucrose. *Transplantation* 1989;47(5):767-71.
573. Wahlberg JA, Southard JH, Belzer FO. Development of a cold storage solution for pancreas preservation. *Cryobiology* 1986;23(6):477-82.
574. Fukuse T, Hirata T, Nakamura T, Ueda M, Kawashima M, Hitomi S, et al. Role of saccharides on lung preservation. *Transplantation* 1999;68(1):110-7.
575. Hopkinson DN, Odom NJ, Bridgewater BJ, Hooper TL. University of Wisconsin solution for lung graft preservation: which components are important? *J Heart Lung Transplant* 1994;13(6):990-7.
576. Hopkinson DN, Odom NJ, Bridgewater BJ, Hooper TI. Comparison of saccharides as osmotic impermeants during hypothermic lung graft preservation. *Transplantation* 1996;61(12):1667-71.

577. Collins GM, Green RD, Halasz NA. Importance of anion content and osmolarity in flush solutions for 48 to 72 hr hypothermic kidney storage. *Cryobiology* 1979;16(3):217-20.
578. Jamieson NV, Sundberg R, Lindell S, Claesson K, Moen J, Vreugdenhil PK, et al. Preservation of the canine liver for 24-48 hours using simple cold storage with UW solution. *Transplantation* 1988;46(4):517-22.
579. Ploeg RJ, Goossens D, Vreugdenhil P, McAnulty JF, Southard JH, Belzer FO. Successful 72-hour cold storage kidney preservation with UW solution. *Transplant Proc* 1988;20(1 Suppl 1):935-8.
580. Swanson DK, Pasaoglu I, Berkoff HA, Southard JA, Hegge JO. Improved heart preservation with UW preservation solution. *J Heart Transplant* 1988;7(6):456-67.
581. McAnulty JF, Southard JH, Belzer FO. Comparison of the effects of adenine-ribose with adenosine for maintenance of ATP concentrations in 5-day hypothermically perfused dog kidneys. *Cryobiology* 1988;25(5):409-16.
582. Buhl MR, Kemp G, Kemp E. Adenosine stimulated postimplantational regeneration of 5-adenine nucleotides in rabbit kidney grafts. *Life Sci* 1976;19(12):1889-96.
583. Pegg DE, Wusteman MC, Foreman J. Metabolism of normal and ischemically injured rabbit kidneys during perfusion for 48 hours at 10 C. *Transplantation* 1981;32(5):437-43.
584. Delmas-Beauvieux MC, Gallis JL, Rouse N, Clerc M, Canioni P. Phosphorus-31 nuclear magnetic resonance of isolated rat liver during hypothermic ischemia and subsequent normothermic perfusion. *J Hepatol* 1992;15(1-2):192-201.
585. Delmas-Beauvieux MC, Gallis JL, Clerc M, Canioni P. <sup>31</sup>P NMR studies of rat liver cold preservation with histidine-buffered lactobionate solution. *Cryobiology* 1993;30(6):551-61.
586. Hiraoka K, Kuroda Y, Tanioka Y, Matsumoto S, Fujino Y, Morita A, et al. Adenosine is a key component in preservation of ischemically damaged canine pancreas by the two-layer cold storage method. *Transplant Proc* 1994;26(2):953-5.
587. Li JM, Fenton RA, Cutler BS, Dobson JG, Jr. Adenosine enhances nitric oxide production by vascular endothelial cells. *Am J Physiol* 1995;269(2 Pt 1):C519-23.
588. Pi F, Badosa F, Sola A, Rosello Catafau J, Xaus C, Prats N, et al. Effects of adenosine on ischaemia-reperfusion injury associated with rat pancreas transplantation. *Br J Surg* 2001;88(10):1366-75.

589. Vaida AM, Tang DG, Allen C, Wise RM, Higgins RS, Cohen NM. Novel protection strategy for pulmonary transplantation. *J Surg Res* 2003;109(1):8-15.
590. Ritzel RA, Hansen JB, Veldhuis JD, Butler PC. Induction of beta-cell rest by a Kir6.2/SUR1-selective K(ATP)-channel opener preserves beta-cell insulin stores and insulin secretion in human islets cultured at high (11 mM) glucose. *J Clin Endocrinol Metab* 2004;89(2):795-805.
591. Nolan MS, Lindsey NJ, Ingram NP, Herold A, Slater DN, Fox M. Hypothermic preservation of the rat pancreas with a view to maintaining endocrine function using either cold storage or pulsatile perfusion. *Transplant Proc* 1984;16:807.
592. Florack G, Sutherland DE, Heil J, Squifflet JP, Najarian JS. Preservation of canine segmental pancreatic autografts: cold storage versus pulsatile machine perfusion. *J Surg Res* 1983;34(5):493-504.
593. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. *Lancet* 1967;2(7515):536-8.
594. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. *Lancet* 1969;2(7632):1219-22.
595. Baumgartner D, Sutherland DE, Heil JE, Zweber B, Awad EA, Najarian JS. Cold storage of segmental canine pancreatic grafts for 24 hours. *J Surg Res* 1980;29(3):248-57.
596. Dreikorn K, Horsch R, Rohl L. 48- to 96-hour preservation of canine kidneys by initial perfusion and hypothermic storage using the Euro-Collins solution. *Eur Urol* 1980;6(4):221-4.
597. van Schilfgaarde R. Pancreas transplantation 1983: where do we stand? *Transplant Proc* 1984;16(5):1287-9.
598. Serrou B, Solassol C, Michel H, Gelis C, Pujol H, Romieu C. Eight- and twenty-four-hour canine pancreas preservations using a simple gel cooling technique. *Transplantation* 1973;16(5):398-402.
599. Toledo-Pereyra LH, Bock G, Schneider A, MacKenzie G. Pancreas preservation with TP-IV: a hyperosmolar colloid solution. *Cryobiology* 1985;22(1):40-6.
600. Sutherland DE, Moudry KC, Elick BA, Goetz FC, Najarian JS. Pancreas transplant protocols at the University of Minnesota: recipient and donor selection, operative and postoperative management, and outcome. *Clin Transpl* 1987:109-26.
601. Barr D, Munn SR, Carpenter HA, Perkins JD. A prospective comparison of two preservation solutions in human pancreaticoduodenal transplantation. *Transplant Proc* 1990;22(2):529-31.

602. Abouna GM, Sutherland DE, Florack G, Najarian JS. Function of transplanted human pancreatic allografts after preservation in cold storage for 6 to 26 hours. *Transplantation* 1987;43(5):630-6.
603. Abouna GM, Sutherland DE, Florack G, Heil J, Najarian JS. Preservation of human pancreatic allografts in cold storage for six to 24 hours. *Transplant Proc* 1987;19(1 Pt 3):2307-9.
604. Largiader F, Baumgartner D, Uhlschmid G. Ischemic tolerance of human pancreatic transplants. *Transplant Proc* 1984;16:1282.
605. Tyden G, Lundgren G, Ost L, Kojima Y, Gunnarsson R, Ostman J, et al. Progress in segmental pancreatic transplantation. *World J Surg* 1986;10(3):404-9.
606. Wahlberg JA, Love R, Landegaard L, Southard JH, Belzer FO. Successful 72 hours' preservation of the canine pancreas. *Transplant Proc* 1987;19(1 Pt 2):1337-8.
607. Pegg DE, Klempnauer J, Diaper MP, Taylor MJ. Assessment of hypothermic preservation of the pancreas in the rat by a normothermic perfusion assay. *J Surg Res* 1982;33(3):194-200.
608. Viste A, Schlumpf R, Munn S, Heil JE, Condie R, Sutherland DE. 96-hour cold storage preservation of the canine pancreas. *Transplant Proc* 1990;22(2):537-8.
609. D'Alessandro AM, Sollinger HW, Hoffmann RM, Pirsch JD, Melzer JS, Kalayoglu M, et al. Experience with Belzer UW cold storage solution in simultaneous pancreas-kidney transplantation. *Transplant Proc* 1990;22(2):532-4.
610. D'Alessandro AM, Stratta RJ, Sollinger HW, Kalayoglu M, Pirsch JD, Belzer FO. Use of UW solution in pancreas transplantation. *Diabetes* 1989;38 Suppl 1:7-9.
611. Belzer FO, Ploeg RJ, Knechtle SJ, D'Alessandro AM, Pirsch JD, Kalayoglu MM, et al. Clinical pancreas preservation and transplantation. *Transplant Proc* 1994;26(2):550-1.
612. Sollinger HW, Vernon WB, D'Alessandro AM, Kalayoglu M, Stratta RJ, Belzer FO. Combined liver and pancreas procurement with Belzer-UW solution. *Surgery* 1989;106(4):685-90; discussion 690-1.
613. Todo S, Nery J, Yanaga K, Podesta L, Gordon RD, Starzl TE. Extended preservation of human liver grafts with UW solution. *Jama* 1989;261(5):711-4.
614. Hartgrink HH, van Bockel JH, Hansen B, Thorogood J, Hermans J, de Meester J, et al. Effect of blood group and HLA matching on pancreas graft survival with the use of UW solution. *Transpl Int* 1995;8(5):366-73.

615. Ploeg RJ. Preliminary results of the European Multicenter Study on UW solution in liver transplantation. The Study Group. *Transplant Proc* 1990;22(5):2185-8.
616. Ploeg RJ, Boudjema K, Marsh D, Bruijn JA, Gooszen HG, Southard JH, et al. The importance of a colloid in canine pancreas preservation. *Transplantation* 1992;53(4):735-41.
617. Urushihara T, Sumimoto R, Sumimoto K, Jamieson NV, Ito H, Ikeda M, et al. A comparison of some simplified lactobionate preservation solutions with standard UW solution and Eurocollins solution for pancreas preservation. *Transplantation* 1992;53(4):750-4.
618. Moen J, Claesson K, Pienaar H, Lindell S, Ploeg RJ, McAnulty JF, et al. Preservation of dog liver, kidney, and pancreas using the Belzer-UW solution with a high-sodium and low-potassium content. *Transplantation* 1989;47(6):940-5.
619. Ploeg RJ, Goossens D, Sollinger HW, Southard JH, Belzer FO. The Belzer-UW solution for effective long-term preservation in canine pancreas transplantation. *Transplant Proc* 1989;21(1 Pt 2):1378-80.
620. Tojimbara T, Wicomb WN, Garcia-Kennedy R, Burns W, Hayashi M, Collins G, et al. Liver transplantation from non-heart beating donors in rats: influence of viscosity and temperature of initial flushing solutions on graft function. *Liver Transpl Surg* 1997;3(1):39-45.
621. Tullius SG, Filatenkow A, Horch D, Mehlitz T, Reutzel-Selke A, Pratschke J, et al. Accumulation of crystal deposits in abdominal organs following perfusion with defrosted University of Wisconsin solutions. *Am J Transplant* 2002;2(7):627-30.
622. Hesse UJ, Troisi R, Jacobs B, Berrevoet F, De Laere S, Maene L, et al. Cold preservation of the porcine pancreas with histidine-tryptophan-ketoglutarate solution. *Transplantation* 1998;66(9):1137-41.
623. Leonhardt U, Tytko A, Exner B, Barthel M, Stockmann F, Kohler H, et al. The effect of different solutions for organ preservation on immediate postischemic pancreatic function in vitro. *Transplantation* 1993;55(1):11-4.
624. Troisi R, Meester D, Van Den Broecke C, Cuvelier CA, Fiers T, de Hemptinne B, et al. Functional and structural integrity of porcine pancreatic grafts subjected to a period of warm ischemia and cold preservation with histidine-tryptophan-ketoglutarate (custodiol) or University of Wisconsin solution. *Transplantation* 2003;75(11):1793-9.
625. Fridell JA, Agarwal A, Milgrom ML, Goggins WC, Murdock P, Pescovitz MD. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin

- solution for organ preservation in clinical pancreas transplantation. *Transplantation* 2004;77(8):1304-6.
626. Roberts RF, Nishanian GP, Carey JN, Sakamaki Y, Starnes VA, Barr ML. A comparison of the new preservation solution Celsior to Euro-Collins and University of Wisconsin solutions in lung reperfusion injury. *Transplantation* 1999;67(1):152-5.
627. Carrier M, Trudel S, Pelletier LC. Effect of Celsior and University of Wisconsin solutions on myocardial metabolism and function after warm ischemia. *J Cardiovasc Surg (Torino)* 1999;40(6):811-6.
628. Vega JD, Ochsner JL, Jeevanandam V, McGiffin DC, McCurry KR, Mentzer RM, Jr., et al. A multicenter, randomized, controlled trial of Celsior for flush and hypothermic storage of cardiac allografts. *Ann Thorac Surg* 2001;71(5):1442-7.
629. Boggi U, Vistoli F, Del Chiaro M, Signori S, Croce C, Pietrabissa A, et al. Pancreas preservation with University of Wisconsin and Celsior solutions: a single-center, prospective, randomized pilot study. *Transplantation* 2004;77(8):1186-90.
630. Sawada T, Matsumoto I, Nakano M, Kirchoff N, Sutherland DE, Hering BJ. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. *Transplantation* 2003;75(12):1965-9.
631. Ohzato H, Gotoh M, Monden M, Kanai T, Yamamoto H, Kawai M, et al. Intraductal injection of collagenase solution at the time of harvesting: a possible solution for preservation and collagenase digestion. *Transplant Proc* 1990;22(2):782-3.
632. London NJ, Lake SP, Wilson J, Bassett D, Toomey P, Bell PR, et al. A simple method for the release of islets by controlled collagenase digestion of the human pancreas. *Transplantation* 1990;49(6):1109-13.
633. Traverso LW, Abu-Zamzam AM. Activation of pancreatic proteolytic enzymes by commercial collagenases. *Transplantation* 1978;25(4):226-7.
634. Robertson GS, Chadwick D, Thirdborough S, Swift S, Davies J, James R, et al. Human islet isolation--a prospective randomized comparison of pancreatic vascular perfusion with hyperosmolar citrate or University of Wisconsin solution. *Transplantation* 1993;56(3):550-3.
635. Kneteman NM, DeGroot TJ, Warnock GL, Rajotte RV. The evaluation of solutions for pancreas preservation prior to islet isolation. *Horm Metab Res Suppl* 1990;25:4-9.

636. Kneteman NM, Warnock GL, Evans MG, Dawidson I, Rajotte RV. Islet isolation from human pancreas stored in UW solution for 6 to 26 hours. *Transplant Proc* 1990;22(2):763-4.
637. Kneteman NM, Wagner T. Critical components of storage fluid for pancreas preservation before islet isolation. *Transplant Proc* 1992;24(6):2824.
638. Belzer FO, Kalayoglu M, D'Alessandro AM, Pirsch JD, Sollinger HW, Hoffmann R, et al. Organ preservation: experience with University of Wisconsin solution and plans for the future. *Clin Transplant* 1990;4(2):73-7.
639. Westbroek DL, De Gruyl J, Dijkhuis CM, McDicken I, Drop A, Scholte A, et al. Twenty-four-hour hypothermic preservation perfusion and storage of the duct-ligated canine pancreas with transplantation. *Transplant Proc* 1974;6(3):319-22.
640. de Gruyl J, Westbroek DL, Macdicken I, Ridderhof E, Verschoor L, van Strik R. Cryoprecipitated plasma perfusion preservation and cold storage preservation of duct-ligated pancreatic allografts. *Br J Surg* 1977;64(7):490-3.
641. Tersigni R, Toledo-Pereyra LH, Pinkham J, Najarian JS. Pancreaticoduodenal preservation by hypothermic pulsatile perfusion for twenty-four hours. *Ann Surg* 1975;182(6):743-8.
642. Tersigni R, Toledo-Pereyra LH, Najarian JS. Effects of methylprednisolone, glucagon, and allopurinol in the protection of pancreaticoduodenal allografts perfused for twenty-four hours. *Surgery* 1975;78(5):599-607.
643. Brynger H. Twenty-four-hour preservation of the duct-ligated canine pancreatic allograft. *Eur Surg Res* 1975;7(6):341-54.
644. Leeser DB, Bingaman AW, Poliakova L, Shi Q, Gage F, Bartlett ST, et al. Pulsatile pump perfusion of pancreata before human islet cell isolation. *Transplant Proc* 2004;36(4):1050-1.
645. Fischer JH, Czerniak A, Hauer U, Isselhard W. A new simple method for optimal storage of ischemically damaged kidneys. *Transplantation* 1978;25(2):43-9.
646. Kuroda Y, Kawamura T, Suzuki Y, Fujiwara H, Yamamoto K, Saitoh Y. A new, simple method for cold storage of the pancreas using perfluorochemical. *Transplantation* 1988;46(3):457-60.
647. Fujino Y, Kuroda Y, Suzuki Y, Fujiwara H, Kawamura T, Morita A, et al. Preservation of canine pancreas for 96 hours by a modified two-layer (UW solution/perfluorochemical) cold storage method. *Transplantation* 1991;51(5):1133-5.

648. Kawamura T, Kuroda Y, Suzuki Y, Fujiwara H, Fujino Y, Yamamoto K, et al. Seventy-two-hour preservation of the canine pancreas by the two-layer (Euro-Collins' solution/perfluorochemical) cold storage method. *Transplantation* 1989;47(5):776-8.
649. Kuroda Y, Kawamura T, Fujino Y, Suzuki Y, Fujiwara H, Saitoh Y. Successful 72-hour preservation of the canine pancreas. *Transplant Proc* 1990;22(2):539-40.
650. Matsumoto S, Kuroda Y, Saitoh Y. The effect of preservation temperature and period on resuscitation of the ischemically damaged canine pancreas during preservation by the two-layer (University of Wisconsin solution/perfluorochemical) method. *Kobe J Med Sci* 1996;42(1):1-17.
651. Matsumoto S, Kandaswamy R, Sutherland DE, Hassoun AA, Hiraoka K, Sageshima J, et al. Clinical application of the two-layer (University of Wisconsin solution/perfluorochemical plus O<sub>2</sub>) method of pancreas preservation before transplantation. *Transplantation* 2000;70(5):771-4.
652. Tanioka Y, Sutherland DE, Kuroda Y, Gilmore TR, Asaheim TC, Kronson JW, et al. Excellence of the two-layer method (University of Wisconsin solution/perfluorochemical) in pancreas preservation before islet isolation. *Surgery* 1997;122(2):435-41; discussion 441-2.
653. Matsumoto S, Lawrence O, Rigley TH, Lakey JR, Stevens RB, Strong DM. University of wisconsin solution with trypsin inhibitor pefabloc improves survival of viable human and primate impure islets during storage. *Cell Tissue Bank* 2001;2(1):15-21.
654. Matsumoto S, Iwanaga Y, Okitsu T, Noguchi H, Yonekawa Y, Tanaka K, et al. Analysis of large-scale nonhuman primate islet isolations. *Transplant Proc* 2005;37(2):1317-21.
655. Hiraoka K, Trexler A, Eckman E, Stage A, Nevile S, Sageshima J, et al. Successful pancreas preservation before islet isolation by the simplified two-layer cold storage method. *Transplant Proc* 2001;33(1-2):952-3.
656. Tsujimura T, Kuroda Y, Kin T, Avila JG, Rajotte RV, Korbitt GS, et al. Human islet transplantation from pancreases with prolonged cold ischemia using additional preservation by the two-layer (UW solution/perfluorochemical) cold-storage method. *Transplantation* 2002;74(12):1687-91.
657. Matsumoto S, Qualley SA, Goel S, Hagman DK, Sweet IR, Poitout V, et al. Effect of the two-layer (University of Wisconsin solution- perfluorochemical plus O<sub>2</sub>)

method of pancreas preservation on human islet isolation, as assessed by the Edmonton Isolation Protocol. *Transplantation* 2002;74(10):1414-9.

658. Kakinoki K, Fujino Y, Suzuki Y, Li S, Tanioka Y, Sakai T, et al. Superiority of the two-layer method before islet isolation confirmed by in vivo viability assessment. *Transplantation* 2005;79(11):1516-21.

659. Tsujimura T, Kuroda Y, Avila JG, Kin T, Churchill TA, Shapiro AM, et al. Resuscitation of the ischemically damaged human pancreas by the two-layer method prior to islet isolation. *Transplant Proc* 2003;35(7):2461-2.

660. Tsujimura T, Kuroda Y, Churchill TA, Avila JG, Kin T, Shapiro AM, et al. Short-term storage of the ischemically damaged human pancreas by the two-layer method prior to islet isolation. *Cell Transplant* 2004;13(1):67-73.

661. Ricordi C, Fraker C, Szust J, Al-Abdullah I, Poggioli R, Kirlew T, et al. Improved human islet isolation outcome from marginal donors following addition of oxygenated perfluorocarbon to the cold-storage solution. *Transplantation* 2003;75(9):1524-7.

662. Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AM, et al. Influence of pancreas preservation on human islet isolation outcomes: impact of the two-layer method. *Transplantation* 2004;78(1):96-100.

663. Witkowski P, Liu Z, Guo Q, Poumian-Ruiz E, Cernea S, Herold K, et al. Two-layer method in short-term pancreas preservation for successful islet isolation. *Transplant Proc* 2005;37(8):3398-401.

664. Matsumoto S, Zhang G, Qualley S, Clever J, Tombrello Y, Strong DM, et al. The effect of two-layer (University of Wisconsin solution/perfluorochemical) preservation method on clinical grade pancreata prior to islet isolation and transplantation. *Transplant Proc* 2004;36(4):1037-9.

665. Noguchi H, Nakai Y, Matsumoto S, Kawaguchi M, Ueda M, Okitsu T, et al. Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. *Am J Transplant* 2005;5(8):1848-55.

666. Zhang G, Matsumoto S, Newman H, Strong DM, Robertson RP, Reems JA. Improve islet yields and quality when clinical grade pancreata are preserved by the two-layer method. *Cell Tissue Bank* 2006;7(3):195-201.

667. Kin T, Mirbolooki M, Salehi P, Tsukada M, O'Gorman D, Imes S, et al. Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. *Transplantation* 2006;82(10):1286-90.

668. Caballero-Corbalan J, Eich T, Lundgren T, Foss A, Felldin M, Kallen R, et al. No beneficial effect of two-layer storage compared with UW-storage on human islet isolation and transplantation. *Transplantation* 2007;84(7):864-9.
669. Papas KK, Hering BJ, Guenther L, Rappel MJ, Colton CK, Avgoustiniatos ES. Pancreas oxygenation is limited during preservation with the two-layer method. *Transplant Proc* 2005;37(8):3501-4.
670. Kuroda Y, Tanioka Y, Morita A, Hiraoka K, Matsumoto S, Fujino Y, et al. Protective effect of preservation of canine pancreas by the two-layer (University of Wisconsin solution/perfluorochemical) method against rewarming ischemic injury during implantation. *Transplantation* 1994;57(5):658-61.
671. Kuroda Y, Tanioka Y, Matsumoto S, Hiraoka K, Morita A, Fujino Y, et al. Difference in energy metabolism between fresh and warm ischemic canine pancreases during preservation by the two-layer method. *Transpl Int* 1994;7 Suppl 1:S441-5.
672. Kuroda Y, Hiraoka K, Tanioka Y, Matsumoto S, Morita A, Fujino Y, et al. Role of adenosine in preservation by the two-layer method of ischemically damaged canine pancreas. *Transplantation* 1994;57(7):1017-20.
673. Matsumoto S, Kuroda Y, Fujita H, Tanioka Y, Sakai T, Hamano M, et al. Extending the margin of safety of preservation period for resuscitation of ischemically damaged pancreas during preservation using the two-layer (University of Wisconsin solution/perfluorochemical) method at 20 degrees C with thromboxane A2 synthesis inhibitor OKY046. *Transplantation* 1996;62(7):879-83.
674. Iwanaga Y, Suzuki Y, Okada Y, Mori H, Matsumoto I, Mitsutsuji M, et al. Ultrastructural analyses of pancreatic grafts preserved by the two-layer cold-storage method and by simple cold storage in University of Wisconsin solution. *Transpl Int* 2002;15(8):425-30.
675. Miyamoto M, Morimoto Y, Balamurugan AN, Kawakami Y, Gu Y, Satake A, et al. Improvement of modified two-layer preservation method (PFC/Kyoto solution) in islet isolation from breeder pigs. *Transplant Proc* 2000;32(7):1660-1.
676. Brandhorst D, Iken M, Bretzel RG, Brandhorst H. Pancreas storage in oxygenated perfluorodecalin does not restore post-transplant function of isolated pig islets pre-damaged by warm ischemia. *Xenotransplantation* 2006;13(5):465-70.
677. Brandhorst D, Iken M, Brendel MD, Bretzel RG, Brandhorst H. Successful pancreas preservation by a perfluorocarbon-based one-layer method for subsequent pig islet isolation. *Transplantation* 2005;79(4):433-7.

678. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251-306.
679. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998;392(6671):86-9.
680. Rovere P, Vallinoto C, Bondanza A, Crosti MC, Rescigno M, Ricciardi-Castagnoli P, et al. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J Immunol* 1998;161(9):4467-71.
681. Earnshaw WC. Nuclear changes in apoptosis. *Curr Opin Cell Biol* 1995;7(3):337-43.
682. Ashkenas J, Werb Z. Proteolysis and the biochemistry of life-or-death decisions. *J Exp Med* 1996;183(5):1947-51.
683. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev* 1993;14(2):133-51.
684. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980;284(5756):555-6.
685. Cohen JJ, Duke RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* 1984;132(1):38-42.
686. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, et al. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *Embo J* 1993;12(9):3679-84.
687. Kokileva L. Multi-step chromatin degradation in apoptosis. DNA breakdown in apoptosis. *Int Arch Allergy Immunol* 1994;105(4):339-43.
688. Cain K, Inayat-Hussain SH, Wolfe JT, Cohen GM. DNA fragmentation into 200-250 and/or 30-50 kilobase pair fragments in rat liver nuclei is stimulated by Mg<sup>2+</sup> alone and Ca<sup>2+</sup>/Mg<sup>2+</sup> but not by Ca<sup>2+</sup> alone. *FEBS Lett* 1994;349(3):385-91.
689. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, et al. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992;69(1):119-28.
690. Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, et al. Mitochondrial control of nuclear apoptosis. *J Exp Med* 1996;183(4):1533-44.
691. Savill J. Apoptosis in resolution of inflammation. *J Leukoc Biol* 1997;61(4):375-80.
692. Dong Z, Saikumar P, Weinberg JM, Venkatachalam MA. Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death. Involvement of serine but not cysteine proteases. *Am J Pathol* 1997;151(5):1205-13.

693. Fujimoto K, Hosotani R, Wada M, Lee J, Koshiba T, Miyamoto Y, et al. Ischemia-reperfusion injury on the pancreas in rats: identification of acinar cell apoptosis. *J Surg Res* 1997;71(2):127-36.
694. Liu X, Drognitz O, Neeff H, Benz S, Hopt UT. Apoptosis is caused by prolonged organ preservation and blocked by apoptosis inhibitor in experimental rat pancreatic grafts. *Transplant Proc* 2004;36(4):1209-10.
695. Drognitz O, Liu X, Benz S, Obermaier R, Herb T, Schareck W, et al. Ischemia/reperfusion injury induces acinar cell apoptosis in experimental pancreas transplantation. *Transplant Proc* 2002;34(6):2361.
696. Drognitz O, Obermaier R, Liu X, Neeff H, von Dobschuetz E, Hopt UT, et al. Effects of organ preservation, ischemia time and caspase inhibition on apoptosis and microcirculation in rat pancreas transplantation. *Am J Transplant* 2004;4(7):1042-50.
697. Drognitz O, Von Dobschuetz E, Kissler H, Liu X, Obermaier R, Neeff H, et al. Organ procurement in experimental pancreas transplantation with minimal microcirculatory impairment. *Eur Surg Res* 2004;36(4):185-91.
698. Drognitz O, Liu X, Obermaier R, Neeff H, von Dobschuetz E, Hopt UT, et al. Ischemic preconditioning fails to improve microcirculation but increases apoptotic cell death in experimental pancreas transplantation. *Transpl Int* 2004;17(6):317-24.
699. Rosenberg L, Wang R, Paraskevas S, Maysinger D. Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 1999;126(2):393-8.
700. Shirouzu Y, Gu Y, Koga M, Sakurai T, Qi M, Hiura A, et al. Cold preservation of islets in UW solution--with special reference to apoptosis. *J Surg Res* 2006;133(2):167-75.
701. Paraskevas S, Maysinger D, Wang R, Duguid TP, Rosenberg L. Cell loss in isolated human islets occurs by apoptosis. *Pancreas* 2000;20(3):270-6.
702. Berney T, Molano RD, Cattani P, Pileggi A, Vizzardelli C, Oliver R, et al. Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. *Transplantation* 2001;71(1):125-32.
703. Stadlbauer V, Schaffellner S, Iberer F, Lackner C, Liegl B, Zink B, et al. Occurrence of apoptosis during ischemia in porcine pancreas islet cells. *Int J Artif Organs* 2003;26(3):205-10.
704. Ramachandran S, Desai NM, Goers TA, Benshoff N, Olack B, Shenoy S, et al. Improved islet yields from pancreas preserved in perfluorocarbon is via inhibition of apoptosis mediated by mitochondrial pathway. *Am J Transplant* 2006;6(7):1696-703.

705. Cattan P, Berney T, Schena S, Molano RD, Pileggi A, Vizzardelli C, et al. Early assessment of apoptosis in isolated islets of Langerhans. *Transplantation* 2001;71(7):857-62.
706. Avila J, Barbaro B, Gangemi A, Romagnoli T, Kuechle J, Hansen M, et al. Intra-ductal glutamine administration reduces oxidative injury during human pancreatic islet isolation. *Am J Transplant* 2005;5(12):2830-7.
707. Yang Z, Chen M, Ellett JD, Fialkow LB, Carter JD, Nadler JL. The novel anti-inflammatory agent lisofylline prevents autoimmune diabetic recurrence after islet transplantation. *Transplantation* 2004;77(1):55-60.
708. Yang Z, Chen M, Ellett JD, Carter JD, Brayman KL, Nadler JL. Inflammatory blockade improves human pancreatic islet function and viability. *Am J Transplant* 2005;5(3):475-83.
709. Nakano M, Matsumoto I, Sawada T, Ansite J, Oberbroeckling J, Zhang HJ, et al. Caspase-3 inhibitor prevents apoptosis of human islets immediately after isolation and improves islet graft function. *Pancreas* 2004;29(2):104-9.
710. Montolio M, Tellez N, Biarnes M, Soler J, Montanya E. Short-term culture with the caspase inhibitor z-VAD.fmk reduces beta cell apoptosis in transplanted islets and improves the metabolic outcome of the graft. *Cell Transplant* 2005;14(1):59-65.
711. Thomas FT, Hutchings A, Contreras J, Wu J, Jiang XL, Eckhoff D, et al. Islet transplantation in the twenty-first century. *Immunol Res* 2002;26(1-3):289-96.
712. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell Transplant* 2005;14(1):67-76.
713. MacGregor RR, Williams SJ, Tong PY, Kover K, Moore WV, Stehno-Bittel L. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. *Am J Physiol Endocrinol Metab* 2006;290(5):E771-9.
714. Lehmann R, Zuellig RA, Kugelmeier P, Baenninger PB, Moritz W, Perren A, et al. Superiority of small islets in human islet transplantation. *Diabetes* 2007;56(3):594-603.
715. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. *Diabetes* 2002;51(1):66-72.

716. Montolio M, Biarnes M, Tellez N, Escoriza J, Soler J, Montanya E. Interleukin-1beta and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation. *J Endocrinol* 2007;192(1):169-77.
717. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes* 2006;55(7):1907-14.
718. Amoli MM, Larijani B. Would blockage of cytokines improve the outcome of pancreatic islet transplantation? *Med Hypotheses* 2006;66(4):816-9.
719. MacFarlane M, Williams AC. Apoptosis and disease: a life or death decision. *EMBO Rep* 2004;5(7):674-8.
720. Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, et al. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc Natl Acad Sci U S A* 1992;89(12):5408-12.
721. Krammer PH. The CD95(APO-1/Fas)/CD95L system. *Toxicol Lett* 1998;102-103:131-7.
722. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. *Eur J Biochem* 1998;254(3):439-59.
723. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994;76(6):959-62.
724. Nagata S, Golstein P. The Fas death factor. *Science* 1995;267(5203):1449-56.
725. Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, et al. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. *Science* 1996;274(5289):990-2.
726. Itoh N, Nagata S. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem* 1993;268(15):10932-7.
727. Krammer PH. CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol* 1999;71:163-210.
728. Baker SJ, Reddy EP. Modulation of life and death by the TNF receptor superfamily. *Oncogene* 1998;17(25):3261-70.
729. Golstein P. Cell death: TRAIL and its receptors. *Curr Biol* 1997;7(12):R750-3.
730. Loweth AC, Williams GT, James RF, Scarpello JH, Morgan NG. Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation. *Diabetes* 1998;47(5):727-32.

731. Luo KX, Zhu YF, Zhang LX, He HT, Wang XS, Zhang L. In situ investigation of Fas/FasL expression in chronic hepatitis B infection and related liver diseases. *J Viral Hepat* 1997;4(5):303-7.
732. Desbarats J, Duke RC, Newell MK. Newly discovered role for Fas ligand in the cell-cycle arrest of CD4+ T cells. *Nat Med* 1998;4(12):1377-82.
733. Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 1989;245(4915):301-5.
734. Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J Exp Med* 1989;169(5):1747-56.
735. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991;66(2):233-43.
736. Ferguson TA, Griffith TS. A vision of cell death: insights into immune privilege. *Immunol Rev* 1997;156:167-84.
737. Signore A, Annovazzi A, Gradini R, Liddi R, Ruberti G. Fas and Fas ligand-mediated apoptosis and its role in autoimmune diabetes. *Diabetes Metab Rev* 1998;14(3):197-206.
738. Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, et al. Significant role for Fas in the pathogenesis of autoimmune diabetes. *J Immunol* 2000;164(5):2523-32.
739. Mandrup-Poulsen T. beta-cell apoptosis: stimuli and signaling. *Diabetes* 2001;50 Suppl 1:S58-63.
740. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993;75(6):1169-78.
741. Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces apoptosis. *J Exp Med* 1994;179(3):873-9.
742. Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, et al. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. *Lab Invest* 1993;69(4):415-29.

743. Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270(5239):1189-92.
744. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. *Nature* 1995;377(6550):630-2.
745. Griffith TS, Yu X, Herndon JM, Green DR, Ferguson TA. CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. *Immunity* 1996;5(1):7-16.
746. Inoue M, Maeno T, Miki D, Embabi S, Hatchell DL. Survival of subretinal pancreatic islet cell allografts and apoptosis in infiltrating lymphocytes in rats. *Ophthalmic Res* 2004;36(1):31-7.
747. Lau HT, Yu M, Fontana A, Stoeckert CJ, Jr. Prevention of islet allograft rejection with engineered myoblasts expressing FasL in mice. *Science* 1996;273(5271):109-12.
748. Wickelgren I. Muscling transplants into mice. *Science* 1996;273(5271):33.
749. Kang SM, Hoffmann A, Le D, Springer ML, Stock PG, Blau HM. Immune response and myoblasts that express Fas ligand. *Science* 1997;278(5341):1322-4.
750. Zhan W, Cai S, Wang J, He Y, Zheng Z, Peng J. The effect of FasL expression on pancreatic islet allografts. *Chin Med J (Engl)* 2002;115(7):1006-9.
751. Lan P, Zhan W, Wang J, Yan L, Xiao L, Wu X. Immune privilege induced by cotransplantation of islet and allogeneic testicular cells. *Chin Med J (Engl)* 2001;114(10):1026-9.
752. Cai SR, He YL, Huang MJ, Peng JS, Wang JP, Zhan WH. The effect of FasL gene transfer to islet cells on pancreatic islet allografts. *Hepatobiliary Pancreat Dis Int* 2003;2(2):308-12.
753. Klein D, Ricordi C, Pugliese A, Pastori RL. Inhibition of Fas-mediated apoptosis in mouse insulinoma betaTC-3 cells via an anti-Fas ribozyme. *Hum Gene Ther* 2000;11(7):1033-45.
754. Wu Y, Han B, Luo H, Roduit R, Salcedo TW, Moore PA, et al. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. *Diabetes* 2003;52(9):2279-86.
755. Machen J, Bertera S, Chang Y, Bottino R, Balamurugan AN, Robbins PD, et al. Prolongation of islet allograft survival following ex vivo transduction with adenovirus encoding a soluble type 1 TNF receptor-Ig fusion decoy. *Gene Ther* 2004;11(20):1506-14.

756. Xenos ES, Stevens RB, Sutherland DE, Lokeh A, Ansite JD, Casanova D, et al. The role of nitric oxide in IL-1 beta-mediated dysfunction of rodent islets of Langerhans. Implications for the function of intrahepatic islet grafts. *Transplantation* 1994;57(8):1208-12.
757. Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM, Jr., et al. Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* 1998;273(24):15148-56.
758. Mandrup-Poulsen T. The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 1996;39(9):1005-29.
759. Yamamoto H, Sugitani A, Kitada H, Arima T, Nishiyama K, Motoyama K, et al. Effect of FR167653 on pancreatic ischemia-reperfusion injury in dogs. *Surgery* 2001;129(3):309-17.
760. Cailleau C, Diu-Hercend A, Ruuth E, Westwood R, Carnaud C. Treatment with neutralizing antibodies specific for IL-1beta prevents cyclophosphamide-induced diabetes in nonobese diabetic mice. *Diabetes* 1997;46(6):937-40.
761. Giannoukakis N, Rudert WA, Ghivizzani SC, Gambotto A, Ricordi C, Trucco M, et al. Adenoviral gene transfer of the interleukin-1 receptor antagonist protein to human islets prevents IL-1beta-induced beta-cell impairment and activation of islet cell apoptosis in vitro. *Diabetes* 1999;48(9):1730-6.
762. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994;265(5174):956-9.
763. Leung K, Betts JC, Xu L, Nabel GJ. The cytoplasmic domain of the interleukin-1 receptor is required for nuclear factor-kappa B signal transduction. *J Biol Chem* 1994;269(3):1579-82.
764. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 1988;242(4878):540-6.
765. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *J Leukoc Biol* 2005.
766. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *Embo J* 1995;14(12):2876-83.

767. Kwon H, Pelletier N, DeLuca C, Genin P, Cisternas S, Lin R, et al. Inducible expression of IkappaBalpha repressor mutants interferes with NF-kappaB activity and HIV-1 replication in Jurkat T cells. *J Biol Chem* 1998;273(13):7431-40.
768. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994;269(7):4705-8.
769. Hammar EB, Irminger JC, Rickenbach K, Parnaud G, Ribaux P, Bosco D, et al. Activation of NF-kappaB by extracellular matrix is involved in spreading and glucose-stimulated insulin secretion of pancreatic beta cells. *J Biol Chem* 2005;280(34):30630-7.
770. Eizirik DL, Mandrup-Poulsen T. A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 2001;44(12):2115-33.
771. Cardozo AK, Kruhoffer M, Leeman R, Orntoft T, Eizirik DL. Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. *Diabetes* 2001;50(5):909-20.
772. Sekine N, Ishikawa T, Okazaki T, Hayashi M, Wollheim CB, Fujita T. Synergistic activation of NF-kappab and inducible isoform of nitric oxide synthase induction by interferon-gamma and tumor necrosis factor-alpha in INS-1 cells. *J Cell Physiol* 2000;184(1):46-57.
773. Rehman KK, Bertera S, Bottino R, Balamurugan AN, Mai JC, Mi Z, et al. Protection of islets by in situ peptide-mediated transduction of the Ikappa B kinase inhibitor Nemo-binding domain peptide. *J Biol Chem* 2003;278(11):9862-8.
774. Giannoukakis N, Rudert WA, Trucco M, Robbins PD. Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. *J Biol Chem* 2000;275(47):36509-13.
775. Grey ST, Longo C, Shukri T, Patel VI, Csizmadia E, Daniel S, et al. Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. *J Immunol* 2003;170(12):6250-6.
776. Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, Sawada T, et al. Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. *Diabetes* 2004;53(11):2815-23.
777. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. *Faseb J* 2002;16(7):745-7.

778. Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, et al. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 1994;369(6476):156-60.
779. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 1994;265(5173):808-11.
780. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994;76(6):1025-37.
781. Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J Biol Chem* 1997;272(33):20490-4.
782. Hambleton J, Weinstein SL, Lem L, DeFranco AL. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc Natl Acad Sci U S A* 1996;93(7):2774-8.
783. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270(5240):1326-31.
784. Heidenreich KA, Kummer JL. Inhibition of p38 mitogen-activated protein kinase by insulin in cultured fetal neurons. *J Biol Chem* 1996;271(17):9891-4.
785. Ruoslahti E, Reed JC. Anchorage dependence, integrins, and apoptosis. *Cell* 1994;77(4):477-8.
786. Meredith JE, Jr., Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 1993;4(9):953-61.
787. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 1994;124(4):619-26.
788. Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol* 1999;163(2):181-90.
789. Ricordi C, Alejandro R, Rilo HH, Carroll PB, Tzakis AG, Starzl TE, et al. Long-term in vivo function of human mantled islets obtained by incomplete pancreatic dissociation and purification. *Transplant Proc* 1995;27(6):3382.
790. Farney AC, Najarian JS, Nakhleh RE, Lloveras G, Field MJ, Gores PF, et al. Autotransplantation of dispersed pancreatic islet tissue combined with total or near-total pancreatectomy for treatment of chronic pancreatitis. *Surgery* 1991;110(2):427-37; discussion 437-9.

791. Kneteman NM, Warnock GL, Evans MG, Nason RW, Rajotte RV. Prolonged function of canine pancreatic fragments autotransplanted to the spleen by venous reflux. *Transplantation* 1990;49(4):679-81.
792. Lanza RP, Sullivan SJ, Chick WL. Perspectives in diabetes. Islet transplantation with immunoisolation. *Diabetes* 1992;41(12):1503-10.
793. Soon-Shiong P, Feldman E, Nelson R, Komtebedde J, Smidsrod O, Skjak-Braek G, et al. Successful reversal of spontaneous diabetes in dogs by intraperitoneal microencapsulated islets. *Transplantation* 1992;54(5):769-74.
794. van Deijnen JH, Hulstaert CE, Wolters GH, van Schilfgaarde R. Significance of the peri-insular extracellular matrix for islet isolation from the pancreas of rat, dog, pig, and man. *Cell Tissue Res* 1992;267(1):139-46.
795. Bates RC, Lincz LF, Burns GF. Involvement of integrins in cell survival. *Cancer Metastasis Rev* 1995;14(3):191-203.
796. Schwartz SM, Bennett MR. Death by any other name. *Am J Pathol* 1995;147(2):229-34.
797. Raff MC. Social controls on cell survival and cell death. *Nature* 1992;356(6368):397-400.
798. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281(5381):1322-6.
799. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells* 1998;3(11):697-707.
800. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002;2(9):647-56.
801. Strasser A, Puthalakath H, Bouillet P, Huang DC, O'Connor L, O'Reilly LA, et al. The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. *Ann N Y Acad Sci* 2000;917:541-8.
802. Frisch SM, Vuori K, Kelaita D, Sicks S. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. *J Cell Biol* 1996;135(5):1377-82.
803. Stromblad S, Becker JC, Yebra M, Brooks PC, Cheresh DA. Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alphaVbeta3 during angiogenesis. *J Clin Invest* 1996;98(2):426-33.
804. Zhang Z, Vuori K, Reed JC, Ruoslahti E. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A* 1995;92(13):6161-5.

805. Tiberio R, Marconi A, Fila C, Fumelli C, Pignatti M, Krajewski S, et al. Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. *FEBS Lett* 2002;524(1-3):139-44.
806. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, et al. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001;293(5536):1829-32.
807. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999;3(3):287-96.
808. Huang DC, Adams JM, Cory S. The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *Embo J* 1998;17(4):1029-39.
809. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993;74(4):609-19.
810. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995;80(2):285-91.
811. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, et al. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 1997;275(5302):983-6.
812. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci U S A* 1995;92(17):7834-8.
813. Han J, Sabbatini P, Perez D, Rao L, Modha D, White E. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev* 1996;10(4):461-77.
814. Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes. *Cell* 1994;79(2):189-92.
815. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348(6299):334-6.
816. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988;335(6189):440-2.

817. Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med* 1997;3(6):614-20.
818. Simonian PL, Grillot DA, Andrews DW, Leber B, Nunez G. Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death. *J Biol Chem* 1996;271(50):32073-7.
819. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997;139(5):1281-92.
820. Strasser A, Harris AW, Cory S. bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 1991;67(5):889-99.
821. Kane DJ, Ord T, Anton R, Bredesen DE. Expression of bcl-2 inhibits necrotic neural cell death. *J Neurosci Res* 1995;40(2):269-75.
822. Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, et al. Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* 1996;13(1):21-9.
823. Shimizu S, Eguchi Y, Kamiike W, Matsuda H, Tsujimoto Y. Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene* 1996;12(11):2251-7.
824. Tsujimoto Y. Stress-resistance conferred by high level of bcl-2 alpha protein in human B lymphoblastoid cell. *Oncogene* 1989;4(11):1331-6.
825. Nunez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J Immunol* 1990;144(9):3602-10.
826. Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* 1993;361(6410):365-9.
827. Chen-Levy Z, Nourse J, Cleary ML. The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. *Mol Cell Biol* 1989;9(2):701-10.
828. Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, et al. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proc Natl Acad Sci U S A* 1998;95(4):1455-9.
829. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86(1):147-57.

830. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, et al. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 1996;184(4):1331-41.
831. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;275(5303):1132-6.
832. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281(5381):1309-12.
833. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* 1998;95(9):4997-5002.
834. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997;275(5303):1129-32.
835. Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. Channel formation by antiapoptotic protein Bcl-2. *Proc Natl Acad Sci U S A* 1997;94(10):5113-8.
836. Embury J, Klein D, Pileggi A, Ribeiro M, Jayaraman S, Molano RD, et al. Proteins linked to a protein transduction domain efficiently transduce pancreatic islets. *Diabetes* 2001;50(8):1706-13.
837. Liu Y, Rabinovitch A, Suarez-Pinzon W, Mukherjee B, Brownlee M, Edelstein D, et al. Expression of the bcl-2 gene from a defective HSV-1 amplicon vector protects pancreatic beta-cells from apoptosis. *Hum Gene Ther* 1996;7(14):1719-26.
838. Judge TA, Desai NM, Yang Z, Rostami S, Alonso L, Zhang H, et al. Utility of adenoviral-mediated Fas ligand gene transfer to modulate islet allograft survival. *Transplantation* 1998;66(4):426-34.
839. Weber M, Deng S, Kucher T, Shaked A, Ketchum RJ, Brayman KL. Adenoviral transfection of isolated pancreatic islets: a study of programmed cell death (apoptosis) and islet function. *J Surg Res* 1997;69(1):23-32.
840. Giannoukakis N, Mi Z, Gambotto A, Eramo A, Ricordi C, Trucco M, et al. Infection of intact human islets by a lentiviral vector. *Gene Ther* 1999;6(9):1545-51.
841. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. *PG -* 1160-9. *Gene Ther* 1999;6(6):1160-9.

842. Rabinovitch A, Suarez-Pinzon W, Strynadka K, Ju Q, Edelstein D, Brownlee M, et al. Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. *Diabetes* 1999;48(6):1223-9.
843. Rytomaa M, Martins LM, Downward J. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol* 1999;9(18):1043-6.
844. Rosen K, Rak J, Leung T, Dean NM, Kerbel RS, Filmus J. Activated Ras prevents downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. *J Cell Biol* 2000;149(2):447-56.
845. Coll ML, Rosen K, Ladeda V, Filmus J. Increased Bcl-xL expression mediates v-Src-induced resistance to anoikis in intestinal epithelial cells. *Oncogene* 2002;21(18):2908-13.
846. Thomas FT, Contreras JL, Bilbao G, Ricordi C, Curiel D, Thomas JM. Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery* 1999;126(2):299-304.
847. Fenjves ES, Ochoa MS, Gay-Rabinstein C, Ricordi C, Curran MA. Retrovirally transferred genes inhibit apoptosis in an insulin-secreting cell line: implications for islet transplantation. *Cell Transplant* 2004;13(5):489-96.
848. Contreras JL, Bilbao G, Smyth CA, Jiang XL, Eckhoff DE, Jenkins SM, et al. Cytoprotection of pancreatic islets before and soon after transplantation by gene transfer of the anti-apoptotic Bcl-2 gene. *Transplantation* 2001;71(8):1015-23.
849. Sutherland RM, Allison J, Thomas HE, Brady JL, Kay TW, Lew AM. Bcl-2 protection of islet allografts is unmasked by costimulation blockade. *Transplantation* 2004;77(10):1610-3.
850. Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, et al. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J Cell Biol* 1999;144(5):903-14.
851. Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *Embo J* 1998;17(14):3878-85.
852. Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, et al. Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 1997;385(6614):353-7.
853. Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, et al. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997;277(5324):370-2.

854. Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G, et al. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc Natl Acad Sci U S A* 1997;94(21):11357-62.
855. Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, et al. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg<sup>2+</sup> ions. *J Cell Biol* 1998;143(1):217-24.
856. Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* 1998;16(17):2265-82.
857. Valentijn AJ, Metcalfe AD, Kott J, Streuli CH, Gilmore AP. Spatial and temporal changes in Bax subcellular localization during anoikis. *J Cell Biol* 2003;162(4):599-612.
858. Gilmore AP, Metcalfe AD, Romer LH, Streuli CH. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J Cell Biol* 2000;149(2):431-46.
859. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, et al. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999;144(5):891-901.
860. Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* 2001;153(6):1265-76.
861. Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* 2001;276(15):11615-23.
862. Waterhouse NJ, Ricci JE, Green DR. And all of a sudden it's over: mitochondrial outer-membrane permeabilization in apoptosis. *Biochimie* 2002;84(2-3):113-21.
863. Zamzami N, Kroemer G. Apoptosis: mitochondrial membrane permeabilization--the (w)hole story? *Curr Biol* 2003;13(2):R71-3.
864. Rytomaa M, Lehmann K, Downward J. Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling. *Oncogene* 2000;19(39):4461-8.
865. Beattie GM, Leibowitz G, Lopez AD, Levine F, Hayek A. Protection from cell death in cultured human fetal pancreatic cells. *Cell Transplant* 2000;9(3):431-8.
866. Wang P, Valentijn AJ, Gilmore AP, Streuli CH. Early events in the anoikis program occur in the absence of caspase activation. *J Biol Chem* 2003;278(22):19917-25.

867. Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* 1997;385(6617):637-40.
868. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 1995;270(5233):96-9.
869. Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T, et al. Haploid loss of bax leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. *Embo J* 1999;18(10):2692-701.
870. Thomas D, Yang H, Boffa DJ, Ding R, Sharma VK, Lagman M, et al. Proapoptotic Bax is hyperexpressed in isolated human islets compared with antiapoptotic Bcl-2. *Transplantation* 2002;74(11):1489-96.
871. Kim SK, Belzer FO, Southard JH. Loss of mitochondrial respiratory function and its suppression during cold ischemic preservation of rat livers with University of Wisconsin solution. *Hepatology* 1992;16(3):742-8.
872. Leducq N, Delmas-Beauvieux MC, Bourdel-Marchasson I, Dufour S, Gallis JL, Canioni P, et al. Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A. *Biochem J* 1998;336 ( Pt 2):501-6.
873. Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. *J Physiol* 2000;529 Pt 1:11-21.
874. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 2000;10(9):369-77.
875. Ravagnan L, Roumier T, Kroemer G. Mitochondria, the killer organelles and their weapons. *J Cell Physiol* 2002;192(2):131-7.
876. Zamzami N, Kroemer G. The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol* 2001;2(1):67-71.
877. Jassem W, Fuggle SV, Rela M, Koo DD, Heaton ND. The role of mitochondria in ischemia/reperfusion injury. *Transplantation* 2002;73(4):493-9.
878. Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, et al. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr* 1999;31(4):305-19.
879. Halestrap AP, Doran E, Gillespie JP, O'Toole A. Mitochondria and cell death. *Biochem Soc Trans* 2000;28(2):170-7.

880. Regula KM, Ens K, Kirshenbaum LA. Mitochondria-assisted cell suicide: a license to kill. *J Mol Cell Cardiol* 2003;35(6):559-67.
881. Kim JS, He L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* 2003;304(3):463-70.
882. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 1995;181(5):1661-72.
883. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, et al. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 1995;182(2):367-77.
884. Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, et al. Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol* 1996;157(2):512-21.
885. Kroemer G, Petit P, Zamzami N, Vayssiere JL, Mignotte B. The biochemistry of programmed cell death. *Faseb J* 1995;9(13):1277-87.
886. Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, et al. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998;281(5385):2027-31.
887. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, et al. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* 1998;1366(1-2):177-96.
888. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997;90(3):405-13.
889. Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 1997;89(2):175-84.
890. Susin SA, Zamzami N, Castedo M, Daugas E, Wang HG, Geley S, et al. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide- induced apoptosis. *J Exp Med* 1997;186(1):25-37.
891. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, et al. Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J Exp Med* 1999;189(2):381-94.

892. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001;292(5517):727-30.
893. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 1999;399(6735):483-7.
894. Shimizu S, Matsuoka Y, Shinohara Y, Yoneda Y, Tsujimoto Y. Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. *J Cell Biol* 2001;152(2):237-50.
895. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;397(6718):441-6.
896. Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. *Immunol Today* 1997;18(1):44-51.
897. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 1998;60:619-42.
898. Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta* 1998;1366(1-2):151-65.
899. Penninger JM, Kroemer G. Molecular and cellular mechanisms of T lymphocyte apoptosis. *Adv Immunol* 1998;68:51-144.
900. Green D, Kroemer G. The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol* 1998;8(7):267-71.
901. Kuwana T, Smith JJ, Muzio M, Dixit V, Newmeyer DD, Kornbluth S. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. *J Biol Chem* 1998;273(26):16589-94.
902. Chen Q, Takeyama N, Brady G, Watson AJ, Dive C. Blood cells with reduced mitochondrial membrane potential and cytosolic cytochrome C can survive and maintain clonogenicity given appropriate signals to suppress apoptosis. *Blood* 1998;92(12):4545-53.
903. Martinou I, Desagher S, Eskes R, Antonsson B, Andre E, Fakan S, et al. The release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. *J Cell Biol* 1999;144(5):883-9.

904. Quan LT, Tewari M, O'Rourke K, Dixit V, Snipas SJ, Poirier GG, et al. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. *Proc Natl Acad Sci U S A* 1996;93(5):1972-6.
905. Cai J, Jones DP. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J Biol Chem* 1998;273(19):11401-4.
906. Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* 1999;274(25):17941-5.
907. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;91(4):479-89.
908. Pan G, O'Rourke K, Dixit VM. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 1998;273(10):5841-5.
909. Hu Y, Benedict MA, Wu D, Inohara N, Nunez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A* 1998;95(8):4386-91.
910. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 1997;388(6639):300-4.
911. Duckett CS, Li F, Wang Y, Tomaselli KJ, Thompson CB, Armstrong RC. Human IAP-like protein regulates programmed cell death downstream of Bcl-xL and cytochrome c. *Mol Cell Biol* 1998;18(1):608-15.
912. Dohi T, Salz W, Costa M, Ariyan C, Basadonna GP, Altieri DC. Inhibition of apoptosis by survivin improves transplantation of pancreatic islets for treatment of diabetes in mice. *EMBO Rep* 2006;7(4):438-43.
913. Emamaullee JA, Rajotte RV, Liston P, Korneluk RG, Lakey JR, Shapiro AM, et al. XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. *Diabetes* 2005;54(9):2541-8.
914. Plesner A, Liston P, Tan R, Korneluk RG, Verchere CB. The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. *Diabetes* 2005;54(9):2533-40.
915. Rivas-Carrillo JD, Soto-Gutierrez A, Navarro-Alvarez N, Noguchi H, Okitsu T, Chen Y, et al. Cell-permeable pentapeptide V5 inhibits apoptosis and enhances insulin secretion, allowing experimental single-donor islet transplantation in mice. *Diabetes* 2007;56(5):1259-67.

916. Tobiasch E, Gunther L, Bach FH. Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. *J Investig Med* 2001;49(6):566-71.
917. Ye J, Laychock SG. A protective role for heme oxygenase expression in pancreatic islets exposed to interleukin-1beta. *Endocrinology* 1998;139(10):4155-63.
918. Ribeiro MM, Klein D, Pileggi A, Molano RD, Fraker C, Ricordi C, et al. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. *Biochem Biophys Res Commun* 2003;305(4):876-81.
919. Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, et al. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. *Diabetes* 2001;50(9):1983-91.
920. Li Y, Li G, Dong W, Chen J, Lu D, Tan J. Transplantation of rat islets transduced with human heme oxygenase-1 gene using adenovirus vector. *Pancreas* 2006;33(3):280-6.
921. Lemasters JJ. V. Necroptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *Am J Physiol* 1999;276(1 Pt 1):G1-6.
922. Di Lisa F, Canton M, Menabo R, Dodoni G, Bernardi P. Mitochondria and reperfusion injury. The role of permeability transition. *Basic Res Cardiol* 2003;98(4):235-41.
923. Kim JS, Qian T, Lemasters JJ. Mitochondrial permeability transition in the switch from necrotic to apoptotic cell death in ischemic rat hepatocytes. *Gastroenterology* 2003;124(2):494-503.
924. Tsujimoto Y. Apoptosis and necrosis: Intracellular ATP level as a determinant for cell death modes. *Cell Death Differ* 1997;4(6):429-34.
925. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 1997;185(8):1481-6.
926. Lanir A, Clouse ME, Lee RG. Liver preservation for transplant. Evaluation of hepatic energy metabolism by <sup>31</sup>P NMR. *Transplantation* 1987;43(6):786-90.
927. Lee RG, Springer C, Kasulis P, Lanir A, Frazer J, Clouse ME. Nuclear magnetic resonance assessment of adenosine triphosphate (ATP) dynamics in ischemic mouse livers perfused with adenine and ribose. *Invest Radiol* 1987;22(8):685-7.
928. Lee RG, Lanir A, Clouse ME. Liver adenine nucleotide metabolism during ischemia and reperfusion of mice livers studied by phosphorous-31 nuclear magnetic resonance. *Invest Radiol* 1987;22(6):479-83.

929. Elsasser A, Suzuki K, Lorenz-Meyer S, Bode C, Schaper J. The role of apoptosis in myocardial ischemia: a critical appraisal. *Basic Res Cardiol* 2001;96(3):219-26.
930. Saikumar P, Dong Z, Weinberg JM, Venkatachalam MA. Mechanisms of cell death in hypoxia/reoxygenation injury. *Oncogene* 1998;17(25):3341-9.
931. Gill C, Mestral R, Samali A. Losing heart: the role of apoptosis in heart disease--a novel therapeutic target? *Faseb J* 2002;16(2):135-46.
932. Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? *Hepatology* 2001;33(2):397-405.
933. Cursio R, Gugenheim J, Ricci JE, Crenesse D, Rostagno P, Maulon L, et al. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. *Faseb J* 1999;13(2):253-61.
934. Jaeschke H. Reperfusion injury after warm ischemia or cold storage of the liver: role of apoptotic cell death. *Transplant Proc* 2002;34(7):2656-8.
935. Bradbury DA, Simmons TD, Slater KJ, Crouch SP. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *J Immunol Methods* 2000;240(1-2):79-92.
936. Gordon JL, Drummond AH. A simple fluorimetric microassay for adenine compounds in platelets and plasma and its application to studies on the platelet release reaction. *Biochem J* 1974;138(2):165-9.
937. Beckers B, Lang HR, Weber W, Pranada F, Schweizer K. Application of intracellular ATP determination in lymphocytes for HLA-typing. *J Biolumin Chemilumin* 1986;1(2):47-51.
938. Larionov VN, Mokhova EN, Priorova NI, Tarakanova AN. [Effect of biologically active substances on lymphocyte energetics studied by using a simple luminometer-ATP meter]. *Nauchnye Doki Vyss Shkoly Biol Nauki* 1988(2):105-9.
939. Tamulevicius P, Streffer C. Metabolic imaging in tumours by means of bioluminescence. *Br J Cancer* 1995;72(5):1102-12.
940. Imataka K, Handa H, Ishikawa M, Hirai O, Kim SH, Yoshida S, et al. [The sequential changes of energy metabolism following cold-induced brain edema--a histochemical study]. *No To Shinkei* 1987;39(5):447-53.
941. Salla S, Redbrake C, Frantz A. Employment of bioluminescence for the quantification of adenosine phosphates in the human cornea. *Graefes Arch Clin Exp Ophthalmol* 1996;234(8):521-6.

942. Kuzikov AN, Bondarenko VM, Latkin AT. [Use of the bioluminescent method for the determination of bacterial adenosinetriphosphate (ATP-metry) in microbiology]. *Zh Mikrobiol Epidemiol Immunobiol* 2003(1):80-9.
943. Mundwiler KE, Lamberti FV, Hill RS. Rapid and inexpensive method for the quantitative assessment of donor pancreata for islet isolation. *Transplant Proc* 1994;26(6):3427.
944. Kilbert LH, Jr., Schiff MS, Foa PP. The measurement of ATP in pancreatic islets by means of the luciferin-luciferase reaction. *Horm Metab Res* 1972;4(4):242-4.
945. Ishii S, Sato Y, Terashima M, Saito T, Suzuki S, Murakami S, et al. A novel method for determination of ATP, ADP, and AMP contents of a single pancreatic islet before transplantation. *Transplant Proc* 2004;36(4):1191-3.
946. Brandhorst D, Brandhorst H, Hering BJ, Federlin K, Bretzel RG. Large variability of the intracellular ATP content of human islets isolated from different donors. *J Mol Med* 1999;77(1):93-5.
947. Goto M, Holgersson J, Kumagai-Braesch M, Korsgren O. The ADP/ATP ratio: A novel predictive assay for quality assessment of isolated pancreatic islets. *Am J Transplant* 2006;6(10):2483-7.
948. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am J Pathol* 1994;145(6):1323-36.
949. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 1993;53(19):4701-14.
950. Bicknell GR, Shaw JA, Pringle JH, Furness PN. Amplification of specific mRNA from a single human renal glomerulus, with an approach to the separation of epithelial cell mRNA. *J Pathol* 1996;180(2):188-93.
951. Gatzka M, Walsh CM. Apoptotic signal transduction and T cell tolerance. *Autoimmunity* 2007;40(6):442-52.
952. White SA, Swann A, Hughes DP, Contractor HH, London NJ. Different treatment regimens to reduce microbial contamination of purified porcine islets of Langerhans. *Transplantation* 1997;64(5):770-3.
953. Swift S, White SA, Nicholson ML. Islet Isolation Laboratory Quality Management System. In: *Human Islet Transplant Programme*. Leicester; 2003.

954. Kneteman N, Warnock GL, Lakey J, Rajotte R. Pancreas procurement and preservation: impact on islet recovery and viability. In: Ricordi C, editor. *Pancreatic Islet Cell Transplantation*. 1st Edition ed. Georgetown: Landes, R.G.; 1992. p. 72-81.
955. Noguchi H, Ueda M, Nakai Y, Iwanaga Y, Okitsu T, Nagata H, et al. Modified two-layer preservation method (M-Kyoto/PFC) improves islet yields in islet isolation. *Am J Transplant* 2006;6(3):496-504.
956. Tellez-Yudilevich M, Tyhurst M, Howell SL, Sheldon J. Secretory capacity and ultrastructure of rat pancreatic islets after preservation of pancreas in different conditions. *Transplantation* 1977;23(3):217-21.
957. Avila JG, Tsujimura T, Oberholzer J, Churchill T, Salehi P, Shapiro AM, et al. Improvement of pancreatic islet isolation outcomes using glutamine perfusion during isolation procedure. *Cell Transplant* 2003;12(8):877-81.
958. Corlett MP, Scharp DW. The effect of pancreatic warm ischemia on islet isolation in rats and dogs. *J Surg Res* 1988;45(6):531-6.
959. Ching CD, Harland RC, Collins BH, Kendall W, Hobbs H, Opara EC. A reliable method for isolation of viable porcine islet cells. *Arch Surg* 2001;136(3):276-9.
960. Ricordi C, Socci C, Davalli AM, Staudacher C, Baro P, Vertova A, et al. Effect of pancreas retrieval procedure on islet isolation in the swine. *Transplant Proc* 1990;22(2):442-3.
961. Toledo-Pereyra LH, Valgee KD, Castellanos J, Chee M. Hypothermic pulsatile perfusion: its use in the preservation of pancreases for 24 to 48 hours before islet cell transplantation. *Arch Surg* 1980;115(1):95-8.
962. Toso C, Oberholzer J, Ris F, Triponez F, Bucher P, Demirag A, et al. Factors affecting human islet of Langerhans isolation yields. *Transplant Proc* 2002;34(3):826-7.
963. Ohzato H, Gotoh M, Monden M, Dono K, Kanai T, Mori T. Improvement in islet yield from a cold-preserved pancreas by pancreatic ductal collagenase distention at the time of harvesting. *Transplantation* 1991;51(3):566-70.
964. Contractor HH, Johnson PR, Chadwick DR, Robertson GS, London NJ. The effect of UW solution and its components on the collagenase digestion of human and porcine pancreas. *Cell Transplant* 1995;4(6):615-9.
965. Gray DW, Leow CK. Non-automated methods for islet isolation. In: Ricordi C, editor. *Pancreatic Islet Cell Transplantation*. 1st Edition ed. Georgetown: Landes, R.G.; 1992. p. 89-98.

966. Fernandez LA, Hatch EW, Armann B, Odorico JS, Hullett DA, Sollinger HW, et al. Validation of large particle flow cytometry for the analysis and sorting of intact pancreatic islets. *Transplantation* 2005;80(6):729-37.
967. Griffin AD, Hawthorne WJ, Allen RD, Grierson JM, Jablonski P, Howden BO, et al. Twenty-four-hour preservation of canine pancreas allografts using low-cost, low-viscosity, modified University of Wisconsin cold storage solution. *Transplant Proc* 1993;25(1 Pt 2):1595-6.
968. Holmes MA, Clayton HA, Chadwick DR, Bell PR, London NJ, James RF. Functional studies of rat, porcine, and human pancreatic islets cultured in ten commercially available media. *Transplantation* 1995;60(8):854-60.
969. Bertuzzi F, Garancini P, Soggi TC, Nano R, Taglietti MV, Santopinto M, et al. Lessons from in vitro perfusion of pancreatic islets isolated from 80 human pancreases. *Cell Transplant* 1999;8(6):709-12.
970. Chou Q, Russell M, Birch DE, Raymond J, Bloch W. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res* 1992;20(7):1717-23.
971. Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, et al. High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* 2001;50(6):1290-301.
972. Hanke J. Apoptosis in cultured rat islets of langerhans and occurrence of Bcl-2, Bak, Bax, Fas and Fas ligand. *Cells Tissues Organs* 2001;169(2):113-24.
973. Bateman AC, Turner SM, Thomas KS, McCrudden PR, Fine DR, Johnson PA, et al. Apoptosis and proliferation of acinar and islet cells in chronic pancreatitis: evidence for differential cell loss mediating preservation of islet function. *Gut* 2002;50(4):542-8.
974. Ihm SH, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD, et al. Effect of donor age on function of isolated human islets. *Diabetes* 2006;55(5):1361-8.