A Pre-clinical Evaluation of Growth Factor-eluting Stents for the Treatment of Chronic Total Coronary Artery Occlusion

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for Mum and Dad

Abstract

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Objectives

Chronic total coronary artery occlusion (CTO) remains a significant challenge for percutaneous coronary intervention. Using a novel endovascular porcine model of coronary occlusion we investigated a strategy of promoting antegrade collateral vessels without crossing the occlusion. The prolyl-4-hydroxylase inhibitor, di-methyl oxalyl glycine (DMOG) was used to up-regulate a pro-angiogenic transcription factor, hypoxia inducible factor-1α.

Method

DMOG was loaded onto a polymer-coated coronary stent. We developed a novel, entirely endovascular, method of inducing a coronary occlusion. Copper-coated stents were delivered percutaneously to produce CTO lesions in 20 Yorkshire white pigs. DMOG stents were implanted at day 28 and angiographic and physiological data collected on distal coronary and collateral flow. At day 56 the animals were sacrificed and histological analysis performed.

Results

A complete total coronary occlusion was present in all animals at day 28 following implantation of a copper stent. At 56 days there was a greater percentage increase in angiographic collateral area in the DMOG group compared with the control (polymer-only stent) group (84.5±34.5% versus 16.5±5.9%, p=0.057). There was no difference between the groups in a surrogate measure of collateral flow at day 56. Histology revealed a significant increase in collateral vessels around the site of occlusion in the DMOG group compared with controls (29.9±2.6 versus 18.4±3.1, p=0.01).

Conclusions

DMOG increased the number of collateral vessels at the site of vessel occlusion but not in the distal vessel. The angiogenic effect of DMOG appeared to be restricted to ischaemic tissue. Implantation of a copper stent provides a reliable entirely endovascular method of producing a CTO, with marked antegrade collateral formation present at 28 days. Proximal placement of stents delivering angiogenic compounds such as DMOG may provide a clinical management option in resistant CTO lesion.

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Statement of work:
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Abbreviations and acronyms

ABPI	Ankle-brachial artery pressure index
AdVEGF	Adenoviral vector encoding Vascular endothelial growth factor
Adv	Adventitial (pertaining to outer arterial wall)
AGENT	Angiogenic GENe Therapy phase II clinical trial
Ang/ ANGPT	Angiopoietin family of pro-angiogenic protein growth factors
AR1	Amplatz size 1 right coronary catheter
BBE	Bovine brain extract
BMS	Bare-metal coronary stent
CABG	Coronary artery bypass surgery
CAM	Chick chorioallantoic membrane assay
CCR	C-C motif receptor (chemokine receptor)
CFI	Collateral flow index
$CoCl_2$	Cobalt Chloride
CT	Computed tomography
CTO	Chronic total coronary occlusion
DE	Mass of DMOG eluted from stent
DL	Mass of DMOG loaded on to stent
DS	Mass of DMOG stripped from stent by solvent
DAB	Diaminobenzidine (immuno-histochemical stain)
DES	Drug-eluting stent
Dil-Ac-LDL	Acetylated low-density lipoprotein

DMOG	Di-methyl oxalylglycine.
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRI	Distal arterial run-off index
ECGF	Extra-cellular growth factor
ECM	Extra-cellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGM	Endothelial growth medium
EHy926	Strain of human umbilical vein endothelial cells used in culture
EPC	Endothelial progenitor cell
EPO	Erythropoietin
ESA	End-systolic area (of left ventricle)
ESI-MS	Electrospray ionization mass spectroscopy
EtOH	Ethanol
ETT	Exercise tolerance test
FBS	Fetal bovine serum
Fe(II)	Ferrous ion
FFR	Fractional flow reserve
FGF	Fibroblast growth factor
FG-2216	Fibrogen compound 2216, a selective PHD2 inhibitor
FIH	Factor inhibiting HIF
FIRST	FGF-Initiating RevaScularization phase II clinical Trial
Flk-1/KDR	High-affinity VEGF tyrosine kinase receptor for VEGF-1 found on endothelial cells (aka. VEGF receptor 2)

Fli-1	Mutant zebrafish phenotype expressing endothelial green fluorescent protein marker
Flt-1	High affinity VEGF tyrosine kinase receptor for VEGF-1 found on endothelial cells (aka. VEGF receptor 1)
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
Grl	Zebrafish gridlock mutant
GSB(%)	Grid-square branching percentage- measure of extent of endothelial cell tubule formation in culture
GTN	Glycerin trinitrate
H&E	Haematoxylin and eosin stain
HAT	Liquid mixture of sodium hypoxanthine aminopterin and thymidine (cell culture medium supplement)
HBS-EP	Buffer solution (10mM Hepes, 150mM Sodium Chloride, 3mM EDTA, 0.005% Polysorbate 20)
HGF	Hepatocyte growth factor
HI	Hypdroxylase inhibitor
HIF	Hypoxia-inducible factor
HMEC	Human mammary endothelial cells
HUVEC	Human umbilical vein endothelial cell
IC	Intra-coronary
IEL	Internal elastic lamina
IGF	Insulin-like growth factor
IV	Intra-venous
JL3	Judkins size 3 left coronary catheter

kDa	kiloDalton
KDR	Gene encoding kinase insert domain receptor (VEGFR2)
LAD	Left anterior descending branch of left coronary artery
LAO	Left anterior oblique angiographic projection
LCx	Circumflex branch of left coronary artery
LVEDP	Left ventricular end-diastolic pressure
LVEF	Left ventricular ejection fraction
LVESA	Left ventricular end-systolic area
MCP	Monocyte-chemotactic protein
MeOH	Methanol
MMP	Matrix metallo-proteinase
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
NCL-vWFp	Antibody to human endothelial cell Von-Willebrand factor
NI	Neointima
NiCl	Nickel Chloride
NIH	U.S. National Institute for Health
NO	Nitric oxide
NOG	N-oxalyl glycine
NOGA	Electro-mechanical mapping ventricular catheter
2-OG	2-oxaloglutarate
Pa	Arterial pressure measured in aorta or at catheter tip
Pd	Pressure measure in the distal coronary artery

Pv	Central venous (right atrial) pressure
Pw	Distal coronary wedge pressure with proximal balloon occlusion
PEP	Programmed elution polymer
PCI	Percutaneous coronary intervention, or coronary angioplasty and stenting
PDGF	Platelet-derived growth factor
PhVEGF	Plasmid Vascular endothelial growth factor
PHD	Prolyl hydroxylase enzyme
PIL	Home Office personal investigation licence
PnVPA	Poly(vinyl)pyrrolidone-co-vinyl acetate polymer
PR39	Macrophage-derived peptide 39
PTFE	Polytetrafluoroethane
PVB	Poly(vinylbutyral-co-vinyl alcohol-co-vinyl acetate polymer
RAO	Right anterior oblique angiographic projection
RCA	Right coronary artery
REVASC	Phase II clinical trial of intra-myocardial injection of AdVEGF121
RGF	Reduced growth factor (e.g. RGF Matrigel)
RWMA	Regional wall motion abnormality
SAVE	Survival and ventricular enlargement clinical trial
SD	Standard deviation of the mean
SEM	Standard error of the mean
SiRNA	Small interfering RNA
SMC	Smooth muscle cell
STEMI	ST-segment elevation myocardial infarction

SPECT	Single photon-emission computed tomography
TGF	Transforming growth factor
Tie	Family of membrane-bound tyrosine kinase receptors for angiopoietins
TMR	Trans-myocardial revascularization
TOAST-GISSE	Total Occlusion Angioplasty Study
US FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von-Hippel Lindau
VCAM	Vascular cell adhesion molecule
VIVA	Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis- phase II clinical trial
VRI	VEGF receptor inhibitor
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor (aka. Factor-VII related antigen)
Z-fix	Zinc formalin tissue fixative

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Chapter 1

Introduction

1.1 Aims of the thesis

This thesis describes a series of experiments to a proof-of-concept hypothesis: that implantation of a growth-factor eluting stent would improve myocardial perfusion in a porcine model of chronic total coronary artery occlusion. The work involved initial in-vitro evaluation of candidate growth-factor compounds, followed by evaluation of the physiological effect of stabilization of a nuclear transcription factor, Hypoxia inducible factor (HIF).

The rationale for this investigation is the presence of a sizeable minority of patients who are ineligible for conventional revascularization by either percutaneous coronary intervention (PCI) or coronary artery bypass surgery (CABG). These patients may experience limiting angina, often in the context of severe diffuse coronary disease. Up to 50% of patients with significant coronary disease have a chronic total occlusion (CTO) of at least one major epicardial coronary artery, with presence of CTO being a strong predictor against the use of PCI as a treatment strategy.[1] There remains a significant failure rate for conventional PCI in the setting of CTO (around 10-15% even in the best hands), with CTO being a major influence in the decision to refer patients with multi-vessel coronary disease for coronary bypass surgery. There is therefore a potential role for novel angiogenic strategies

1.2 Coronary artery disease and chronic total coronary occlusion

1.2.1 Incidence of coronary artery disease

Estimates of the prevalence of symptomatic angina in the community vary, but the most robust recent data comes from Scotland and puts the prevalence at 28/1000 in men and 25/1000 in women, with a strong association with socio-economic deprivation.[2, 3] In this study the annual incidence in men was 1.8/1000, compared to earlier data in the South of England quoting an estimated annual incidence of 1.13/1000 male patients.[4]

While the rate of non-fatal myocardial infarction or death during the first year after the diagnosis of angina is high at 10-15%, even with optimal treatment, this falls to an annual adverse cardiac event rates of 2-3% in patients with stable symptoms.[5, 6] Improved patient survival among CHD patients and increasing longevity in the population as a whole means that cardiovascular morbidity due to complex coronary disease will continue to increase. It is estimated that around 2 million adults in the UK experience angina pectoris.[7]

1.2.2 Methods of coronary artery revascularization

Treatment methods and rates of revascularization vary widely across Europe, dependent on access to catheterization facilities. In a recent pan-European registry

only 13% of patients presenting with angina had revascularization by percutaneous coronary revascularization (PCI) or coronary artery bypass surgery (CABG), although the figure was higher in those with unstable presentations.[8]

PCI has become the dominant form of revascularization therapy. Just over 80,000 PCI procedures were performed in the U.K. in 2008.[9] A widening of the indications for PCI, particularly in the management of acute coronary syndromes have lead to PCI outstripping CABG in a ratio of 3:1. PCI rates in the UK have increased from 1050 per million population to 1308 pmp in 2008 with a sustained growth rate of around 15% per annum since the publication of the National Service Framework for coronary heart disease in 2000.The UK however still lags some way behind the majority of European nations in terms of PCI provision, with Germany and France leading the way with PCI rates in excess of 2500 per million population.[10] Mortality in elective PCI continues to fall, and currently stands at less than 0.5%.

1.2.3 The challenge of patients not suitable for revascularization

Approximately 5-21% of patients presenting with angina are deemed not to be suitable for either surgical or percutaneous coronary intervention as a result of co-morbidity, coronary anatomy or a combination of these two factors.[11] The variation in estimates of 'non-revascularizable' coronary disease between studies can be largely explained by regional and institutional variability in management

strategies. Evidence from contemporary large-scale U.S. registries suggests increased rates of mortality and recurrent MI in patients with multi-vessel coronary disease undergoing incomplete revascularization compared with complete revascularization by PCI with drug-eluting stents.[12] As the population ages, the proportion of patients ineligible for conventional revascularization by PCI or CABG may increase. Common reasons for non-revascularized myocardium include co-morbid conditions conferring unacceptable surgical risk; poor target vessels for CABG or PCI with diffuse atherosclerosis or small distal vessels; and chronic total occlusion (CTO) of either native vessels or grafts. CTOs therefore form a considerable proportion of lesions in patients who are unsuitable for conventional revascularization techniques. While drug-eluting stents have ameliorated the problem of restenosis following PCI, unique technical challenges exist in the conventional percutaneous treatment of CTOs. The main cause of PCI procedural failure in the case of CTOs remains inability to cross the occlusion point with an angioplasty guide-wire.

The need to develop treatment strategies for patients not suitable for revascularization by CABG or conventional PCI has led to pre-clinical evaluation of a number of strategies designed to achieve therapeutic angiogenesis. To date, mechanical means such as trans-myocardial laser revascularization, angiogenic peptides including the fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGF) and gene therapy have been used clinically, although none has proven ideal.

1.2.4 The clinical impact of Chronic Total Occlusions

A chronic total occlusion (CTO) is defined as a coronary artery that has been occluded for at least 3 months. Precise estimation of the duration of occlusion is difficult, and usually relies on a clinical history of the event that may have been related to vessel occlusion such as acute myocardial infarction or ischemia in the relevant coronary territory. In many patients however the duration of occlusion remains uncertain. The temporal criterion used to define a CTO has varied widely from >2 weeks to > 3 months in previous reports, resulting in diverse descriptions of lesion characteristics and PCI success rates.

1.2.5 Prevalence of CTO

An accurate indication of CTO prevalence in the general population is difficult because some patients with CTO are minimally symptomatic or asymptomatic and therefore never undergo coronary angiography. Among patients with recent onset angina undergoing coronary angiography, 1 or more CTO is found in around 30-50% of cases.[1, 13, 14] The incidence of CTO increases with age, although based on US registry data older patients are less likely to undergo attempted PCI revascularization.[15] While PCI techniques have advanced rapidly in recent years, CTO remains a major cause of referrals for coronary artery bypass surgery.[1, 16]

A significant proportion of patients following acute myocardial infarction will develop a persistently occluded infarct-related artery. The observed frequency of arterial occlusion is a function of the underlying atherosclerotic burden, the type and extent of delay to reperfusion therapy and the point at which coronary angiography is performed to assess arterial patency. In patients with STEMI not treated by reperfusion therapy, an occluded infarct-related artery has been found in 87% of patients within 4 hours, 65% between 12-24 hours, 53% at 15 days and 45% at 1 month.[17] Even with optimal treatment by primary PCI, initial treatment failure or subsequent re-occlusion occurs in 5% to 10% at 6 months.[18, 19]

1.2.6 Clinical presentation

Around 85-90% of patients undergoing attempted PCI for CTO have stable or progressive angina, with minimally symptomatic patients often managed medically.[20, 21] Prior myocardial infarction has been reported in 42% to 68% of patients with CTO.[22]

Even in the absence of a history of prior myocardial infarction, stress induced ischaemia is a frequent finding in patients with CTO. He et al demonstrated reversible perfusion defects using stress myocardial single-photon emission CT (SPECT) in 83% of 71 patients without prior MI with single-vessel disease in the form of a CTO.[23] The techniques of adenosine SPECT and technetium myocardial perfusion scanning appear to be more sensitive than exercise treadmill testing in

detecting ischaemia in patients with CTO.[24] Antegrade collateral vessels of varying size are an almost universal finding at occluded artery sites. These vessels usually result in inadequate myocardial perfusion, preserving myocardial viability but often leaving the patient with symptoms of angina.

1.2.7 Anatomy and histopathology of CTO

The histopathology of the chronically occluded artery has been described in detail by Srivasta and colleagues.[25] CTOs consist of organised fibro-atheromatous plaque with a variable amount of thrombus present dependent on the mechanism and duration of occlusion. Most commonly CTOs arise from a thrombotic occlusion, followed by thrombus organization and tissue aging.[26] A tough fibrous cap is often present at the proximal and distal margins of the occlusion. Around half of angiographic coronary occlusions are in fact <99% stenotic when examined histologically. Often the CTO is traversed by fine endothelialised micro-channels which may allow passage of a hydrophilic angioplasty guidewire. In many cases however, these channels are poorly developed and successful recanalisation by angioplasty is less likely to be successful.

The atherosclerotic plaque of a typical CTO consists of intracellular and extracellular lipids, smooth muscle cells, extracellular matrix and calcium.[27] Collagen-rich fibrous tissue predominates at the proximal and distal ends of the lesion, encasing a softer core of organized thrombus and lipids. The extent of

calcification and degree of inflammation and neovascularization determine the structure of the CTO. Younger occlusions (<1 year old) tend to be 'softer' and are more likely to allow passage of an angioplasty wire directly through the tissue planes or via neovascular channels. Conversely hard plaques are more likely to deflect guide wires into sub-intimal regions, creating dissection planes. The age-related increase in calcium and collagen content of CTOs largely accounts for the progressive difficulty in crossing older CTOs during PCI.

Inflammation is present throughout the occluded arterial wall but most marked in the intima, with inflammatory infiltrates consisting of macrophages, foam cells and lymphocytes. Both negative and positive vessel remodelling occur.

Progressive neovascularization occurs in all CTOs to a variable extent. Angiogenesis is an early event with relatively large (>250 μm) capillaries seen throughout the vessel wall. Neovascularization and inflammation appear to be frequently co-localized within intimal plaque and adventitial although it is not clear whether inflammation is a trigger or simply an effect of new vessel growth. It has been postulated that lymphocytes and monocytes/ macrophages may play an active role in both progression of atherosclerosis and angiogenesis by producing mitogenic and angiogenic factors.[28]

Data from autopsy series suggest than neovascularization in CTOs occurs from the adventitia towards the intima. These vessels often arise from adventitial vasa

vasorum in lesions of >70% stenosis. In CTOs, thrombus-derived angiogenic stimuli may play a role.[29] These micro-channels may recanalise the distal lumen, and are suggested at angiography by the presence of small antegrade collateral vessels. Organization and retraction of thrombus occasionally leads to recanalisation of a previously occluded artery by non-angiogenic means.

1.2.8 Rationale for opening CTOs

Successful PCI of CTOs poses unique technical challenges, mainly in achieving successful passage of the angioplasty guide wire across the occlusion. In addition there has been uncertainty regarding long term benefit and concerns regarding the short and long term patency of the artery following PCI. These concerns have led to limited interest in PCI for CTO with the proportion of PCI procedures undertaken for single-vessel CTOs remaining static at around 9% from 1992-2008.[9] Recent data reporting improvements in long-term outcome following successful opening of CTOs, along with advances in technique and equipment with the development of dedicated angioplasty guide wires, has led to a rekindling of interest in CTO angioplasty.[20, 30] Even with optimal technique and equipment however, success rates are much lower than in conventional angioplasty, at around 75-80%.[30-32]

A number of studies attest to the survival benefit bestowed by having an open artery following myocardial infarction. The Survival and Ventricular Enlargement

(SAVE) investigators reported that persistent occlusion of an infarct-related artery was associated with a relative risk of 1.47 in adjusted 4 year mortality.[33] This suggested that late recanalisation may improve outcomes.

Several more recent retrospective observational studies have confirmed the benefit of opening CTOs. The largest series from the U.S. comes from the Mid America Heart Institute, consisting of 2007 patients undergoing intended PCI of a non-acute CTO between 1980 and 1999.[31] Data show a progressive improvement in procedural success rates until the mid-1990s when procedural success rates reached a plateau of around 75%. Successful opening of a CTO significantly improved long-term outcome (10 year survival 73.5% with CTO success versus 65.0% with CTO failure; p=0.001) with failure to open a CTO an independent predictor of reduced survival (hazard ratio 1.4; p=0.0003). Additionally survival was similar in successfully treated CTO patients to a matched cohort undergoing angioplasty of non-occluded arteries. This benefit in terms of late mortality with successful PCI of CTO was replicated in a more contemporary British Columbia Cardiac Registry, and in the prospective Total Occlusion Angioplasty Study (TOAST-GISSE). This later study demonstrated that successful PCI significantly reduced symptoms of angina, and was the only non-surgical treatment for patients with CTO which reduced adverse cardiac events at 1 year (odds ratio 0.24; p=0.018).[20]

The most common indication for PCI of a CTO is to relieve angina pectoris or inducible myocardial ischaemia as an alternative to CABG. Berger et al reported 87% of patients to be asymptomatic at six months after successful opening of a CTO[34] while Ivanhoe et al reported that 69% were symptom-free at 36 months.[35]

There is evidence that successful PCI of CTOs improves left ventricular function in patients with inducible ischaemia.[36] Improvements of left ventricular ejection fraction (LVEF) from 62% to 67% ($p<0.001$), mainly in areas supplied by the occluded artery, have been reported.[37] While improvements In LVEF can be detected among those patients with previous myocardial infarction, these were only statistically significant in those patients with well developed collateral vessels prior to PCI for CTO. Collateral vessels may be a marker for so-called 'hibernating' myocardium where the contractile apparatus is in a dormant state and recovers once myocardial perfusion improves. These observations are compatible with previous data demonstrating improved survival in those patients who spontaneously recanalise the infarct-related artery following myocardial infarction.[33, 38] Improvement in LV function following PCI for CTO seems to be more marked where the occlusion is of shorter duration (<6 weeks), and is dependent on sustained vessel patency at follow-up.[37, 39] Completed trans-mural infarction is unsurprisingly associated with failure to achieve improvement in myocardial function following revascularization. Contrast MRI, SPECT, stress echocardiography and nuclear perfusion techniques have all shown utility in

identifying patients with hibernating myocardium who might benefit from revascularization of a CTO.

The role of PCI in the 'asymptomatic' patient who is demonstrated to have an occluded coronary artery following a myocardial infarction was clarified in 2006 by the Occluded Artery Trial. In this study 2166 patients who were identified as having an infarct-related coronary occlusion at day 3 to 28 post MI were randomised to PCI or optimal medical therapy. There was no benefit of PCI in terms of adverse cardiac events over four year follow-up, emphasising that intervention should be ischaemia or symptom driven.[40, 41] The patients studied in this trial are however distinct from patients with CTO (coronary occlusion of at least 3 months duration). OAT patients presented with acute MI-related coronary occlusion and therefore the clinical results from this trial are not directly applicable to patients with CTO.

1.2.9 Drug-eluting stents and restenosis following PCI

Arterial restenosis is caused by injury-induced neo-intimal hyperplasia and is clinically relevant in up to 15% of patients treated by bare metal stents.[42] Attempts to limit restenosis by limiting hyperplasia eventually lead to the development of stents designed to elute anti-proliferative drugs. The first drug used was Sirolimus or Rapamycin, a macrolide antibiotic which inhibited smooth muscle cell proliferation. Early clinical trials showed almost complete abolition of

re-stenosis, results largely replicated in a plethora of subsequent clinical trials and in registry studies demonstrating a fall in restenosis rates to approximately 5-10%.[43-47]

The first drug-eluting stent (DES) was granted US FDA approval in April 2003 and rapidly entered widespread clinical use. There are currently 7 distinct DES approved for use in Europe, with developments anticipated in the design of the delivery platform and the possibility of absorbable or bio-neutral stents in the future. The delay in re-endothelialisation of stent struts that anti-proliferative drugs cause mandates a prolonged duration of dual anti-platelet therapy (currently aspirin plus the thienopyridine-derivative Clopidogrel). The latest data from meta-analysis suggests that drug-eluting stents are safe, reducing the need for target lesion revascularization by 30% compared with bare-metal stents. While there is a suggestion of excess late (>30 days) stent thrombosis with paclitaxel-eluting stents there is no such hazard with sirolimus-eluting stents.[48]

1.2.10 A new method for treating CTO

Successful revascularization of a CTO by PCI in appropriate patients relieves angina and improves myocardial performance. These benefits depend on maintenance of arterial patency. While the advent of drug-eluting stents has improved long-term patency rates, restenosis remains a significant problem. Even if the artery is opened by PCI, several series suggest restenosis rates of around

10% of patients.[30, 49] This compares to restenosis rates of 3-5% with DES in most other lesions.[50] Failed PCI in this setting of a CTO is strongly associated with adverse events and a frequent cause of referral for coronary artery bypass surgery.

While the era of drug-eluting stents has largely addressed the problem of restenosis, DES cannot hope to address the limiting factor in 'conventional' PCI for CTOs, namely the difficulty of crossing a resistant occlusion with an angioplasty guide-wire. There have been technological advances in this area recently with dedicated guide wires and techniques such as accessing the occlusion point via retrograde collaterals from a contra-lateral coronary artery. Such advanced techniques require a high level of skill however and success rates remain around 80-85% in the best hands.[51] Equally, a proportion of patients unsuitable for revascularization by PCI will neither be fit to undergo the stress of coronary bypass surgery. Development of an alternative strategy focused on augmenting the natural process of collateralization to improve distal myocardial perfusion, without the need to cross the occlusion point of the target artery, is an unmet need.

The main aim of this project was to investigate the feasibility of using a pro-angiogenic growth factor to promote the growth of antegrade collateral vessels from the occlusion point of a CTO to improve perfusion of the distal myocardium. This was investigated in a porcine model of chronic total occlusion with

angiographic and physiological measures of myocardial perfusion. The intention was to determine proof-of-principle in the porcine model.

1.3 Endothelial cell biology

A basic appreciation of endothelial cell structure and function forms the background to understanding the processes involved in new vessel formation. Vascular endothelial cells form a monolayer lining all blood vessels and via complex interactions between neighbouring cells and the extra-cellular matrix initiate and control physiological processes such as the initiation of coagulation, leukocyte adhesion and activation, active and passive transport of molecules and the angiogenesis cascade. Endothelial cells number about 1 to 6 x 10^{13} in adult humans and weigh around 1kg.[52] While the basic anatomical architecture of the circulatory system is relatively constant, endothelial cells exhibit wide morphological heterogeneity depending on local tissue needs.[53, 54]

The heterogeneity of endothelial cell phenotype underlines the complexity of new vessel formation and has implications for attempts at therapeutic angiogenesis. Key to the function of vessels are mural cell which support the endothelium. Arteries and veins are covered by a variable layer of smooth muscle cells which maintain the integrity of the vessel and expand or contract to control blood flow. Pericytes are pluripotent mesenchymal cells associated with capillaries and post-capillary venules which support the endothelium and have the capacity to

differentiate into smooth muscle cells, fibroblasts or osteoblasts under specific stimuli.[55] Smooth muscle cells proliferate in response to injury and have a vital role in synthesising the connective tissue matrix of the vessel wall which is composed of elastin, proteoglycans and collagen.

1.4 Endothelial cell interactions

Central to endothelial cell function are interactions between endothelial cells and between endothelial cells, the extra-cellular matrix and leukocytes.

1.4.1 The extra-cellular matrix in vascular development

Perhaps most important cellular interactions in terms of new vessel formation are the complex interactions between endothelial cells and the extra-cellular matrix (ECM). The ECM is organized in two layers: a vascular basement membrane and smooth muscle cells, and an interstitial matrix composed of fibrillar collagens and glycoproteins such as fibronectin.[56] One of the first processes to occur under the influence of angiogenic stimuli is the production of matrix metalloproteinases (MMP) by endothelial cells. MMPs degrade ECM components and alter interactions with endothelial cells in preparation for the formation of new blood vessels. Integrin $\alpha_v\beta_3$ has been extensively studied and upregulates MMP-2 activity.[57] The same integrin directly regulates expression of VEGF receptor-2 and is itself induced by angiogenic growth factors such as VEGF and bFGF.

Following an angiogenic stimulus by VEGF, bFGF, Platelet-derived growth factor (PDGF) or similar chemokines, pericytes become detached and endothelial cells invade through the basement membrane and ECM with the help of MMPs. During this stage matrix components such as collagen, laminin and fibronectin promote angiogenesis, while other ECM components such as endostatin and canstatin predominate and exert anti-angiogenic effects once a critical concentration of new vessels have formed.[58]

1.5 Processes of new vessel formation and collateral vessel formation

The importance of collateral vessels within the coronary circulation has long been recognized by clinicians. Collateral vessels occur to a variable extent among patients with coronary artery stenoses, their development dependent on a number of co-morbid factors. More extensive collateralization is thought to be promoted by sustained ischaemia due to gradual development of a high-grade stenosis or occlusion, with new vessel formation typically occurring at the interface between normal and ischaemic tissue. Such new vessel growth may permit sufficient perfusion of blood to maintain cellular viability, or even preserve myocardial function. Emulation or enhancement of this process therefore appears to be an attractive therapeutic strategy.

The pathological consequences of neovascularization were first elucidated by Folkmann in 1971 describing the production of angiogenic cytokines by tumour

cells and the role of new vessel growth in tumour cell survival.[59] The field of angiogenesis research first attracted attention with the realisation that anti-angiogenic therapies would be effective in targeting tumours greatest vulnerability, namely their blood supply. Much more recently evidence for therapeutic augmentation of angiogenesis in humans has been established in the context of critical limb ischaemia.[60, 61] Although these clinical trials recruited only small numbers of subjects and reported somewhat subjective end-points, they were widely considered to provide a vindication of the concept of therapeutic angiogenesis and provided an impetus to investigation of severely symptomatic patients with coronary disease unsuitable for conventional revascularization.

1.5.2 Definition of angiogenesis

The process of neovascularization in the adult is highly complex and poorly understood, involving the interaction of both pro and anti-angiogenic cytokines, the endothelium and extra-cellular matrix. The terminology surrounding new vessel formation has been inconsistent, mirroring the uncertainties around the processes involved. *Angiogenesis,* is the process of new vessel formation from pre-existing vasculature, with formation of thin-walled, endothelium lined structures which lack a smooth muscle layer.[62] New capillaries sprout by a process of migration and proliferation of previously undifferentiated endothelial cells. This has been likened to a process of capillary 'budding' from enlarged venules. These capillary buds become divided by peri-endothelial cells or trans-

endoothelial cell bridges to form capillaries. This process requires vasodilatation and increased permeability to allow extravasation of proteins to form an extracellular matrix, and differentiation of endothelial cells following proliferation and migration in response to local tissue signals. 'Angiogenesis' is the predominant process seen in healing wounds and at the border of myocardial infarctions. *Vasculogenesis* is the process of formation of new vascular structures *de novo* from endothelial progenitor cells or stem cells called angioblasts which fuse together and differentiate into mature endothelial cells: this is thought to the predominant process in utero. The primitive network then differentiates by angiogenesis with sprouting, branching and intussusceptive growth from established capillaries. Until recently vasculogenesis was thought to be confined exclusively to embryonic development, with angiogenesis thought to be the sole process involved in adult revascularization. Realisation of the role of circulating bone-marrow derived endothelial progenitor cells (EPCs) has revised understanding of new vessel growth in adults. EPCs have been shown to circulate in peripheral blood, migrate to and incorporate into foci of neovascularization in adult animals and proliferate under ischaemic conditions.[63-65] Thus both vasculogenesis and angiogenesis-related processes are active in adult neovascularization.

1.5.3 Definition of arteriogenesis

Arteriogenesis refers to stabilization by laying down of mural cells and remodelling into larger blood vessels such as those seen in advanced coronary disease. Although remodelling of existing vessels is the major factor in 'arteriogenesis', it is thought that a process similar to angiogenesis may also play a role: this is poorly understood but thought to involve smooth muscle cell migration and differentiation into a contractile phenotype.[66] The potential for collateral development and response to ischaemia varies widely among species and among individuals of the same species, at least in part due to differences in underlying endothelial function. The stimulus for collateral formation is poorly understood but thought to include increased blood flow velocity and intra-luminal shear stress that may result within branches of an occluded epicardial coronary artery.[67]

Angiogenesis in adults is stimulated during wound healing, tissue ischaemia and regeneration and the female reproductive cycle, and tightly controlled by positive and negative regulators. While the above distinctions are important in attempting to understand the process of new vessel growth, it is undoubtedly true that the term 'angiogenesis' is often used in the literature to describe a general process of neovascularization including the processes of vasculogenesis and angiogenesis. (*fig. 1.1)*

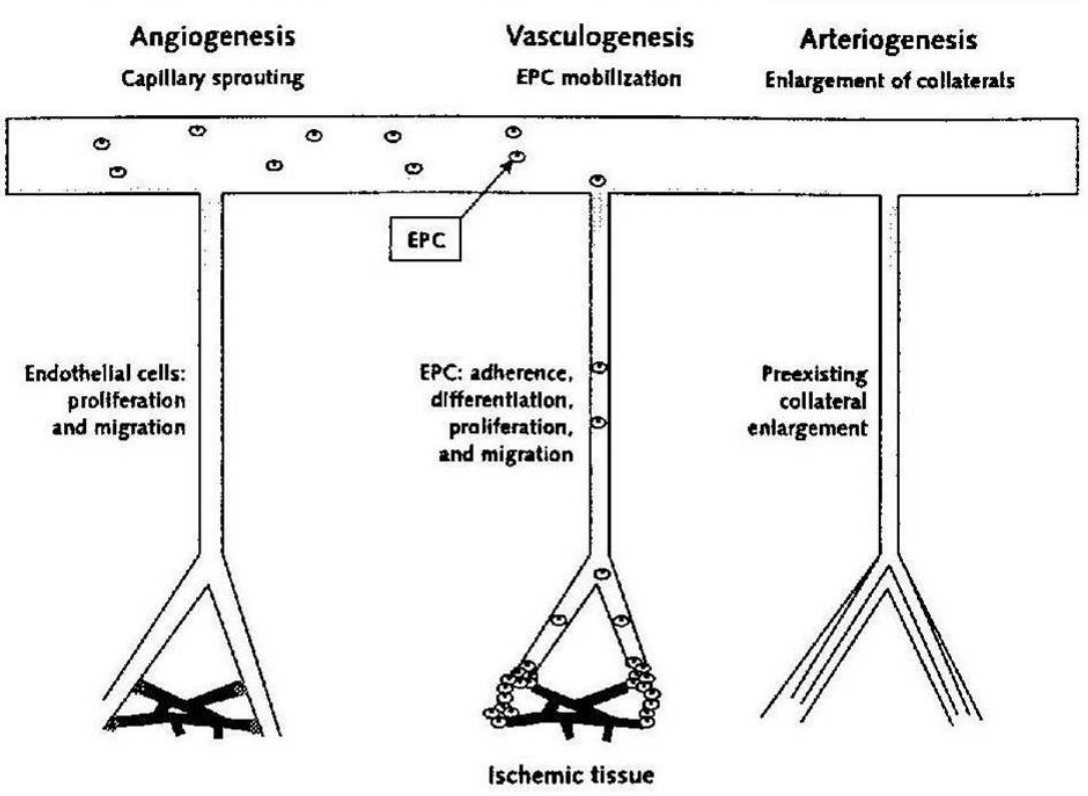


Figure 1.1 Mechanisms of Neovascularization: Angiogenesis involves capillary sprouting by proliferation of fully differentiated endothelial cells. Vasculogenesis involves bone-marrow derived endothelial progenitor cells (EPC), which naturally circulate in peripheral blood, home to areas of ischaemia and incorporate into neovascular foci. Arteriogenesis involves enlargement of pre-existing collateral vessels. Adapted from: Therapeutic angiogenesis for coronary artery disease. Ann Intern Med. 2002;136:55.

1.5.4 Stages involved in new vessel formation

The formation of new tissue requires reliable delivery of an adequate supply of oxygen and nutrients. To achieve new vessel formation, endothelial cells must undergo 4 cardinal steps:

1. Invasion of endothelial cells through the basal lamina surrounding existing blood vessels
2. Migration of endothelial cells towards a source signal, a process likely critically dependent on local cytokine gradients.
3. Endothelial cell proliferation
4. Tube formation

As with most processes in homeostatic cell systems, angiogenesis is under complex regulatory control. Several angiogenic growth factors have been identified, many of which influence all four steps listed above.

1.6 The history of growth-factor research

1.6.1 Tumour-derived growth factors- fibroblast growth factor (FGF)

The presence of tumour-derived growth factors was first suggested by the 'tumour window' experiment of Sandeson and Ide in 1937, where implantation of a tumour in a rabbit's ear promoted a marked angiogenic response.[68] *(fig. 1.2)*

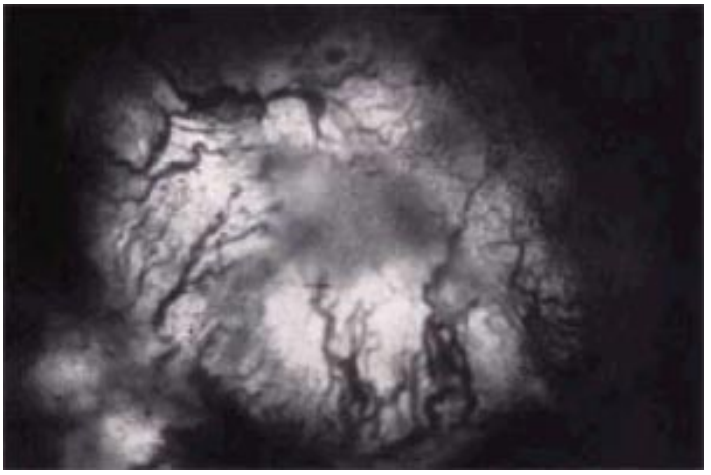


Figure 1.2. Original image reproduced from Ide et al (1939) showing an extensive vascular network in transplanted rabbit epithelioma. Ide AG, Baker NH, Warren SL. Experimental stimulation of new blood vessel growth in the rabbit. Am J Roentgenol. 1939;42:891-5.

Subsequent correlation of tumour size with vessel density hinted at the intimate relationship between tumour growth and angiogenesis. In 1968 Greenblatt and Ehrmann prove the presence of diffusible tumour-released growth factors by interrupting of vessel growth by placing a filter between an implanted tumour and its host.[69] Three years later Judah Folkman isolated a soluble 'tumour angiogenic factor' with mitogenic and tube-forming effects on endothelial cells.[59] Basic Fibroblast growth factor (bFGF) or FGF-2 confirmed Folkman's theory that tumours are dependent on production of growth factor mediated angiogenesis. [70] The fibroblast growth factor (FGF) family of proteins are the most extensively

investigated angiogenic cytokines apart from Vascular endothelial growth factor (VEGF), and the only other protein growth factors to have been investigated in clinical trials. Acidic FGF (FGF-1) and basic FGF (FGF-2) are potent and pleiotropic cell mitogens that act as ligands promoting proliferation of endothelial cells, smooth muscle cells and myoblasts.[71, 72] FGFs bind rapidly to cell-surface heparin sulphate proteoglycans and like VEGF effect intracellular signalling via tyrosine kinase. Like VEGF, FGFs induce endothelial cell production of proteases to facilitate digestion of the extracellular matrix as an initial step in angiogenesis and may act synergistically with VEGF. It is now recognized that there are multiple molecular targets for a wide variety of growth factors and extra-cellular matrix proteins, all of which act in concert to facilitate neovascularization. *(table 1.1)*

	MOLECULAR TARGETS	EFFECTS ON PROGENITOR CELLS
Growth Factors		
VEGF	VEGFR on endothelial cells, monocytes, haematopoietic stem cells	Mobilization, survival, differentiation of EPCs; tube formation
PlGF	VEGFR-1 and 2	Mobilization of EPCs
FGF	FGFR on endothelial cells, SMCs and myoblasts	Stimulates cell proliferation
Angiopoietin-1	Tie-2 receptor expressed on endothelial cells	Vital 'switch' co-ordinating vessel maturation
HGF	c-met receptor on endothelial cells and cardiac myocytes	Attracts tissue-resident cardiac stem cells
IGF	IGF-R on vascular and satellite cells	Skeletal muscle regeneration
Erythropoietin	EPO-R on EPC, endothelial cells haematopoietic stem cells, cardiac myoctes	Mobilizes EPCs, increases cell survival
GM-CSF	Activates monocytes	Mobilizes haematopoietic stem cells and EPCs
MCP-1	Stimulates CCR-2 receptor on monocytes	Attracts EPCs to foci of neovascularization
EC Matrix Proteins		
CCN family	Integrin interaction	Regulates extra-cellular matrix during angiogenesis
Del-1	Regulates homeobox genes and thus integrin binding	Regulates extra-cellular matrix during angiogenesis
Transcription Factors		
HIF-1	Activates gene expression (VEGF, VEGFR-2, EPO, IGF-2, NO synthase, etc	Angiogenic 'masterswitch' co-ordinating angiogenic response to hypoxia

Table1.1. Potential candidates for therapeutic angiogenesis: EPC= Endothelial Progenitor Cells, VEGF= Vascular Endothelial Growth Factor, PlGF= Placenta Growth Factor, HGF= Hepatocyte Growth Factor, GM-CSF= Granulocyte Macrophage Colony Stimulating Factor, MCP-1= Monocyte Chemoattractant protein 1, CCR= Chemokine Receptor, HIF= Hypoxia Inducible Factor, NO= Nitric Oxide. Adapted from Therapeutic Angiogenesis and Vasculogenesis for ischaemic disease: Circulation 2004;109:2488.

1.6.2 Isolation of Vascular Endothelial Growth Factor

A second protein was isolated in 1983 which induced vasodilatation and markedly increased vascular permeability, and was thus called 'vascular permeability factor'.[73, 74] Ferrara and others later identified and in 1989 cloned a specific endothelial cell mitogen which was eventually shown to be identical to 'vascular permeability factor'.[75] This protein was named 'Vascular endothelial growth factor' or VEGF.

The observation that ischaemic areas within glioblastoma multiforme tumours contained significantly higher concentrations of VEGF than non-ischaemic areas led to the realisation in 1992 that hypoxia is a potent environmental trigger for VEGF production and for neovascularization in tumours as well as in adult tissue repair.[76] Around the same time, the intracellular pathway of VEGF induced signal transduction was elucidated with the characterisation of the trans-membrane tyrosine-kinase VEGF receptors VEGFR-1 (FLT1) and VEGFR-2 (KDR). [77]

1.7 Review of peptide growth factors

1.7.1 Vascular Endothelial Growth Factor (VEGF).

VEGF is the most extensively studied of the protein growth factors. It consists of a family of six structurally-related glycoproteins which co-ordinate the growth, maintenance and repair of vascular structures by promoting endothelial cell proliferation, migration and protection from apoptosis.

VEGF was initially described as Vascular Permeability Factor[78] (VPF) or Vasculotropin[79]. Early work in the field of cancer research followed from the realization that growth factors play a central role in the progression of cancers. The importance of VEGF in controlling the regeneration of blood vessels in ischaemic tissues means that manipulation of growth factors may be of clinical relevance to many diseases.

1.7.2 Structure of VEGF

VEGF is a heparin-binding endogenous 46kD homo-dimeric peptide. It is one of a family of peptides (VEGF-A to E) which includes the related placenta growth factor (PLGF). *(table 1.2)*

The VEGF (i.e. VEGF-A) form is by far the most thoroughly studied. It occurs in five isoforms of 121, 145, 165, 189 and 206 amino acids, which are formed by alternative splicing. The protein *in vivo* is found as a glycoprotein, although removal of the carbohydrate moiety does not appear to affect its physiological effects.

VEGF is synthesized in smooth muscle cells and macrophages (amongst others). It is almost exclusively bound by endothelial cells. Importantly however, receptors for VEGF have been detected in human atherosclerotic artery tissue[80]. This suggests that, despite its name, VEGF is not solely active on the endothelial cell and may have effects on other cells within the vascular wall. These effects may include a paradoxical *stimulation* of the atherosclerotic process.

VEGF Family Members	Receptors	Functions
VEGF (VEGF-A)	VEGFR-2,VEGFR-1, neuropilin-1	Angiogenesis Vascular maintenance
VEGF-B	VEGFR-1	Not Established
VEGF-C	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-D	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-E (viral factor)	VEGFR-2	Angiogenesis
PLGF	VEGFR-1, neuropilin-1	Angiogenesis & Inflammation

Table 1.2. VEGF peptides, their receptors and functions.

1.7.3 The VEGF receptors and their ligands

The VEGF receptors span the endothelial cell membrane and incorporate an intra-cellular tyrosine kinase enzyme which is responsible for intra-cellular signal transduction. After binding of the appropriate VEGF ligand, adjacent receptors dimerize by a process of auto-phosphorylation with activation of the tyrosine kinase pathway.[81]

VEGFR 1 and 2 are primarily involved with angiogenesis, with VEGFR-2 predominant in the adult.[77] While VEGFR-2 (flk-1)undoubtedly plays the dominant role in terms of signalling endothelial proliferation, migration and survival, VEGFR-1 does induce the production of lytic enzymes such as tissue-type plasminogen activator and matrix metalloproteinase-9 to aid cellular invasion.[82] VEGFR-3 is associated with lymphangiogenesis. VEGFR-2 is found on almost all endothelial cells, with VEGFR-2 and 3 confined to distinct vascular

beds. The interactions of these receptors and their ligands are shown below (*fig.1.3*)

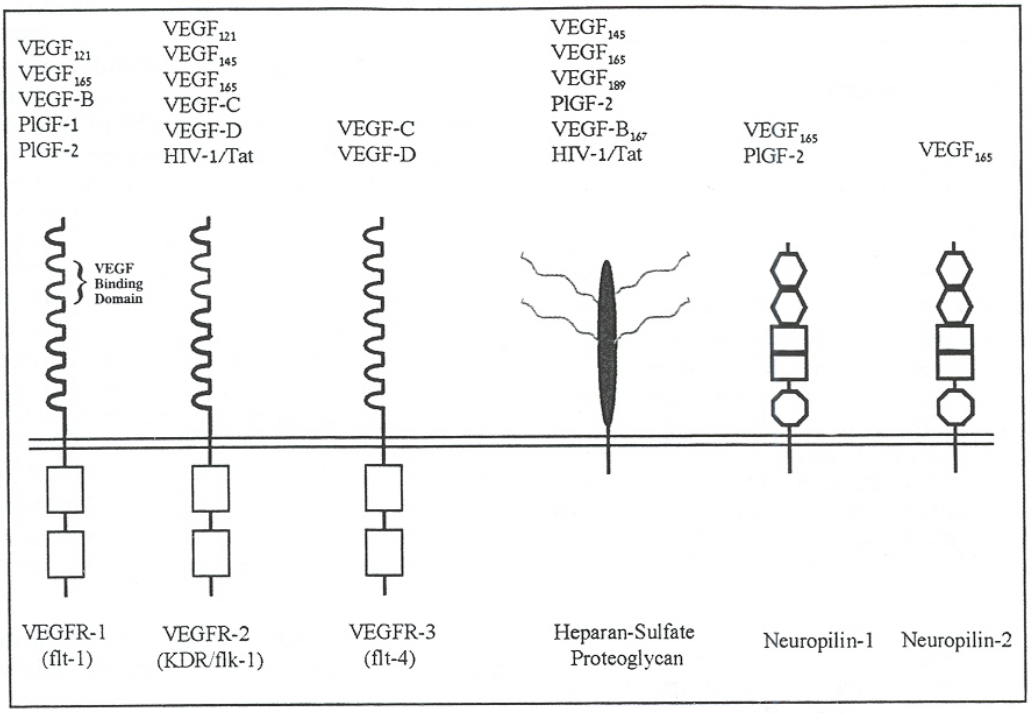


Figure 1.3: The VEGF receptor family and their specific ligands. PlGF is Placental Growth Factor.(adapted from Neufeld et al*, FASEB 13, Jan 1999 p10[81]).*

1.7.4 Actions of VEGF

VEGFs react with a series of different tyrosine kinase receptors to promote several stages important in angiogenesis, lymphangiogenesis and regulation of embryonic cardiovascular development. Angiogenic stimuli are largely regulated through the binding of VEGF-1 to VEGF receptor 2. (VEGFR2).

VEGFs are expressed in cardiac myoctes and vascular smooth muscle cells, and their expression is upregulated by vascular injury, ischaemia and hypoxia.[83] Activation of tyrosine kinase pathways increases local production of nitric oxide, leading to increased vascular permeability, increased endothelial cell growth and survival and formation of tubular structures.

1.7.5 Regulation of VEGF

Chemical conditions, *in vitro* at least, have shown effects on VEGF production in cardiac myocytes. Hypoxia appears to be of importance in the stimulation of VEGF production. VEGF is often found in the relatively ischaemic and hypoxic microenvironment of the interior of a solid tumour. *In vitro*, hypoxia induces endothelial cells to produce VEGF.[84] A transcription promoter for the VEGF gene has been identified that upregulates VEGF synthesis in response to hypoxia-inducible factor (HIF-1)[85]. HIF-1 is also responsible for the upregulation of erythropoietin, emphasising the close functional similarities between the angiogenic and haematopoietic growth factors.

Other stimulatory factors for VEGF production include glucose[86], and the transition metals cobalt and manganese[87]. Reactive oxygen species[88] and oxidised-LDL[89], which promote the progression of atherosclerotic plaques, both potentiate the production of VEGF.

1.8 Related angiogenic pathways

The pro-angiogenic angiopoietin (Ang) family comprises four protein ligands for the endothelial cell tyrosine kinase receptor Tie-2.[90] Ang-1 is primarily involved in developmental angiogenesis and promotes cell sprouting and vessel survival. Ang-2 is pro-angiogenic only in the presence of VEGF and may promote vessel regression in isolation.[91]

Transforming growth factor (TGF) is, like FGF, a pleiotropic cell mitogen. TGF-β has complex effects on tumour growth, initially suppressing cell proliferation but in later stages promoting passage through the cell cycle and thereby angiogenesis. TGF-β is implicated in progression of several chronic inflammatory and fibrotic conditions via its action to stimulate production of extra-cellular matrix.[92]

A number of other growth factors are potential candidates for therapeutic angiogenesis. Hepatocyte growth factor is a potent multifunctional protein acting on endothelial cells and haematopoietic stem cells. Like VEGF, erythropoietin and angiopoietin-1 have been shown to mobilize EPCs from the bone marrow[93, 94]. The combination of hepatocyte growth factor and IGF-1 has been shown to mobilize cardiac stem cells, offering a potential future therapeutic strategy for myocyte regeneration

1.9 Angiogenesis in disease

Much of the interest in manipulation of angiogenic pathways has been in the field of tumour angiogenesis. Angiogenesis however is an important part of many diverse processes include wound healing, the female reproductive cycle, diabetic retinopathy and nephropathy, and rheumatoid arthritis and related inflammatory diseases. The role of hypoxic signalling in ischaemic myocardium and the potential for manipulation of erythropoietin and VEGF levels via the HIF pathway has attracted attention and been the subject of recent reviews.[95]

1.9.2 Limitations in the use of single-protein growth factors

While understanding of the complex systems involved in regulation of angiogenesis has increased, pre-clinical and clinical trials to date have investigated only the use of single-protein growth factors, mainly VEGF or FGF, delivered either as recombinant protein or by means of injected genetic material. More recently this approach has been criticised as naïve, the complexity of neovascularization demanding a more sophisticated and integrated therapeutic approach than simply addition of single growth factors. While these growth factors may control aspects of the process of neovascularization, observers have theorized that they would be unable to co-ordinate the array of stages involved in new vessel growth.

The doubts raised over the potential efficacy of protein growth factor therapy have led to attention being turned to the use of transcription factors which promote angiogenesis by targeting and activating various angiogenic genes. These so-called 'master-switch genes' are known to encode various proteins involved in angiogenesis, and therefore conceptually have a greater likelihood of effecting a co-ordinated pro-angiogenic response by acting at an earlier stage in the angiogenic cascade. Target genes for transcription factors include early growth response protein-1 and hypoxia-inducible factor-1α.[96] Intervention targeting HIF-1α underpins the experimental work in this thesis.

1.9.3 Delivery of peptide growth factors

The use of single-protein growth factors (VEGF and FGF) dominated early trials of therapeutic angiogenesis. Two different approaches to growth factor administration have been pursued: injection of recombinant protein and gene transfer.

1.9.4 Peptide growth factor therapy

Intra-myocardial injection of recombinant protein (eg. VEGF) has the advantages of accurate control the amount of protein delivered, predictable pharmacokinetics and tissue levels, and absence of unexpected side effects. The main drawbacks to protein injection include the short half-life of most proteins and the cost of

recombinant molecules. Attempts to engineer sustained delivery systems have led to the adoption of heparin alginate capsules for the delivery of peri-vascular FGF-2 in surgical angiogenesis trials.[97] In animal studies to date, protein has been administered systemically, and the high plasma concentrations required to achieve adequate myocardial uptake has led to the development of significant adverse effects. These have included tissue oedema and hypotension with VEGF[98, 99] and renal and bone marrow toxicity with FGF[100, 101]

1.9.5 Gene therapy

Gene transfer relies upon the ability of injected genetic material to sustain production of the desired protein in the target tissue. The target cells in effect become 'factories' for the desired angiogenic cytokines. The advantages of gene transfer include prolonged expression of the angiogenic protein, ability to express transcription factors, and ability to express multiple genes simultaneously. The major drawback of gene transfer is the lack of long-term experience with regard to safety of viral vectors, and the potential for adverse effects due to excess production of protein. Potential risks with over-expression of angiogenic protein include local and remote pathological angiogenesis and the possibility of inflammatory reactions to the delivery vector.

Various vectors exist to facilitate gene transfer into cells. When naked DNA comes into contact with the cell membrane, only a small amount enters the cell.

The efficiency of transfection can be increased by using a plasmid or liposomal carrier molecule, but with this approach the duration of trans-gene expression is limited. Much greater transfection efficiency can be achieved by the use of modified adenovirus-type vectors which enter the cell via specific receptors. All such viral vectors are subject to a host antibody response which ultimately limits their use. More recently replication-deficient retroviruses have been used as vectors, although their use is limited to dividing cells and there are safety fears over the accidental production of replication-competent retroviruses. Lentivirus has shown recent promise as a vector able to transfect both diving and quiescent cells.[102]

1.9.6 Methods of delivery

Existing pre-clinical and clinical trials have used a variety of different delivery strategies. Broadly these can be classified as (i) systemic (intravenous), (ii) local or regional infusion (intra-coronary or via peripheral artery in limb studies), (iii) local peri-adventitial delivery (catheter based or at time of surgery) and (iv) intra-muscular (either myocardium or peripheral muscle, via catheter or open surgery). Although much data have been accumulated, in particular regarding the angiogenic potency of single-protein growth factors in animal models, no consensus has been achieved as to the optimal method of growth factor delivery. Wide variations exist in the amount and duration of delivery of the active agents, and in the precise route of delivery. In different studies of myocardial

angiogenesis intra-coronary, epicardial and endocardial injections of growth factors have been used. The variability in methodology between studies has made interpretation of results difficult, especially in the few clinical studies where minor improvements in 'soft' clinical end-points have been claimed.

1.10 Pre-clinical studies with VEGF

VEGF has been shown *in-vitro* and *in-vivo* to have mitogenic effects on the vascular endothelium mediated through the VEGFR-2 receptor.[82] VEGF promotes the process of angiogenesis by direct effects on endothelial cells, and indirectly through the production of matrix metalloproteinases such as interstitial collagenase. In vitro, VEGF has been shown to stimulate this enzyme's production from HUVECs.[103] Blockade of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin receptors by antibodies has largely abolished the angiogenic effects of VEGF.[104]

VEGF is not expressed in detectable quantities in normal arteries, but is expressed following the arterial injury of balloon angioplasty when levels of VEGF correlate with the extent of adventitial angiogenesis in the *vasa vasorum*.[105] VEGF is also detectable in coronary sinus blood samples from patients with complete coronary artery occlusions, whereas VEGF levels in patients with severe stenosis are much lower. It has been postulated that this may represent the natural angiogenic response to an ischaemic stimulus in the total occlusion group and that VEGF may be central to the development of collateral vessels.[106] Increased VEGF

expression has been implicated in neovascularization of atherosclerotic plaques.[107] This raises the possibility that VEGF, including exogenously delivered VEGF, may contribute to an accelerated atherosclerotic process.

1.10.1 Rabbit models

In a rabbit hind-limb model of ischaemia a single intra-arterial bolus of 500-1000μg has been shown to promote collateral growth and improve ankle-brachial blood pressure index.[108] The histological increase in collateral density was associated with increased distal blood flow following the VEGF bolus.[109] These results have been confirmed using a rabbit femoral artery ligation model: collateral density was increased after VEGF was infused into the iliac artery over a 28-day period.[110] Locally delivered VEGF gene therapy in the rabbit hind-limb model has shown similar results.[111]

1.10.2 Porcine models

Significant inter-species differences exist between larger animals with regards to coronary and myocardial anatomy. The most commonly used animal has been the pig, which has few existing arteriolar connections compared with the dog. As a result coronary ligation in the pig tends to produce larger trans-mural infarctions compared to the smaller infarcts seen in dogs. The most common method used to induce myocardial ischaemia is gradual occlusion of an epicardial coronary artery

using an ‘ameroid constrictor’. The constrictor device is a partial steel ring with a central core of ameroid, a gel-like substance which slowly absorbs water. The device is sutured over the coronary artery and gradually occludes the coronary artery by means of external pressure. This technique has the major disadvantages of requiring a thoracotomy with associated surgical morbidity, and of being non-physiological in terms of the histopathological features of the stenosis or occlusion produced.

In one pig study, 2μg VEGF was administered extra-luminally after ameroid constrictor placement to the left circumflex artery (LCx). VEGF animals showed higher blood-flow in the LCx territory.[112] Separately, a high dose of 2mg VEGF was injected into the left coronary ostium 28 days after ameroid constrictor placed around LCx. Half of these animals died due to extreme hypotension, while the survivors showed improved perfusion.[98] In a separate study Yorkshire pigs underwent placement of an ameroid occluder to LCx and three weeks later were randomized to either VEGF (20μg) via an intra-coronary delivery system, intra-coronary bolus infusion, epicardial injection, or intra-coronary saline. All three VEGF treatment groups, but not the control animals, demonstrated a significant increase in collaterals and myocardial bloodflow.[113] Another study in pigs produced a dose-dependent increase in coronary blood flow after a single intra-coronary bolus injection of VEGF, without increase in artery diameter.[114]

In a canine[115] models of chronic coronary ischaemia, VEGF showed enhanced collateral vessel density and blood flow when delivered as a local infusion, although not when administered systemically.[116] VEGF has been implicated in the angiogenic proliferation seen following trans-myocardial laser revascularization (TMR). The effects of TMR are augmented by the addition of VEGF in ischaemic pig hearts.[117] Studies have suggested that the effect of collateral growth may be most marked when VEGF is co-administered with bFGF both *in vitro*[118] and *in vivo*.[119] VEGF has been delivered injecting it directly into ischaemic muscle. VEGF protein injected daily for ten days in high doses showed a dose-dependent improvement of collateral growth in the rabbit model.[120] Intramuscular injections for the plasmid encoding for VEGF show successful transfection of the DNA into striated muscle cells. Synthesis of VEGF was demonstrated for up to two weeks following the procedure despite only 2% of cells having incorporated the plasmid.[121] However, another study demonstrated that injection of an entirely unrelated gene into the myocardium of pigs also caused upregulation of VEGF expression.[122] This raises the possibility that the trauma of injection is itself a stimulus for VEGF production, undermining the scientific rationale for performing the treatment. Other workers have tried to replicate the results obtained in the rabbit hind-limb model of ischaemia and have found that while VEGF plasmid injection did seem to be correlated with increased angiogenesis, no actual VEGF could be detected in the tissues.[123]

1.10.3 Quantification of treatment effect

Quantitation of angiogenesis has been challenging, as new vessels typically measure in the region of 200 μm in diameter and are difficult to visualize at angiography. A number of direct and surrogate measures of angiogenesis have been employed. The effectiveness of angiogenesis has been measured directly either by histological techniques: assessment of the calibre and number of new vessels or immunohistochemistry to quantify endothelial cells markers. More commonly, investigators have attempted to detect surrogate physiological markers of improved myocardial perfusion. Left ventricular contractility has been estimated using simple determination of maximal dp/dt via intra-cavity pressure measurement or by means of standard echocardiography. Dobutamine-stress echocardiography has been used to quantify the extent of ischaemia in the territory supplied by the occluded coronary artery. Finally, measures of coronary blood flow or collateral flow reserve have been possible using a variety of small intra-coronary pressure transducers.

Most published animal studies have reported evidence of enhanced angiogenesis following the intervention. *(table 1.3)*

AGENT	ROUTE OF ADMINISTRATION	ANIMAL	OUTCOME	REF
FGF-2	IC injection	Dog	↑collateral flow	[116, 124-128]
FGF-2	Peri-vascular	Pig	↑ LV function	[129, 130]
FGF-2	Intra-pericardial	Pig	↑ CFR	[131-133]
VEGF165	IC injection	Pig	Neutral	[98]
VEGF165	IC balloon catheter	Pig	Neutral	[134]
VEGF165	Peri-adventitial pump	Pig	Neutral	[135]
VEGF165	Intra-myocardial	Pig	Neutral	[136]
VEGF165	28 days IC	Dog	Neutral	[137]
VEGF165	IV	Dog	Ineffective	[138]

Table 1.3. Pre-clinical trials of recombinant protein growth factors in models of myocardial ischaemia. IC= Intra-coronary, CFR= Coronary collateral flow reserve. Notes: IC injection of FGF-2 in (e) improved CFR with measurable effects persisting for 5 weeks. Previous studies had shown FGF-1 to be ineffective in the dog, possibly due to rapid inactivation.

1.11 Pre-clinical studies of gene therapy

Several pre-clinical studies have investigated the therapeutic use of genes which encode growth factors. Experience with FGF gene transfer is limited to two studies. A single intra-myocardial injection of naked DNA encoding FGF-1 was shown to improve perfusion in a rabbit hind-limb occlusion model[139], and intra-coronary adenoviral transfer of the FGF-5 gene was effective in a porcine model of coronary occlusion.[140] A number of studies have been performed with genes encoding isoforms of VEGF. Intra-myocardial injection appears to produce higher myocardial gene levels and higher VEGF levels than intra-coronary administration, particularly where adenoviral vectors are used. Intra-myocardial injection of plasmid DNA encoding VEGF 165[141], VEGF-2 [142] or adenovirus encoding VEGF 121[143, 144] improved measures of collateral flow and left ventricular contractility in a pig ameroid model of ischaemia. In these studies the intra-myocardial injection of the plasmid DNA was performed via a lateral thoracotomy. More recently intra-myocardial injection of plasmid VEGF 165 has been achieved percutaneously via a catheter and has achieved suitable gene expression[145, 146]

The ameroid constrictor is a surgically-implanted device that exerts external occlusive pressure around an artery as a hygroscopic casein protein gradually expands within a metal casing. *(fig. 1.4)* Crottogini et al. implanted ameroid constrictors at thoracotomy in the left circumflex arteries of 16 pigs to produce

coronary occlusions.[147] After 3 weeks basal perfusion was measured with a nuclear (99mTc sestamibi) scan and the animals were randomised to receive trans-epicardial injection of 3.8mg plasmid VEGF-165 (n=8) or placebo. Five weeks later there was histological evidence of a greater number of small (40-50m diameter) collaterals and evidence of increased regional perfusion on repeat nuclear perfusion scanning. The authors reported this as evidence of an arteriolar angiogenic effect in a large animal model.

Intra-myocardial injection of plasmid encoding VEGF-2 gene has subsequently been investigated in multiple clinical trials.[148]

Figure 1.4. Ameroid constrctor. The device is placed around an artery and gradually occludes it as the hygroscopic casein material within the metal ring swells. Placement around a coronary artery requires surgical thoracotomy.

1.12 Trials of therapeutic angiogenesis in human subjects

Initial attempts to demonstrate proof-of-concept of therapeutic angiogenesis in humans centred on patients with peripheral vascular disease. This patient group was chosen primarily as the peripheries afford easier access for both administration of study drugs and assessment of neovascularization and blood flow. Additionally the lack of effective medical treatment for peripheral arterial disease created a pool of patients where the risk-benefit ratio was favourable for evaluation of a novel treatment. In 1996 Isner and colleagues reported evidence of improved ankle-brachial pressure index and angiographic evidence of new collateral formation in 8 patients with critical limb ischaemia treated with intra-muscular injection of VEGF plasmid DNA.[60, 61, 149] Although this study has been criticised for its small size and inadequate control group, it was widely taken to adequately demonstrate proof-of-concept.

1.12.2 Trials of therapeutic myocardial angiogenesis- VEGF protein

Clinical trials of myocardial angiogenesis in humans have used both protein and gene-based therapy. No large Phase III efficacy trials have as yet been reported. Promising pre-clinical results in the early to mid-1990s led to the investigation of several agents for angiogenesis. Initial clinical Phase I studies of safety and tolerability focused on protein-based therapy and were encouraging with intra-coronary and intravenous infusion of VEGF protein appearing safe, albeit with

limiting dose-dependent hypotension. This effect was predicted and due to the marked effect of VEGF on capillary permeability.

A level of enthusiasm was engendered at this point which perhaps proved detrimental to the subsequent scientific reputation of therapeutic angiogenesis. Although not presented as efficacy studies it was noted that most patients in VEGF dose-escalation studies improved symptomatically, and that their symptom relief was associated with evidence of improved perfusion.[150, 151] Further studies using single-photon emission CT (SPECT)-Sestamibi myocardial perfusion imaging demonstrated a reduction in stress-induced ischaemia and a significant reduction in rest defects.[152] These results did provide objective evidence of improved myocardial perfusion after therapeutic neovascularization, perhaps via recruitment of hibernating myocytes.

Despite editorials from leading investigators urging caution in interpretation of these results, there was great expectation awaiting the results of the first randomised, double-blind, controlled Phase II trial of intra-coronary and intravenous VEGF.[153] The negative results of the VEGF in Ischaemia for Vascular Angiogenesis (VIVA) trial therefore delivered a significant blow to the scientific reputation of therapeutic angiogenesis. A total of 178 patients with non-revascularizable severe symptomatic coronary disease were randomised to low-dose, high-dose or placebo groups with VEGF delivered by an intra-coronary infusion followed by intravenous infusions at three day intervals. After 60 days of

follow-up there was no significant improvement in treadmill exercise time or reported symptoms. At one year there was a non-significant trend towards improvement in angina class in the treatment groups. The VEGF was well tolerated, with no increase in cancer or myocardial infarction. Notably a large placebo effect was demonstrated with significant improvements in all measured indices of function across all three groups, a finding that has been persisted for up to 30 months in several other trials involving patients with end-stage coronary artery disease.[154]

1.12.3 Trials of therapeutic myocardial angiogenesis- FGF-2 protein

Six Phase I studies and one Phase II evaluation of FGF-2 protein have been conducted, all using either direct peri-adventitial or intra-myocardial injection at time of thoracotomy or intra-coronary and intravenous infusions in a manner similar to the VEGF trials. The initial first-in-man study was a randomized placebo-controlled study of 24 patients undergoing CABG surgery with a viable but un-graftable are of myocardium. Peri-adventitial delivery of FGF-2 (10 or 100 mcg) was achieved by implantation of heparin alginate beads which released the agent over 3 weeks.[97, 155] A significant improvement in symptoms and a reduction in ischaemic area using nuclear perfusion and MRI imaging was seen at 90 days between the 100mcg FGF-2 and control groups. However with prolonged follow-up to 16 months the differences became smaller and non-significant as endogenous collateralization occurred.[156]

The next obvious step was percutaneous delivery of FGF-2 to no-option patients as monotherapy. Fifty-two patients deemed unsuitable for conventional revascularization by PCI or CABG surgery received open-label intra-coronary FGF-2 at doses ranging from 0.33mcg/kg to 48mcg/kg. Significant improvements were seen at 6 months in symptomatic and ischaemic parameters including quality of life scores, exercise time, nuclear perfusion assessment of infarct size and wall thickening scores by MRI imaging.[157, 158] Limitations regarding the study design, in particular the lack of a control group and the knowledge of the 'no-option' treatment placebo effect prevented a definitive conclusion from being drawn regarding efficacy. Nevertheless, the physiological data from this Phase I trial were widely taken as confirmation of the potential of therapeutic myocardial angiogenesis. Again hopes and expectations were raised regarding the results of pending Phase II trials.

The FIRST trial was a multi-centre randomized, placebo-controlled, double-blind Phase II trial with striking similarities in design and in particular agent delivery to the VIVA trial with VEGF. Like VIVA this FGF-2 trial produced disappointingly negative results.[131, 159] 337 'no-option' patients were randomized to placebo or 0.3, 3, or 30mcg/kg of single-bolus intra-coronary FGF-2. At three months a uniform placebo effect was seen but no significant difference between the groups in terms of symptoms or myocardial perfusion.

1.12.4 Trials of therapeutic myocardial angiogenesis- Gene therapy

Many more trials have been performed with gene therapy than with isolated injection of VEGF or FGF protein. Clinical evaluation of gene therapy for therapeutic myocardial angiogenesis remains at an early stage, with mixed results to date. Phase I trials began in the late 1990s and largely involved intra-myocardial injection at thoracotomy, with one study employing a novel electromechanical mapping catheter (NOGA system, Biosense-Webster, J&J) to delineate viable from infracted myocardium.[160] These studies have again shown good evidence of tolerability safety, with no excess of malignancy, myocardial infarction or hypersensitivity and only mild inflammatory side-effects related to viral vectors in a few patients. End-points were recorded but without prior formal power calculations and largely concentrated on 'soft' measures such as GTN use.[161, 162] Although some physiological effect was seen following VEGF gene transfer, for example in increased myocardial tissue Doppler velocity[163], it is not possible to draw firm conclusions regarding clinical efficacy from these studies.

The Angiogenic Gene Therapy trial (AGENT) was a Phase I/II double-blind, placebo-controlled trial using intra-coronary infusion of increasing doses of replication defective adenovirus (Ad) containing a human fibroblast growth factor (FGF) gene encoding FGF-4. 79 patients with Canadian cardiovascular society (CCS) class 2 or 3 angina were randomized to placebo (19) or one of five doses of Ad5-FGF4, resulting in small numbers of patients in each group. The primary

end-point of exercise tolerance was not significantly different between groups until stratified to include only those patients most severely limited at baseline (in patients with baseline ETT < 10 minutes improvement was 1.6 versus 0.6 minutes, P=0.01, n=50).[164] The trial was originally planned to recruit 450 patients, but was grossly underpowered for the chosen primary end-point and enrolment was voluntarily terminated after 200 patients when an interim analysis showed no statistical possibility of detecting efficacy.

The AGENT-2 trial recruited 52 patients with stable angina and high (>9%) ischaemic burden on SPECT imaging. This was a randomized, double-blind, placebo-controlled trial of intra-coronary injection of adenoviral particles containing a gene encoding fibroblast growth factor (Ad5FGF-4) in an attempt to determine an objective effect on myocardial perfusion. At eight weeks there was a significant reduction in the resting perfusion defect in the treatment group (from 32.4% to 28.2%, p<0.001) but not among the controls. There was also a trend towards greater reduction in reversible perfusion defect size (4.2% vs. 1.6%, p=0.14) which the authors reported became significant after removal of one outlier. Nonetheless the study was not wholly conclusive.[165]

Recruiting around the same time as AGENT-2, the Kuopio Angiogenesis Trial (KAT) used infusion of either a VEGF plasmid liposome or VEGF adenovirus via an infusion catheter at the time of PCI and reported an increase in myocardial

perfusion in the VEGF adenovirus group compared with control, but no influence on restenosis.[166]

The REVASC study by Stewart et al. was a phase 2 randomized, controlled trial of AdVEGF(121) versus maximum medical treatment.[167] Sixty-seven patients with severe angina due to coronary artery disease and no conventional options for revascularization were randomized to AdVEGF121 gene transfer via mini-thoracotomy or continuation of maximal medical treatment. Exercise time to 1 mm ST-segment depression was significantly increased in the AdVEGF121 group compared to control at 26 weeks (P=0.026), as was total exercise duration and time to moderate angina at weeks 12 and 26, p=0.001. There was however no demonstrable difference in reversible ischaemia on perfusion scanning.

Ripa et al. followed a similar protocol to the REVASC study in 32 randomised patients but with the addition of granulocyte colony stimulating factor to intra-myocardial injection with plasmid VEGF-165. At 3 months there was no difference in myocardial perfusion between the treatment or control groups.[168] While the studies above required a limited thoracotomy to achieve intra-myocardial injections of VEGF plasmid, Fuchs et al. in a 2006 phase 1 pilot study described a catheter based method of endomyocardial injections using a non-fluoroscopic, 3-dimensional mapping and injection (NOGA) catheter-based system.[169] This advance should facilitate recruitment into future studies of intra-

myocardial delivery of new angiogenic agents and means that treatment may be applicable to a wider patient population.

More recently Reilly et al. have reported 2 year follow-up data for 30 consecutive non-randomised patients with intractable severe angina and no revascularization option who received intra-myocardial VEGF 2.[170] These patients underwent direct gene transfer of VEGF naked DNA via limited thoracotomy at total doses of 0.2, 0.8 or 2.0mg. At a mean follow-up of 2 years there were 4 deaths (13.8%), 5 myocardial infarctions (17.2%) and 7 revascularization procedures (24.1%). There were 15 hospitalizations in 12 patients and two new diagnoses of cancer. At the end of follow-up there was an improvement in angina scores with no patients having CCS grade IV symptoms, 3 patients had class III symptoms, and 23 (88.5%) had class I or II symptoms. As there was limited objective assessment made the possibility of a significant placebo effect cannot be discounted. The authors cite the results as a promising response to treatment and are currently undertaking a larger scale randomised clinical trial.

The largest phase II clinical trial to date is the Euroinject trial which randomised 80 patients to percutaneous intra-myocardial plasmid gene transfer of vascular endothelial growth factor (phVEGF-A(165) to assess effects on myocardial perfusion, left ventricular function, and clinical symptoms.[171] 0.5mg of VEGF-165 or control plasmid was targeted via NOGA catheter to the areas of myocardium showing reversible ischaemia on perfusion imaging. After three

months there were no differences in perfusion imaging between the groups. VEGF gene transfer did however improve indices of regional left ventricular wall motion as assessed by NOGA and contrast ventriculography. As evidence of the placebo effect associated with all these trials, exercise duration improved to a similar degree in both groups. Subsequent analysis of the area of impaired perfusion as assessed by NOGA catheter alone (as opposed to the entire myocardium) suggested VEGF treatment improved perfusion compared with control but did not normalize perfusion in the treated areas.[172] The major clinical trials of VEGF and FGF gene therapy are summarized in table 1.4.

AUTHOR	YEAR	AGENT	DESIGN	OUTCOME
Symes JF. et al.	1999	phVEGFA165	PhI open	↑MP
Sylven C. et al.	2001	phVEGFA165	PhI/II open	↓symptoms
Sarkar N. et al.	2001	phVEGFA165	PhI open	↓symptoms
Losardo DW. et al	2002	phVEGF2	Ph1/2 RCT	↓symptoms
Grines C. et al	2002	AdvFGF4	Phase I RCT	↔ ET
Grines C. et al	2003	AdvFGF-4	Phase II RCT	↔ MP overall
Hedman M. et al	2003	phVEGF liposome/ AdvVEGF	Phase II RCT	↑MP (in AdvVEGF group)
Kastrup J. et al.	2005	phVEGFA165	Phase II RCT	↔MP ↑RWM score
Gyongyosi M. et al	2006	phVEGFA165	Phase II RCT	↑MP (in ischaemic myocardium)
Ripa RS. et al	2006	phVEGFA165 +G-CSF	Phase I/II RCT	Safe No MP effect
Fuch S. et al	2006	AdVEGF121 (percutaneous)	Phase I RCT	Safe
Stewart DJ. et al	2006	AdVEGF121	Phase II RCT	↓symptoms ↔MP

Table 1.4. Clinical trials of VEGF and FGF gene therapy. phVEGF= plasmid encoding VEGF. AdvVEGF= adenovirus encoding VEGF. Ph I= Phase I. RCT= Randomimsed clinical trial. MP= Myocardial perfusion. ETT= exercise tolerance (time). RWM score=Regional wall motion score.

1.13 Stem cell therapy as a treatment for ischaemic heart disease

A detailed review of the rapidly expanding field of cellular biological therapies for ischaemia and heart failure is beyond the scope of this chapter. Injection of autologous bone marrow precursor cells has been used in trials on the failing heart in the hope that the pluripotent stem cells will differentiate into functional myoblasts and improve ventricular performance when exposed to local factors. While there have been several such trials in the heart failure population, stem cell therapy as an angiogenic tool has been less extensively investigated. Beeres et al. randomised 20 patients with refractory angina and determined that autologous bone marrow-derived mononuclear cell injection in patients with ischemia is safe, reduces angina symptoms and improves myocardial perfusion.[173] In a similar phase I/II study Losordo et al. showed improvements in 'soft' end-points such as GTN use and exercise time following intra-myocardial injection of CD34-positive autologous stem clls.[174]Most recently a phase II study by Van Romhurst et al. randomized 50 patients to intra-myocardial bone marrow cell injection or placebo and demonstrated a statistically significant but modest improvement in myocardial perfusion.[175] In the near future a strategy of combined stem cell and gene-based therapy may be attempted.

1.14 Discussion of the results of early clinical trials in myocardial angiogenesis

Over a decade of clinical investigation has been performed in the field of myocardial angiogenesis. In-vitro assessment of pro-angiogenic compounds is technically challenging (with in-vitro testing perhaps better suited to assessment of angiogenesis inhibitors) and has often produced inconsistent results, but most agents have been successful in promoting neovascularization in animal models of tissue ischaemia. In-vitro evaluation of pro-angiogenic compounds has proved technically challenging, leading to widespread adoption of in-vivo techniques such as the chick chorionic membrane assay and the sponge-matrigel assay.[176, 177]

Small-scale pilot studies in humans have demonstrated that intra-myocardial delivery of these agents is safe, while increased myocardial perfusion demonstrated in these studies has translated into variable improvements in clinical outcomes in larger-scale Phase II clinical trials. Overall, gene therapy studies (such as Euroinject[171]) have shown some evidence of objective benefit, while protein delivery studies (such as VIVA[153]) on the whole have not.

Many commentators have postulated reasons to explain the disparity between encouraging pre-clinical findings and the disappointing clinical results in Phase II trials such as VIVA.[178, 179] Angiogenesis is a complex process requiring the co-ordinated action of multiple growth factors and modulators and many

commentators have felt it naïve to suppose that a single growth factor could be sufficient to provoke a sustained angiogenic response.

Inadequate delivery and/or persistence of VEGF probably contributed to the negative results of the phase II clinical trials. Recombinant proteins in their native state have a short half-life in-vivo. Simple intra-coronary injection appears to be inadequate: in animal studies only a very small proportion of VEGF administered by intra-coronary injection (<1%) is detected in the myocardium at 1 hour, and this is rapidly degraded over 24 hours.[133] The majority of VEGF and FGF given intravenously is taken up by the liver.[133] Endocardial penetration after intra-pericardial delivery has been shown to be limited, with direct myocardial injection providing the best rates of drug retention, although this amounts to around 20% of the delivered drug after 24 hours.[131] This may of course explain the apparent superiority of viral vectors where a genomic 'protein factory' is administered to provide more persistent elevations in growth factor.

1.14.2 Relevance of pre-clinical models to human physiology

A further important issue to consider when designing pre-clinical studies is selection of an animal model as a surrogate of human atherosclerotic disease. The predominant animal angiogenesis models are murine, rabbit and porcine. The juvenile animals typically used for these experiments are pre-pubertal, normo-lipidaemic and have normal endothelial function. Conversely endothelial

dysfunction is ubiquitous in patients with coronary artery disease and a feature inadequately addressed by most animal models. There is evidence linking endothelial dysfunction with impaired angiogenic response in both humans and in a hyper-cholesterolaemic animal model with induced myocardial ischaemia.[180-182] Endothelial dysfunction has been put forward by some as a potential explanation for the disparity between positive pre-clinical but negative human clinical trials of angiogenic therapy. Small-animal models do however demonstrate that while collateral formation is attenuated by hyper-cholesterolaemia, it may still be successfully augmented, thus offering hope that therapeutic angiogenesis may be similarly achievable in adult humans.[183]

1.14.3 Outcome measures in clinical trials

A further possible explanation for the negative results of clinical studies to date may be that outcome measures were insufficiently sophisticated or sensitive to detect the small changes in myocardial perfusion induced by growth factor therapy. The number of subjects required to generate sufficient statistical power to detect differences in adverse cardiac event rates would be prohibitive, and therefore a combination of quality of life or exercise tolerance measures in combination with measures of myocardial perfusion are the realistic limits of phase III clinical trials. It is likely that a combination of imaging modalities such as magnetic resonance imaging, echocardiographic myocardial contrast perfusion imaging and nuclear techniques will be required to assess future clinical trial

outcomes. While considered ‘soft end-points’, the patient group eligible for therapeutic myocardial angiogenesis will likely be patients with limiting symptoms and no other revascularization option, in whom modest improvements in myocardial perfusion may be of important symptomatic benefit. The presence of a powerful placebo effect, lasting up to 2 years, means that adequately powered, randomized, placebo controlled trials to test efficacy will be essential.[181]

1.15 Steps in the development of a therapeutic angiogenic agent

Figure 1.5. The critical steps in the development of a successful agent for therapeutic angiogenesis are outlined below Adapted from Lee SU et al.

Angiogenesis: Bench to Bedside, Have we Learned Anything. Toxicologic Pathology 2006;34:3-10.

Central to the present thesis is the concept that single-protein growth factors, while capable of promoting angiogenesis, are likely to be inferior to a strategy of 'up-stream' modification of genes which are able to generate multiple growth-factors and co-ordinate numerous steps involved in the angiogenic cascade. This approach, using transcription factor modulators such as our main study drug, Di-methyl oxalyl glycine (DMOG) is as yet untested for myocardial angiogenesis.

1.16 An alternative strategy to the use of peptide growth factors: Hypoxia Inducible factor (HIF) modulation

1.16.1 Metabolic regulation of angiogenesis

Understanding of the mechanisms underlying the co-ordinated control of oxygen delivery to metabolically active tissues has advanced rapidly in recent years. Both hypoxia and oxygen toxicity at cellular level must be avoided, and several organ systems combine to ensure appropriate oxygen delivery. Simple diffusion is inadequate in complex organisms such as humans, where in excess of 10^{14} respiring cells rely upon adequate oxygen homeostasis.

The observation by Krogh in 1919 that capillary density within skeletal muscle varies in proportion to different species basal metabolic rate was the first indication that angiogenesis may be subject to metabolic regulation.[184] Manipulation of environmental oxygen can have obvious physiological effects in humans: retinal blood vessel development is attenuated in premature infants exposed to high levels of inspired oxygen, with subsequent return to normoxic ventilation associated with a risk of blindness due to pathological retinal neovascularization, a condition known as retrolental fibroplasia.[185]

The importance of neovascularization to cancer cells in maintaining oxygen delivery to foci of rapidly dividing malignant cells is well recognised. By virtue of their rapid and chaotic growth such cells are often relatively distant from capillary beds and have been demonstrated to secrete angiogenic factors in response to a hypoxic environment.[59] Similarly a restorative angiogenic response is seen at the edges of wounds, where capillary supply has been interrupted.[186] Initial recognition of a secreted angiogenic factor came from wound-repair research: macrophages are central to this process, and hypoxic culture of macrophages led to the characterization of several angiogenic growth factors. Expression of mRNA encoding platelet-derived growth factor and vascular endothelial growth factor (VEGF) was noted in cell culture experiments under hypoxic conditions.[76] Furthermore similar processes were observed at the hypoxic 'watershed' areas surrounding tumours, suggesting that angiogenesis might be promoted by local tissue hypoxia. [187]

1.16.2 Oxygen sensing in tissues

Evidence for a specific oxygen-sensing system came indirectly from observations of the hormonal control of haemoglobin concentration by the haematopoietic growth-factor erythropoietin (EPO). Unlike the slow time-course of angiogenic responses in-vivo, circulating levels of EPO rise by several hundred fold within hours of hypoxic stimulation, strongly suggesting a specific feedback mechanism rather than merely the influence of basal metabolic processes. The curious finding of apparent occupational polycythaemia in U.S. cobalt miners prompted evaluation of the effects of cobaltous ions, which unlike other non-specific mitochondrial inhibitors were shown to upregulate EPO levels.[188, 189]

In 1992, Semenza and colleagues first described both a 50 nucleotide sequence of the human erythropoietin gene which when stimulated caused a seven-fold upregulation in EPO production, and a nuclear factor which bound to this sequence under hypoxic conditions. The latter peptide was named 'Hypoxia inducible factor' of HIF.[190]. Subsequently it has been shown that transcriptional regulation of not only EPO but a number of key angiogenic growth factors are under linked control mediated by the transcriptional complex HIF-1.[191] Thus angiogenesis is controlled, at least in part, by local oxygen availability. Several excellent recent review articles have covered recent advances in the understanding of the HIF system and its relationship to angiogenesis.[192-196]

HIF-1 is an αβ heterodimer of two proteins, each consisting of a basic helix-loop-helix domains. Three forms of the α subunits of HIF (HIF 1α, HIF 2α and HIF 3α) are encoded in humans by distinct genes. The HIF-1αβ heterodimer binds to core DNA sequences in so-called hypoxia response elements (HREs) of target genes, activating the genes at a transcriptional level. Over 40 genes have been identified that undergo HIF-1 mediated gain-of-function or loss-of-function under hypoxic conditions, and have a functionally-essential HIF-1 binding site in the gene.

The regulation of gene transcription by HIF-1 represents the most highly developed method of oxygen sensing seen in vertebrates. HIF 1β is constitutively expressed, but HIF 1α concentrations rise exponentially as 0_2 levels fall. Oxygen-dependent hydroxylation of distinct proline residues within HIF-α allows interaction with the von Hippel-Lindau (VHL) suppressor, part of a ubiquitin E3 ligase which targets the HIF-α subunits for proteosomal degradation. HIF-α subunits are therefore destroyed by proteolysis in the presence of oxygen. In order to respond rapidly to hypoxia, HIF-1 is constantly synthesized, ubiquitinated and degraded within cells under non-hypoxic conditions. Under hypoxic conditions, the degradation of HIF-1α is inhibited, resulting in accumulation of HIF-1α protein; this promotes dimerization with HIF-1β, binding of this dimer to the HREs of target genes, and activation of gene transcription. HIF therefore acts as a highly responsive tissue oxygen sensor. *(fig. 1.6.)*

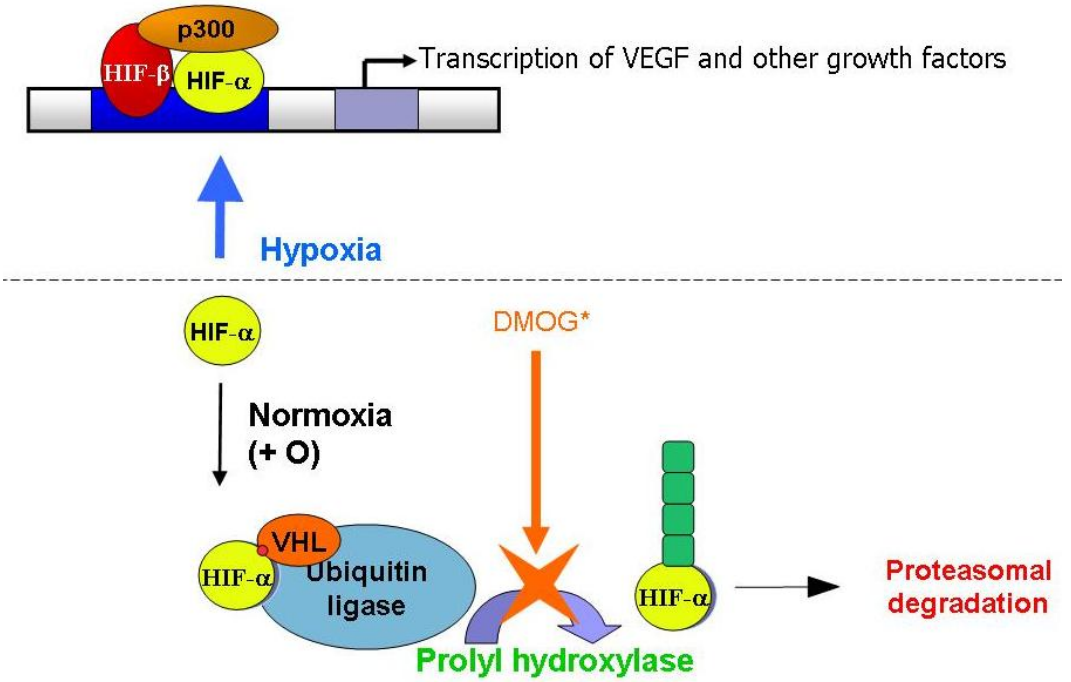


Figure 1.6. Regulation of hypoxia-inducible factor under conditions of varying tissue oxygen concentration. Under conditions of normoxia Prolyl-4-hydroxylase enzymes induce conformational change which 'tags' HIF for proteosomal degradation. VHL= Von Hippel Lindau protein complex. DMOG= di-methyl oxalyl glycine. P300 is a protein transcription co-factor. Image courtesy of Prof. C. Schofield.

1.16.3 HIF homeostasis

HIF-1α functions as an effective and highly responsive 'oxygen sensor' in tissues by virtue of its breakdown being dependent on cellular oxygen concentrations. Prolyl hydroxylation, the signal for proteolytic degradation of HIF, is catalysed by three oxygenases termed PHD1, PHD2 and PHD3 (PHD, prolyl hydroxylase domain enzymes). Recently a fourth related hydroxylase P4H-TM has been described.[197] The HIF hydroxylases are all Fe(II) and 2-oxoglutarate dependent di-oxygenases that require molecular oxygen to function and therefore act as a sensitive oxygen sensor- themselves inactive under hypoxic conditions: hypoxia therefore stabilizes HIF. The HIF-1α prolyl hydroxylases use 0_2 as a substrate with a *K*m that is slightly above atmospheric 0_2 concentration so that enzymatic activity varies across the physiological range of oxygen tension. The Fe(II) at the catalytic centre is readily displaced by other metals, such as cobaltous ions, with loss of catalytic activity and upregulation of HIF: this explains the polycythaemia seen with cobalt and iron chelator exposure in humans.

A further oxygen-dependent hydroxylation reaction (this time of an Asparagine residue in HIF-1α and HIF-2α) catalysed by 'factor-inhibiting HIF' (FIH) inhibits translational activation by interfering with the binding of a co-factor (p300) which promotes transcription. Thus, in the presence of adequate local oxygen, transcriptional activity is inhibited by a dual mechanism involving enhanced

proteosomal degradation of HIF-α, mediated by oxygen-dependent 'tagging' of HIF-α by the PHD enzymes, and by a separate oxygen-dependent inhibition of transcriptional activity. Angiogenic regulation is thus linked directly to metabolic demand and tissue oxygen availability. Other transcriptional pathways, such as that involving p53, are influenced by hypoxia but how these interact with the HIF hydroxylation pathway has not been fully established.[198] Furthermore, hypoxic inhibition of prolyl-hydroxylation appears to function as an oxygen sensor in other metabolic pathways, such as in VHL-dependent ubiquitylation of RNA polymerases.[199] The PHD enzymes may also have a hydroxylase-independent negative feedback action in antagonizing hypoxia-induced endothelial cell proliferation.[200] Recently, the interferons, in particular interferon γ have been shown to regulate transcription of HIF-1α in human endothelial cells by inducing PHD3, thus linking immunity and hypoxia.[201]

1.16.4 Angiogenesis and hypoxia

Angiogenesis is a complex process involving the co-ordinated activity of multiple genes and gene products. These include nitric oxide synthase, growth factors such as VEGF, FGF, angiopoietins and their receptors, and genes involved in maintenance of the extracellular matrix such as matrix metalloproteinases, collagen prolyl hydroxylase and plasminogen activators and inhibitors.[202, 203] Genes for all the above have been shown to be activated by hypoxia. Hypoxia has

also been shown to induce several individual steps important in angiogenesis in cell culture models, including cell migration and endothelial cell tube formation.[29]

The pivotal role of the HIF pathway has been further recognised following cell culture experiments using cells bearing inactivating HIF mutations, while hypoxia response elements for HIF-1α have been identified on several key genes involved in angiogenesis.[204] *(table 1.5)*

In rat cardiomyocytes there is evidence of widespread induction of HIF-1α and HIF-2 α following exposure to environmental hypoxia or coronary arery ligation. There was prolonged transcriptional response to ischaemia sustained over 4 weeks and mediated by HIF.[205]

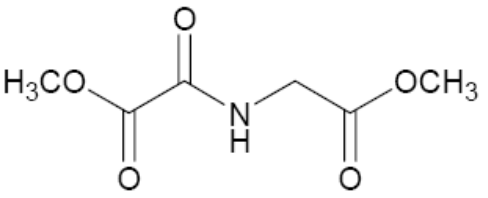
Interventions directed at the wider hypoxic response therefore seem to offer greater hope of therapeutic angiogenesis than simple addition of a single protein growth factor, perhaps by augmenting the natural peri-ischaemic response of collateral vessel formation.

ABCG2 (*STEM CELL MARKER)*	HGTD-P(*PRO-APOPTOTIC MITOCHONDRIAL PROTEIN)*
α1β-Adrenergic receptor	ID2 (*Inhibitor of DNA-binding, promotes progression through cell cycle)*
Aldolase A (*Glycolytic enzyme)*	Integrinβ2 (*Plasma membrane protein)*
Adrenomedullin (*Vasodilator)*	Intestinal trefoil factor (*Promotes epithelial cell migration and tissue repair)*
Atrial Natriuretic Peptide	Lactate dehydrogenase A
Carbonic anhydrase 9	Lactase
CD18 (*Leukocyte Integrin*)	Leptin (*Metabolic regulatory hormone)*
Ceruloplasmin (*Hepatic copper carrier)*	Membrane type-1 matrix metalloproteinase
C-MET (*Proto-oncogene)*	Multi drug-resistance 1 (ABCB1)
Connective tissue growth factor	Myeloid cell-factor 1 (MNL1)
CYP3A6 (*Liver detoxification enzyme)*	Nitric oxide synthetase 2
CXCR4 (*Chemokine receptor)*	NIP 3 (*Pro-apoptotic factor, dimerizes Bcl-2)*
DEC1 (*Female gematogenesis)*	NUR 77 (*Regulates cortisol production)*
DEC2 (*Female gematogenesis)*	P35sj (CITED2) (*Regulates co-activators of HIF-1 gene transcritption: p300/CBP)*
Ecto-5'-nucleotidase (*Intestinal permeability)*	Phosphoglycerate kinase 1
Endocrine gland-derived VEGF (*Endothelial cell mitogen)*	6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase-3 (PFKFB3)
Endoglin (*Part of TGFβ complex on endothelial cells, vascular development)*	6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase-4 (PFKFB4)
Endothelin-1 (*Vasoconstrictor peptide)*	Plasminogen activator 1 (*Serine protease)*
Enolase-1 (*Glycolytic enzyme)*	Procollagen prolyl-4-hydroxylase α(I)
ENOS (*Endothelial Nitric Oxide Synthetase)*	RORα (*Retinoid-related orphan receptor alpha, nuclear receptor, may modulate inflammatory response)*
Erythropoeitin (*Glycoprotien hormone, cytokine for erythrocyte precursors)*	Stromal-derived factor 1 (SDF-1) (*Mobilizes haematopoieic stem cells)*
ETS-1 (*Proto-oncoprotein, involved in regulation of vascular angiogenesis)*	Telomerase (*Regulates cell senescence)*
Glucose transporter 1	Transferrin (*Plasma glycoprotein Iron carrier)*
Glyceraldehyde-3-phosphate dehydrogenase	Transferrin receptor
Glucose regulated protein, 94-kDa (*Implicated in malignant cell transformation)*	Transforming growth factor β (*Control of cellular proliferation and differentiation)*
Haem oxygenase-1	Vascular endothelial growth factor (VEGF)
HIF-1α prolyl hydroxylase PHD2 (EGLN 1) (*Facilitates proteolytic degradation of HIF-1 under hypoxic conditions)*	VEGF receptor-1 (Flt-1) (*tyrosine kinase receptor for VEGFβ,PlGF, regulates cell proliferation, stem cell recruitment and vasculogenesis)*
HIF-1α prolyl hydroxylase PHD3 (EGLN3) (*Facilitates proteolytic degradation of HIF-1 under hypoxic conditions)*	

Table 1.5. Products of genes known to be directly regulated by HIF-1. Adapted from Hirota K, Semenza G. Regulation of angiogenesis by hypoxia-inducible factor 1. Critical reviews in Oncology/Haematology 2006;59:15-26

1.17 Novel angiogenic compounds- Di-methyl oxalyl glycine

A novel compound, d-methyl-oxalyl-glycine (DMOG) effectively upregulates HIF by inhibiting its proteosomal degradation pathway, while also reducing levels of an inhibitor of HIF. DMOG is a small molecule ($C_6H_9NO_5$with molecular weight of only 175.1 *(fig. 1.7)*



PHYSICAL APPEARANCE: Off-white solid (m.p. 46-48°C)

MOLECULAR FORMULA: $C_6H_9NO_5$ MOLECULAR WEIGHT: 175.1

PURITY: 99% (TLC: 10% Methanol/methylene chloride; R_f = 0.71)

SOLUBILITY: Soluble in DMSO (>25 mg/mL) and ethanol (>25 mg/mL)

Figure 1.7 Chemical structure of di-methyl oxalyl glycine (DMOG)

1.17.1 Physiological actions of DMOG

DMOG is a non-selective cell permeable prolyl-4-hydroxlase inhibitor which upregulates HIF activity and stimulates angiogenesis in animal models.[206-208] DMOG also inhibits FIH (Factor Inhibiting HIF), an asparaginyl hydroxylase, which enhances the HIF response.[209] Prior to this current project, DMOG had not been stent-loaded for intra-coronary delivery.

There are theoretical advantages to the use of such a molecule, in terms of its ability to upregulate a 'master-switch' transcription factor and so activate several angiogenic factors The lipid solubility of DMOG can be altered by compounding it with a lipophilic 'tail' to potentially delay elution from any stent-based polymer reservoir.

A major driving force behind the research interest in PHD is the potential for therapeutic targeting in various diseases. PHD inhibitors are the focus of active pre-clinical research in stroke medicine[210] and osteoarthritis[211].

In humans, DMOG has undergone (as yet unpublished) phase I testing as a potential inducer of erythropoietin and thus treatment for renal-failure related anaemia. An orally available PHD inhibitor, FG-2216 has been shown to promote erythropoietin production in rhesus monkeys by upregulating HIF.[212] A related orally active HIF prolyl-hydroxylase (PHD) inhibitor (coded FG-4592) is

currently recruiting for a phase II clinical trial for renal anaemia coordinated by Fibrogen, Inc CA, USA. (Limited US FDA approval, see http://clinicaltrials.gov/ct2/show/NCT00761657?term=fibrogen&rank=1)

More selective molecules in terms of targeting individual prolyl-hydroxlases are in development and may be investigated in future studies. While cell culture models have suggested pro-angiogenic effects of these compounds, there is limited in-vivo experience with these inhibitors.[207, 213] DMOG has the advantage of an evidence base showing effectiveness in promoting vascular growth in the setting of ischaemia which is relevant to the use of this kind of inhibitor in clinical practice.

1.18 Evidence for the biological activity of prolyl 4-hydroxylase inhibitors and HIF modulation in therapeutic angiogenesis

The biological activity of HIF-1α has been confirmed in experiments using non-ischaemic rat cardiac cells where exposure of cells to an adenovirus containing constitutively active HIF-1α led to increases in the expression of genes encoding angiogenic growth factors including VEGF, angiopoietin-1 (ANGPT1), ANGPT2, placental growth factor and platelet-derived growth factor-B.[214] Further cell culture work has confirmed the ability of DMOG to induce stabilization of both HIF-1 α and HIF-2 α proteins and induce downstream gene expression.[215] These findings support the concept of HIF as a 'master regulator' of angiogenesis.

Several approaches have been used in an attempt to stabilize HIF and promote angiogenesis. Evidence for the developmental importance of HIF includes early embryonic lethality in a transgenic mouse strain homozygous for a null allele at the HIF-1α locus, associated with failed vascularization.[204] Conversely, transgenic expression of constitutively active HIF-1α molecule can be produced by deletion of the oxygen-dependent degradation domain. Expression of such a molecule in the skin of mice results in marked neo-vascularization, with capillaries that unlike those formed under the influence of exogenous VEGF are not excessively leaky.[216] This suggests that HIF upregulates the expression of several critical angiogenic regulators in addition to VEGF and thus may invoke a therapeutic response more closely approximating that seen in ischaemic tissue.

An alternative approach has been gene therapy: fusion of the N-terminal DNA binding and dimerization domain of HIF-1α to the trans-activation domain of a herpes simplex virus as gene therapy. Administration of this naked DNA provoked collateral vessel growth and blood supply in a rabbit hind-limb model of ischaemia, and in a rat myocardial infarction model.[217, 218]

Further methods of stabilizing HIF-1α have included attempts to block its degradation. This has been attempted with a number of different molecules. A macrophage-derived peptide, PR39, has been shown to interfere with proteosomal activity and increase peri-infarct neovascularization in a murine cardiac model.[219]

A similar approach has used over-expression of peptides to competitively inhibit the VHL-binding prolyl hydroxylation sites of HIF to stabilize the molecule and promote angiogenesis.[220]

Normoxic HIF-1 preservation has been achieved in murine models using small interfering RNA (si-RNA) to inhibit PHD2. Wild-type mice infused with PHD2 si-RNA showed increased levels of HIF-1 and nitric oxide synthase (iNOS), and demonstrated smaller infarct size in an ex-vivo ischaemia-reperfusion model using isolated Langendorff-perfused hearts, possibly via a preconditioning effect of HIF-1.[221]

Systemic administration of DMOG has been used in rabbit and murine models. Ockaili et al. demonstrated downstream activation of the haem-oxygenase pathway due to upregulation of HIF-1 in rabbits treated with large systemic doses (20mg/kg) of DMOG. In the same study DMOG was associated with a reduction in infarct size following in-vivo coronary ligation and with attenuated pro-inflammatory chemokine production (IL-8) following induction of inschaemia.[222] Milkiewicz et al. demonstrated similar findings in a murine femoral artery ligation experiment, with increased expression of HIF-1 in association with increased capillary density following systemic administration of DMOG (20mg/kg). Of particular relevance to the present study, increased neovascularization, HIF upregulation, and expression of the VEGF Flk-1 receptor were seen to a greater

extent in the presence of both DMOG and ischaemia than with either DMOG or ischaemia alone.[208]

The systemic effects of DMOG have not been fully investigated but any potential human treatment would seek to minimize systemic exposure. In animal studies toxicity has not been manifest when concentrations of systemic DMOG in the millimolar range have been given. Potential exists for adverse effects however given the importance of oxo-glutarate in intermediary metabolism. All PHDs are potential tumour suppressors and therefore concern has been expressed as to the possibility of a pro-malignant effect from long-term exposure to PHD inhibitors. The existence of multiple PHDs however means it may be possible to exploit differential tissue expression profiles and HIF-specificity to achieve the best therapeutic outcomes while minimizing side effects.

1.19 Angiogenesis models

1.19.1 Endothelial cell culture

Endothelial cells exhibit considerable genetic, phenotypic and functional heterogeneity depending on the tissue in which they are found.[223] The ideal source of endothelial cells for culture in experiments designed to test the behaviour of microvessels is therefore capillary endothelial cells form the tissue of interest. In practical terms this presents numerous problems. Techniques have been

developed to isolate endothelial cells form capillaries employing mechanical disruption of the tissue followed by enzymatic digestion of cells from the matrix by means of collagenase, trypsin or similar enzymes.[224] Purification steps using gradient density centrifugation or mesh filters are then required to obtain a pure colony of endothelial cells.[225] Alternatively more complex but effective techniques for obtaining capillary endothelial cells include immunological separation techniques using antibody-linked paramagnetic beads or fluorescence activated cell sorting. Commonly used antigens include CD31, CD36, factor VIII-related antigen, CD146 and VCAM-1.[226, 227]

1.19.2 Human umbilical vein endothelial cell culture

Perhaps the most extensively studied source of endothelial cells are those form human umbilical veins (HUVECs). This is largely due to the ease of collection of cells which are readily isolated by perfusion of the vein with trypsin.[228]. Primary endothelial cells have a limited number of replication cycles prior to senescence and a process of immortalization is required to produce multiple cell lines and allow prolonged cell culture.[229] Several 'immortalized' cell lines, such as HMEC-1 are commercially available. Even with the use of specialized cell lines HUVEC phenotype tends to alter over time in culture and it is preferable to use early-passage number cells whenever possible. Phenotypic drift is especially relevant in analysis of gene expression but can also affect functional assays.[230] Gene expression studies overall show that exposure of cultured cells to growth factors

such as VEGF results in upregulation of genes involved in the cell cycle, apoptosis and metabolism.[231]

1.20 Types of angiogenesis assay: In-vivo, ex-vivo and in-vitro

Much research effort has been expended over the last 30 years in the search for an ideal angiogenesis assay. Such an assay would be cheap, widely applicable, reproducible and readily adapted for different applications. The field of angiogenesis research however encompasses two extremes in terms of desired outcome however: inhibition of angiogenesis and testing of anti-angiogenic therapies and attempts to promote neovascularization. Many more assays are optimized for the testing of anti-angiogenic than pro-angiogenic compounds. Given the characteristic of endothelial cells to exhibit plasticity depending on their environment, the ideal assay would be an in-vivo one, where the role of subtle micro-environmental stimuli (such as growth factor gradients) and the function of pericytes, stromal cells and epithelial cells could be assessed.

However in-vivo assays are expensive and *in-vitro* assays, while deficient in many of the above regards allow rapid, large-scale screening of compounds. In-vitro assays either measure cellular proliferation, measure migration of cells through artificial barriers in response to growth factors, or attempt to quantify the early stages of cellular differentiation into vessels (tube forming assays). Complex in-vitro assays have also been developed to examine component parts of

the angiogenic process, rather than quantify magnitude of overall angiogenic effect. These include experiments to elucidate membrane and intracellular signalling events and endothelial-mural interactions. Many of these latter assays utilize cells from transgenic animals expressing endothelial reporter genes and involve co-culture with mural cells.[232] A third group of *ex-vivo* vessel-outgrowth assays employ tissue or organ culture of intact tissue such as mouse, rat, chick or pig aorta in an attempt to more closely approximate in-vivo conditions.

Several in-vivo models have been developed, either employing sub-cutaneous implantation of a scaffolding compound (such as in the 'sponge-matrigel' assay) [233, 234] or exploiting a physical characteristic of a particular organism. In the latter category are the chick chorioallantoic membrane (CAM) assay,[235] the murine dorsal air sac model,[236] the corneal angiogenesis model [237]and the numerous models developed using the zebrafish, *Danio rerio*. All the in-vivo models largely rely upon easily visualization of new vessels by light or fluorescent microscopy as a result of tissue transparency. Some examples of each type of assay, relevant to the methods in chapter 2, are outlined below.

1.21 Zebrafish in angiogenesis research

The zebrafish, *Danio Rerio,* has several features which make it particularly suited to its role as a model for study of cardiovascular development. We investigated the zebrafish as a potential bio-assay for DMOG (section 2.9.9.) It is a teleost that

is capable of producing hundreds of offspring per mating. Embryos develop rapidly and are transparent, allowing easy visualization of the vasculature by light microscopy. As a result of their small size (c.3mm long at 5 days post fertilization) embryos receive enough oxygen via passive diffusion from water to survive for several days without a functional cardiovascular system, thus facilitating the study of genetic mutants. The zebrafish is unique in that aspects of both vasculogenesis and angiogenesis can be studied over several days. The differentiation and migration of angioblasts from axial vessels along the trunk is considered vasculogenesis while angiogenesis is represented by development of segmental vessels.

Various techniques have been described to aid visualization of the vasculature. These range from the simple such as intravascular injection of dyes or resin casting of the vascular tree to more sophisticated techniques employing immunohistochemistry and transgenic animals. Fluorescent microscopy after injection of fluorescein into early-stage embryos has allowed lineage tracing experiments and identification of the lateral plate mesoderm as a major site of vascular progenitors, as it is in other vertebrates.[238] Confocal micro-angiography using intravascular injection of fluorescent micro-spheres allows 3-dimensional rendering of the entire vascular tree.[239]

Vascular hybridization markers in the zebrafish include pan-endothelial gene markers such as Fli-1, Flk-1, Tie1, Tie2 and VE-cadherin; artery-specific markers

such as Ephrin B2, Gridlock (Grl), and notch-5; and vein-restricted marker Ephrin B4.[240-242] The location and function of these genes are similar to those seen in other vertebrate models.[243] As in the mouse, knock-down experiments in the zebrafish have confirmed the crucial role of VEGF-A in vascular development. Using morpholino-modified oligonucleotides a dose-dependent effect of VEGF was noted, with low levels of VEGF resulting in a lack of all vessels except the heart and yolk sac vessels, and moderate decreases abolishing segmental but not axial vessel development.[244] The functional similarity between zebrafish and mammalian cell markers such as the plexin family of cell-guidance receptors further demonstrates the effectiveness of the zebrafish as a model for mammalian vascular development.[245]

1.21.2 Fluorescent transgenic zebrafish model

Real-time in-vivo imaging of vascular gene expression is possible in the zebrafish by the use of transgenic lines expressing green fluorescent protein (GFP). Several zebrafish lines have been developed which express enhanced GFP (eGFP) in developing vasculature and allow imaging of vessels over extended periods of time.[246, 247]

The zebrafish offers the only vertebrate model that allows high-throughput mutagenesis screening for the purpose of identifying genes involved in vasculogenesis. Several mutations have been created which are specific to the

cardiovascular system. These include gridlock (Grl), heart of glass (heg), out of bounds (obd) and cloche (clo).[248-250] These mutants express a range of phenotypes varying from almost complete loss of endothelial and haematopoeic systems (clo) to derangement of inter-segmental vessels (obd). Cloning of these genes and their products has lead to elucidation of new pathways of vascular development.

1.21.3 Gridlock mutant zebrafish

Perhaps the most exciting potential role for the zebrafish is the use of transgenic mutants in the screening of small-molecules and peptides with a view to development of novel therapies for cardiovascular or malignant disease. Gridlock (grl) mutants lack any aortic or trunk blood flow due to lack of a gene analogous to the mammalian hey2 gene. Compounds have been screened against the grl phenotype and a small number found capable of rescuing the phenotype via increased VEGF signalling.[251]

1.22 Ex-vivo vessel outgrowth cell-culture assays

The *ex-vivo* organ culture assay forms a bridge between complex and expensive in-vivo animal models and in-vitro studies. The development of these models represents the realisation that it is impossible to accurately replicate the complex interactions between endothelial cells and their surrounding environment by means of an in-vitro assay. Ex-vivo assays are diverse in form and represent

various stages of angiogenesis from simple assays of cell proliferation through assays of migration to those examining microvessel formations, the latter involving culture of tissue explants in a 3-dimensional matrix for 7-14 days. Several different tissues have been used from several species, all with the aim of producing a reproducible high-output assay for the screening of compounds affecting angiogenesis. As with other types of angiogenesis assay it is generally more straightforward to produce reproducible assays for testing of angiogenesis inhibitors as these do not rely on stimulation of supra-maximal vessel outgrowth.

SPECIES	ORGAN TYPE	ADVANTAGES	DISADVANTAGES
Rat	Aorta	Easy to handle	Large vessel, variation between species
	Inferior vena cava	Good microvessel sprouting	Difficult to handle
Mouse	Aorta	Ability to study transgene function	Large vessel
Chick	Embryonic aorta arch	Microvascular tissue Rapid assay	Need growing embryos
Human	Placental blood vessel	Species specific data	Limited supply
	Saphenous veins	Species specific data	Heterogenous donor characteristics
Porcine	Carotid	Good supply, rapid assay	Large vessel

Table 1.6 Types of ex-vivo vessel-outgrowth (organ culture) models. Adapted from Angiogenesis Assays, Staton C ed, Wiley Publishing.[252]

1.22.2 The rat aortic ring assay

This assay was originally devised by Nicosia [253] and involves prolonged culture of isolated rat aorta cut into 1mm cross-sections and embedded in a sandwich of matrix along with a basal medium optimized for microvascular endothelial cells. Microvessel outgrowth is stimulated by injury caused during the dissection process and mediated by endogenous growth factors produced from the aorta, however microvessel proliferation is minimal in the absence of exogenous sera or growth factors. Obtaining uniform sections is important in standardizing vessel outgrowth and interpretation of results, and harvesting of tissue needs to be performed in strictly aseptic conditions to prevent culture infection. Air-borne fungal infection is a particular problem with organ culture models.[254]

1.22.3 Selection of matrix material

Several substances have been tried to simulate the properties of the extra-cellular matrix, and the effects of these on microvessel outgrowth have been assessed using tissue culture models. Matrix-replacement substances include plasma clot, type I and type IV collagen, and matrix matrigel, a solubulized basement membrane preparation. Microvessel growth-curves have been determined for each of the above. Collagen promotes vessel outgrowth in a dose-dependent manner with growth maximal at around 7 days before regressing. [255] Matrigel has become the most widely used matrix material and has the advantage of allowing shorter

culture times with micro-vessels formation apparent after 5 days. Sprouts have been shown to stain positive for factor VIII-related antigen confirming endothelial origin. Several reports of microvessel ultra-structure using this assay report changes consistent with lumen formation.[256, 257] Other related organ culture assays include the mouse aorta assay which allows assessment of the gene function in transgenic mice, with vessel formation being shown to be dependent on the genetic background of the animal.[258] The more recent chick aortic arch assay has the advantage of producing rapid microvessel outgrowth in low-serum conditions.[259]

1.22.4 Matrigel

Matrigel (BD Matrigel™). is the trade name for a gelatinous protein mixture secreted by Englebreth-Holm-Swarm mouse sarcoma cells, a tumor rich in ECM proteins. The preparation is liquid below (4°C) and rapidly polymerizes to form a gel at room temperature. The gel is designed to resemble mammalian basement membrane and consists of laminin, collagen IV, heparin sulphate proteoglycans and enactin.[260] These structural proteins provide cells with adhesive peptide sequences to aid proliferation and migration, and three-dimensional cell behaviour can be observed within thicker layers of gel. As such matrigel provides a good surface for the attachment and differentiation of a number of anchorage-dependant cells, including endothelial cells, smooth muscle cells and fibroblasts. The major components of matrigel are laminin, collagen IV, heparin sulphate

proteoglycans, entactin and nidogen.[261] Matrigel contains small concentrations of a number of growth factors including Platelet-derived growth factor, Insulin-like growth factor-1 and transforming growth factor-β. [262] While collagen has been used in tissue-culture previously, matrigel is well suited to use in angiogenesis assays. The basement membrane is an important mediator of angiogenesis, and Matrigel has been successfully used in cell-culture based angiogenesis assay using human umbilical vein endothelial cells (HUVECs) and tissue-culture assays.[263, 264] In order to provide better modeling of the effect of exogenous pro-angiogenic factors, a reduced growth-factor (RGF) matrigel has recently become available with around half the concentration of growth factors compared with standard matrigel. Notably some non-endothelial cells including fibroblasts can form tubules on matrigel which means caution is required in interpreting organ-culture results.[265]

Amounts of Growth Factors (GF) Present in BD Matrigel™ Matrix vs. GFR BD Matrigel™ Matrix			
Growth Factor	Range of GF Concentration in BD Matrigel Matrix	Average GF Concentration in BD Matrigel Matrix	Typical GF Concentration in GFR BD Matrigel Matrix
EGF	0.5-1.3 ng/ml	0.7 ng/ml	< 0.5 ng/ml
bFGF	< 0.1-0.2 pg/ml	n.a.*	n.d.**
NGF	< 0.2 ng/ml	n.a.*	< 0.2 ng/ml
PDGF	5-48 pg/ml	12 pg/ml	< 5 pg/ml
IGF-1	11-24 ng/ml	16 ng/ml	5 ng/ml
TGF-β	1.7-4.7 ng/ml	2.3 ng/ml	1.7 ng/ml
** n.a. - not applicable **n.d. - not determined*			

Table 1.7 Matrigel product information, communication from Becton Dickinson

1.22.5 The porcine carotid artery assay

Stiffey-Wilusz et al. in 2001 described a modification of the rat aortic ring assay using commercially-sourced porcine carotid tissue.[266] The group tested a number of aortic and venous tissues and determined that porcine aortas most closely approximated human aortic tissue and gave the best combination of robustness of growth, size, ease of handling and reproducibility of results. The artery was splayed open and cut by hand into 1.5mm square sections before being sandwiched between layers of matrigel matrix and cultured in an endothelial cell growth medium containing 2% serum. Optimal sprouting was observed at 12-14 days. The assay shows promise as a high-throughput screening test, but it is important to note that the authors describe the effects only of inhibitors of angiogenesis on microvessel sprouting, and the capacity of the assay to discriminate the effects of the addition of exogenous growth factors on microvessel growth remained untested.

1.22.6 Applications of whole or partial vessel outgrowth assays

Organ culture models are widely used to screen potential anti-angiogenic compounds, and have been used to detect the presence of both anti- and pro-angiogenic compounds in serum and plasma.[267] Furthermore the rat-aortic ring assay has been used as a bio-assay for anti-angiogenic agents.[268] An advantage of organ culture assays over cell-based techniques is that they more closely replicate

in-vivo angiogenesis as triggering of micro-vessel sprouting occurs from essentially quiescent endothelial cells, rather than those that are actively proliferating in culture and are modified after repeated passages.[269] The assays also benefit from the presence of mural cells such as pericytes and smooth muscle cells with a surrounding matrix. Drawbacks to organ culture include technical difficulty in maintaining contaminant free culture over the prolonged period of culture required, and the use of large vessels rather than more physiologically appropriate microvessels.

1.23 In-vitro- tubule formation assays

In-vitro cell-based assays are the most common type of angiogenesis assay used to screen angiogenesis inhibitors or stimulators. These assays are considered to represent the later stages of cellular differentiation during angiogenesis. Human umbilical vein endothelial cells (HUVECs) are widely used as they are readily available on a commercial basis and have relatively low maintenance costs. While primary cell lines exhibit senescence after around 10 passages, the use of immortalized cell lines has largely circumvented this problem at the expense of some alteration of cellular characteristics.[230] However in vivo, angiogenesis only occurs in microvascular cells. Human (dermal) microvascular endothelial cells, or H(D)MECs, are more expensive to purchase but in theory offer greater physiological authenticity.

In-vitro tubule formation assays utilize the fact that endothelial cells are capable of differentiation into a network of tubules which resemble a capillary network when cultured on matrigel, fibrin or some collagens. Endothelial cells of all types observe 'cobblestone' morphology in culture and are capable of forming tubules in vitro if allowed long enough to allow them to lay down extracellular matrix components.[270] The degree of tubule formation is dependent on the concentration of growth factors present and thus tubule formation assays have become widely used to assay angiogenic stimulating or inhibiting compounds. Experiments in the early 1980s determined that endothelial cells are capable of laying down ECM components and forming tube-like structures after prolonged culture. By depriving the cells of endothelial cell growth factor (ECGF), growing on a fibronectin matrix and adding proteases, the rate of tubule-formation could be greatly increased, analogous to the pivotal role of proteases in early in-vivo angiogenesis.[271]

1.23.2 Tubule formation on matrigel

Once plated onto matrigel endothelial cells produce dynamic cellular processes and migrate towards each other in a phagocytic manner to form capillary-like cords. Tight-junctions have been noted between these aggregates of cells. [272] Over time these structures appear to develop a central lumen, using actin polymerization and myosin-dependent contractility to differentiate into tubules.[273] There is disparity among authors as to how similar these tubules are to capillaries.

Some contend that the lumen forms by central apoptosis while others describe a process of cellular 'folding' to produce a lumen.[274] Electron microscopy has been used to prove the presence of lumina [273, 275] although others dispute the presence of lumen in cellular aggregates in culture.[276] Apoptosis is accepted by most authorities as a central step in lumen formation, with tubule formation inhibited by caspase.[277] Tubule formation on matrigel is not inhibited by alkylating drugs indicating that neither transcriptional or translational events are required, however the inhibitory effects of a protein kinase C inhibitor (H7), a microtubule inhibitor (nocodazole) and general growth factor receptor inhibitors (suramin) suggest that post-translational mechanisms are important.[278] Tubule formation on matrigel is thought to be controlled by differential gene expression, while the mechanism of tubule formation appears to vary depending on the matrix substrate.[279]

1.23.3 Matrigel formulations and methods

There is no clear consensus on methodology when using matrigel. Factors influencing the extent of microvessel formation include the type and volume of matrix material, cell type and the density at which cells are plated onto the wells.

Reduced growth factor (RGF) matrigel has recently become available with markedly reduced levels of stimulatory growth factors and cytokines. Use of GFR matrigel allows for more selective determination of the efficacy of pro-angiogenic compounds. A comparison of different matrix materials confirms that GFR-matrigel promotes slower and less extensive tubule formation than matrigel, and

importantly also confirms that addition of VEGF increased tubule length and total area only in the RGF-matrigel system and not in the standard matrigel assay.[265] This result underscores the importance of using RGF-matrigel when testing pro-angiogenic compounds. The precise amount of matrigel added to each well and the density of cells plated per well both strongly influence the formation of tubules, and thus reproducible results are highly dependent on experimental technique with a moderately long learning curve with regards to culture methods.[269, 280]

1.23.4 Co-culture assays

Assays have been developed in an attempt to merge the advantages of an ex-vivo assay in terms of physiological relevance with the convenience of a cell-based assay. Co-culture of endothelial cells with mural cells such as fibroblasts and smooth muscle cells offers some advantages of both systems with mural cells contributing to the ECM. Such co-culture systems appear to produce tubules with lumens and a phenotype closer to capillaries than that seen with endothelial culture but require longer durations of culture to produce tubules, often up to 14 days.[265, 281] The utility of this system in the screening of angiogenesis-modifying compounds has not been ascertained, nor have the complex relationships between the cells in culture.

1.23.5 Quantification of tubule formation: in ex-vivo tissue culture assays

Quantification of vessel outgrowth is challenging and must be standardized for each assay. The lack of a consensus method makes comparison of results between groups more difficult. The general concept is to derive a measure of vessel length and/or area. The relative merits of different quantification techniques in the tissue culture angiogenesis assay are considered further in section 2.2.7.

1.23.6 Quantification of tubule formation: in-vitro cell-based assays

While cell-based assays allow easy visualization of tubules with a standard inverted light microscope, difficulties arise in trying to obtain standardized data on the extent of tubule formation. Light microscopy allows clear detection of tubules against the background matrix, although some groups use either H&E staining or immunohistochemical techniques with CD31or vWF antibody stains to improve contrast.[282] Off-line digital image analysis is preferable, and the use of 96-well plates allows a single digital photograph to capture the entire well.[269] The five variables that are used in varying combinations to assess extent of tubule formation are total tubule length, number of tubules, average tubule length, number of confluence points, and total tubule area.

Measurement of total tubule length is largely delegated to automated image analysis software packages which vary in their accuracy in differentiating tubules

from the background. Similarly publications have depended solely on tubule number which is perhaps easier to measure by hand.[283] Tubule area lends itself well to semi-automated analysis, however assumes that all cells within the area outlined are differentiated, and is a two-dimensional measure of three-dimensional sprout outgrowths. Furthermore total area alone is inaccurate as in some circumstances area coverage can decrease with increasing angiogenesis and differentiation of cellular cords into more mature tubules. This phenomenon has been demonstrated with HDMEC culture under the influence of VEGF.[284]

Inherent sources of error exist within image analysis programs themselves and relate largely to the process of 'thresholding' by which the programme attempts to differentiate tubules from a grey-scale background. Several programs appear in the literature including freeware such as Image J (Stanford University).[285] In the absence of a consensus the best compromise is to use an automated technique such as area detection in concert with a manual measure, such as tubule length or number of confluence points or nodes. The latter has been advocated as a measure of angiogenic differentiation into tubules.[286]

1.24 Coronary stents as a delivery system for angiogenic compounds

1.24.1 Polymer coated stents

Polymers have been used extensively since the advent of drug-eluting stents as the delivery vehicle for a variety of compounds. Initially the intention was that polymers would lend greater biocompatibility for metal stents by providing an inert non-thrombogenic covering. Development of biocompatible polymers proved challenging however and polymers are now primarily used as a reservoir for drug or molecules. This is the concept of the stent as a local drug delivery device for the endothelium.

In recent years polymer-coated drug-eluting stents have been implicated in hypersensitivity reactions and coronary thrombosis although no direct causal link to polymer has been made.[287, 288] Recent multiple meta-analyses however suggest that polymer-based DES are safe in general clinical use.[289, 290]

1.24.2 Polymer biocompatibility

In vivo assessment of new polymers is essential, as the polymer will usually interact and stimulate components of the coagulation cascade, increasing the risk of thrombosis. Hypersensitivity reactions or low-grade inflammatory responses increase the likelihood of clinically significant restenosis.[291] Polymers, including

polyurethane and polytetrafluoroethane, which have appeared to be inert and biocompatible *in vitro* have subsequently induced significant inflammatory response and neointimal hyperplasia in vitro.[292]

Perhaps the polymer in widest clinical use is phosphorylcholine, a phospholipid that is naturally found as part of the cell membrane bilayer. Phosphorylcholine-coated stents initially proved to be biologically neutral in rabbits and pigs, but showed a neutral effect on restenosis at six months[293]. Other polymer coatings including fluorine-acryl-styrene-urethane-silicone and polyamine/dextran sulphate have shown promise, not just *in vitro* but *in vivo*, with no increase in thrombosis or intimal hyperplasia in animal studies.[294, 295] Heparin-adsorbed polymer surfaces have long been known to reduce the adhesion of platelets.[296] A heparin-bound polymer coated stent was used clinically in the BENESTENT II trial, and showed favourable clinical outcomes to historical controls.[297] Subsequently advances in oral anti-platelet therapy negated the use of heparin-coated stents.

1.24.3 Polymer coated stents as drug delivery devices

The advantages of using a coronary stent as a delivery device are that:

- Drug delivery is targeted to local tissues and high concentrations can be achieved.
- By varying the polymer composition 'programmed' elution of a drug over time can be achieved.

- Fewer drug systemic side effects than with higher-dose systemic dosing.

Disadvantages of local drug delivery include:

- A variable proportion of drug is lost in the circulation rather than being delivered locally. This is dependent on drug pharmacokinetics and adherence to the polymer. In-vivo elution characteristics are variable and difficult to accurately profile in-vitro.
- There may be an initial "bolus effect" when the stent is implanted, with the potential for increased toxicity.
- For any agent here will be a limited total dose per unit area of stent.
- Stent struts cover only 5 -12% of the arterial surface area within any stented segment[298], therefore drug delivery to any given area of endothelium is unlikely to be uniform.[299]
- The polymer coating may be non-biocompatible and produce adverse effects.

Many experimental agents have been delivered in pre-clinical and clinical studies including heparins[295, 300], glycoprotein receptor antagonists[301, 302], steroids[303], NO donors[304], growth factors[305, 306] and anti-proliferative drugs[307-309]. Most of these investigations have been with the aim of reducing restenosis but only stents eluting anti-proliferative drugs have persisted into widespread clinical practice. The same concept has even been used on bioprosthetic heart valves where polymeric matrices have delivered Diphosphonates *in vitro* to reduce valvular

calcification.[310] The polymer-based drug-eluting stents in current clinical use predominately elute anti-proliferative, anti-restenotic drugs (mainly sirolimus analogues and taxol derivatives). An endothelial progenitor cell capturing antibody stent has been marketed[311]. Bio absorbable stents of magnesium[312, 313] or poly-lactylic acid[314] and bio-absorbable polymers[315] are the subject of active clinical trials in an attempt to avoid the potential negative effects of polymer use.

1.25 Animal models of chronic total occlusion

The use of animal models has been an essential intermediate step in the 'bench to bedside' development of new cardiovascular technologies. All animal procedures in the UK are registered and closely regulated by the Home Office. Translational research relies heavily upon both the insights into pathological processes and therapeutic effects that animal models provide. The development of contemporary DES involved extensive pre-clinical investigation, primarily involving porcine angioplasty models.[316-318] There is no consensus as to the best animal model of restenosis, and both the peripheral rabbit iliac model, canine models and the porcine model have been extensively studied.

1.25.2 Porcine models of coronary artery disease

The pig has become widely accepted as the optimal large animal model for coronary artery studies. In terms of histological findings the porcine model is

perhaps most analogous to human patho-physiology with identical neo-intimal hyperplasia seen within 28 days of balloon injury to the artery wall.[319, 320] In pigs, as in humans, the extent of neo-intimal hyperplasia is proportional to the extent of injury and this allows semi-quantitative assessment of treatment effects.[321, 322] Details of the extensive pre-clinical work in the field of restenosis is beyond the scope of this introduction but several excellent reviews are available.[323] A technique of percutaneous cut down to the external carotid artery under general anaesthesia has become standard and have the advantage in the pig of facilitating coronary angiography with routine angiography catheters. A further advantage of the pig is its robustness and ability to recover from repeated procedures, although animal husbandry requirements are moderately onerous and require experienced animal handlers and close veterinary supervision.

1.25.3 Pre-clinical models of CTO

Until recently attempts to develop targeted technologies to assist in percutaneous treatment of CTOs have been hampered by a lack of reproducible animal models. In addition the pathophysiology of CTO remains poorly understood. In recent years specific equipment for PCI of CTO has become available with both dedicated micro-catheters and angioplasty guide wires as well as devices which attempt disobliteration by laser and mechanical means.[324, 325] Reliable pre-clinical CTO models are essential to help develop new technologies.

The earliest attempts to produce an induced CTO involved external pressure by means of a ligature or ameroid constrictor.[326] The latter device, originally used therapeutically in veterinary medicine, consists of hydroscopic casein protein lining a C-shaped metal ring. Implantation around the coronary requires thoracotomy and the resultant CTO has none of the pathological features of inflammation and atheroma seen in the disease state. Furthermore it is not possible to recanalise an occlusion so caused.

1.25.4 Endovascular methods of inducing CTO

Endovascular approaches to induce CTO have varied widely in their approach and success rates. Several groups have attempted to produce occlusion by injection of material into the coronary. Strauss et al. modified a thrombin injection model by infusing collagenase.[327] This was successful in producing an occlusion with close histological correlation with native disease, namely mature fibrous tissue, multiple intra-luminal micro-channels, extracellular lipid deposits and disruption of the internal elastic lamina. They suggested that the extent of micro-channel development may influence the likelihood of successful guide-wire crossing during PCI.[328] Other mechanical techniques for inducing CTO have included a variety of partially occluded and constricted stents and direct injection of intra-coronary alcohol.[329]

Accurate reproduction of the disease state has been difficult as it is challenging to produce accelerated pathological change including intimal infiltration with atheroma and medial calcification. Histology of CTOs suggests that an inflammatory component is invariable and required to mimic the human condition.[25, 26] This degree of inflammation is not invoked by the majority of mechanical or angioplasty-derived approaches.

The lack of biocompatibility of some previously tested polymers which were previously investigated and rejected as stent coatings has been used to provoke CTOs.[330] Prosser et al. described the use of a poly-L lactic acid plug placed into canine and porcine coronary arteries. After 28 days the polymer resorbs leaving a micro-channel CTO.[331] Suzuki et al. have recently achieved similar results with calcified CTOs obtained in pig coronary arteries using apatite-coated bio-absorbable polymer sponges.[332, 333] Other published methods include delivery of thermal energy to the coronary via heated angioplasty balloons: this method produced excess neo-intimal hyperplasia but not complete arterial occlusion.[334]

1.25.5 The copper stent model of CTO

Most recently delivery of a copper-coated coronary stent has been shown to be highly effective in producing CTOs with favourable histological features including intra-luminal microvessels.[335, 336] Our experience with this technique is that early stent thrombosis can be a problem due to the exuberant inflammatory

reaction caused by local copper. Step-wise experimentation was required to determine the optimal dose and formulation of copper, for example coating only the abluminal stent surface to minimize thrombosis. Dual anti-platelet therapy is essential to avoid fatal stent thrombosis, and mortality is significantly lower when larger (40Kg) juvenile animals are used, the left anterior descending artery is avoided (as acute occlusion of the LAD is invariably fatal), and balloon injury is kept to a minimum. If these recommendations are followed a CTO success rate of >90% at 28 days with low mortality can be achieved. As this model closely mimics human disease it is appropriate for testing of novel PCI equipment as well as novel biological treatments, as in this thesis study. The intense inflammatory reaction seen following copper stent implantation may however affect the response of the endothelium to subsequent stimulation, an issue that is relevant to this thesis and requires further study.

1.26 Summary

Symptomatic coronary occlusion remains a significant management challenge. While major advances have occurred in percutaneous coronary intervention, a significant number of patients remain unsuitable for angioplasty and stenting as it is not possible to cross the chronic coronary occlusion in order to deliver coronary stents and open the artery. While CTO remains a major cause of referral for coronary bypass surgery, many affected patients are precluded from cardiac surgery due to co-morbidity or diffuse coronary disease. Treatment of occlusive coronary disease by augmentation of existing antegrade collateral vessels in order to relieve angina is a new concept. The use of pharmacological agents and growth factors which may promote angiogenesis has been discussed. Many of these have seemed promising in initial trials only to disappoint in clinical use and the ideal agent has not yet been identified. A coordinated therapeutic response might be achieved by use of a 'master-switch' transcription factor modulating-agent, one which can activate a number of genes involved in neo-angiogenesis. The possibility of using novel compounds to up-regulate a key component of oxygen homeostasis, hypoxia-inducible factor, has been discussed. Local drug delivery using polymer-coated stents allows high local concentration to be achieved while avoiding potential systemic side-effects. In summary, it has been demonstrated that there is an un-met need for development of alternative treatments for chronic coronary occlusion.

1.27 Original hypothesis

In a newly developed porcine copper-stent model of CTO, up-regulation of HIF will result in neo-angiogenesis with increased collateral density and measurable improvements in myocardial perfusion. The prolyl-4-hydroxylase inhibitor di-methyl oxalyl glycine has been selected to test this hypothesis, in part due to availability of this compound and due to it being the PHD inhibitor with the most pre-clinical evidence of angiogenic efficacy.

Chapter 2 and chapter 3 deal with the methodology and the results of the *in-vitro* experiments. These include tissue-culture and cell-culture angiogenesis assays, and the loading and elution of DMOG onto coronary stents in preparation for a pre-clinical trial. Chapters 4 and 5 describe the methods and results of a randomized controlled trial of DMOG in porcine chronic total occlusion, and the development of a novel, entirely percutaneous chronic occlusion model. Discussion points specific to methods are dealt with in these chapters. Chapter 5 is a summary of the major findings of the work and a general discussion thereon, including suggestions for further study.

Chapter 2

Methodology of in-vitro experiments

2.1 Background

Before embarking on in-vivo evaluation of potential pro-angiogenic effects of DMOG we conducted a series of in-vitro experiments with DMOG and related prolyl-4-hydroxylase inhibitors to determine whether a pro-angiogenic effect was present.

The phases of in-vitro work were as follows:

- *Investigation of the effects of DMOG in-vitro, using the following assays:*

(A) Tissue culture (microvessel outgrowth) assay using porcine carotid artery.

(B) Cell culture using Human umbilical vein endothelial cell (HUVEC) tubule-formation assay.

- *Fabrication of polymer-coated DMOG eluting stents (performed by PolyBioMed Ltd, Sheffield)*

- *Measurement of DMOG elution:*

(A) Directly using physical methods

(B) Using a cell-culture based bio-assay.

(C) Zebrafish Fli-1 and gridlock mutant assays.

2.2 Tissue culture

2.2.1 Compounds investigated in tissue culture

Four compounds known to increase tissue levels of hypoxia-inducible factor under normoxic conditions were investigated in tissue culture. *(table 2.1)* Di-methyl oxalyl glycine (DMOG), and its substitution ester N-oxalyl glycine (NOG) are both non-specific inhibitors of prolyl-4-hydroxylases. FG-2216 is a more specific inhibitor of prolyl-4-hydroxylase. All of these were supplied by Professor. C. J. Schofield, Chemistry research laboratories, Oxford. Cobalt Chloride 10mM solution was obtained from Sigma-Aldrich and VEGF 10µg from Invitrogen. DMOG and NOG (0.1 to 5mM) were made from serial dilutions of a 50mM stock solution. The stock solution was made by dissolving solid compound in sterile PBS after passing through a 20micron filter. FG-2216 and cobalt chloride which are less hydrophilic were diluted in small volumes of DMSO before further dilution with PBS. Glycine was used as a control compound for the HIF HI compounds, with phosphate buffered-saline used as a control with VEGF.

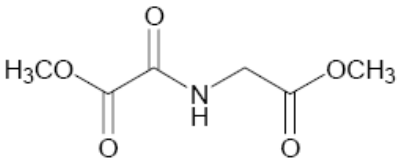
DI-METHYL OXALYLGLYCINE (DMOG) $C_6H_9NO_5$ 
N-oxalyl glycine (NOG) $C_4H_5NO_5$
FG-2216
Cobalt Chloride ($CoCl_2$)
Vascular Endothelial Growth Factor (VEGF)

Table 2.1. Compounds investigated in tissue culture. The above compounds, with the exception of VEGF, inhibit one or more of the prolyl-hydroxlase enzymes involved in haemostatic regulation of the hypoxia-inducible factors (HIF).

2.2.2 Tissue culture assay using porcine carotid artery

These experiments used a modification of a previously published tissue-culture model developed by Stiffey-Wilusz and colleagues[266] which was itself a modification of the rat aortic-ring model first described by Nicosia[253]. Tissue culture models, while technically challenging, are considered to more closely approximate physiological processes seen in the intact organism than cell culture models.[272] These processes include endothelial cell proliferation, migration and microvessel formation.[269] This may be of particular importance in the field of angiogenesis where complex paracrine effects are thought to underpin angiogenic processes.[259] Such paracrine activity is unlikely to involve a single cell type and therefore may not be adequately assessed by culture of a single cell-type such as isolated endothelial cells.

Most tissue-culture or cell-culture techniques have to date involved assays aimed at evaluation of angiogenesis *inhibitors* in the field of cancer research. To evaluate *promotion on* of angiogenesis in a large animal model we felt a tissue culture assay would be most appropriate.

2.2.3 Tissue harvesting and preparation

We further modified the rat aortic-ring type assay in an attempt to develop an in-vitro means of quantifying induced angiogenesis. Porcine carotid tissue was

obtained. I harvested the tissue using aseptic techniques from a local abattoir (J Morris & Sons, South Kilworth, Leicestershire) and transported the tissue to the laboratory in Dulbecco's Modified Eagle medium on ice. Preparation of tissue was started within 2 hours.

Tissue samples were prepared on a sterile field in a laminar-flow culture hood to reduce the risk of bacterial or fungal contamination. The tissue was rinsed in phosphate-buffered saline (PBS) and kept on ice during processing.

Short lengths of carotid artery, around 9mm in diameter, were open longitudinally with a scalpel. Initial attempts to use a standardized biopsy cutter proved unsuccessful with sprouting largely absent or present but highly asymmetrical (results not shown). This was likely due to the biopsy cutter denuding the fragile endothelial layer. The use of an automated tissue cutter proved similarly unsuccessful. Tissue was therefore cut by hand into $1mm^2$ fragments using a pressing motion with great care taken to preserve the endothelium.

2.2.4 Experimental procedure- tissue culture

100 µl of reduced growth-factor (RGF) Matrigel (Becton Dickenson) was layered into the 48 inner wells of a 96-well cell culture plate. To the outer wells were added 500µl of PBS to reduce evaporation. The matrigel was allowed to polymerize at room temperature for 20 minutes. Tissue fragments were then

added, endothelial surface uppermost, and immediately covered with a further 100µl of RGF Matrigel.

The tissue/ matrigel 'sandwiches' were then covered with a standard endothelial growth medium (EGM), a modification based upon EGM Bullet Kit (Cambrex Ltd). The basal EGM was supplied without growth factors. To this we added standard concentrations of human endothelial growth factor (EGF), ascorbic acid, gentamyin and amphoteracin-B. Although supplied as part of the bullet-kit, bovine brain extract (BBE) and hydrocortisone were not added: BBE contains fibroblast growth factor which we felt would make interpretation of sprouting extent difficult. Hydrocortisone has been shown to influence growth of HUVECs in-vitro.[253, 337] The major determinant of rapidity of endothelial cell growth in cell-culture is the concentration of added Fetal Bovine Serum (FBS).[338] FBS was therefore added in variable concentrations in an attempt to determine a dose-concentration curve for endothelial sprouting in this model. Study compounds (DMOG, NOG, FG-2216, VEGF and Cobalt Chloride) were prepared to pre-specified concentrations. 200µl of the study treatment was added to the basal growth medium.

2.2.5 Fetal Bovine Serum (FBS) dose-response experiment

I conducted experiments over a period of 12 weeks, using in total approximately 50ml of RGF matrigel. Availability of porcine carotid tissue and RGF-matrigel and was sporadic throughout this time due to abattoir closure and problems with contamination of matrigel batches (information on file at BD Biosciences). As there was no published literature on the likely effect of adding exogenous growth factors to this model, it was not possible to calculate sample size necessary to determine a statistically significant pro-angiogenic effect. Preliminary work (not shown) suggested that 6 experiments (i.e. 6 wells) were sufficient to obtain reproducible mean values for sprouting using similar tissue culture assays under any given set of basal conditions. I used this number of wells as the basis for the FBS dose-ranging experiments and the addition of VEGF. For the test compounds, we performed as many experiments as feasible with the aim of producing a standard deviation of sprout area $\leq 0.5 mm^2$ in each experiment.

A series of preliminary experiments were conducted to determine the optimal duration of culture. These experiments determined that no visible sprouting occurred before day 9 of culture and became maximal around day 12. Prolonged culture did not appreciably increase the density or area of sprouting but was associated with a higher incidence of bacterial and fungal infection of the culture medium. Sprouting was therefore evaluated on day 9 and 12.

The culture plate was incubated at 37C with 5% CO_2 for up to 12 days. The plates were inspected daily to detect sprouting. EGM +/- treatments was changed every 48 hours without disturbing the underlying matrigel. Experiments were performed on four occasions using RGF Matrigel and EGM from the same lots to avoid batch to batch variability. Based on the results of the FBS standard curve, a concentration of FBS 5% was added to the basal EGM. This offered the best compromise between preserving tissue viability long enough to allow sprout growth and reducing background growth factors to a level where any additional effect produced by addition of the study compounds might be detected.

2.2.6 Preparation of reagents

DMOG was reconstituted from dry powder by serial dilution after dissolving 87.5mg in 10ml PBS to make a stock solution of 50mM. Previous published work suggests that systemic concentrations of 0.1 to 1.0mM DMOG have resulted in stabilization of hypoxia inducible factor expression (as determined by western blotting) in HUVEC cell culture[207], and have promoted collateralization in ischaemic tissue in a murine subcutaneous sponge-matrigel model. We therefore investigated the effect of DMOG at 0.05mM, 0.1mM, 0.25mM, 0.5mM, 1mM, 2mM, 5mM and 50mM.

N-oxalyl glycine (NOG, MW 145) was investigated as NOG is an intracellular metabolite of DMOG and having slightly greater lipophilicity may better

penetrate endothelial cells. NOG is a prolyl-hydroxylase (PHD) inhibitor with greater specificity for PHD-2 than DMOG. Solutions were again prepared by serial dilution using sterile filtered PBS. Basal medium contained FBS 5%.

FG-2216, a specific PHD inhibitor, was first dissolved in a small volume of DMSO then concentrations made by serial dilution in sterile filtered PBS. Basal medium contained FBS 5%.

Vascular endothelial growth factor (VEGF) 10µg was diluted in sterile filtered PBS. The final concentration used was 0.25 µg/ml or 0.5µg/ml based on concentrations used in previously published cell-culture studies. Basal medium contained FBS 5% as before.

2.2.7 Evaluation of sprouting

Sprout area was measured from digital photographs of the culture plates. Many methods have been used to quantify angiogenesis in this type of model, with recent publications utilizing computer-assisted measurement of sprout area.[339, 340]

Plates were imaged on an inverted fluorescent microscope (Axiovert, Zeiss) with an digital imaging system (Olympus AZ500) and 2.5X objective which gave a final image magnification of 35X. The images were recorded using C14 Image

acquisition software (Apple inc) for later analysis with Image J quantification software (Stanford University).

Using the area quantification feature of Image J, the edge of the sprouting area was detected by semi-automated edge detection, and corrected manually where there were obvious discrepancies. The tissue fragments were calibrated against a standard 1mm measure. The output was 'Corrected sprout area':

Corrected sprout area (mm^2) = (Total sprout area - tissue fragment area)

2.2.8 Immunohistochemistry

Immunohistochemical analysis was performed to determine whether the sprouts were of endothelial or smooth-muscle cell origin.

Standard tissue fragments averaging 120mg in weight were rapidly frozen at -80C prior to tissue culture for later histological analysis. On completion of culture, culture-plate wells were collected for histological analysis. The plates were frozen at -80C. 500 ml of zinc formalin (Z-fix) was added at room temperature for 6 hours, then the matrigel scooped out into cassettes for processing. Histological processing was performed at the Histology department of Leicester Royal Infirmary and supervised by myself. Once placed in cassettes, samples were fixed in Z-fix for 1 to 3 weeks before being processed and embedded in paraffin.

Sections (4.5microns) were cut and stained with haematoxylin and eosin (H&E), factor VIII-related antigen (von Willebrand factor) antibody, NCL-vWFp. In addition transverse cuts of the matrigel were taken containing sprouts and the matrigel was dissolved off using 5 ml matrigel cell recovery solution (BD Biosciences). The antibody was used at a dilution of 1:200. Diluent was used as a negative control. Slides were pre-treated in a 0.01M citrate buffer, pH 6 for 18 minutes in a 1000W microwave pressure cooker. Signal was detected using a Vectastain Elite ABC kit. DAB substrate was used to stain the vWF positive cells brown. Further staining for alpha-smooth muscle actin was undertaken to determine whether the sprouts were of smooth muscle origin.

2.2.9 Statistical analysis

Data were analysed using Prism4 (Graph Pad software). Continuous variable were compared using a 2-sided t-test. The Kolmogorov goodness-of-fit test was used to derive a correlation co-efficient for the FBS dose-ranging experiment.

2.3 Cell culture tubule formation assay

After determining that the tissue-culture assay was not suitable for assessment of the potential pro-angiogenic effect of the study compounds (see chapter 3), we designed a modified cell culture assay based on previously published models of cellular migration and tube-formation using matrigel as a growth substrate.

2.3.1 Matrigel method

There is no clear consensus on methodology when using matrigel. Factors influencing the extent of microvessel formation include the type and volume of matrix material, cell type and the density at which cells are plated onto the wells.[252]

Reduced growth factor (RGF) matrigel has recently become available with markedly reduced levels of stimulatory growth factors and cytokines. Until RGF matrigel was widely available it was difficult to detect the effect of pro-angiogenic compounds using tubule formation assays as the background levels of growth factors (mainly fibroblast growth factor) present in matrigel tended to render the assay insensitive to weak angiogenesis promoters. Use of GFR matrigel allows for more selective determination of the efficacy of pro-angiogenic compounds. A comparison of different matrix materials confirms that RGF-matrigel promotes slower and less extensive tubule formation than matrigel, and importantly also confirms that addition of VEGF increased tubule length and total area only in the RGF-matrigel system and not in the standard matrigel assay.[265]

This result underscores the importance of using RGF-matrigel when testing pro-angiogenic compounds.

EHy926 cells, a HUVEC-derived endothelial cell line were supplied by Professor Alison Goodall (University of Leicester). These were cultured for three weeks in order to obtain a sufficient stock. They were grown on 75ml flasks in a pyruvate-free high glucose Dulbecco's modified eagle medium (Invitrogen, Gibco 41965) with 2% HAT solution (Gibco Cat No. 21060), 2% Penicillin/ Streptomycin and initially 5% Fetal bovine serum (Amersham). After passage 2 a standard cell culture medium was prepared as above but using 2% FBS.

Cells at passage 4-5 were used for the migration assay. To 24 inner wells of a 96-well culture plate was added a thin layer of 50ul reduced growth-factor (RGF) Matrigel (BD Bioscience) equivalent to 1.76µl/mm^2.

2.3.2 HUVEC cell preparation

20,000 Ehy926 cells were added after calculation of the total number of cells per Falcon tube by haemocytometer, giving a cell density of 556 cells/mm^2 (well surface area 36mm^2). Unused wells were filled with 100µl of sterile PBS to prevent evaporation. Trial and error determined that the best technique to ensure even coverage of cells over the well was to reconstitute 1-2x10^6 cells in 2ml of cell culture solution after the precise number of cells present in the centrifuged

pellet had been calculated by haemocytometer. Using a pasteur pipette the column of cells in solution were gently agitated then swirl-mixed for 10 seconds. Immediately, without allowing the cells to settle the correct volume was drawn up using a calibrated pipette. A drop was allowed to form and drop over the centre of each well. The plates were gently agitated for one minute on a rocker table to promote uniform dispersion of the cells within each well. A top layer of 100µl of the standard cell culture medium was added after each cell inoculation. The wells were visually inspected by light microscopy to ensure even cell coverage and technical failures (ie. grossly uneven cell coverage) were rejected.

To each well was then added a known concentration of a study compound. Control wells were treated with glycine 250µM.

The wells were visually inspected every two hours from 0-12 and 24-36 hours. At 8, 24 and 48 hours the cell culture plate was formally assessed for evidence of cellular migration and tube-forming activity. The plates were imaged with a 25x inverted fluorescent microscope (Axiovert, Zeiss) with and without addition of Dil-Ac-LDL, a specific endothelial cell marker. The images were captured by a digital imaging system (Olympus AZ500) linked to a PC running C14 image analysis software (Apple, inc) for subsequent off-line quantitative analysis using Image J software (Stanford Univ.).

2.4 Analysis of tubule formation in the cell-based assay

Analysis of the extent of tubule formation is performed either manually or using image analysis programs after photography of each individual culture well. An inverted microscope attached to a digital camera will allow the entire area of a single well of a 96-well plate to be captured on each image. Five main indices have been used by research groups to determine the extent of tubule formation: total or average tubule length, number of tubules, tubule area, percentage tubule coverage, and number of tubule branching points.

Tubule area is commonly used as it lends itself to the use of automated image analysis programs [269, 341] However area of tubules is dependent on initial plating density of cells, and under high density of plating the observed tubule area may fall as cells differentiate into tubules, thus giving a false impression as to the extent of angiogenesis.[191] A recent adaptation by Sanz et al.[286] demonstrated improved reproducibility in assessment of the extent of tubule formation by combining tubule area with an assessment of the proportion of the total culture area containing a branching tubule formation or 'node'.

I elected to use the area covered by tubules as the primary measure of tubule formation. Assessments of total tubule length and number are difficult and time-consuming and require manual counting which does not lend itself to repeated experiments.

Using the grid-overlay function of Image J and both manual and automatic edge-detection functions, the area covered by any tubule-like structure was determined. Additionally the number of 10x10 grid squares in each well containing a branching tubule-like cellular structure (the grid-square branching percentage, GSB%) was recorded. GSB% is a measure of the proportion of the well containing distinct tubule-like structures (i.e. tubule coverage) and is distinct from overall cell coverage which includes clumped cells. *(fig. 2.1)*

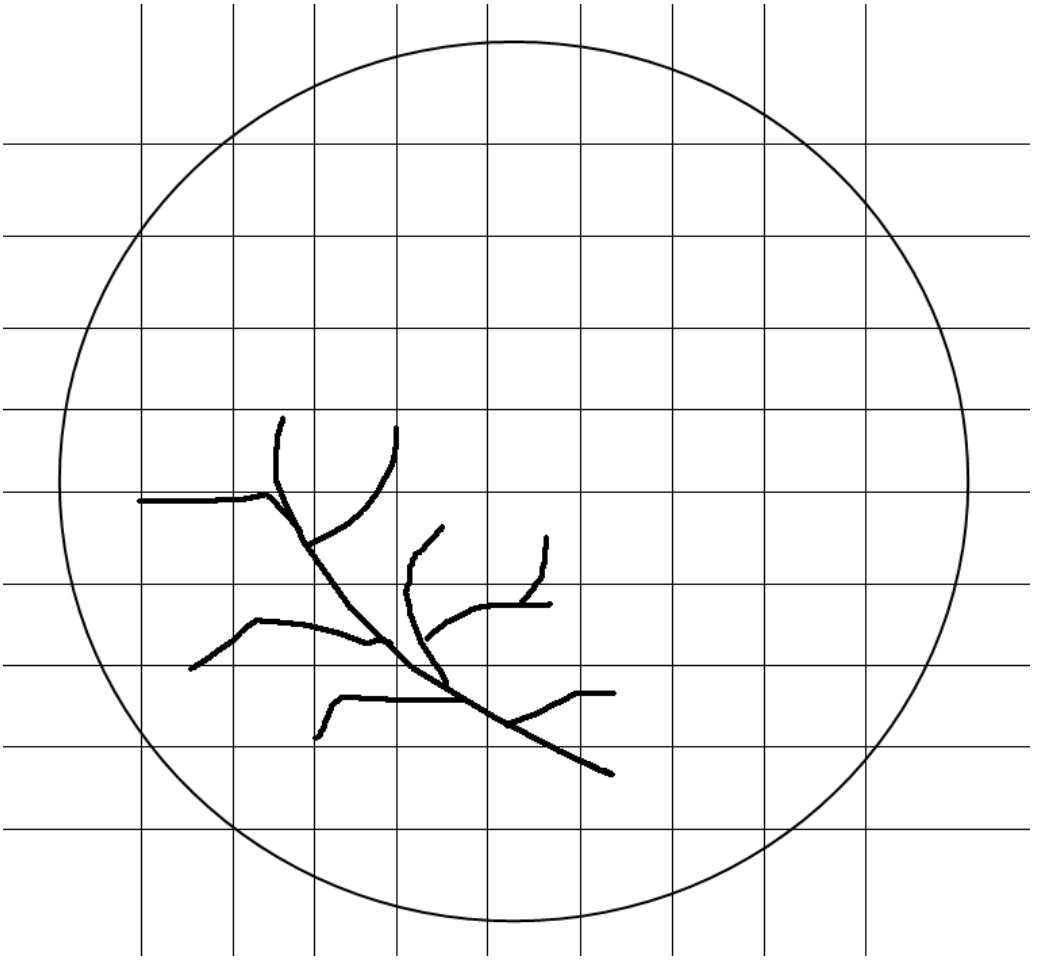


Figure 2.1 Schematic representation of the grid overlay system. This divides the well photograph into 100 segments. The proportion of segments containing a branching tubule-like structure (as opposed to clumped cells) is counted to give the grid-square branching percentage (GSB%), a measure of tubule coverage. In this schematic example GSB%=18.

2.5 Compounds investigated in Tube-formation cell assay

The same compounds that were tested in the tissue culture assay were tested in the cell culture tubule formation assay: di-methyl oxalyl-glycine (DMOG), N-oxalyl glycine (NOG) $C_4H_5NO_5$, FG-2216, Cobalt Chloride ($CoCl_2$) and VEGF.

2.5.1 DMOG dose-ranging experiment

In order to determine a dose-effect curve a series of preliminary experiments were run using differing concentrations of di-methyl oxalyl glycine. Based on previous reports[252] a standard set of conditions was used with 50µl RGF-matrigel per well and a cell plating density of approximately 556 cells/mm2 of plate (20,000 cells per well of a 96-well plate). Initial proving experiments showed that use of lower amounts of matrigel (<50µl) led to incomplete and irreproducible well coverage and that higher cell density produced unpredictable amounts of cellular clumping with poor tubule formation. *(fig. 2.2)*

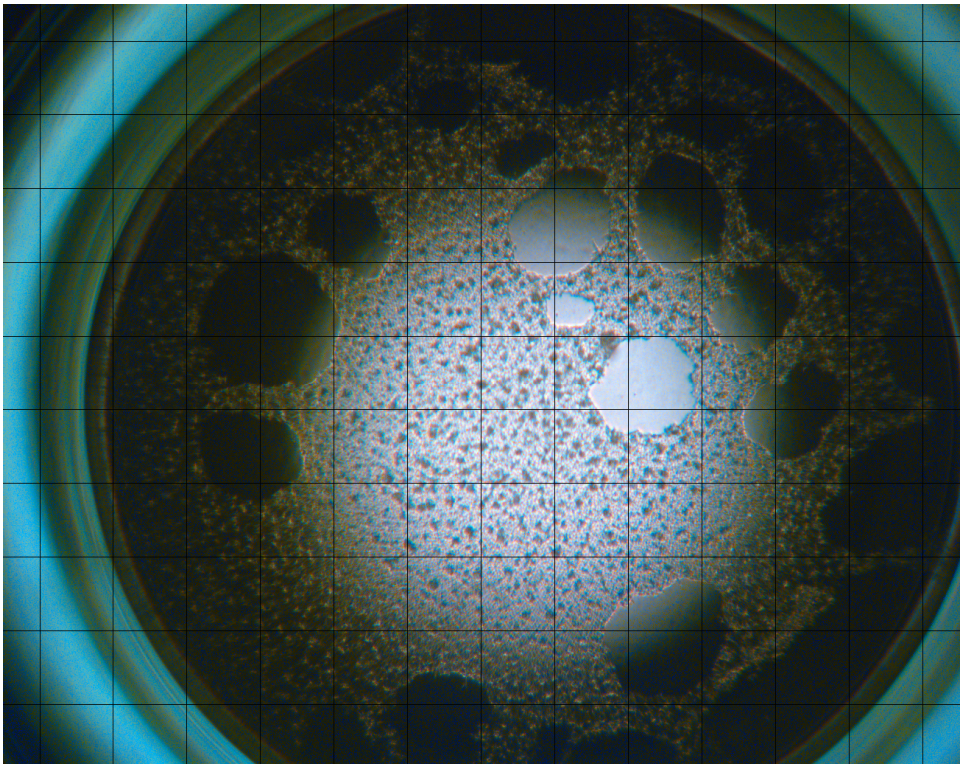


Figure2.2 Example of a technical failure due to excessively high cell density (50,000 cells) demonstrating some tubule formation at 16 hours but a large central area of undifferentiated cell clumping promoted by the excessively high cell density present. Image represents a single well of a 96-well cell culture plate at 10x magnification. Percentage of well coverage with branching tubule formation (GSB%) = 22%, cell coverage 74%, area tubule formation= 4.8mm^2

Initially six wells each were incubated with either control (Glycine 250µM) or one of four different concentrations of DMOG (DMOG 100µM, 200µM, 500µM and 1mM). There is very limited available data on the use of DMOG in cell culture and the concentrations of DMOG chosen were based on an empiric lower dose range and on the level of DMOG that produced toxic effects during culture of porcine carotid tissue.

2.5.2 DMOG compared to VEGF and DMOG with a VEGF 2 receptor inhibitor

We compared the extent of tubule formation under control conditions with, based upon the results of the dose-ranging experiments 125µM DMOG and 250µg VEGF (based upon published cell culture data). Experimental set up using reduced growth factor matrigel and culture medium additives was identical to that in the DMOG dose-ranging experiments. A known concentration (160µM) of a VEGF receptor 2 inhibitor (VRI, Calbiochem Cat No. 676495**)** was added to alternate wells. Digital photographs were taken at 4, 8, 16, 24, 36 and 48 hours for off-line analysis as before. The proportion of grid squares containing branching tubule-like structures was noted.

2.6 Statistical analysis

Statistical analysis was performed to compare tubule formation at 36 hours between control conditions and test compounds. Mean, standard deviation and standard error of the mean were calculated. An unpaired two-tailed student t-test was performed to compare groups (Prism 4, Graph Pad software).

2.7 DMOG stent: loading and measurement of elution

2.7.1 Stent polymers

DMOG was loaded into a proprietary PEP polymer by Polybiomed Ltd.. The maximum amount of DMOG that could be loaded onto each stent was established by experiments carried out by Polybiomed.

A programmable elution profile (PEP™) polymer was used. This patented polymer is designed to allow release of a biological compound such as DMOG in a predictable fashion over a pre-determined period of time. The polymer is composed of two compounds A &B.

Polymer A is poly(vinyl pyrrolidone-co-vinyl acetate, PnVPA) with an average MW of 50,000 *(fig. 2.3)*

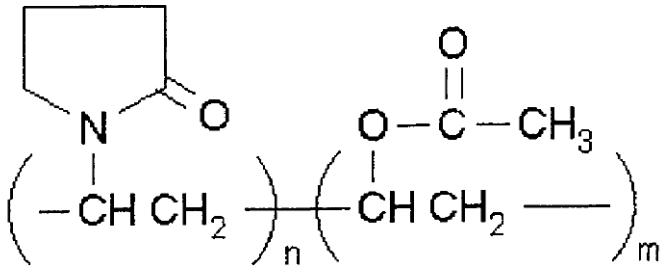


Figure 2.3 Chemical structure of PnVPA polymer

Polymer B is poly(vinylbutyral-co-vinyl alcohol-co-vinyl acetate, PVB) with a molecular weight from 50,000 to 80,000. *(fig 2.4)*

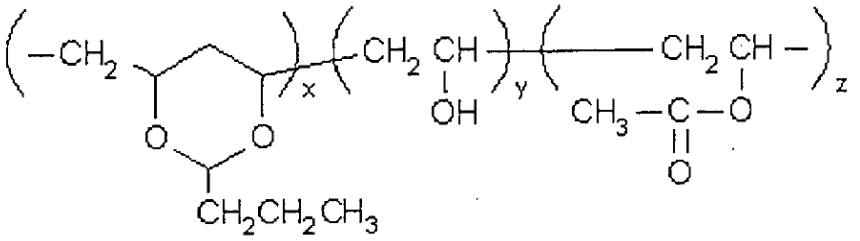


Figure 2.4 Chemical structure of PVB polymer

2.7.2 DMOG stent preparation

The polymer/ DMOG solution is applied to the bare metal stents by spraying. PVB is predominantly hydrophobic and comprises the bulk of the formulation. Small additions of PnPVA give the coating 'programmability' on terms of being

able to control the release of rate of drugs. The total thickness of polymer was around 10-12 microns.

The DMOG was dissolved in chloroform and mixed with the co-polymers. The mixture was dried at 40°C under vacuum. Based on previous work with the PEP system and sirolimus a mixture of polymer PVB:PnPVA of 94:6 was used to produce an estimated linear elution profile of 28 days in-vivo for the entire dose of loaded DMOG.

The stents used were S7 stainless steel stents (Medtronic Inc; Minneapolis, MN) size 2.75mmx 18mm. These were supplied as unloaded stents (i.e. not loaded on balloons) and had a surface area of 77.83 mm^2. The stents were cleaned by immersion in IPA for 30 minutes with agitation by ultrasound and dried overnight at 100°C. A coating solution was prepared in chloroform with a ratio of PVB/PnVPA of 94:6 (PEP 94). DMOG was added to this formulation so that by weight the composition was 75% polymer and 25% DMOG. The stents were weighed, spray coated with PEP94/DMOG and dried overnight at 40°C under vacuum. (*fig. 2.5)*

A finite amount of DMOG can be loaded into a given amount of polymer with high levels of polymer coating increasing the likelihood of polymer cracking when the stent is deployed. Several stents with different coating weights were prepared using scanning electron microscopy to examine the stent. The optimal

thickness of polymer was determined to be around 6μm which resulted in minimal polymer cracking (*fig. 2.6)* This determined the total amount of DMOG that could be loaded and equated to 400μg DMOG per stent. The stents were supplied and coated as unexpanded and uncrimped tubes. It was not possible for to coat balloon-mounted stents with polymer. We did not examine the effects upon the polymer of subsequent hand crimping onto balloons or the effect on the polymer of subsequent balloon expansion. Stent over-expansion may be associated with polymer cracking, this risk was reduced by our practice in the in-vivo study of implanting the stents at a maximum diameter of 3.5mm (on a 3.5mm balloon at nominal pressure), which is within the limits of the S7 stent cell design. As DMOG was mixed with the polymer rather than applied as a surface coating, manual crimping was unlikely to alter stent composition. While we did not perform electron microscopy on expanded stents, microscopy of fully expanded stents coated with the same polymer load during development of the polymer did not show significant polymer disruption (data on file with PolyBioMed Ltd.)

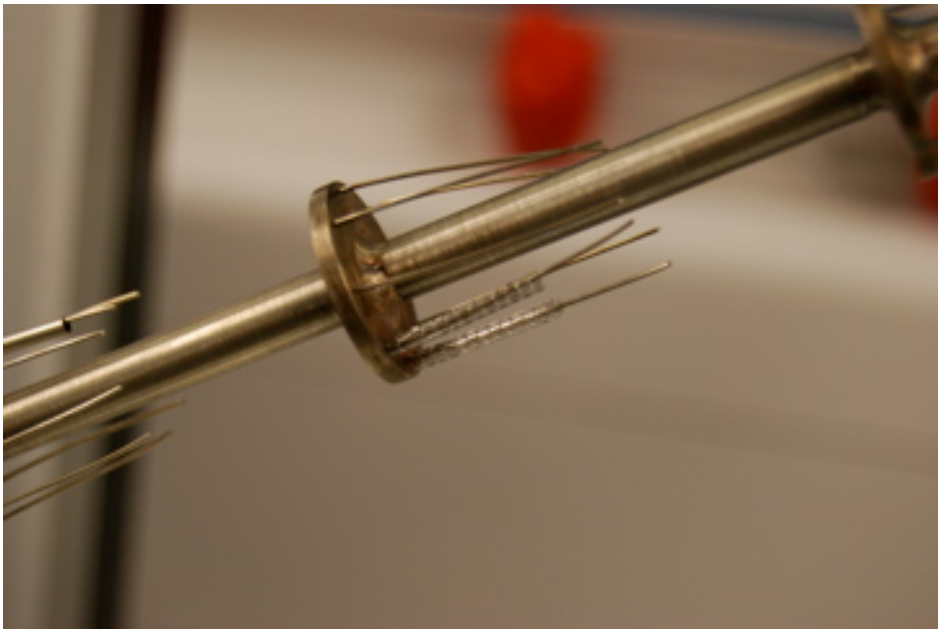


Figure 2.5 Bare-metal Medtronic S7 stents loaded on a rig for spraying with polymer/ DMOG at the PolyBioMed laboratory.

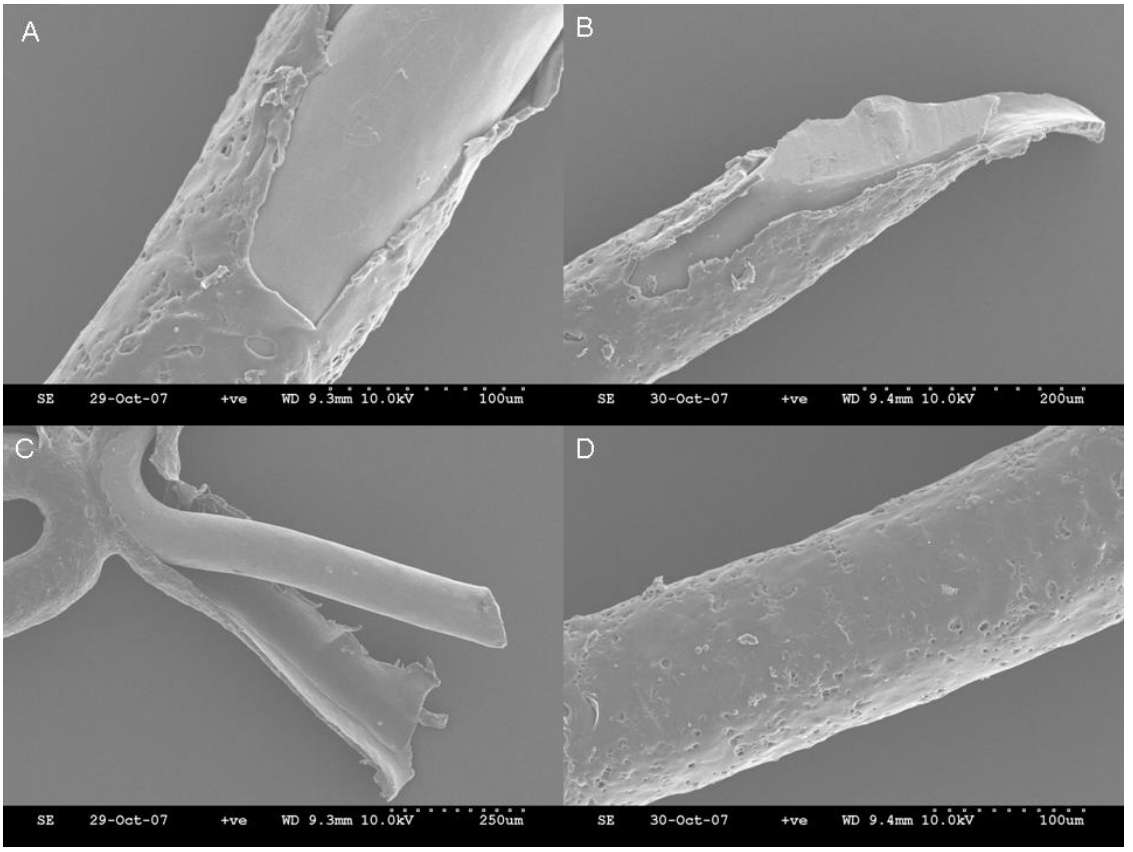


Figure 2.6 Scanning electron microscopy of the S7 stents demonstrating the effects of different polymer thickness. (A) Stent strut coated with 10μm polymer showing extensive polymer cracking and loss. (B) Cut end of same stent in (A). (C) Stent wire showing 10μm polymer stripped from stent due to mechanical shearing (cutting of the stent). (D) Stent with 6μm thick polymer as used in the final in-vivo experiments. This thickness gave good coverage with minimal cracking.

2.8 Elution testing

2.8.1 DMOG stent elution experiment

We wished to simulate the situation in an occluded coronary artery and therefore felt a continuous recirculating model was not appropriate. Each stent was placed on a hook and suspended in a vial containing 8ml of 40% human serum albumin solution with a 5mm magnetic flea. We used gentle agitation with vials incubated at 37°C on a multipoint stirrer set at 100 rpm.

After a given time interval ranging from 1 to 28 days the release medium was analysed. Stents were eluted for 1, 3, 5, 7, 14, 21 and 28 days. All the eluant samples were available for analysis by electrospray ionisation mass spectrometry (ESI-MS) on the same date.

2.9 Detection of DMOG

2.9.1 Direct detection of DMOG in solution

Initially we conducted a series of experiments to determine whether it was possible to directly detect DMOG in solution. The difficulty in comparison to relatively large polypeptide molecules such as VEGF lies with the small size of DMOG (only 75 kDa molecular weight). Molecules below 1000 MW often are

difficult to detect reliably using standard techniques based on mass spectroscopy. DMOG detection experiments using electrospray ionisation mass spectrometry (ESI-MS) were conducted by Dr. Jasmin Mecinovic and Professor Christopher Schofield at the University of Oxford Chemical research laboratories.

2.9.2 DMOG Calibration curve

We first obtained a calibration curve representing the intensity of DMOG from freshly made solutions of known DMOG concentration. Initially, electrospray ionisation mass spectrometry (ESI-MS) run in the positive ion mode was used for the detection of DMOG. DMOG was dissolved in methanol (MeOH) and data were acquired on a Bruker micrOTOF ESI-MS machine (Bruker Daltonics, Coventry, UK). The capillary exit was 150 V and a flow rate 3 µl/min. The pressure at the interface between the athmospheric source and high vacuum region was fixed at 3.56 mbar. External instrument calibration was achieved using NaI. Data were processed with Data Analysis (Bruker Daltonics). A calibration curve for DMOG in methanol solution was obtained.

2.9.3 Analysis of eluant from elution experiments

Next we attempted to detect DMOG from eluant samples from the stent elution experiment using similar methods to those used to generate the calibration curve.

2.9.4 DMOG stent 'stripping' to determine residual stent DMOG

Finally we analysed samples of polymer/DMOG 'stripped' from the loaded stent to determine the residual amount of DMOG left on the stent following a period of elution. A stent was placed in a vial with 8 ml of MeOH, rolled for 2 hrs on a roller mixer. The stent was removed and dipped in fresh ethanol to wash off any stripping solution and dried in an oven at 100°C. A further experiment was performed as above but with the stents agitated by sonication by an ultrasonic source. After cooling the stent was reweighed and examined under a scannnig electron microscope (40x magnification) to check for coating residues. The MeOH stripping solution was then analysed by ESI-MS. Subsequently the PEP polymer was found to be partially soluble in MeOH, but fully soluble in EtOH. EtOH was therefore used as a stripping solvent in a second group of experiments. Both MeOH and EtOH were used as solvents for the stent elution assays due to their volatility and compatibility with ESI-MS analyses. HBS-EP (Biacore, 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) buffer was also investigated but was found to be inappropriate for ESI-MS presumably due to the lack of volatility.

Elution of DMOG from the stent (D_E) was calculated as the difference between the amount of DMOG loaded (D_L) initially (by weighing before and after coating) - the amount of DMOG detected after stripping the stent (D_S). As stripping is not

100% complete the amount of DMOG eluted into the solution is likely to be overestimated.

$$\text{i.e.} \quad D_E = D_L - D_S$$

2.9.5 Elution Bioassay

An alternative approach to direct measurement of DMOG was the use of a cell culture bioassay. Semi-quantitative assessment of the presence of DMOG was made by means of a modification to the cell culture tubule-formation model. Two sources of DMOG were co-cultured with HUVEC cells

(a) Eluant from the stent elution experiments.

(b) Fragments of sterile stent wire were prepared from the stents agitated in human albumin solution. Stent wire fragments were prepared from stents that had been agitated for 1, 3, 5, 7, 14, 21 and 28 days. The total number of experiments run was limited by the cost of fabricating the DMOG stents.

2.9.6 Eluant test

The experimental set-up for the elution bio-assay was similar to that for the tube-formation angiogenesis assay outlined above (pyruvate-free high glucose Dulbecco's modified eagle medium (Invitrogen, Gibco 41965) with 2% HAT solution (Gibco Cat No. 21060), 2% Penicillin/ Streptomycin) except the standard

medium was used without the addition of Fetal bovine serum. This serum free medium had previously been shown to induce very minimal or absent tubule formation with HUVECs cultured on reduced growth-factor matrigel.[269] Samples of eluant from stents eluted for 1, 3, 5, 7, 14, 21 and 28 days were tested.

2.9.7 Blinded stent-wire test

To investigate the ability of the bioassay to successfully differentiate between control (polymer-only) and DMOG-loaded stents we were provided with a sample of 12 stent wires. 6 were polymer-only and 6 polymer/DMOG. The assay was tested for its ability to correctly identify the DMOG loaded stent wires prior to testing DMOG-loaded stent wires which had undergone a period of elution.

2.9.8 Eluted stent wire

The added agent (eluant or stent wire) was considered to be biologically active if tubule formation was seen at 36 hours around the stent wire fragments. The extent of tubule formation that was considered positive was determined based on the results of the earlier DMOG cell culture experiments with DMOG concentrations in the range of 100-150μM. 40% human serum albumin or polymer-only stent wire fragments were cultured as a control. The stent wire fragments were prepared at Polybiomed Ltd and blinded to the investigators performing the cell culture experiment. The extent of tubule formation was graded in a semi-

quantitative manner as this was an indirect test of whether residual active DMOG remained on and eluting from the stent after a period of elution. Tubule formation was graded as absent, minimal (+) or extensive (++). Extensive tubule formation was taken to be ≥60% area coverage or grid square branching coverage (see *fig. 2.2* or *fig. 3.15*). 3 stent wire fragments were eluted over each time-point.

2.9.9 Zebrafish Fli-1 and gridlock mutant assays

The zebrafish (Danio Rerio) is a small freshwater fish that has found widespread application in cardiovascular research. Zebrafish were supplied by, and the experiments supervised by Dr. Tim Chico, University of Sheffield. We investigated for the first time the vascular effects of DMOG on zebrafish embryos in order to determine whether it was possible to use the zebrafish as an alternative bio-assay for detection of DMOG. The embryonic stage of the zebrafish develops rapidly and is transparent, allowing ready appreciation of vascular development. The 'grid-lock' mutant form (GM) has been developed to have a proximally occluded aorta, and produces collateral thoracic vessels at a variable rate over 2-5 days.

We conducted an experiment to determine the effects of exposure of early-stage zebrafish embryos to a range of concentrations of DMOG.

2.9.9.1 Gridlock mutant zebrafish embryos

Gridlock mutant zebrafish embryos (GM), n=320, were produced by insertional mutagenesis and expressed a phenotype with an proximal aorta in association with a variable degree of distal collateral formation via inter-segmental vessels. GM embryos,3 days post fertilization were exposed to a range of DMOG concentrations (25μM, 50μM, 100μM, or 250μM) in E3 medium and the proportion of embryos displaying distal aortic flow via collateral vessels was quantified by light microscopy.

2.9.9.2 Fli-1 mutant zebrafish

Transgenic zebrafish embryos (Fli-1 mutants) expressing green fluorescent protein as an endothelial cell marker (n=956), were collected 3 hours post fertilization and exposed to increasing doses of DMOG up to 100μM. The effect upon arteriogenesis was observed using confocal microscopy with the intention of developing a DMOG bio-assay.

Chapter 3

Results of in-vitro experiments

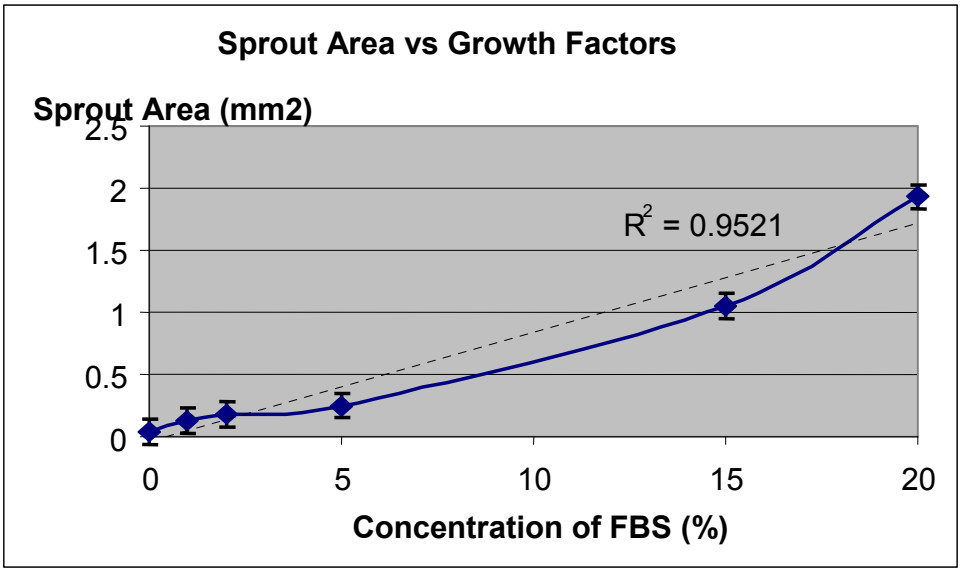
3.1 Tissue culture assay using porcine carotid artery

3.1.1 Fetal Bovine Serum (FBS) dose-response experiment.

The FBS dose-ranging experiment was performed in order to check the reproducibility of our methods of tissue culture, and to determine the optimal concentration of FBS to use in the subsequent angiogenesis assay. A description of the methods is in section 2.2. First a dose-ranging experiment was conducted in the porcine carotid model with varying doses of fetal bovine serum (FBS). 12 wells were assessed under 0%, 1%, 2%, 5%, 15% and 20% concentrations of FBS. FBS was obtained from a single lot (Amersham Bioscience) and frozen at -20C until required. The standard endothelium growth medium (without bovine brain extract) was used throughout. The experimental procedure followed the outline above but no additional treatments were added. The sprout area is derived from image analysis on day 12 *(table 3.1)*. There was a linear relationship between the cultured concentration of FBS and the degree of sprouting, with a correlation co-efficient of 0.95. *(figs. 3.1-3.4)* There was minimal sprouting seen with a serum-free medium.

	SPROUT AREA (MM^2)	SPROUT AREA (MM^2)	SPROUT AREA (MM^2)	SPROUT AREA (MM^2)	SPROUT AREA (MM^2)
FBS (%) / **Experiment**	0%	1%	2%	5%	20%
1	0	0.1	0.1	0.4	1.8
2	0	0.2	0.2	0.3	2.1
3	0	0.1	0.3	0.4	2.5
4	0.1	0.1	0.2	0.2	2
5	0	0	0.1	0.4	0.6
6	0	0.1	0	0.1	2.4
7	0.1	0.1	0.2	0	2.3
8	0	0.2	0.4	0.5	2.2
9	0.1	0.1	0	0.2	1.1
10	0.2	0.2	0.2	0.2	2.3
11	0	0.2	0.3	0.2	2
12	0	0.1	0.2	0.1	1.9
Mean	**0.01**	**0.13**	**0.18**	**0.25**	**1.93**
SD	0.07	0.07	0.12	0.15	0.23
p value	NS	0.0045	0.0016	0.002	<0.0001

Table 3.1. Corrected sprout area under variable concentrations of Fetal Bovine Serum. Statistical analysis compared to serum-free (0% FBS) medium. SD= standard deviation. (fig. 3.1)



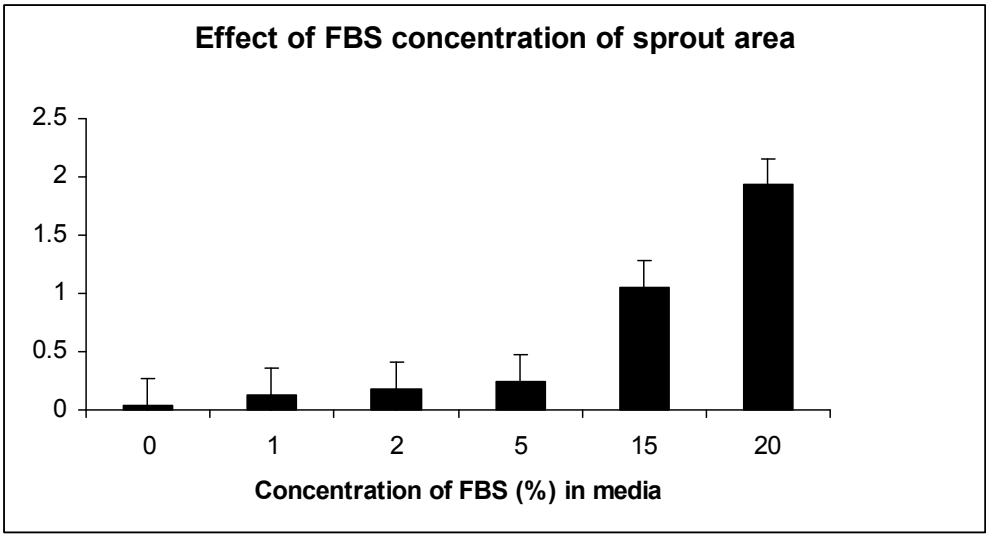


Figure 3.1. Linear relationship between sprout area on day 12 and concentration of Fetal Bovine Serum. Co-efficient (R) value indicates a linear relationship between sprout growth and serum concentration. Error bars represent ± standard error of the mean (SEM).

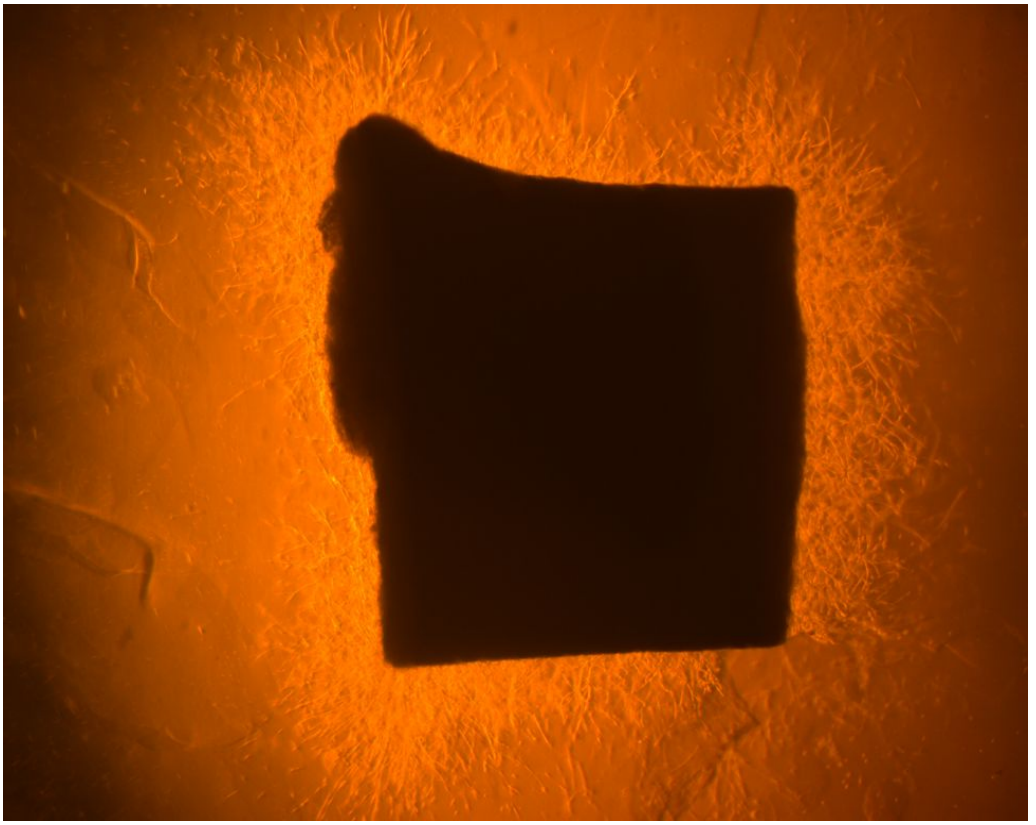


Figure 3.2. Sample Image: Well 1, FBS 15% at 35X Magnification. Total sprout area 1.82 mm^2, tissue area 0.99 mm^2, perimeter 4.0mm. Corrected Sprout area 0.8mm^2

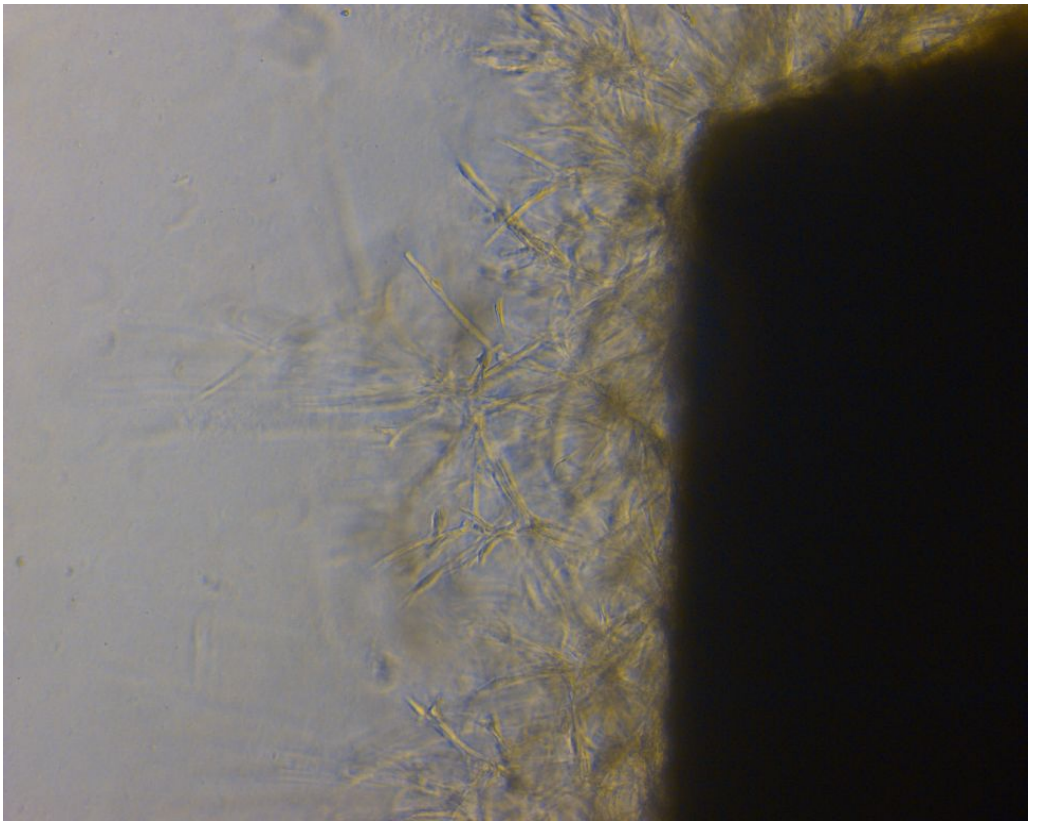


Figure 3.3. High power (350X) image of sprouts from Well 1, FBS 5%.

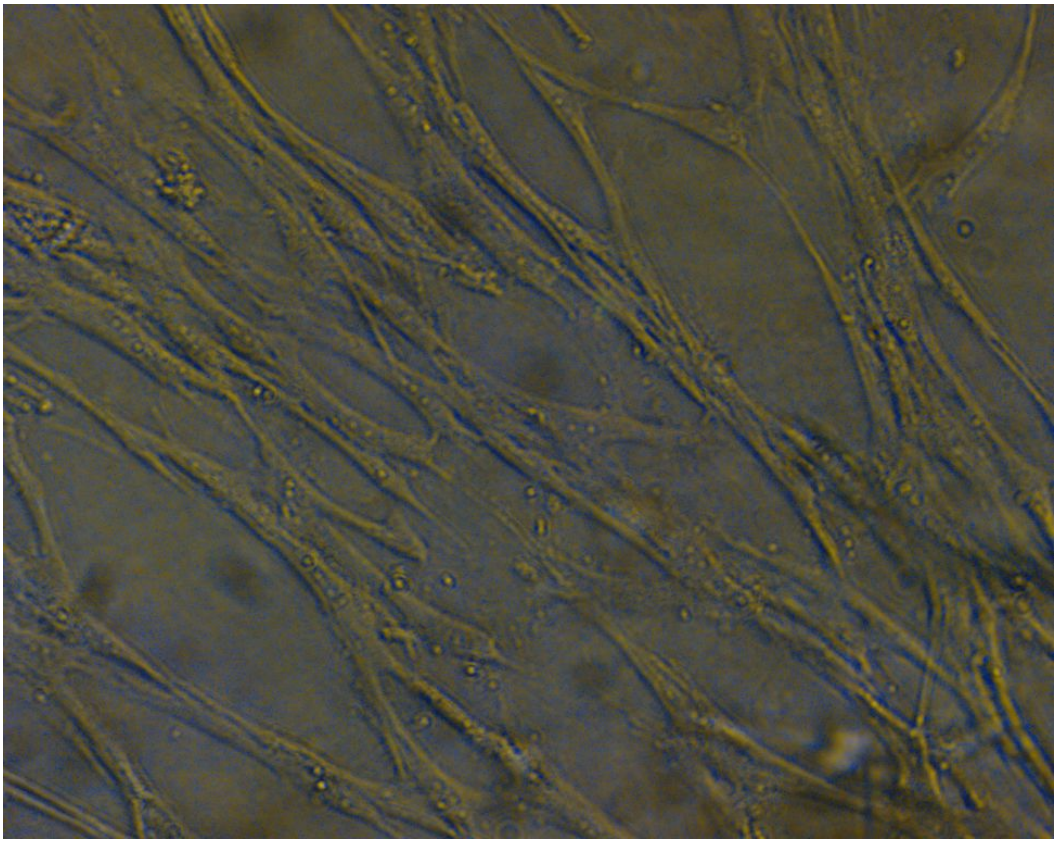


Figure 3.4. High power (1400X) from the same sample as in figure 3.3

3.2 Tissue culture (Porcine carotid) culture

3.2.1 Response of tissue culture to study compounds

DMOG, NOG, VEGF and FG-2216 were added to the standard tissue culture basal medium containing 5% FBS and compared to a glycine control.

There was no difference in the extent of micro-vessel outgrowth from the carotid tissue with addition of DMOG or NOG in the concentration range 0.05mM to 1mM after 12 days culture. *(table 3.2 & 3.3, fig. 3.5& 3.6)* There was no difference in sprouting with addition of VEGF 0.5µg/ml. *(table 3.4, fig.3.7).* Similarly there was no effect discernable following addition of FG-2216 *(table 3.5, fig.3.8)*

Results of the tissue outgrowth assay with the study compounds at optimal concentrations are summarized below.*(table 3.6, fig. 3.9)* There was no difference in micro-vessel outgrowth detected with any of the tested compounds in this assay.

	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)
DMOG (mM)	**Control**	**0.05mM**	**0.1mM**	**0.25mM**	**1mM**	**2mM**	**5mM**	**50mM**
1	0.2	0.2	0.2	0.1	0.2	0.1	0	0
2	0.3	0.1	0.4	0.3	0.3	0.1	0.1	0
3	0.1	0.2	0.3	0.4	0.2	0.1	0	0
4	0.3	0.1	0.1	0.2	0.3	0.2	0.1	0.1
5	0.4	0.2	0.2	0	0.1	0.1	0	0
6	0.4	0.4	0.4	0	0.2	0	0	0
7	0.1	0.3	0.3	0.3	0.1	0.1	0.1	0
8	0.1	0.3	0.3	0	0.2	0.2	0.1	0
9	0.1	0.1	0.3	0.4	0.1	0	0	0
10	0.2	0.4	0.4	0.5	0.1	0.2	0.1	0
11	0.3	0.2	0	0.3	0.2	0.2	0.1	0
12	0.3	0.1	0.1	0.3	0	0.1	0.1	0
Mean	**0.23**	**0.22**	**0.25**	**0.23**	**0.17**	**0.12**	**0.06**	**0.008**
SD	0.12	0.11	0.12	0.17	0.09	0.07	0.05	0.03
p=		0.72	0.75	1	0.13	0.007	<0.001	<0.001

Table 3.2 Corrected sprout area after culture for 12 days with DMOG at variable concentrations. Basal medium contained FBS 5%. There was no difference in sprout area with addition of DMOG 0.05mM to 5

1mM. Concentrations of DMOG higher than 2mM inhibited growth. Mean sprout area was compared to a glycine control. (fig. 3.5)

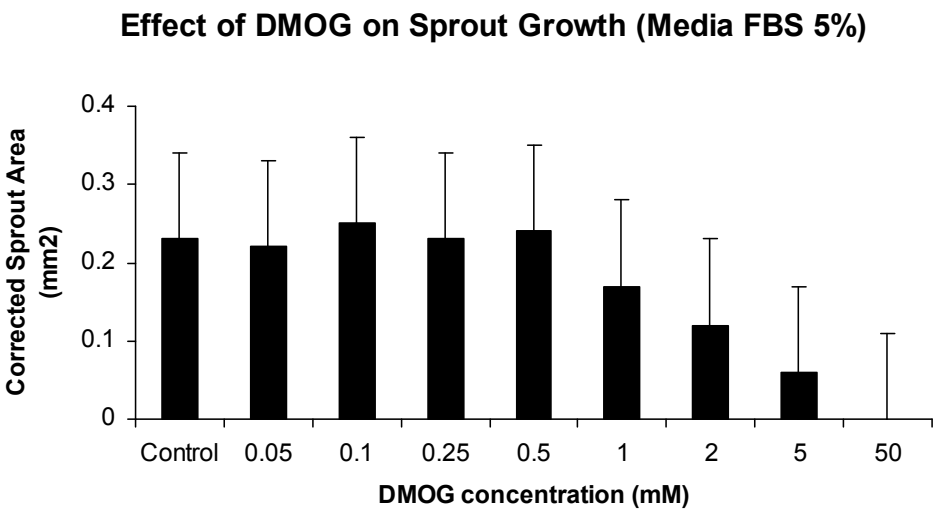


Figure 3.5 There was no difference in extent of sprouting in the tissue culture model with addition of DMOG in the range 0.05mM to 1mM. Higher doses of DMOG inhibited growth. Error bars represent ± standard error of the mean (SEM).

	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprou t area (mm2)
NOG (mM) / Experiment	**Control**	**0.05mM**	**0.1mM**	**1mM**	**2mM**	**5mM**
1	0.3	0.2	0.3	0.3	0.2	0
2	0.2	0.3	0.4	0.2	0.2	0.1
3	0.4	0.3	0.4	0.2	0.1	0
4	0.3	0.1	0.3	0.1	0.2	0.1
5	0.1	0.2	0.3	0.3	0.3	0.1
6	0.3	0.2	0.3	0.2	0.2	0
Mean	**0.27**	**0.22**	**0.33**	**0.22**	**0.2**	**0.05**
SD	0.10	0.08	0.05	0.08	0.06	0.05
p=		0.36	0.19	0.36	0.21	0.01

Table 3.3 Corrected sprout area after culture for 12 days with NOG at variable concentrations. Basal medium contained FBS 5%. There was no difference in sprout growth with addition of NOG. Mean sprout area was compared to a glycine control. (fig. 3.6)

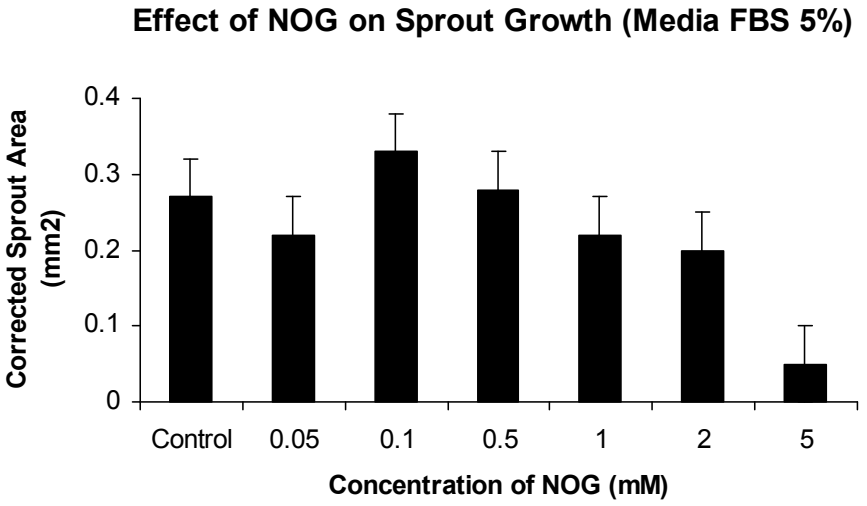


Figure 3.6 Corrected sprout area after culture for 12 days with NOG at variable concentrations. Basal medium contained FBS 5%. There was no difference in sprout growth with addition of NOG in the concentration range 0.05mM to 2mM, with NOG 5mM inhibiting growth. Mean sprout area was compared to a glycine control.

	Sprout area (mm2)	**Sprout area (mm2)**	**Sprout area (mm2)**
VEGF (µg/ml) / Experiment	**Control**	**0.25**µg/ml	**0.5**µg/ml
1	0.4	0.3	0.3
2	0.1	0.2	0.2
3	0.3	0.3	0.6
4	0.2	0.2	0.4
5	0.2	0.2	0.1
6	0.3	0.5	0.2
Mean	**0.25**	**0.28**	**0.3**
SD	0.10	0.12	0.18
p value		0.61	0.57

Table 3.4 Corrected sprout area after culture for 12 days with VEGF at variable concentrations. FBS was 5%. There was no difference in growth with addition of VEGF up to 0.5µg/ml. Mean sprout area was compared to a glycine control. (fig. 3.7)

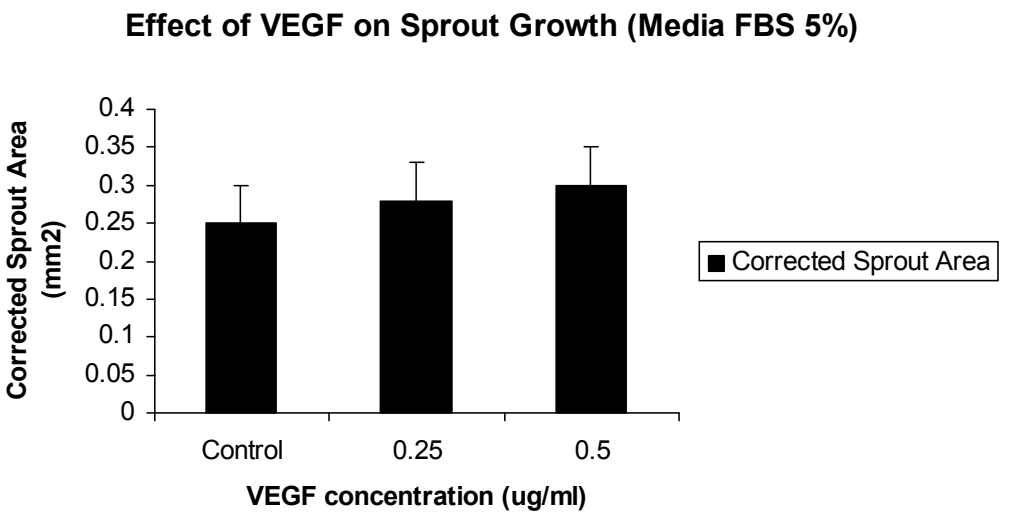


Figure 3.7 Corrected sprout area after culture for 12 days with NOG at variable concentrations. FBS was 5%. Mean sprout area was compared to a glycine control.

	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)	Sprout area (mm2)
FG2216 (mM) Experiment	**Control**	**0.1mM**	**0.5mM**	**1mM**
1	0.4	0.3	0.3	0.2
2	0.1	0.2	0.2	0.3
3	0.1	0.1	0.6	0.2
4	0.2	0.2	0	0.1
5	0.2	0.2	0.1	0
6	0.3	0.5	0.2	0.1
Mean	**0.22**	**0.25**	**0.23**	**0.15**
SD	0.12	0.14	0.21	0.1
p value		0.66	0.87	0.33

Table 3.5 Corrected sprout area after culture for 12 days with FG 2216. There was no difference in sprout growth with NOG. FBS was 5%. Mean sprout area was compared to a glycine control. (fig. 3.8)

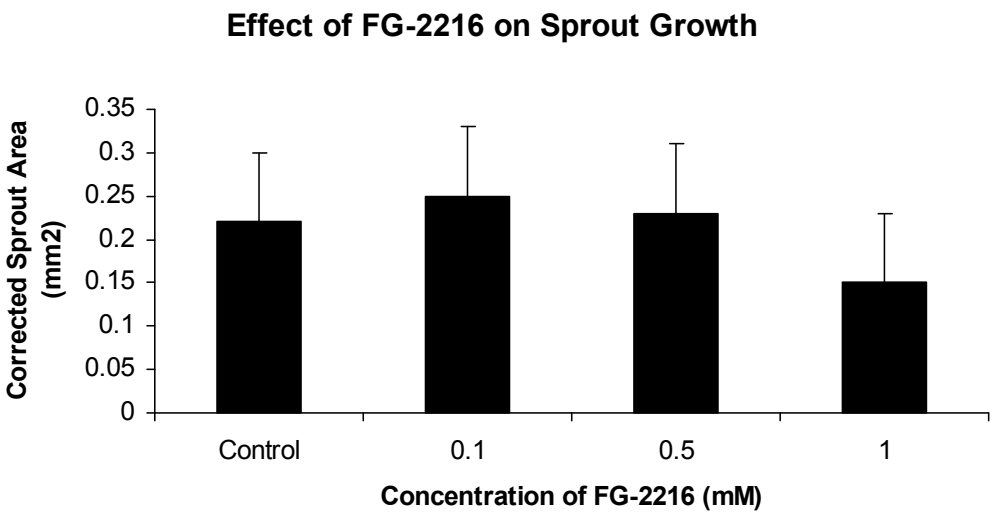


Figure 3.8 Corrected sprout area after culture for 12 days with FG 2216. There was no difference in sprout growth with FG-2216. FBS was 5%. Mean sprout area was compared to a glycine control.

COMPOUND	*SPROUT AREA*	*P VALUE**
Control	***0.25***	**-**
VEGF	***0.3***	***0.57***
DMOG	***0.25***	***0.75***
FG-2216	***0.25***	***0.76***
NOG	***0.25***	***0.66***
Cobalt Chloride	***0***	***<0.001***

*Table 3.6. Vessel outgrowth at optimal study compound concentrations. CSA= Corrected sprout area (mm2). SD=Standard deviation. VEGF 0.25µg/ml, DMOG 0.1mM, FG-2216 0.1mM, NOG 0.1mM, Cobalt Chloride 0.1mM. *statistical comprison to glycine control. (fig 3.10)*

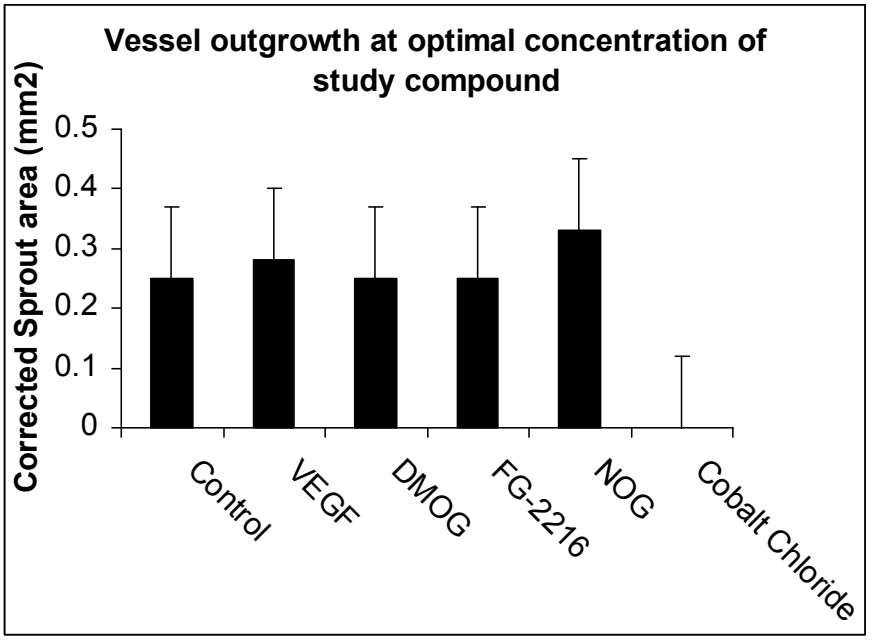


Figure 3.10. Vessel outgrowth at optimal study compound concentration. VEGF 0.25µg/ml, DMOG 0.1mM, FG-2216 0.1mM, NOG 0.1mM, Cobalt Chloride 0.1mM.

3.2.1 Response to Cobalt Chloride

Cobalt chloride was investigated as it acts as a non-specific prolyl-hydroxylase inhibitor. Cobalt chloride was added to the standard basal medium at concentrations of 0.1 to 1mM. These concentrations have been shown to produce upregulation of HIF in cell culture.[342] Even at the lower concentration range of 0.1mM cobalt chloride inhibited sprout growth, with minimal sprouting seen. *(fig.3.9)* Cobalt chloride caused dissolution of the polymerized matrigel at concentrations above 0.5mM. It appears therefore that Cobalt chloride at concentrations of 0.1 to 1mM is not a suitable agent to use in assays containing matrigel.

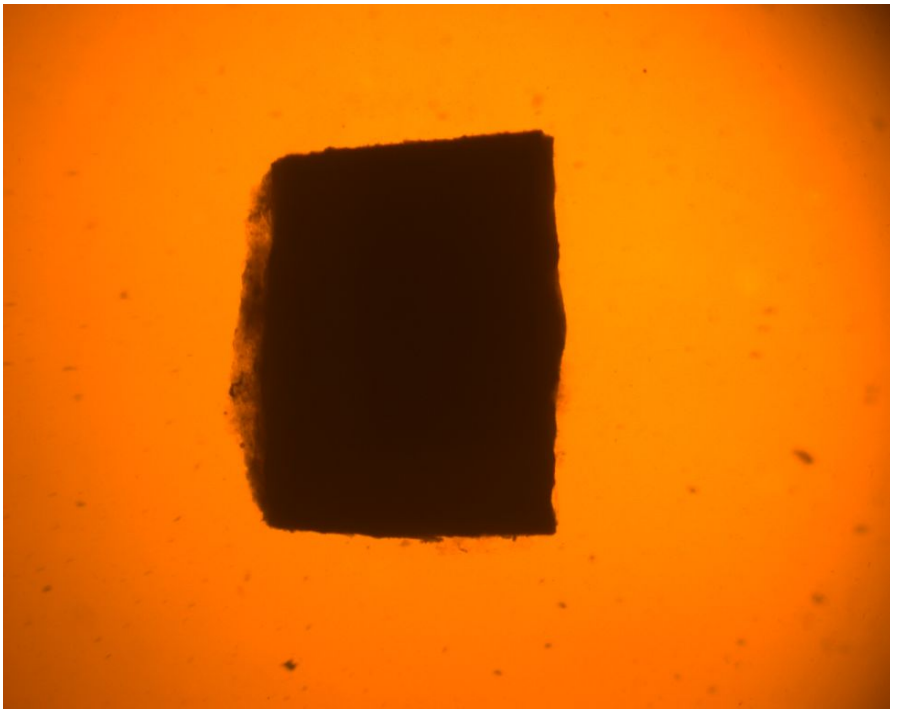


Figure 3.9. There was complete Inhibition of sprout growth at day 12 with $CoCl_2$ 0.1mM

3.3 Immunohistochemistry

Transverse sections were cut through the carotid tissue cubes as outlined in section 2.2.2. A thin blue-staining endothelial layer representing presence of the endothelium-specific antibody NCL-vWFp is seen. Using an α-smooth muscle actin antibody, smooth muscle strands re appreciated in the underlying tunica intima and media. *(fig. 3.12)* This confirms that the surface endothelial cell layer remains intact following processing. It was technically impossible to obtain a transverse section of the very delicate frond-like micro-vessels which sprouted from the carotid tissue. Despite multiple sections it was not possible to achieve a satisfactory longitudinal section capturing the delicate endothelial cell sprouts which were approximately 100μm in diameter. To demonstrate the cellular origin of the sprouts a cell suspension was therefore recovered from depolymerised matrigel containing sprout cells and the cellular origin was confirmed by positive staining for factor VIII-related antigen and negative staining with α-smooth muscle actin.

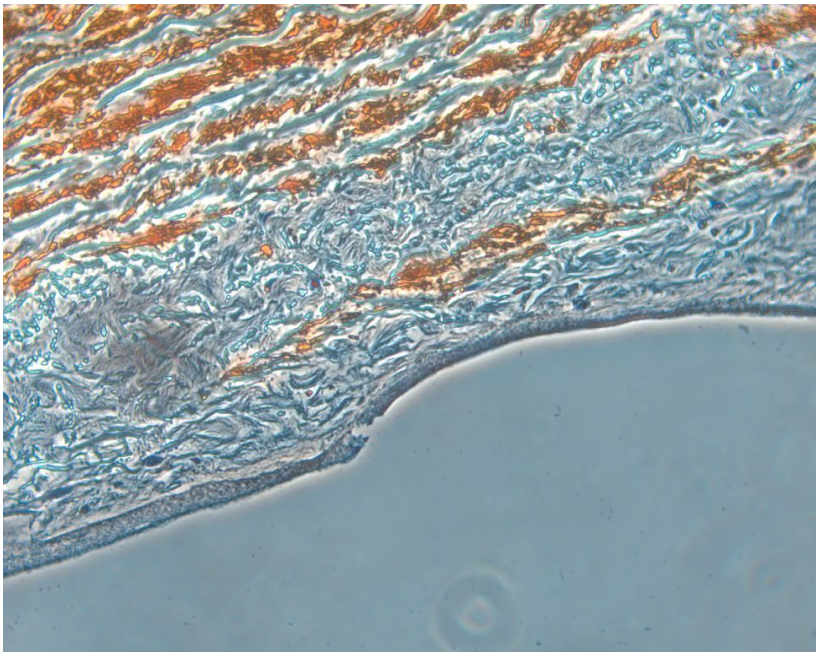


Figure 3.11. Transverse section of porcine carotid tissue NCL-vWFp (factor VIII-related antigen) staining demonstrates positive staining (blue) in a thin layer of surface endothelium. α-smooth muscle actin (brown) co-staining demonstrates intimal strands of smooth muscle.

3.4 Cell culture (tubule formation) angiogenesis assay

3.4.1 Results- DMOG dose-ranging experiments

For each concentration of DMOG 12 well experiments were run. Results presented are the mean values from 12 wells of the extent of tubule formation. (*Table 3.7, fig. 3.12, 3.13).* Compared to a glycine control there was a significant increase in tubule formation seen with addition of DMOG in the concentration range 100μM to 250μM, with higher doses of DMOG appearing to inhibit cell growth.

	EXTENT OF TUBE FORMATION MEAN COVERAGE (GSB%) N=12 WELLS					
Compound	**8h**	**16h**	**24h**	**36h**	**48h**	**p value**
Control (Glycine 100µM)	0	13.3	31.7	**31.6**	31.3	-
DMOG 50µM	0	7	32.1	**31.8**	29.6	0.22
DMOG 100µM	0.3	29.3	42.2	**70**	68.3	<0.001
DMOG 125µM	1.3	31	47.3	**73.3**	69.8	<0.0001
DMOG 150µM	0.8	28.5	43	**62.3**	61.8	<0.0001
DMOG 200µM	0.5	26	40.5	**42**	42.3	<0.001
DMOG 250µM	0	16.5	39	**44**	43.7	<0.001
DMOG 500µM	0	12.8	26.7	**26.4**	26.3	0.23
DMOG 1000µM	0	4.8	5.7	**14.8**	5.9	<0.001

*Table3.7 Initial dose-ranging experiment with DMOG. GSB%= Percentage of well containing branching tube structure, ie. % tubule coverage. *p values for test compound compared with control conditions at 36 hours. Based on the initial data further dose-ranging experiments were performed at increments around 50-200µM DMOG. (fig. 3.12)*

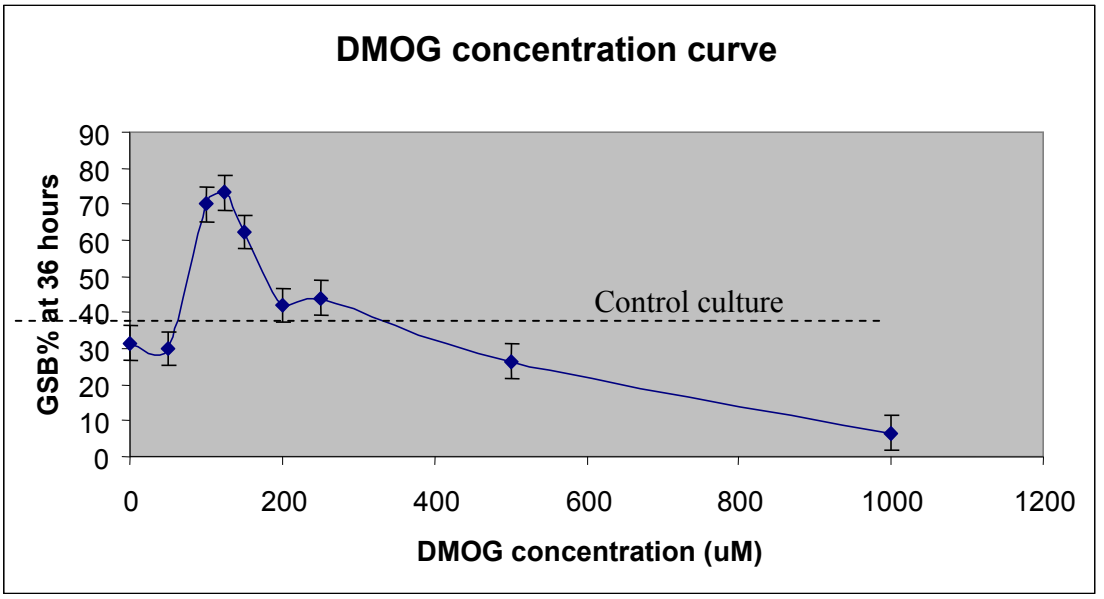


Figure 3.12. Dose ranging DMOG experiment. Tubule formation assessed at 36 hours. Dashed line indicates the degree of tubule formation observed under control conditions.

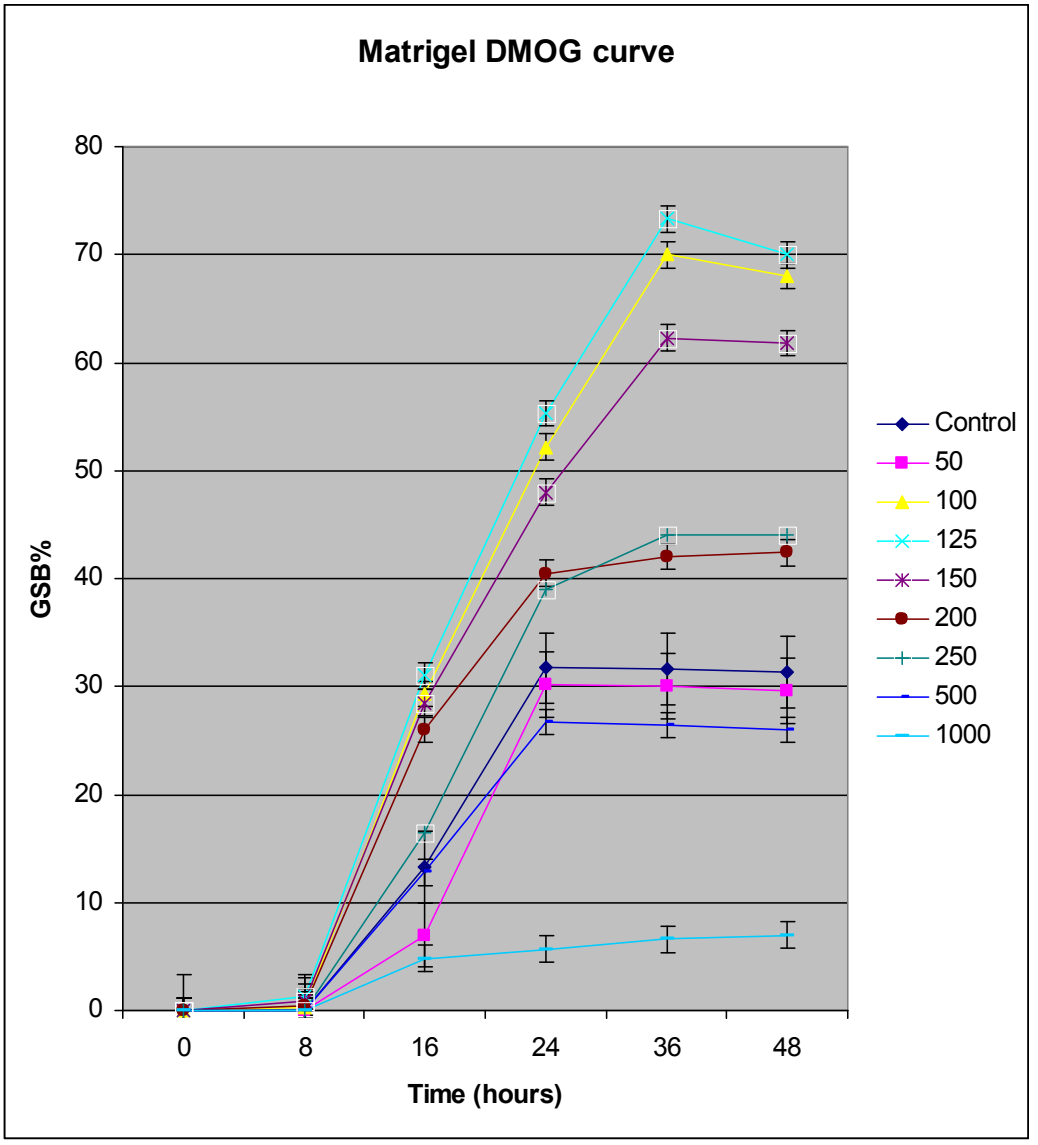


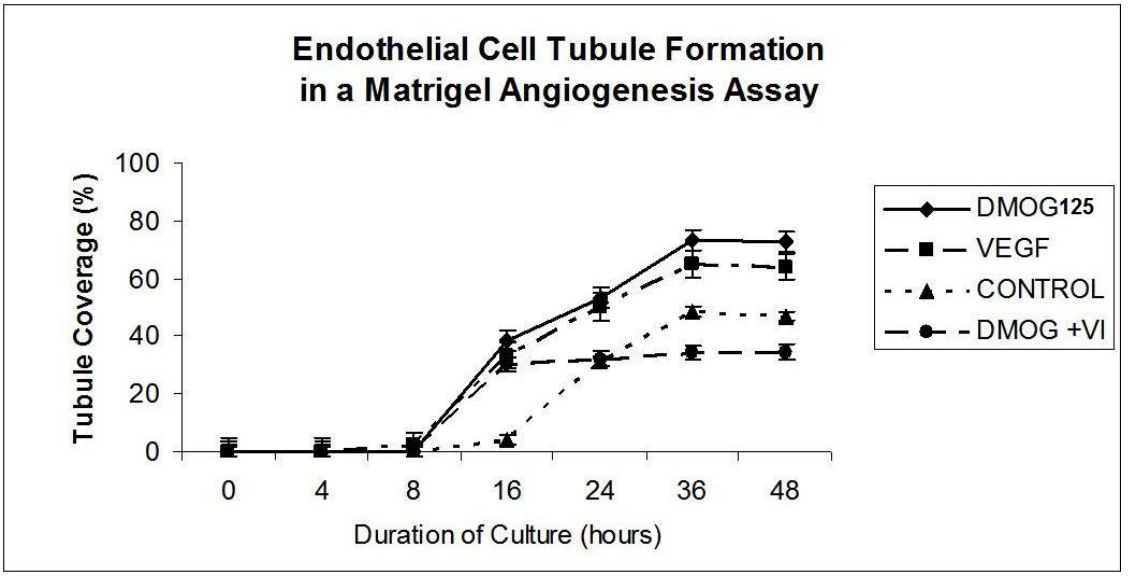
Figure 3.13. Tubule formation over time with differing concentrations of DMOG.

3.4.2 Result of DMOG in comparison to VEGF and with VEGF 2 receptor inhibitor

In a separate series of experiments, DMOG was first added to the cell-culture assay as in the experiment described in section 2.7.1. A VEGF receptor 2 inhibitor (VI) was added to alternate wells of a 96-well plate. Results presented are the mean values from 12 wells. Minimal tube-formation was present at 8 hours. By 16 hours DMOG culture produced similar tubule formation (expressed as GSB%) to VEGF but more than control, Mean (SD): DMOG 38.7(3.6), VEGF 33.4(4.6), p=0.60; Control 4(1.7), p=0.012. Addition of VI reduced mean GSB% at 16 hrs in DMOG culture to 30.3(2.5), p=0.038; and essentially abolished HUVEC migration under VEGF culture, GSB% 0.5(0.7), Cell migration was complete in all cultures by 36 hours. *(table 3.8, fig.3.14, 3.15)*

	EXTENT OF TUBE FORMATION MEAN GSB% N=12 WELLS					
	8h	**16h**	**24h**	**36h***	**48h**	**p value**
Control	0.2	4	30.2	43	41	-
DMOG 50µM	0.1	6.2	38.5	52.2	50.8	0.002
DMOG 125µM	3	38.7	53	75.6	74.4	<0.0001
DMOG 125 + VI	0	30.3	32	32.5	36	<0.0001
VEGF 250µg	0	33.4	51	70.1	68	<0.0001
VEGF + VI	0	0.5	6	33.9	33.7	<0.0001

*Table 3.8. Extent of tubule formation assessed by off-line digital image analysis. GSB%= the number of 10x10 grid squares containing part of a branching tubule-like structure (i.e. Grid Square Branching %).. DMOG= Di-methyl oxalylglycine. VEGF= Vascular endothelial growth factor. VI= VEGF receptor 2 inhibitor. * stastically significant result. Statisitcal test by means of paired 2-way Student's t-test comparing GSB% at 36 hours with control compound. (fig. 3.14).*



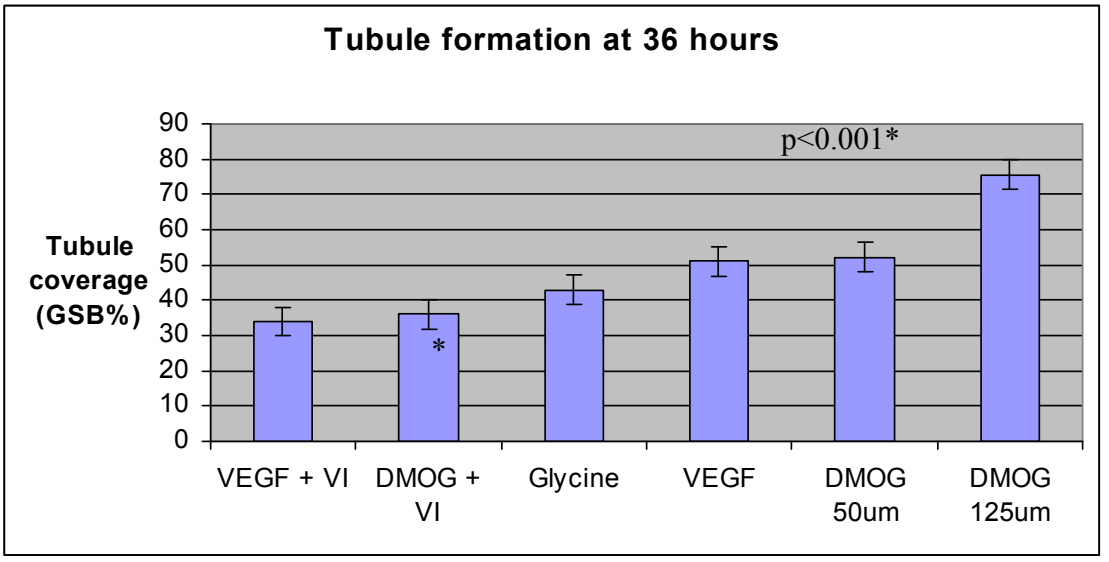


Figure 3.14. Extent of tubule formation over time. VI= VEGF receptor 2 inhibitor.

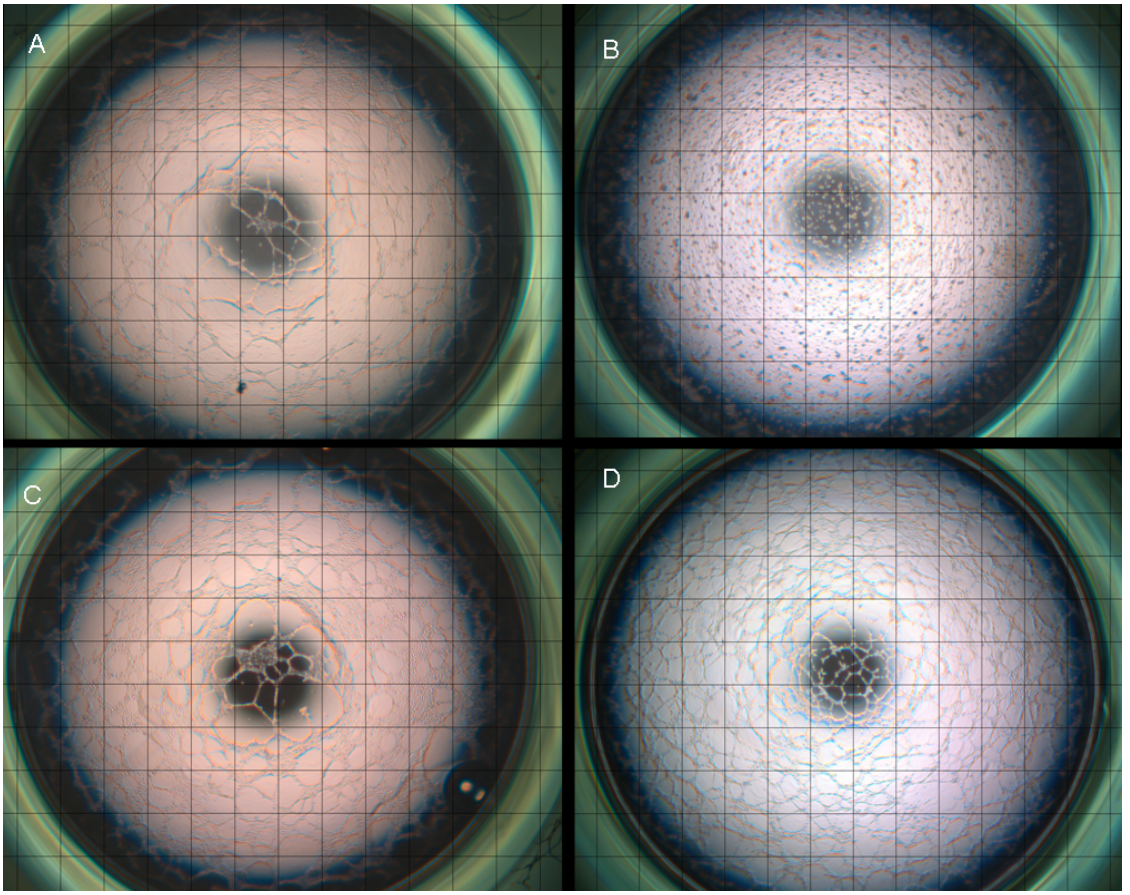


Figure 3.15. HUVEC/ matrigel tubule-formation experiments. (A) Control glycine, 36 hours. GSB%= 52. (B) VEGF + VEGF receptor 2 inhibitor 160μM, 24 hours. There is minimal tubule formation GSB%=4. (C) VEGF 250μg, 36 hours, with extensive tubule formation, GSB%= 78. (D) DMOG 125μM, 36 hours, extensive tubule formation, GSB%=82.

3.5 DMOG stent: loading and measurement of elution

3.5.1 Fabrication of stents

The process of fabrication of the DMOG-loaded stents is covered in Chapter 4, Methodology of In-vivo studies. The amount of DMOG loaded is proportional (in approximately a 1:3 ratio) to that of the total weight of polymer loaded on to the stent. After weighing, the average coated weight of the S7 stainless steel polymer-coated stents was 1480µg yielding a DMOG content of 370µg ± 25.5 µg or approximately 4.8µg/mm^2 of stent area.

3.5.2 Elution testing

Direct detection of DMOG in solution was attempted by electrospray ionisation mass spectrometry (ESI-MS) by Dr. J. Mecinovcic and Prof. C.J. Schofield at the Univeristy of Oxford Chemistry research laboratory.

3.5.2.1 Calibration curve

First we determined a calibration curve for known concentrations of freshly reconstituted DMOG in solution. DMOG was dissolved in (a) methanol and (b) ethanol and analysed by electrospray ionisation mass spectrometry (ESI-MS). The calibration curve suggested that the detected intensity of DMOG was linear in

the range of 200 ng/ml to 10μg/ml. These data suggested that the lowest detection limit for DMOG using this methodology was about 100 ng/ml. Both methanol and ethanol were suitable as solvents for the purpose of constructing a calibration curve.

3.5.2.2 Analysis of eluant from elution experiments

Analysis of the eluant from the DMOG stent elution experiment revealed inconsistent results with evidence of DMOG instability in EtOH due to ester exchange with formation of an ethylmethyl ester of N-oxalyl glycine (NOG). It was thought that rapid DMOG esterification was responsible for the inconsistent low levels of DMOG detected in the eluant samples. Direct measurement of DMOG from the elution experiment eluant was therefore not possible. As measurement of DMOG in the eluant was unreliable for technical reasons, we attempted to measure the amount of residual DMOG left on the stents following a period of elution and thus determine how much DMOG had eluted from the stents during that period.

3.5.2.3 DMOG stent 'stripping' to determine residual stent DMOG

The amount of DMOG loaded onto the stent could be accurately estimated as the total polymer loading x 0.30. The weight estimate was accurate to +/- 50 micrograms of polymer (hence DMOG to +/- 15 micrograms).

Polymer/DMOG loaded oronary stents were then stripped by rolling in methanol (MeOH) as described in Chapter 2. MeOH stripping solution was then analysed by electrospray ionisation mass spectrometry (ESI-MS). The process was then repeated using ethanol as a stripping solvent.

The results revealed that the amount of detected DMOG was much smaller than expected, by a factor of 5-6 times. Both MeOH and EtOH were found to be useful solvents for calibration curve experiments, but failed for the stent stripping experiments. The concentration of DMOG detected after stent stripping into EtOH appeared to be 3-4 fold lower than expected. *(table 3.9)*

The ESI-MS results were not reproducible, and tended to underestimate the amount of DMOG, as determined by the amount of polymer loaded onto the stent, by a factor of between 3 and 8. It was determined that the polymer coating was fully soluble in methanol and ethanol, and that the most likely explanation for these findings was variable esterification of DMOG into an ethylmethyl ester of N-oxalyl glycine (NOG). The direct measurment experiments did confirm that DMOG was loaded onto the stents but were not suficiently accurate or reproducible to allow construction of an elution curve.

SAMPLE	DESCRIPTION OF SAMPLE	CONCN BY MS (µG/ML)	CONCN BY WEIGHT (µG/ML)	WEIGHT CONC./ MS CONC.
A	PEP only control stent stripped in ethanol	0	0	
B	DMOG in methanol	15.6	18.4	1.2
C	DMOG in ethanol	25	7	0.3
D	PEP94/DMOG stripped in ethanol	2.3	15.4	6.7
E	PEP94/DMOG stripped in ethanol	4.2	28.6	6.8
F	PEP94/DMOG stripped in ethanol	6.5	55.1	8.5
G	PEP94/DMOG stripped in ethanol	4.6	16.6	3.6
H	DMOG/PEP97 in ethanol	6.7	19.4	2.9
I	DMOG stent sonicated	8.2	16.6	2

Table 3.9. Results of analysis of 'stripped' stents by mass spectroscopy. The results obtained by MS were inaccurate and varied by up to a factor of 8.5 from the expected concentration as determined by weighing of the stents.

3.5.2.4 Elution Bio-assay

These were semi-quantitative experiments based on the tubule-forming angiogenesis assay. As outlined in Chapter 2, a medium without added fetal bovine serum was used to minimize basal tubule formation. Biological effect was considered present if there was tubule formation seen before day 9 in culture or in the region of the stent wires. As the medium was serum-free we would, based on previous experiments, expect no or minimal basal tubule formation.

Using a semi-quantitative score,
0 was no tubule formation
+ was equivalent to minimal tubule formation within the well
++ was equivalent to 2-4 branching structures within a quadrant of the well or within a quadrant adjacent to the stent wire.
+++ was equivalent to >4 branching structures within a quadrant of the well or within a quadrant adjacent to the stent wire.

3.5.2.5 Eluant experiments

The result of the bio-assay using samples of eluant was inconsistent, although compatible with the attempts to directly measure DMOG in solution. There was tube-forming activity seen with addition of eluant from stents eluted for 1,3, and 7 days only. Based on the earlier results of mass spectroscopy, we believe that

esterification of DMOG occurs in solution to a variable extent producing both the ethyl ester, N-oxalyl glycine and other degradation products which may explain the relative inactivity of the eluant in cell culture.

NUMBER OF DAYS STENT ELUTION	*TUBULE FORMATION*
0	0
1	+
3	+
5	0
7	+
14	0
21	0
28	0

Table 3.10. Result of the semi-quantitative assay of the extent of tubule formation seen after cell culture with eluant. There was no clear relationship between duration of elution and tubule formation.

3.5.2.6 Blinded stent wire identification experiment

As a result of the inconsistent results of the eluant experiments we proceeded to test stent wire segments with a view to obtaining a semi-quantitative guide to the amount of DMOG left on the stents after a period of elution. As a first step I was provided with a set of polymer or polymer/DMOG stent wires (12 in total) from PolyBioMed Ltd, to determine whether the cell culture assay could differentiate the active (DMOG) stent wires from the controls.

Serum-free (No FBS) medium was used with the cell culture assay set-up otherwise identical to that in section 2.3.2. Biological activity was considered to be present if there was ++ or +++ branching structure formation within a quadrant of the wire adjacent to the stent wire after co-culture for 8 days.

There was 92% accuracy in identifying the presence of DMOG in a blinded sample of DMOG versus control polymer-only stents. *(table 3.11)* On reviewing sample D it was apparent that cell clumping led to error in attributing a biological effect. On the basis of this experiment we proceed to test for the presence of residual DMOG using the stent-wire assay with co-culture for 8 days of stent wires that had been eluted for a period of time in human serum albumin.

SAMPLE	DESCRIPTION	ASSAY	CORRECT ALLOCATION
A	PEP only	0	Y
B	PEP/DMOG	++	Y
C	PEP only	0	Y
D	PEP only	++	N
E	PEP/DMOG	++	Y
F	PEP/DMOG	++	Y
G	PEP only	0	Y
H	PEP/DMOG	+++	Y
I	PEP/DMOG	++	Y
J	PEP only	0	Y
K	PEP only	0	Y
L	PEP/DMOG	++	Y

Table 3.11. Blinded stent-wire experiment used to determine whether the stent-wire bio-assay was feasible. There was 92% (11/12) accuracy in identifying whether the stent wires were DMOG coated or control.

3.5.2.7 Elution of DMOG-coated stent wires

The final iteration of the bio-assay was co-culture of stent wires after they had been eluted in agitated human serum albumin for between 1 and 28 days (3 samples per time-point). The assay was semi-quantitative and sought to determine whether there was biologically active DMOG remaining on the stent wires after elution. There was evidence of extensive tubule formation with co-culture of stent wires that had been eluted for up to 21 days. These findings were consistent for each of the 3 samples, except at day 5 where 2 of the samples showed minimal tubule formation. None of the samples eluted for 28 days showed significant tubule formation. *(table 3.12, figs. 3.16-3.18)*

NO. OF DAYS ELUTION	*TUBULE FORMATION*
0	++
1	++
3	++
5	+
5	++
7	++
14	++
21	++
28	0

Table 3.12. These semi-quantitative results are suggestive that DMOG continues to elute in amounts that produce biological effects in this bio-assay after elution for 21 days in agitated human serum albumin.

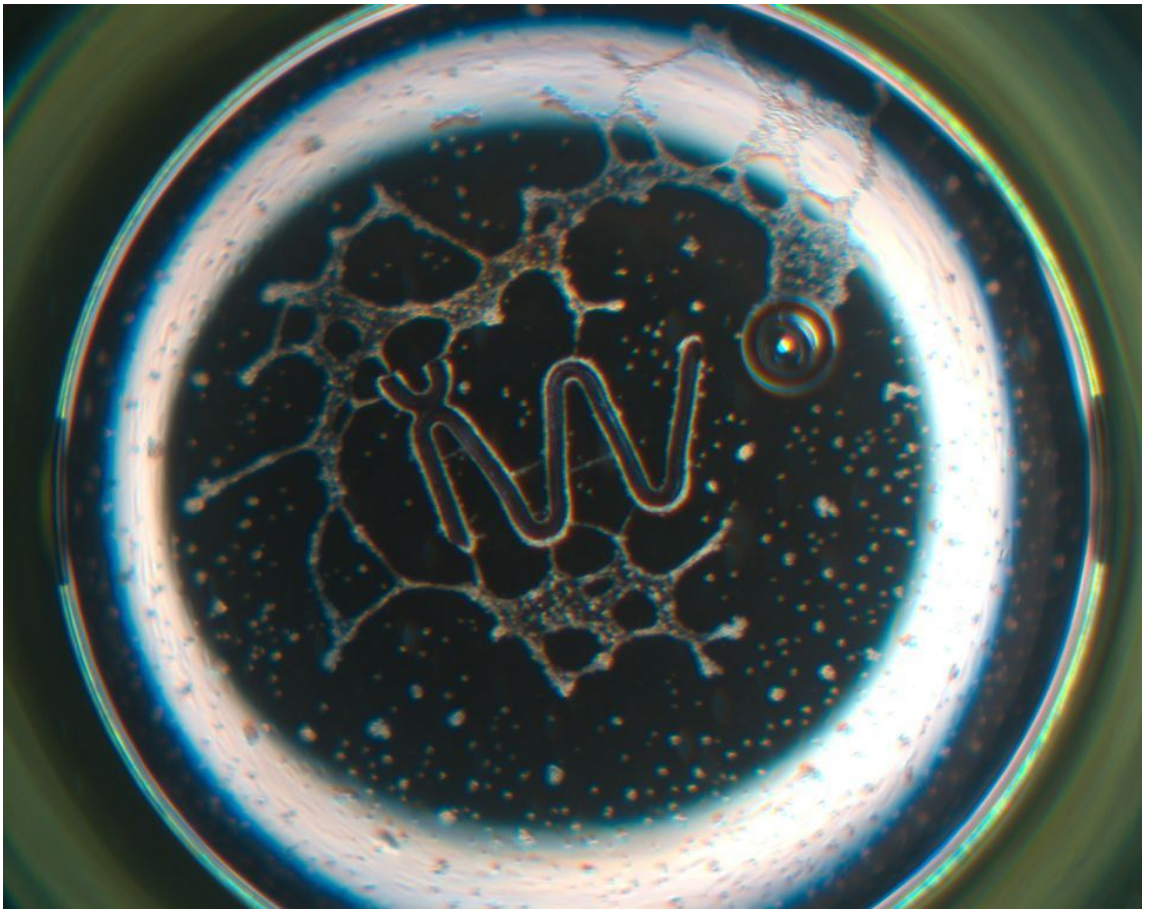


Figure 3.16. DMOG-loaded stent wire after 3 days elution exhibiting tubule formation (+ on the semi-quantitative scale) indicative of a residual biological effect.

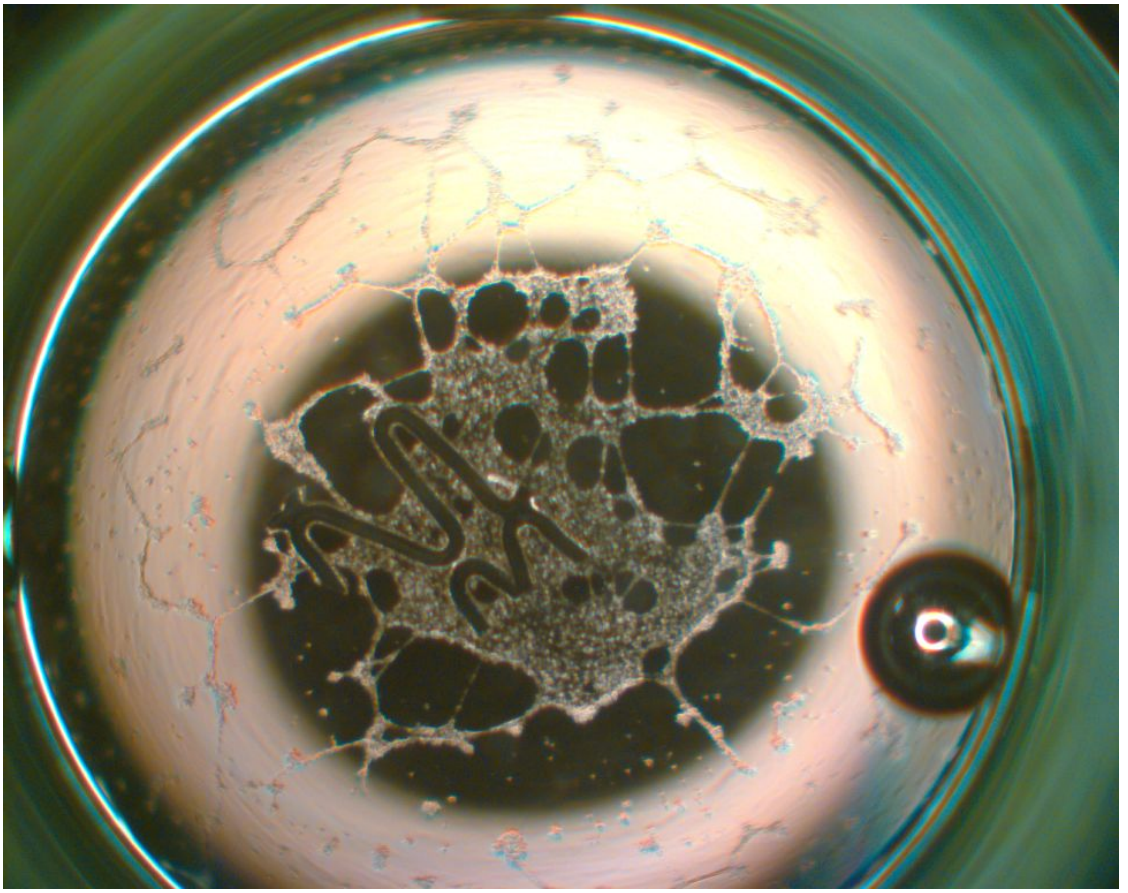


Figure 3.17. DMOG-loaded stent wire after 7 days elution exhibiting tubule formation (++ on the semi-quantitative scale) indicative of a residual biological effect.

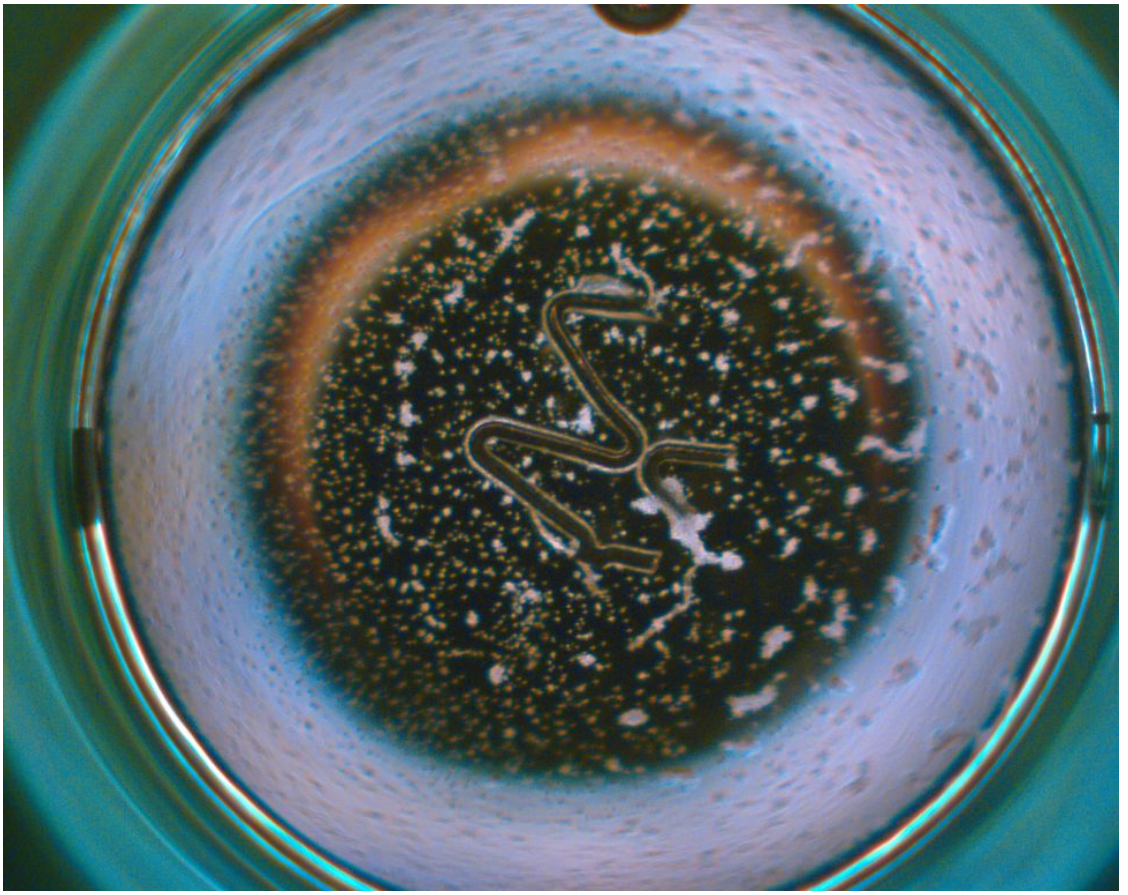


Figure 3.18. DMOG-loaded stent wire after 28 days elution exhibiting no appreciable tubule formation (0 on the semi-quantitative scale).

3.5.2.8 Zebrafish experiments

DMOG did not affect the rate of collateral recruitment among grid-lock mutant (GM) embryos. At 5 days post fertilization there was no difference in the extent of collateralization in embryos exposed to DMOG 100μM and control GM embryos: distal aortic flow was seen in 38.5% of those in DMOG and 49.5% of embryos in control medium, $p=0.38$.

A dose-dependent relationship was observed between DMOG concentration and altered arteriogenesis in the Fli-1 mutant embryos (n=956). At 27 hours post fertilization 5.7% of the control embryos displayed evidence of alteration in development of the aorta or inter-segmental vessels with ectopic vasculature and arterio-venous fistulation. In DMOG 50μM 41.4% displayed altered vasculature, $p=0.003$; rising to 96.8% of embryos exposed to DMOG 100μM , $p<0.001$. Structural changes were observed in the aorta and in the adjacent notochord, a structure which is known to signal embryonic arteriogenesis via Vascular Endothelial Growth Factor (VEGF). While these changes were of interest and suggested a dose-dependent biological effect of DMOG, for practical reasons (the number of embryos required to run the assay) we did not develop the assay further as a bio-assay for DMOG elution from stents.

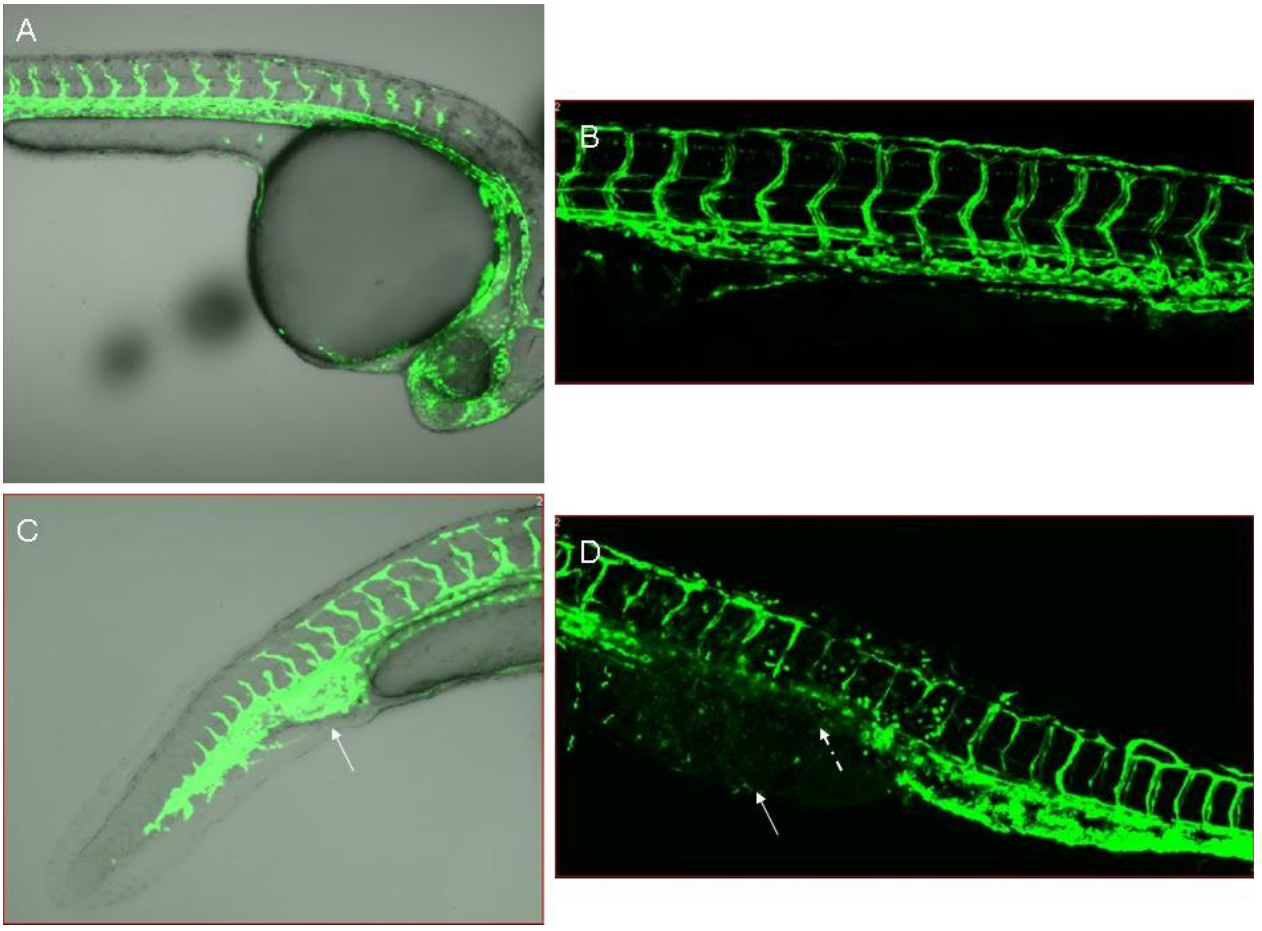


Figure 3.19. Confocal microscopy. (A) Control Fli-1 mutant zebrafish embryo at 29 hours post fertilization (hpf). (B) Close-up view of mid-tail section of control embryo, 29hpf. (C) Tail segment of embryo in DMOG 50μM at 29hpf showing arterio-venous fistulae (arrow). (D) Close up of mid-tail segment, DMOG 50μM, 29hpf. There is a fine network of ectopic vessels and neovascularization around an disrupted notochord.

3.6 Discussion of in-vitro experiments

Efforts to develop reproducible assays suitable for high-throughput screening of anti-angiogenic compounds have been only partially successful, and tissue culture remains technically challenging. Furthermore, quantification of promotion of angiogenesis is generally considered to be more difficult than quantification of angiogenesis inhibition. This is because to maintain viability of the tissue explant requires a culture media rich in growth factors. While it is relatively easy to achieve maximal tissue growth in culture, deliberately creating conditions of sub-maximal growth while preserving tissue viability poses a major challenge.

Published work with tissue culture in this field to date has concentrated largely on assays designed to screen angiogenesis inhibitors, with no commercially available system marketed specifically to quantify the effect of an angiogenesis promoting drug. Furthermore, a search of the literature fails to reveal any account of attempts to develop such an in-vitro system. To date, early evaluation of potentially pro-angiogenic compounds has involved systemic application of the compound in small animals, or a hybrid approach using an implanted sub-cutaneous culture medium such as sponge-matrigel in mice.[343]

3.6.1 Porcine carotid vessel-outgrowth assay

Our results demonstrate a striking linear relationship between corrected sprout growth area and the concentration of fetal bovine serum (FBS). This is consistent with the presence of growth factors including VEGF, bFGF and bovine brain extract in FBS. We have demonstrated that microvessel sprouting in this assay is reproducible and dependent on the concentration of local growth factors present.

Most angiogenesis assays to date have been concerned with high-volume screening of potential anti-angiogenic compounds. This application merely relies on production of consistent maximal vessel sprouting from which a conclusion could be drawn as to the efficacy of the angiogenesis inhibitor under investigation. Tissue under culture is ischaemic and requires a threshold concentration of growth factors in order to remain viable.

We hypothesised that by adapting a tissue culture model initially designed to investigate angiogenesis inhibitors, we could determine whether a positive pro-angiogenic effect due to our study compounds. Bovine brain extract, used in previous studies, was omitted due to its high concentration of fibroblast growth factor. The results show no significant increase in sprouting with any of the study drugs, albeit with a significant inter-sample variability. Despite the relatively large standard deviations seen, there was no significant augmentation in sprouting seen with addition of the study drugs to a standard growth medium containing 5%

FBS. This concentration of FBS was chosen as the best compromise between maintaining tissue viability and allowing detection of any pro-angiogenic effect.

Given the consistency of the results showing no pro-angiogenic effect with either the investigative compounds or VEGF, it can be hypothesised that this model is insufficiently sensitive to detect a pro-angiogenic effect in the presence of sufficient concentrations of FBS required to maintain tissue viability. The culture medium is essentially 'flooded' with basal growth factors so that demonstrating an additional effect is difficult.

The linear response to FBS seen in our experiments demonstrates that the porcine carotid matrigel model may be a suitable platform for the testing of angiogenesis inhibitors, but appears unsuited to the assessment of pro-angiogenic compounds for the reasons outlined above. This is a significant finding that was not addressed either in original descriptions of the Nicosia rat aortic-ring assay or in subsequent descriptions of modifications to this assay. Indeed, several authors suggest that matrigel-based whole or partial vessel outgrowth assays may be suitable for rapid screening of both anti-angiogenic and pro-angiogenic compounds, while only describing their use in assessment of anti-angiogenic compounds such as Batimastat or Suramin. Our results would suggest that matrigel-based tissue culture assays are unlikely to be suitable for assessment of pro-angiogenic compounds. The explanation for this is that intact tissue in culture requires a

threshold level of growth factors to maintain viability, and that detection of incremental angiogenic effect above this level of growth factors is not possible.

We strived to conduct our experiments in an environment with minimal intrinsic growth factors, and as such required prolonged culture periods to produce sprout outgrowth. The results of the FBS dose-ranging experiment clearly show a threshold below which only minimal vessel outgrowth occurs (around 5% FBS concentration). Furthermore we observed a high incidence of culture plate infection. We can hypothesis this was a result of obtaining tissue from a commercial abattoir rather than from a dedicated scientific supplier of animal tissue. Despite adhering to strict aseptic technique during harvesting of the tissue approximately 25% of plates exhibited growth of fungal hyphae which had the effect of inhibiting vessel outgrowth.

Tissue preparation was in our experience critical to achieving successful sprout outgrowth. Most authors have made no distinction between cutting tissue by hand or the use of automated cutting techniques. In our experience only careful hand cutting using a pressing motion with a scalpel maintained endothelial integrity. Use of punch forceps or automated cutting machines, while producing more uniform tissue fragments, resulted in highly asymmetric or absent sprouting, most likely due to disruption of the delicate endothelial layer. Immunohistochemical techniques confirmed the presence of a surface endothelial layer where sprouting was present, and cell recovery from sprouts invading into the matrigel confirmed

that the sprouts themselves were composed of endothelial cells without smooth muscle cells. Precise visualization of the sprouts was not possible, although it can be postulated that these consist of simple layers of endothelial cells. Some controversy remains over the architecture of sprouts seen in this model, with the predominant view being that they consist of endothelial cells join by tight cell junctions to form rope-like cords. Some authors have observed lumina which are though to form by apoptosis of centrally lying cells. The diversity of reports in the literature reflect the technical difficulty of visualising sprouts in these assays.

3.6.2 Quantification of angiogenesis

Several methods have been described for quantification of angiogenesis. This involves quantification of the extent of 'micro-vessel outgrowth' in tissue culture assays or 'tubule formation' in cell-culture based assays. Corrected sprout area was selected as the end-point in our tissue-culture assay after experimentation with a number of different off-line techniques. The lack of a consensus method makes comparison of results between groups more difficult. As sprouts lie in a three-dimensional orientation and often clustered together simple counting of the number of sprouts is impractical, and often the area covered by the sprouts is taken.[269] More detailed quantification can be performed by measuring the number of intersection or confluence points of sprouts, and various methods have been described to integrate these measures into an overall score.[340] Both manual and automated methods are described in the literature. Manual processing essentially

involved either counting the number of vessels and branch points, or the number of confluence points per unit area: both methods are imperfect however.[253]

Alternatively sprout area can be determined manually or with manual tracing of a pointer around an off-line digital image. The sprout area is then expressed as a ratio over the original area of the tissue.[344] Various automated edge-detection techniques have been described using several different computer image-analysis programmes which attempt to differentiate sprouts from background pixels.[339] While these methods are developing continuously and offer faster throughput than entirely manual techniques, they all require a degree of manual over-ride and are highly sensitive to changes in experimental conditions such as lighting intensity and white-balance of the captured digital image. No consensus exists as to the best method. Accepting that analysis based on a 2-dimensional photograph of a 3-dimensional process is inherently limited we opted in this assay to assess total micro-vessel outgrowth area. This was compared to values obtained by manually measuring number *x* length of sprouts (in mm) with good correlation and was considerably quicker to perform. Furthermore manual counting of sprouts, sprout volume or confluence points in this assay is arguably less applicable than overall area where a dense 'blush' of vessels is present and it is often not possible to differentiate individual sprouts. Finally, our method of area calculation has been used in published work describing similar assays.[345]

Our results suggest that contrary to previous untested suggestions in the literature the porcine carotid tissue culture angiogenesis assay is not suitable for the assessment of pro-angiogenic compounds. The assay is technically demanding and requires prolonged tissue culture with a threshold level of basal growth factors to maintain tissue integrity. It was not possible to determine any incremental benefit of exogenous growth factors, including known pro-angiogenic compounds such as VEGF. High concentrations of DMOG or Cobalt were toxic to endothelial sprouting. In order to assess and attempt to quantify the effects of growth factors alternative in-vitro angiogenesis assays are required. We therefore turned our attention to development of an alternative cell-based angiogenesis assay.

3.6.3 Matrigel

The precise amount of matrigel added to each well and the density of cells plated per well both strongly influence the formation of tubules, and thus reproducible results are highly dependent on experimental technique with a moderately long learning curve with regards to culture methods. [269, 280] Plating density has been shown to affect tubule response to growth factors, with high densities leading to 'clumps' of undifferentiated cells and complicating image analysis (where clumped cells may resemble early tubule-like structures). Similarly low plating densities can inhibit tubule formation.[191] A review of 31 research papers using matrigel revealed a wide disparity in methods with regards to culture conditions

and the amount of matrigel used per unit area of culture plate. The groups used 6, 12, 24, 48 and 96-well plates with volumes of matrigel ranging from 0.17µl/mm^2 to 3.53µl/mm^2.[53, 280] Carolyn Staton's group report that use of too thin a layer of matrigel (less than 1.2 µl/mm^2) leads to a well effect due to surface tension with pooling of cells in the centre of the wells. In the above review of research methods, cell plating density ranged from 56.6 - 2829.7 cells/mm^2 with an average of 563 cells/mm^2**.** Groups also varied in the interval chosen befor e analysis: this ranged from 4 to 24 hours.

3.6.4 Cell culture (tubule formation) angiogenesis assay

Our experiments suggest a significant increase in tubule formation with DMOG 125µM compared with control. Addition of a specific VEGF2-receptor inhibitor abolishes this effect. This suggests that DMOG is acting via an effect in up-regulating expression of VEGF. Use of reduced growth factor matrigel and a relatively low (5%) added fetal bovine serum is necessary to avoid excessive basal tubule formation.

There is to date a single report of increased tubule formation with the addition of VEGF to a cell-based matrigel assay.[346] More recent evaluations of tube-forming matrigel assays have failed to demonstrate significant effects with exogenous VEGF, with the authors postulating that the 'background' levels of VEGF within the matrigel may have elicited maximal stimulation of tubule formation and

prevented production of any further stimulation of tubule formation by added VEGF. [265]

While this assay has potential use as a screening tool for evaluation of pro-angiogenic compounds it has the limitation of being very sensitive to set-up. Maintenance of a uniform cell plating density is crucial in achieving reproducible results. The manufacturers of matrigel also advise against comparing results from experiments using different batches of matrigel due to variations in the levels of basal growth factors present.

Ultimately this cell-based assay, while useful as a screening tool and for detection of pro-angiogenic compounds, is only a basic representation of the far more complex processes occurring in an intact organism.

3.6.5 Zebrafish experiments

DMOG affected arteriogenesis in the normal embryonic zebrafish in a dose-dependent manner. Interestingly changes were induced both in the notochord and in the vasculature. VEGF is known to signal notochord development, and also to be produced by the notochord in order to stimulate arteriogenesis. There was however no effect on collateral recruitment in the gridlock-mutant zebrafish model. This suggests that this embryological mutation may not be responsive to exogenous growth factors, and is not suitable as a bio-assay for DMOG.

3.6.6 DMOG detection by mass spectroscopy and elution bio-assay

Several methods to directly measure DMOG in solution were discounted at a planning stage: advice from our collaborators in the Chemistry research laboratory of Oxford University was that radio-labelling would be practically difficult in the case of C14 (a difficult substitution reaction) and would lead to great variation in solubility and lipophilicity in the case of Iodine 131. Most of the difficulty was a result of the very low molecular weight of DMOG (MW 175.14) in comparison to polypeptide molecules such as VEGF in which radio-labelling has less effect on pharmacokinetics. As a method of measuring elution, radio-labelling was therefore discounted.

Attempts to directly measure DMOG by electrospray ionisation mass spectrometry (ESI-MS) were conducted over several months. Although a calibration curve for DMOG freshly reconstituted into solution (ethanol or methanol) was constructed it proved impossible to obtain reproducible results for DMOG concentrations in solution. It was thought this was due to variable esterification of DMOG in ethanol and methanol.

The alternative approach of a bio-assay was novel, based in part on previous work by Professor Gershlick's group where a similar bio-assay was used to confirm biological activity of vascular endothelial growth factor (VEGF) eluted from a

polymer-coated stent.[345] The bio-assay was first assessed by its ability to successfully discriminate between blinded DMOG or control stent wires. Subsequently biological activity was seen with stent wires following up to 21 days elution. Although this approach limited assessment at each time-point to the presence or absence of biological activity, there was clear evidence of tubule formation in a serum-free matrigel model with an effect equivalent to 100-150μM DMOG.

Chapter 4

Methodology of in-vivo experiments

4.1 Background

This chapter describes the methodology of the in-vivo programme of work culminating in an in-vivo pre-clinical study of local administration of hypoxia-inducible factor hydroxylase inhibitor (di-methyl oxalyl glycine, DMOG) to an occluded coronary artery. Two main stages in this process are described:

- Stage 1: Development of a novel, entirely endovascular model of porcine coronary occlusion
- Stage 2: Trial design and procedure of a randomized, controlled and blinded study of a DMOG-eluting coronary stent compared to a placebo stent in the setting of an occluded porcine coronary artery (CTO)

4.2 Development of endovascular porcine coronary occlusion model (stage 1)

The pig coronary artery is currently the standard model for the development of PCI technologies such as stents, but there is no widely accepted endovascular animal model that allows chronic total coronary occlusions (CTOs) to be studied biologically or allows interventional developments to be tested. External coronary constrictors have been used for over 25 years in animal models, but these require a thoracotomy.[347] Copper-coated stents were first used as a means of producing arterial stenosis to study arterial remodeling, copper producing an

intense inflammatory reaction with exuberant neo-intimal hyperplasia.[348, 349] Our aim was to develop a simple, reliable, survivable model of a CTO in a porcine coronary artery.

4.2.1 Background to the development of the porcine CTO model

The porcine coronary occlusion model was developed and refined at the Sheffield University field laboratory. A programme of work dating from 2004 had evaluated a number of devices potentially capable of producing a survivable CTO by percutaneous means. Initial experiments were performed in sequential groups of 3-4 animals each, to allow incremental improvements to be made in the protocol, after discussion with the Home Office Inspector. In contrast with external constrictors, these devices were deliverable percutaneously. At the start of this M.D. project (late 2006), experiments were ongoing using constricted stent grafts in an a attempt to produce a CTO, but no survivable and effective percutaneous CTO model existed. Development of such a model with Dr. Julian Gunn formed a major part of this programme of work towards this thesis.

4.2.1.1 The Jostent GraftMaster™

In these experiments the Jostent GraftMaster™ (Abbott Vascular Devices, Redwood City, CA) was used first. This is a constricted, balloon-expandable, stent graft consisting of a polytetrafluoroethane (PTFE) membrane sandwiched

between two stainless steel balloon-expandable stents. This device was modified by applying a 2-0 vicryl ligature around the central portion of the graft, so that, upon balloon expansion, a constriction remained within the graft. (*fig 4.1)* The intention was to allow some residual blood flow whilst the constriction could act a nidus for platelet aggregation, thrombosis, and neointimal hyperplasia which could progress to a CTO.

4.2.1.2 The Symbiot™ stent

The second device to be tested was the Symbiot™ stent (Boston Scientific Corporation, Natick, MA). This was a constricted, self-expanding, nitinol stent coated with a membrane of PTFE. The manufacturers modified the expansion characteristics of the stent for the authors by applying a Prolene ligature of diameter 1mm at the centre of the stent inside the delivery sheath. *(fig 4.2)*

The stent graft devices were tested in a total of 10 pigs. Whilst vessel occlusion was achieved, both devices were bulky and there were significant difficulties in tracking the devices and deploying them within the coronary arteries. Furthermore there was a high early mortality with both devices due to stent thrombosis. This was mitigated, but not abolished, by use of the RCA rather than the LAD. Despite modification of the Symbiot device to try to overcome these difficulties the stent graft approach remained technically difficult and was abandoned.

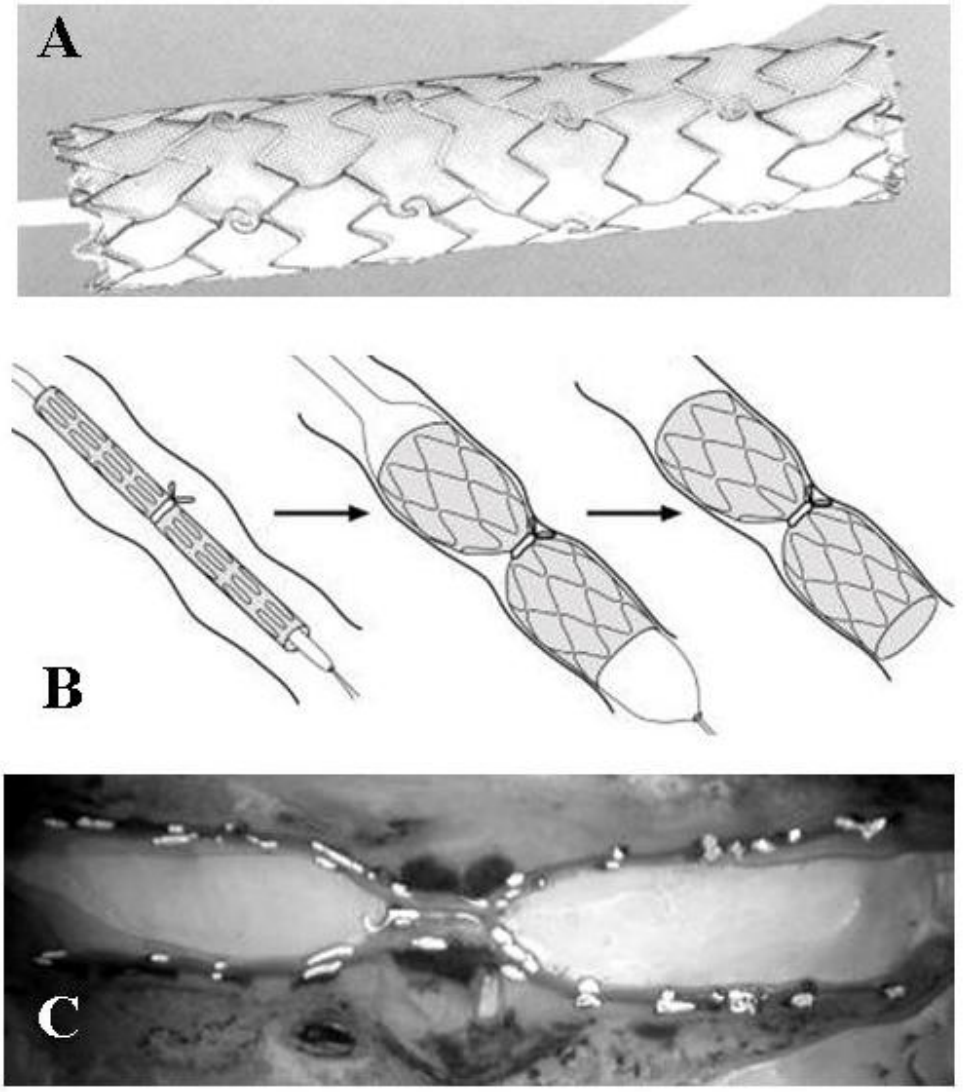


Figure 4.1: The Jostent GraftMaster™ (Abbott Vascular Devices) covered stent. This consists of a PTFE membrane contained within a sandwich of 2 stainless steel stents. (A) The device is shown after balloon inflation, without a constricting ligature. (B) Cartoon depicting deployment of the stent graft with an externally applied ligature, from positioning (left), through balloon inflation (center), to balloon removal (right). (C) Longitudinal section of a constricted stent graft from a pig which survived for 16 days. The suture material can be seen around the constriction, which is completely occluding the lumen of the vessel. Neointima lines both ends of the graft.

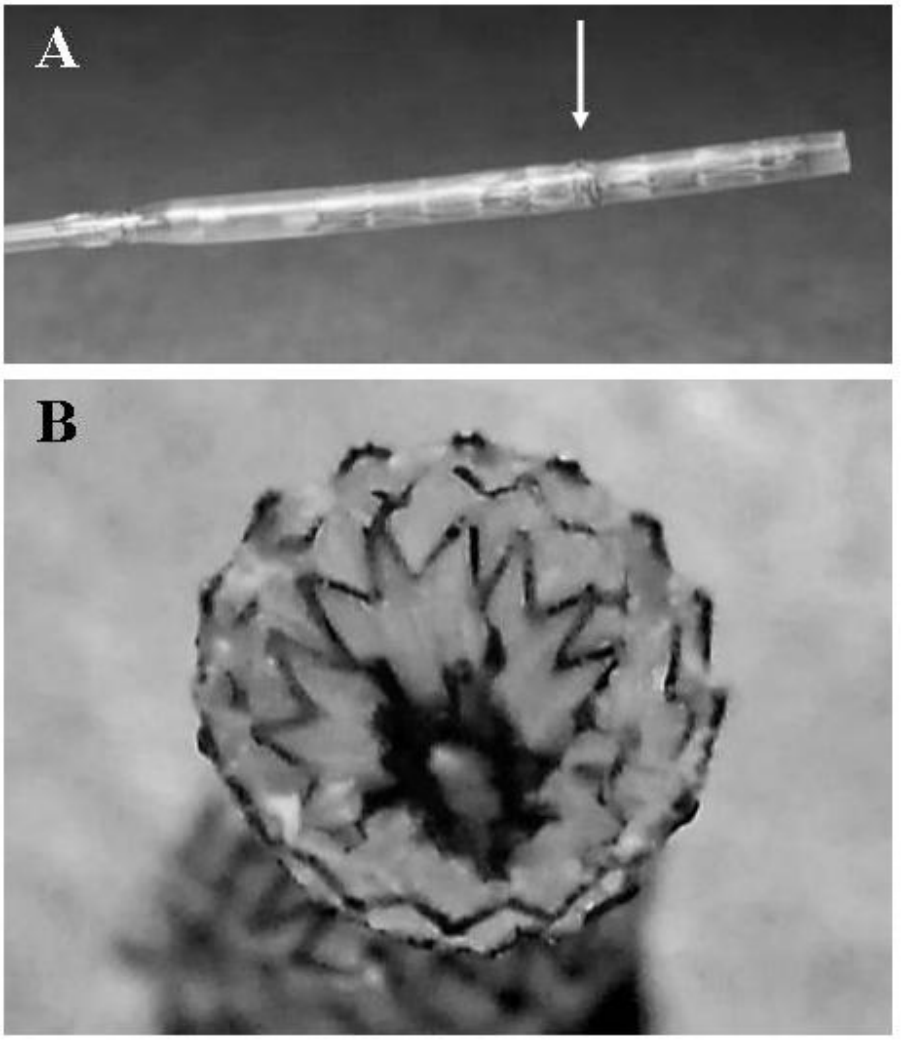


Figure 4.2: The Symbiot™, PTFE-covered, nitinol stent (Boston Scientific Corporation). (A) The device is shown loaded in its constraining sheath. The ligature applied around the stent graft is visible through the sheath at the arrow. (B) View of the stent graft from one end, showing the nitinol frame fully expanded at the end, and the central constriction which is 1mm in diameter.

4.2.2 Balloon-expandable, copper stent

The copper stent was the device then selected for testing in the attempted production of a CTO model. The basis of this device was the Bio*divYsio*™ stent, formerly manufactured by Biocompatibles Ltd, Farnham, UK. This is a balloon-expandable, stainless steel stent. It is no longer in clinical use. Copper coating was performed by Brivant Medical Engineering Ltd (Galway, Ireland). The phosphorylcholine coating was removed, a nickel coating was applied as a base layer and then the surface was electroplated with copper. This was achieved in a bath of nickel chloride, initially using a nickel plate as one electrode and the stent as another. The nickel plate was used as the anode at first, leading to removal of the phosphorylcholine in a process akin to electropolishing. Then, by reversing the polarity, a nickel 'strike' was applied to the stripped surface of the stent. Finally, by replacing the nickel with a copper plate, the stent was electroplated with copper after 30 minutes' immersion. Copper was transferred from a copper plate (anode) to the nickel-plated stainless steel (cathode) through electrolysis.*(fig 4.3)* In its final version, the electroplating was applied only to the abluminal aspect of the stent. This was achieved by passing a current of 0.2A through a heated solution of Nickel Chloride with the stent suspended in solution between two sources of copper.*(fig 4.4)* Brivant Ltd. reported an approximate abluminal dose (by weight) of 200mg copper per stent. The stent was then re-sterilized ready for use.

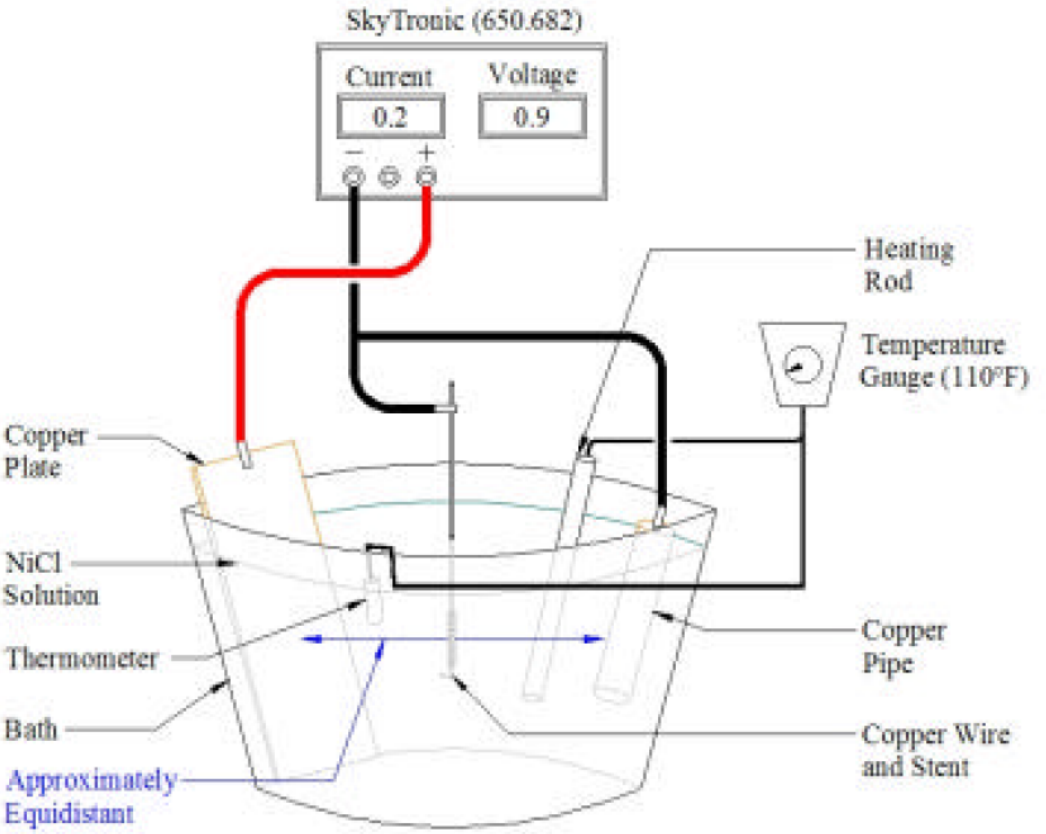


Figure 4.3: Copper-plating electrolysis apparatus (image courtesy of Brivant, Ltd).

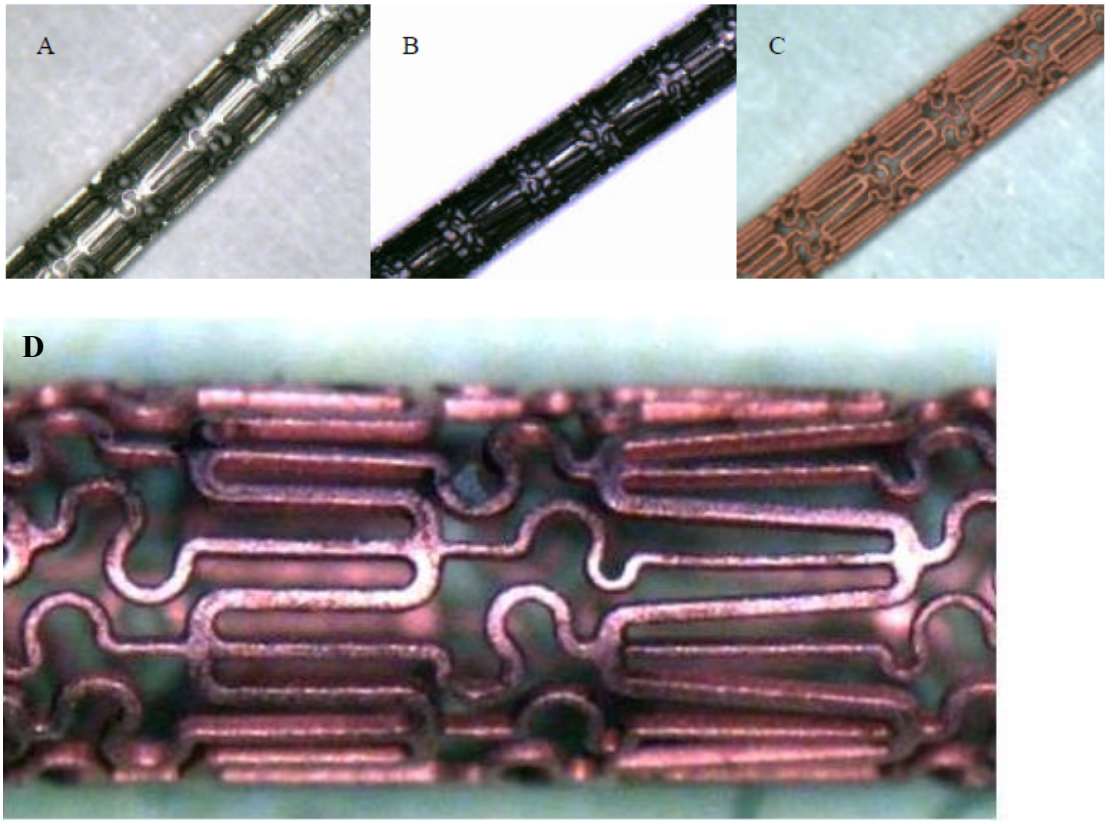


Figure 4.4: Process of plating the copper stents. The balloon-expandable, copper-plated stent. A BiodivYsio™ stent (Biompatibles Ltd) is plated with a nickel 'strike' after stripping off the phosphorylcholine coating. It is then electro-plating with copper. (A) Bare metal stainless steel stent; (B) Nickel-plated stainless steel stent; (C) Copper-plated stainless steel stent. (D) Close-up view of stent cell design.

4.3 Interventional procedure- CTO model development

The following describes initial procedures used during development of the CTO model and during experiment 1 of the subsequent pre-clinical trial. Experimental procedures were performed according to UK Home Office regulations (Personal Investigation License PIL 40/8433). Yorkshire White pigs were used, weighing approximately 30kg. Smaller animals (around 18Kg) had been shown to be less resilient to stent implantation and more prone to fatal sub-acute stent thrombosis. This was in part due to smaller artery size dictating larger balloon: artery size ratios and hence more arterial injury. (Dr J. Gunn, personal communication).

The final protocol for the CTO model was as follows. 20 Yorkshire White pigs weighing approximately 30kg (range 25-35 Kg) were used. All procedures were performed in accordance with UK Home Office regulations. The pigs were pre-treated for 7 days with aspirin 37.5mg and clopidogrel 37.5mg in their feed. Sedation was initiated with intramuscular azaperone and general anaesthesia was induced with intravenous propofol and maintained with spontaneously inhaled enflurane and oxygen. Vascular access was gained via the right carotid artery and a bolus dose of sodium heparin 2500IU administered. Under fluoroscopic guidance standard clinical coronary artery angioplasty guide catheters (left Judkins JL3 for the RCA and Amplatz AR1 for the LCA) were used to obtain left and right coronary angiograms in 2 projections. This was done for as baseline for comparison with subsequent angiograms to look for collateral vessel formation,

antegrade or retrograde. The images were archived. Stent implantation was in the RCA only. After passage of a floppy 0.014" coronary guidewire, a copper-electroplated, stainless steel, balloon-expandable stent *(fig. 4.4)* was directly implanted, under fluoroscopic guidance and without post-dilatation, in mid-vessel. The delivery balloon had an expansion diameter of approximately 3.5mm at 12 atmospheres pressure. The segment of vessel selected for implantation had a diameter similar to this. After implantation, the wounds were closed and the animal allowed to recover. Dual anti-platelet therapy was continued throughout the study period. At 28 days, the animals were re-anesthetized, and the angiograms repeated in the same recorded views. The animals were then killed with an intravenous overdose of thiopentone, and the RCA excised, fixed in 10% formalin, embedded in T8100 resin, cut with a diamond-tipped saw, and ground and polished to produce multiple cross-sections. Sections were stained with hematoxylin and eosin and prepared for microscopy. The study endpoints were the creation of a CTO, with assessment both angiographically and histologically. This histological preparation has been described previously by our group.[350]

4.4 Preparation of DMOG or control stents for a randomized, controlled trial after establishment of CTO with the copper stent (stage 2).

The process of elution testing of the DMOG stents is described in Chapter 2. In brief the DMOG was dissolved in a small amount of an inorganic solvent and mixed with a proprietary polymer (Programmed elution polymer, PEP 94). This was sprayed onto 2.75 x 18mm bare-metal stainless steel stents (S7 stents, Medtronic Inc, Minneapolis, MN) with a surface area of 77.83 mm^2. The maximal amount of DMOG possible was loaded without necessitating an excessively thick layer of polymer. The final amount of DMOG loaded was 400µg per stent (approximately 5.1µg DMOG /mm^2) with the elution programmed to occur in a linear fashion over 28 days. The results of a stent-wire elution bio-assay are in Chapter 2, demonstrating metabolically active levels of DMOG in matrigel cell-culture assay, equivalent to 100-150µM DMOG, after 21 days elution of the stent.

4.5 Randomized study of DMOG or control stents in CTO

Following validation of the CTO model, a series of three experiments were planned for each of 20 animals (10 randomized at day 28 to implantation of a di-methyl oxalyl glycine (DMOG)-loaded PEP polymer coated stent and 10 randomized to implantation of a control PEP polymer only stent) over a 56 day period.. The aims of the study were:

- To deliver loaded active or placebo stents proximal to the occlusion to determine whether the eluting agent could enhance antegrade collateral formation.
- To determine the functional effect of any DMOG-stimulated antegrade collaterals.

Stent allocation was randomized independently (by Polybiomed Ltd) and blinded to the investigators until final histological analysis was complete. Basic experimental procedures below were common to all three study days (*fig 4.5)*

- Experiment 1, Day 0:

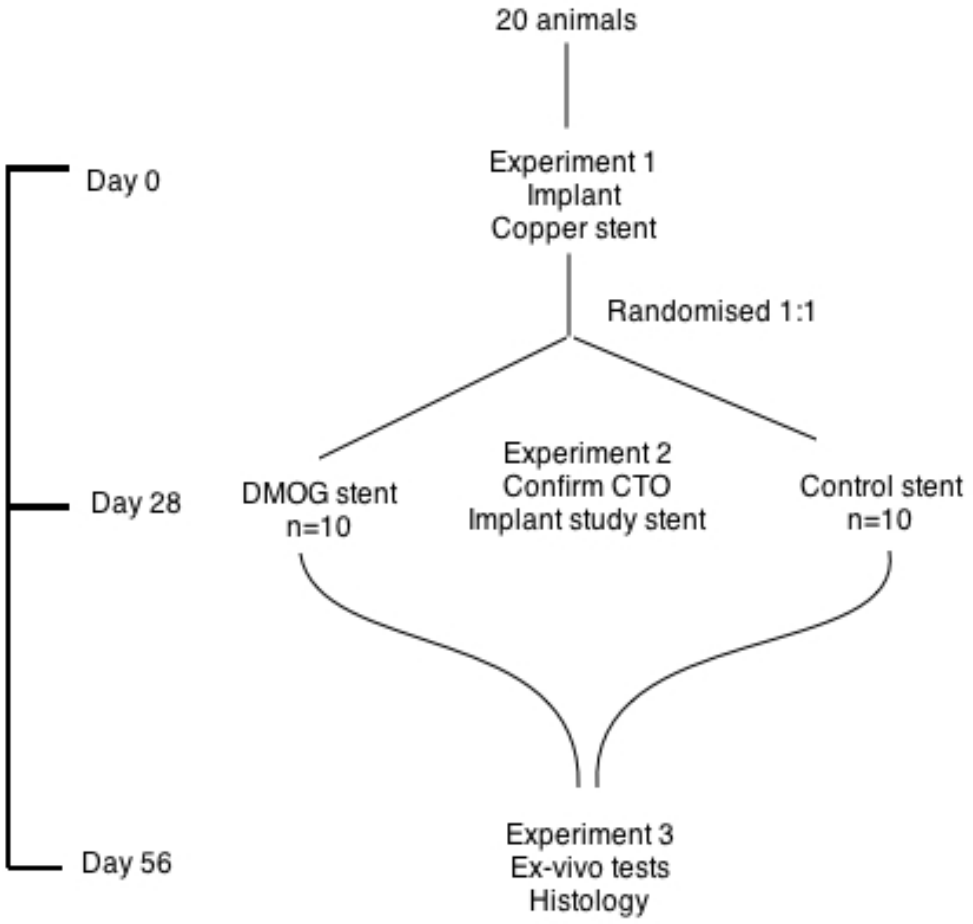
  Initial angiography, left ventriculography, and estimation of collateral flow index (CFI) with pressure wire. Copper stent implanted to promote vessel occlusion.

- Experiment 2, Day 28:

  Repeat angiography, left ventriculography, and estimation of 'Distal arterial run-off index' (DRI), a surrogate measure of CFI using the pressure wire. Study stent (DMOG or control) implanted. The aim was to place the study stent proximal to and abutting the copper stent. The exact distance from the distal edge of the study stent to the proximal edge of the copper stent was recorded.

- Experiment 3, Day 56:

Repeat angiography, left ventriculography, and estimation of Distal arterial run-off index with pressure wire. Sacrifice, ex-vivo flow studies, and histological sampling.



	Angio	LV	Pressure wire	Copper stent	Study stent	Ex-vivo tests	Histology
Day 0	X	X	X	X			
Day 28	X	X	X		X		
Day 56	X	X	X			X	X

Figure 4.5: Study flow-chart and interventions

4.5.1 Sample size consideration

The in-vivo study was an exploratory proof-of-concept study for the concept of transcription factor up-regulation. As the first trial of its type no formal sample size calculation was undertaken. Based on previous pig work (both published studies and histological studies from the Sheffield field laboratory) it was determined that at least 8 animals per group would be required to draw conclusions on histological vessel density.[350-353] The primary end-points were planned to be (a) histological quantification of luminal micro-vessels and adventitial microvessel number and (b) angiographic assessment of antegrade collateral area. (c) Physiological assessment of distal arterial run-off (Distal run-off index or DRI, a surrogate of collateral flow index).

4.5.2 Basic experimental procedures during experiments on day 0, 28 and 56.

4.5.2.1 Interventional procedure

Experimental procedures were performed according to UK Home Office regulations. Yorkshire White pigs were used, weighing approximately 30kg. The following methods were employed at each study day.

4.5.2.2 Coronary angiography

Sedation was initiated with intramuscular azaperone and general anaesthesia was induced with intravenous propofol and maintained with spontaneously inhaled enflurane and oxygen. By means of surgical cut-down, vascular access was gained via the right carotid artery and, under fluoroscopic guidance, the left coronary arteries was intubated with a 6 French Amplatz right, and the right coronary artery with a 6F left Judkins guide catheter. Coronary angiography was performed in LAO 35° and RAO 30° projections. Left ventriculograms were obtained in standard RAO 30° and LAO 30° projections to allow for comparison of estimates of left ventricular ejection fraction. A 2 pence coin was placed in the angiographic field of view to provide a constant diameter for sizing reference.

The table height was fixed at 27.5cm from floor level with the intensifier positioned 22cm from the subject to ensure constant magnification. As before a 2 pence piece was placed in the angiographic field of view, on the surface of the chest, to act as a fixed visual reference for vessel size, a round coin having the property of presenting a constant fixed maximum diameter (25.9mm) irrespective of the angle of angiographic image acquisition. Images were acquired with an image intensifier and digitally archived as Dicom files using via a Philips Xcelera workstation. *(fig 4.6)*

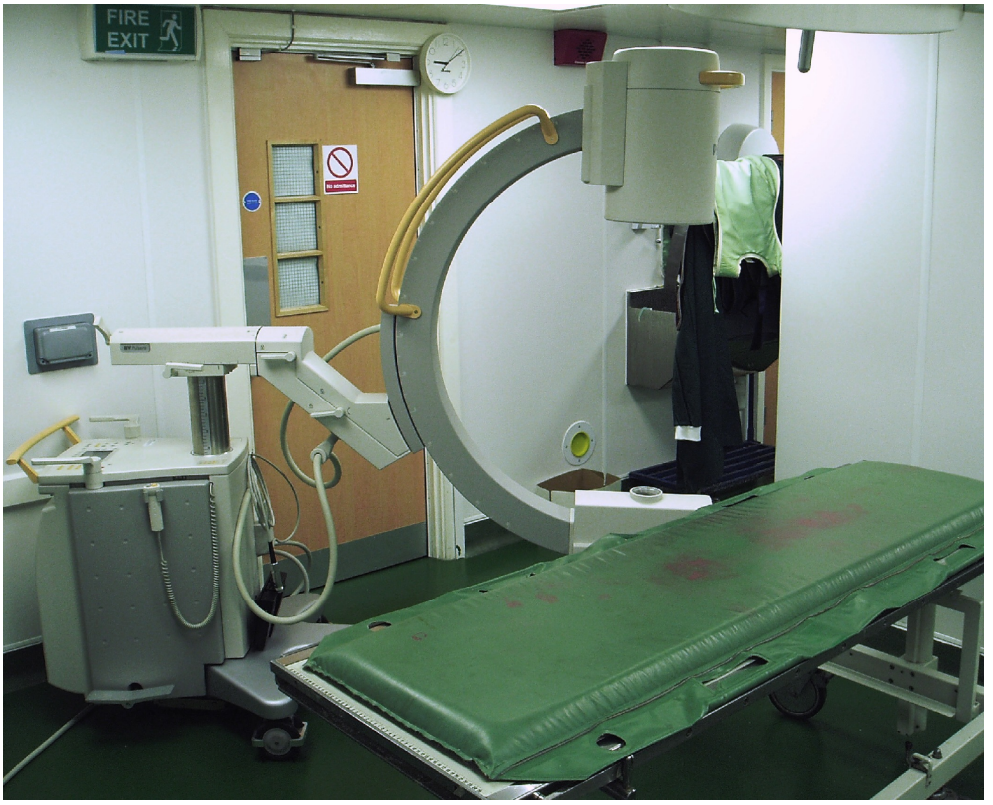


Figure 4.6. Field laboratory X-ray equipment and operating table.

4.5.2.3 Analysis of digital images

Dicom files were exported from the Philips Xcelera workstation for off-line analysis using Image J64 (NIH software).

4.5.2.4 Assessment of left ventricular function

Left ventricular function was assessed using ImageJ64. The automated edge-detection function was used to highlight the left ventricular cavity in diastole and systole in orthogonal projections. Corrections to this estimation were made by hand.

Whilst the constant angle of projection, magnification and table height aimed to minimize inter-acquisition differences in LV volume a 2 pence piece again acted as a size reference.

Left ventricular volumes were obtained in end-diastole and end-systole in both the RAO 30° and LAO 30° projections.
Left ventricular ejection fraction (LVEF) was expressed as the average of LVEF in the RAO 30° and LAO 30° projections. LVEF was estimated from 2-D LV area as follows:

$$LVEF = \frac{\text{End-diastolic area}^3 - \text{End-systolic area}^3}{\text{End-diastolic area}^3}$$

4.5.2.5 Angiographic collateral assessment

Dicom images were viewed with Osirix viewer v 3.5.1. Digital subtraction images were created to highlight small collateral vessels. Angiography allowed clear resolution of vessels down to around 200μm (the upper range of collateral vessel diameter). Small collateral vessels (down to 40-200μm) were therefore below the resolving limit of angiography. Analysis of histological images (chapter 5) suggests that 40-50% of adventitial microvessels are <200μm in diameter: while these vessels cannot be individually counted angiographically, multiple vessels are visible as a 'sheath' of vessels at angiography. In the angiographic assessment collateral area was therefore an important measure.

Collaterals (antegrade from proximal to distal RCA) were graded by:

1 Recipient filling grade (Rentrop Classification)

Grade 0- No recipient vessel filing

Grade 1- Minor side-branch filling but no epicardial recipient

Grade 2- Complete side-branch and partial epicardial recipient filling

Grade 3- Complete epicardial recipient filling

2 Collateral connection grade

CC0- No continuous connection between donor and recipient artery

CC1- Continuous threadlike connection

CC2- Continuous small side-branch like size of collateral throughout its course.

3 Collateral frame count (Coronary washout score)

=Number of frames for 15 ml of dye to clear proximal artery after deflation of proximal occlusion balloon

At day 28 and day 56 a simple coronary contrast washout test was performed as a measure of antegrade collateral flow. This was based on the technique described by Seiler at al.[354] After confirmation of an occlusion at the site of copper stent implantation, the proximal artery (proximal to the first curvature, within 20mm of the ostium) was occluded with a 4mm balloon inflated at low pressure. 15ml of contrast were rapidly injected via the guiding catheter, and the balloon was deflated. The number of frames taken until contrast washed out from the proximal artery was recorded. This measure is a surrogate for collateral flow, the number of frames taken for dye to was out of the artery being inversely proportional to collateral flow index.[354]

4 Simple counting of the number of continuous donor-recipient collaterals

Total number of continuous antegrade collaterals >0.4mm. This size was chosen as it is the limit of discrimination of most modern x-ray equipment and below this diameter a ‘blush’ of vessels is seen that is difficult to distinguish from

background ‘quantum mottle’. Branches >0.4mm were counted as a separate collateral.

5 Collateral area

The program Image J (Stanford University) was used to calculate the volume of antegrade collateral vessels. First a standardized degree of magnification was applied to the digital angiogram image based on the size of the 2p marker. The 2p had a diameter of 25.9mm, area 527mm^2. The line of proximal occlusion and the proximal part of the filling distal artery were included in the image to be analysed. An overlay grid was applied to the image such that each square had a width of 0.8mm, area 0.66mm^2. This size of overlay box offered the best balance between ease of use and discriminatory power. Each square that was completely filled by a collateral vessel that formed part of a complete bridging antegrade collateral was marked. *(fig 4.7)* The volume of the antegrade collateral network was calculated by multiplying the number of marked squares by 0.66mm^2.

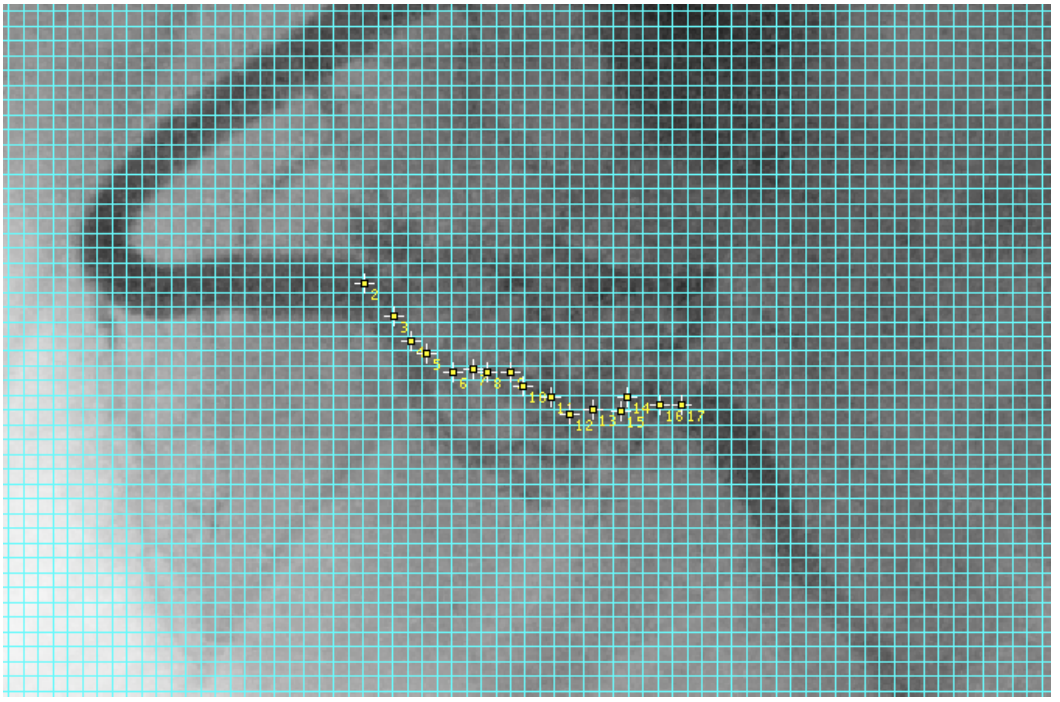


Figure 4.7. Example of overlay grid (each box 0.66mm^2) used to calculate collateral volume.

4.5.2.6 Coronary physiological measurements

An estimation of collateral flow was made at baseline (day 0) prior to copper stent implantation, at day 28 prior to study stent implantation and at day 56 prior to sacrifice.

During experiments 1 & 2 no attempt was made to cross any coronary occlusion that may be present after implantation of the copper stent during experiment 1. A surrogate of collateral flow index (CFI) was therefore calculated: this was

effectively a measure of arterial run-off distal to a proximal balloon occlusion. The primary reasons for our decision not cross the occlusion (as would be required for a true estimation of CFI) were practical ones. As a result of initial experience in the development of the CTO model, we learnt that pig survival was significantly reduced with lengthy PCI procedures and prolonged anaesthesia. The CTO produced by the copper stent often consists of a long, dense 'cap' of fibrous tissue which is challenging to cross even with specialist CTO guide wires, and initial attempts to cross copper-stent CTOs prior to the study proper were unsuccessful. As histological quantification of micro-vessel collaterals was the primary outcome measure, we were concerned that histological appearances might be altered by prolonged attempts at guide wire passage through the CTO.

We therefore adopted a proximal balloon occlusion technique (with maximal hyperaemia using intra-coronary adenosine and measurement of Ra pressure) to determine a surrogate 'Pw' with the transducer placed as far distal in the RCA as possible, but proximal to the occlusion point. 'Distal arterial run-off index' or DRI was calculated as (Pw-Pv)/(Pa-Pv) where Pv= right atrial pressure. We recognize that this surrogate measure of Pw is distinct from true Pw obtained by placing the transducer through the CTO into the distal vessel, and will be influenced by side-branch run-off proximal to the occlusion. The concept of using distal wedge pressure to assess coronary run-off has been described in the context of occluded coronary arteries.[337] DRI is not a true representation of distal collateral perfusion as would be obtained were it possible to pass the pressure

transducer distal to the occlusion point, but it does give a reproducible indication of antegrade perfusion. Our main aim was to compare serial changes in coronary perfusion within each animal, and there was therefore an effective internal control for side-branch run-off at each time-point. We refer to our surrogate measure of collateral flow as 'Distal arterial run-off index' (DRI)

Physiological measurements were taken before stent implantation (i.e. copper stent on day0, study stent on day 28). *(figs 4.8 & 4.9)* A 0.014-inch pressure monitoring guidewire (Volcano Therapeutics, Inc. Rancho Cordova, Ca.) was used to calculate the distal arterial run-off index (DRI). The pressure wire was calibrated in the guiding catheter. Aortic pressure was measured via the guiding catheter using a CardioMed Flowmeter system (CardioMed A/S, Oslo, Norway). Pressure wire measurements were transduced and recorded via a WaveMap (Endosonics Corp, Rancho Cordova, Ca.).

Mean aortic pressure (Pa), coronary distal pressure (Pd) and coronary wedge pressure (Pw) were recorded both at baseline and after a bolus intra-coronary (IC) injection of adenosine 60μg to produce maximal hyperaemic vasodilatation of the coronary capillary bed. The decision to proceed using IC adenosine was a pragmatic one based on the difficulties we encountered during previous experiments in achieving reliable intra-venous infusion of adenosine in the pig. Previous attempts to cannulate the internal jugular vein had resulted in bleeding and subsequent haematoma formation while maintaining peripheral access (via

friable veins on the dorsal aspect of the pinna) was challenging and often the veins 'tissued' during infusion. While intra-coronary injection of adenosine is widely used during clinical measurement of fractional flow reserve, there are general limitations to this approach and limitations specific to the measurement of collateral flow reserve. When delivered as an IC bolus there is a brief window of maximal hyperaemia (10-15 seconds) during which to record Pa/ Pd, after which the adenosine effect wears off (due to rapid hydrolysis and inactivation in the bloodstream). More specific to the present study there is a significant risk of not achieving maximal hyperaemia in the context of poor collateral filling of the distal coronary bed, due to inadequate antegrade penetration of the adenosine. This would have been circumvented by IV adenosine steady-state infusion. The use of IC adenosine, while necessiatated for technical reasons, will therefore have resulted in inaccurate measurements of CFI/ DRI due to inadequate hyperaemia (thus tending to an over-estimate of collateral flow). As such the results obtained must be interpreted with caution.

Right atrial pressure (Venous pressure, Pv) was measured via a 6F diagnostic catheter after cannulation of the internal jugular vein.

Fractional flow reserve (FFR) was calculated as (Pd-Pv)/(Pa-Pv). In order to evaluate the instantaneous recruitability of the collateral circulation the 'Distal arterial Run-off Index' (DRI) was calculated as: DRI= (Pw-Pv)/(Pa-Pv). *(fig 4.8)* In measuring DRI it is especially important to take into account right atrial

pressure (Pv) as the coronary wedge pressure (Pw) is low where there is a CTO and poor distal vessel perfusion (i.e. values of Pw tend to be lower than those of Pd in non-CTO vessels). This means that Pv exerts a greater influence upon the numerator when measuring DRI than it does during measurement fractional flow reserve {(Pd-Pv)/(Pa-Pv) in an unobstructed but stenosed artery. While in clinical practice a working approximation of FFR is often taken as Pd/Pa (ignoring the contribution of Pv), assessment of CFI/ DRI mandates incorporation of Pv to ensure accuracy.

The sequence of physiological studies were as follows:

- Measure Aortic (Pa), right atrial pressure (Pv) and distal coronary pressure (Pd)
- Measure Pa and Pd after bolus 60µg IC adenosine
- Inflate proximal 3.5mm balloon to occlude artery
- Measure Pa and coronary wedge pressure (Pw)
- Measure Pa and coronary wedge pressure (Pw) after bolus 60µg IC Adenosine

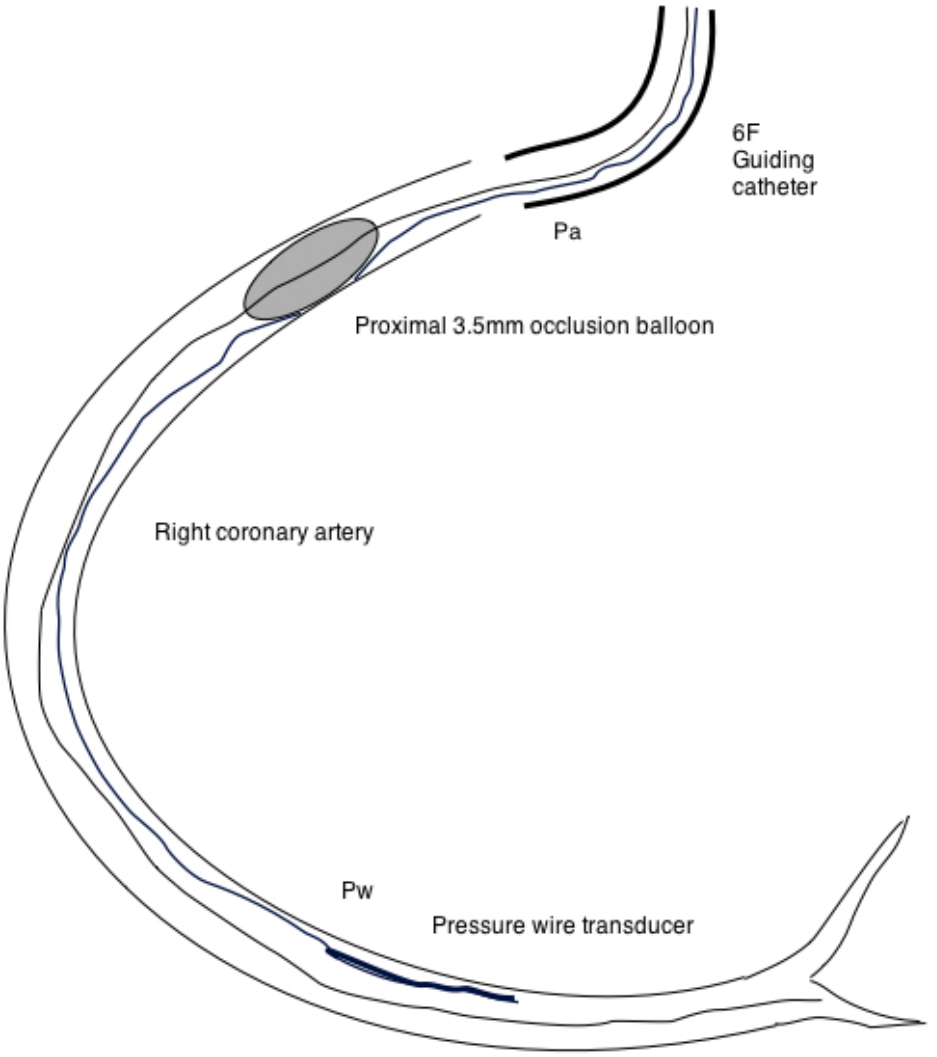


Figure 4.8 Schematic representation of experimental protocol for recording aortic pressure (Pa) and coronary wedge pressure (Pw).

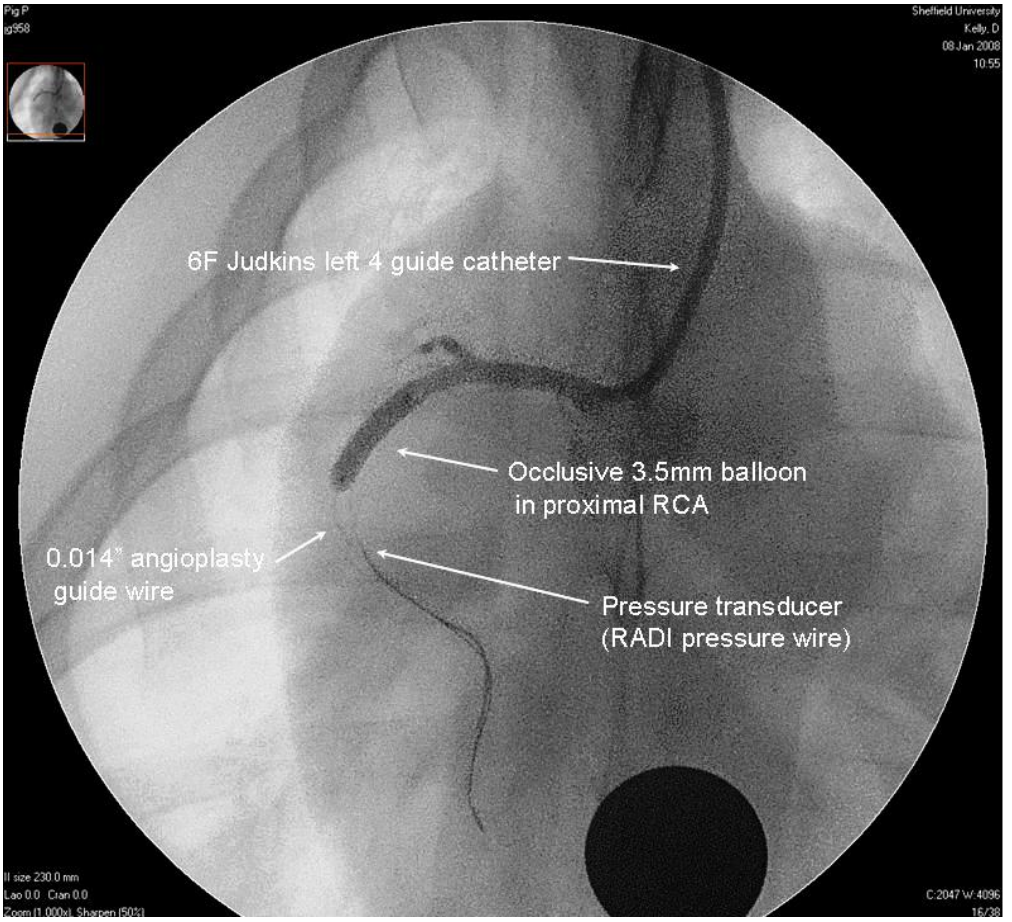


Figure 4.9 Angiographic image of experimental protocol for recording aortic pressure (Pa) and coronary wedge pressure (Pw) at day 0 (prior to copper stent implantation) by means of proximal balloon occlusion of the coronary artery.

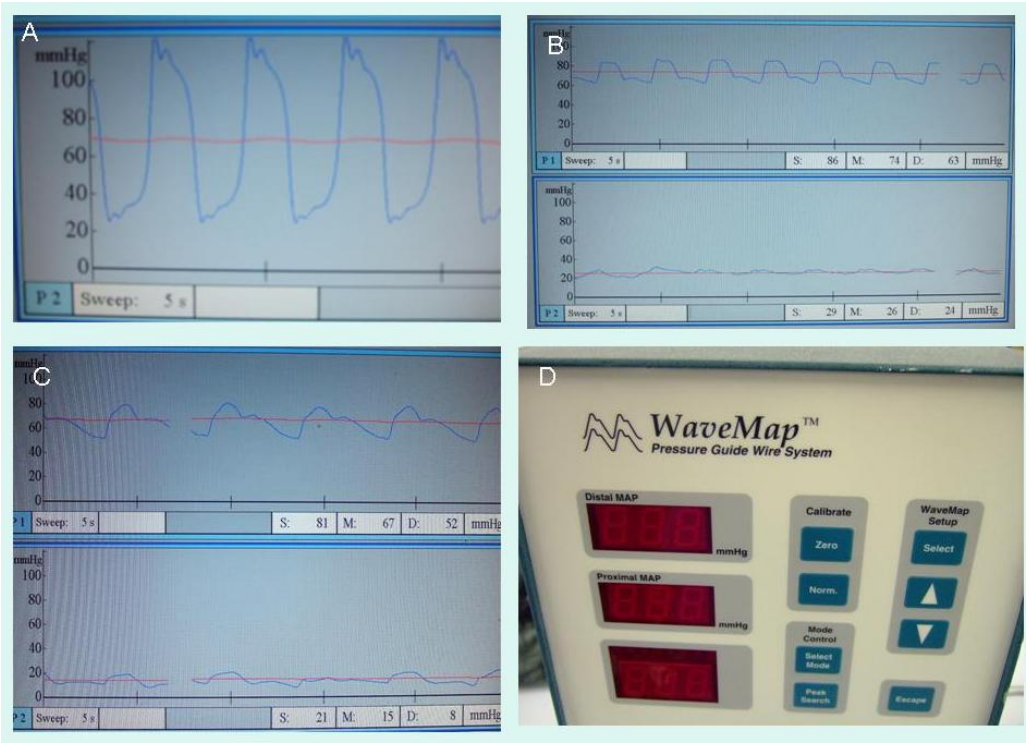


Figure 4.10 Screen photographs of invasive pressure monitoring [instantaneous pressure (blue) and average pressure (red)] from experiment 1. (A) left ventricular pressure, pig P; (B Upper screen shows aortic pressure measured via the guiding catheter tip and lower screen shows simultaneous coronary artery wedge pressure, pig P; (C) Aortic and coronary wedge pressure traces from Pig Q; (D) Numerical output of pressure wire readings were obtained via a WaveMap console.

4.5.2.7 Additional protocols for the primary study days

Day 0

In addition to the basic experimental procedures a copper stent was implanted in the mid RCA during this experiment. After passage of a floppy 0.014" coronary guidewire, a copper-electroplated, stainless steel, balloon-expandable stent was implanted, under fluoroscopic guidance, in mid-vessel. The delivery balloon had an expansion diameter of approximately 3.5mm at 12 atmospheres pressure. The quantitative coronary angiography function (QCA) of the Philips Xcelera workstation was used to measure the RCA diameter and select a segment of vessel of 3.5mm in diameter in which to implant the stent with a balloon to vessel ratio of 1:1. Oversize was not used because preliminary work with the copper stent had shown a high sub-acute occlusion (and death) rate with oversize at 1.25:1. Left and right coronary angiograms and left ventriculograms were recorded in 2 orthogonal views. After implantation, the wounds were closed and the animal allowed to recover. Dual anti-platelet therapy was continued throughout the study period.

Day 28

The procedures involved in production and testing of the DMOG-loaded stents are discussed in detail in Chapter 2, in-vitro experiments. The DMOG or control

stent was based on a 2.75 x 18mm bare metal stent platform (S7 stent, Medtronic Ltd., Minneapolis, MN) coated with proprietary PEP polymer (Polybiomed Ltd). This stent size was used for practical reasons of availability and has the same cell design as the S7 3mm and 3.5mm diameter stents. These stents were supplied as unmounted stents and after coating were hand-crimped on to 3.5x18mm Monorail Maverick balloons (Boston Scientific Inc., Natick, MA). Using information from QCA analysis of the landing-zone segment of vessel the stent was implanted at or just above nominal pressure so as to achieve 1:1 sizing relative to the arterial diameter. The study stent (DMOG or control) was implanted proximally as close as possible to the copper stent. The shape of the balloon tip and difficulties of stent delivery in tortuous coronary arteries made it difficult to achieve contiguity. The mean distance between the two stents did not however differ between the groups when images were subsequently analysed: DMOG group 3.5mm (range 2-5mm) vs. control 2.9mm (2-4mm), p=0.17.

Day 56

No further stent implantation was performed during the terminal experiment on day 56. In addition to basic experimental procedures the following post-mortem procedures were undertaken. Following angiographic and physiological testing sacrifice occurred at day 56 under general anaesthesia by administration of an overdose of thiopentone. The great vessels were clamped and divided and the heart excised.

4.5.2.9 Direct estimation of coronary flow

A watertight coronary infusion apparatus was made by suturing a 14G intravenous cannula around the coronary os of the explanted heart, taking care not to occlude the artery. To this was attached an intravenous giving set and a 500ml bag of 0.9% saline. The saline bag was suspended at a fixed height of exactly 4 feet (122 cm). Infusion was commenced and the proximal insertion site checked for leaks. The heart was placed on a set of electronic balance scales (EH-5000H balance) and the scales reset to zero. The infusion was started at its maximum infusion rate and the time taken for the scales to register 100g (i.e. 100 ml saline) was noted. From this a standardized coronary flow rate (ml/min) was estimated. *(fig 4.11)*

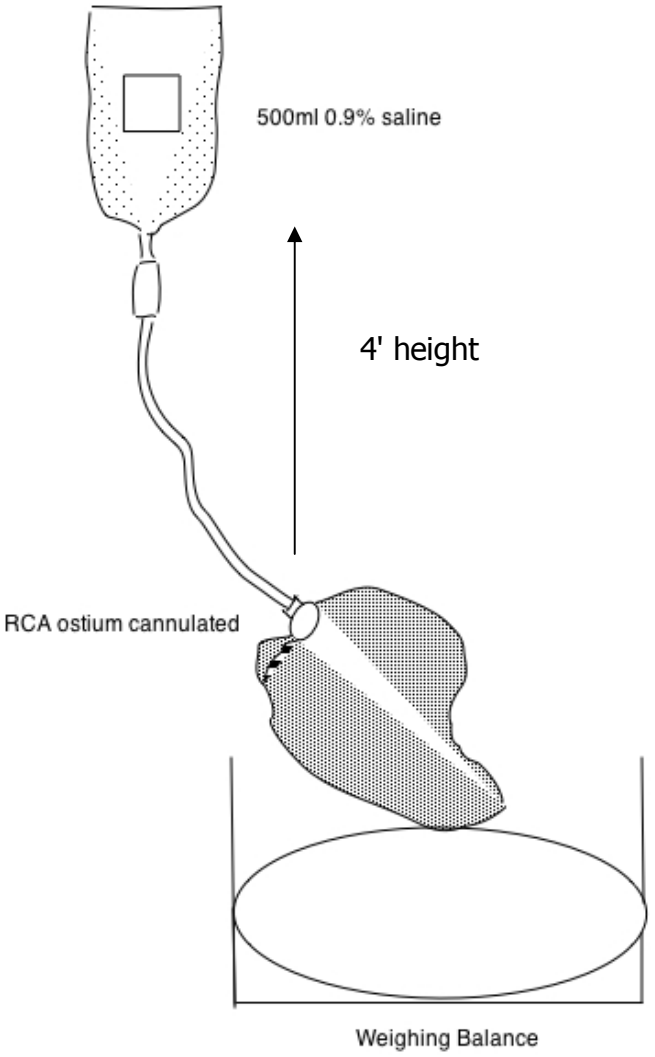
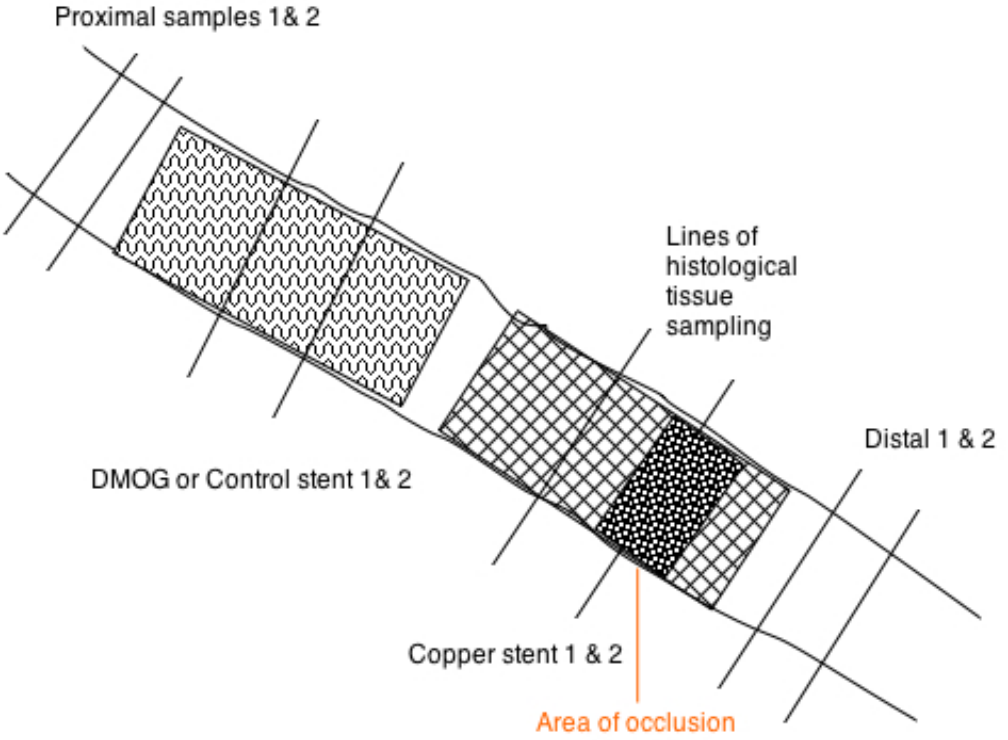


Figure 4.11. Experimental set-up for estimation of ex-vivo coronary flow

4.5.2.10 Histological processing

The coronary arteries were carefully dissected free with a minimum of surrounding tissue and flushed with saline. The two adjoining stents (study stent most proximal, copper stent distal) were identified. A scalpel blade was used to

cut down onto the copper stent and the artery was opened out in a flat sheet, preserving the stent and its contents. Sections were taken as follows; 2 immediately proximal to the study (DMOG or control) stent, 2 at the mid point of the study stent, 2 at the mid point of the copper stent, and 2 immediately distal to the study stent. Sections were also taken from the mid left anterior descending artery as controls.



TYPICAL HISTOLOGICAL SAMPLING WITH SEGMENT OF RCA OPENED OUT & LAID FLAT

Figure4.12. Location of tissue samples taken from excised RCA during terminal experiment.

The tissue samples were labeled and stored in formalin for subsequent staining with haematoxylin and eosin (H&E) and processing for immunohistochemistry.

After processing the tissue samples above the copper stents and their neointimal contents sections were fixed in 10% formalin for 24 hours, embedded in T8100 resin, cut with a diamond-tipped saw, and ground and polished to produce multiple cross-sections. Sections were stained with H&E and prepared for microscopy. This histological preparation has been described previously by our group.[350]

Slides were photographed using a Zeiss Axiovert microscope at 40x magnification and Olympus digital camera with C14 image analysis software. To assess the number of collateral vessels present, the central lumen of the RCA was first identified at low power. The total number of micro-vessels was counted in 40 high power fields (10 high power fields in each of 4 quadrants centred on the RCA lumen). This process was repeated for both sections in each sampling position and an average value obtained. Poor quality or damaged slides were discounted. The microvessels were categorized as small (<100μm) or large (≥100 μm). Analysis was performed on the total number of angiographically visible vessels and the number of large vessels.

4.5.2.11 Statistical analysis

Data were analysed using Prism4 (GraphPad software). Unpaired Student's t-test (2-tailed) was used for comparison of continuous variables of angiographic collateral number and area, and number of collaterals at histological analysis. A p-value of <0.05 was considered significant.

Chapter 5

Results of in-vivo coronary studies

5.1 Introduction

Results are presented for 2 distinct stages of the in-vivo work:

Stage 1: Development of a novel, entirely endovascular model of porcine coronary occlusion

Stage 2: Randomized, controlled and blinded study of a DMOG-eluting coronary stent in the setting of an occluded porcine coronary artery (CTO)

5.2 A novel endovascular porcine coronary occlusion model (stage 1)

A programme of work, described in Chapter 4, led to the refinement of the CTO model using copper stents for which the results are presented. As described in chapter 4 two designs of stent graft were undergoing testing at the commencement of this M.D. thesis programme. Modified versions of a two covered stents, the Jostent GraftMaster™ (Abbott Vascular Devices) stent, and the Symbiot™, PTFE-covered, nitinol stent (Boston Scientific Corporation) were tested by Dr. Julian Gunn between 2004 and 2006. The stents were modified by addition of a silk ligature to partially constrict the stent lumen and promote vessel occlusion. Although the Symbiot device was effective in producing coronary occlusion *(fig 5. 1)*, both proved difficult to deliver to the coronary artery and produced a high rate of early stent thrombosis. The copper-coated stent was thus selected as the device to be used in the in-vivo studies. *(fig 5.2)*

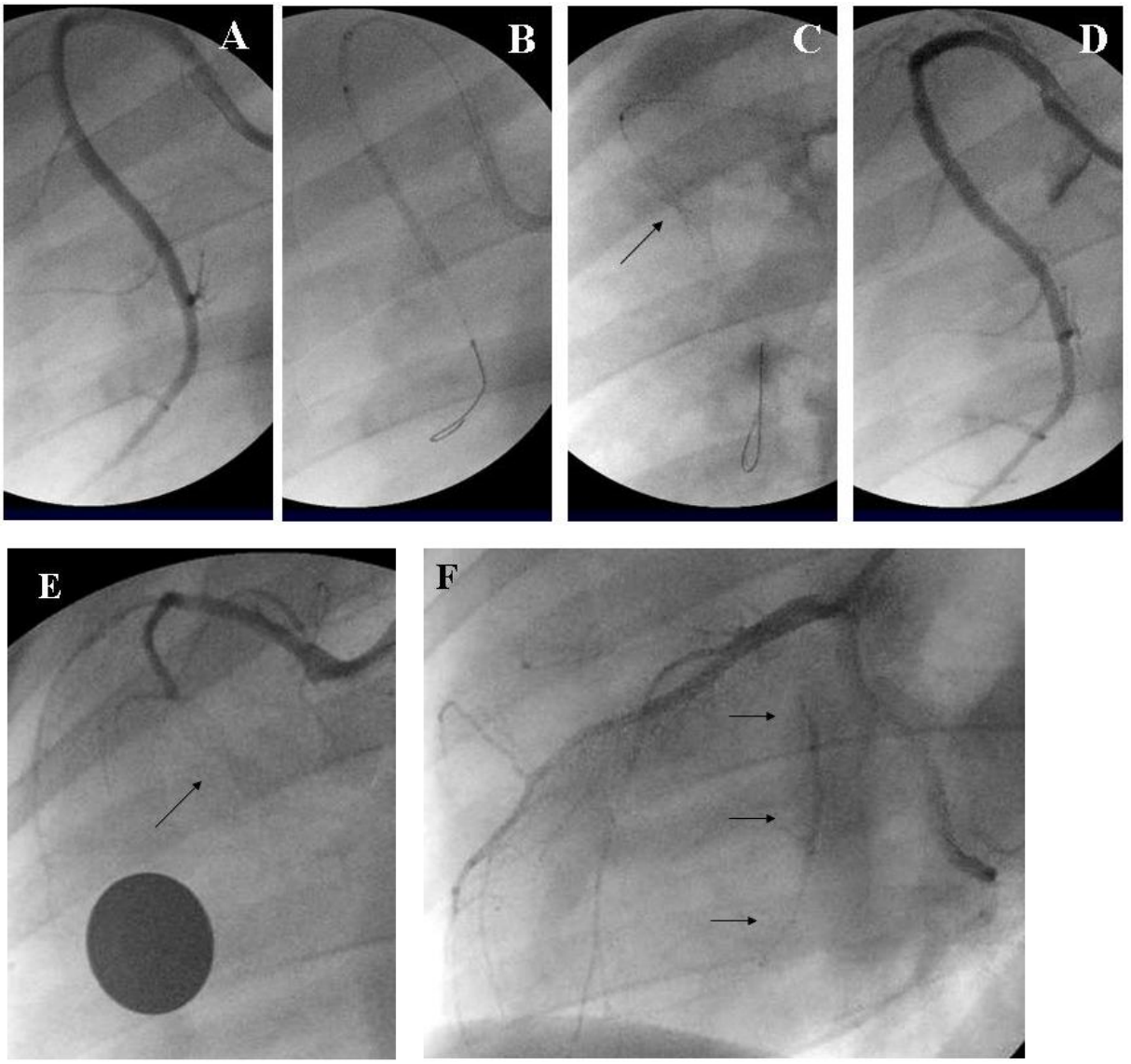


Figure 5.1: Symbiot stent angiography sequence. (A) RCA angiogram at baseline. (B) Positioning of the Symbiot stent graft. (C) The deployed, suture-constrained, stent graft (arrow indicates the constriction). (D) Post-deployment angiogram. (E) RCA angiogram at 37 days showing a CTO, beyond which is seen an outline of the constrained Symbiot™ stent graft. (F) The left coronary angiogram demonstrates filling of a patent distal RCA via retrograde collateral vessels (arrows). The elliptical object is a coin placed over the heart and used for calibration.

5.3 Results of the endovascular porcine occlusion model using the copper stent

In all 20 animals used to validate the model, copper stent deployment was successfully achieved. Survival was complete to planned sacrifice at 28 days, a full set of pre-implantation and pre-sacrifice coronary angiograms was obtained and a CTO was produced both angiographically and histologically at the site of implantation of the copper stent in the RCA. *(fig 5.3)* Day 28 was selected as the time-point for implantation of the study stent based on experience with earlier iterations of the copper stent model. Histological examination from animals succumbing to stent thrombosis demonstrated intense inflammatory cell infiltrate and exuberant neo-intimal hyperplasia around the copper stent struts (Personal communication from Dr. J. Gunn on work completed prior to the start of this thesis). In animals surviving to 28 days there was a dense occlusion with almost complete resolution of active inflammation resulting in an occluded central lumen of fibrotic tissue and organised thrombus. The histological features of those animals succumbing to stent thrombosis may not be wholly representative of the natural history of vessel occlusion in this model and we recognise that further study with serial histological sampling is required to fully elucidate the time-course of vessel occlusion in this model and to determine the optimal time-point for intervention to the occluded vessel.

In all 20 cases, antegrade (RCA to RCA) but not contra-lateral (LCA to RCA) collateral vessels were visible angiographically around the CTO. The neointima filling the stents was consistent, and appeared similar to neointima described previously by Schwartz[355], with abundant cells having the appearance of vascular SMCs and some intercellular matrix. Positive staining for α-actin confirmed the identity of the cells. Microvascular channels were seen both within the neointima and in the adventitia around the stented segment. Histological cross-sections of pig coronary arteries at different time-points after deployment of a copper stent are shown. These are from the CTO model validation experiments and demonstrate progressive luminal occlusion due neo-intimal hyperplasia with dense inflammatory infiltrate. *(figs 5.3. to 5.6)* Colour reproduction of histological sections helps to demonstrate the presence of luminal microvessels which are a feature of this model and mimic human CTOs. The copper stents are coated with copper on the abluminal surface only to reduce stent thrombosis, dual anti-platelet therapy with aspirin and clopidogrel (a thienopyridine anti-platelet agent) is mandatory. *(fig 5.9)*

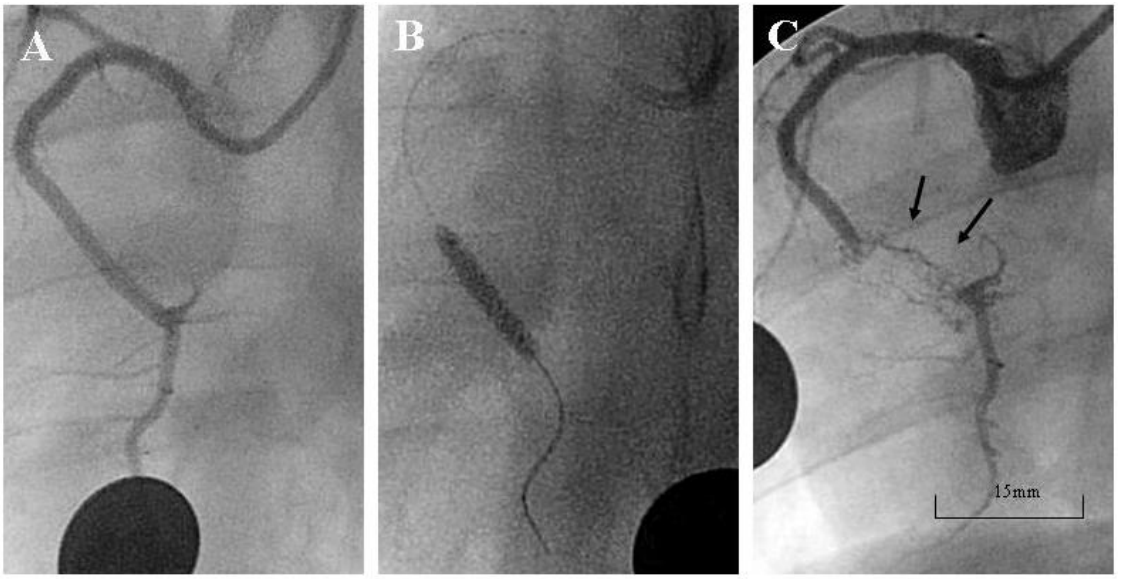


Figure 5.2: (A) RCA angiogram prior to copper stent placement in a 30kg pig in the final (validation) group. (B) Implantation was on a 3.0mm diameter balloon, and the segment of artery selected for implantation was also 3.00mm in diameter. (C) Angiogram 28 days later, during which time dual anti-platelet therapy was given, showing a CTO within the stent, with a patent vessel visible beyond, and many antegrade collateral vessels. The left coronary angiogram did not show any retrograde collaterals

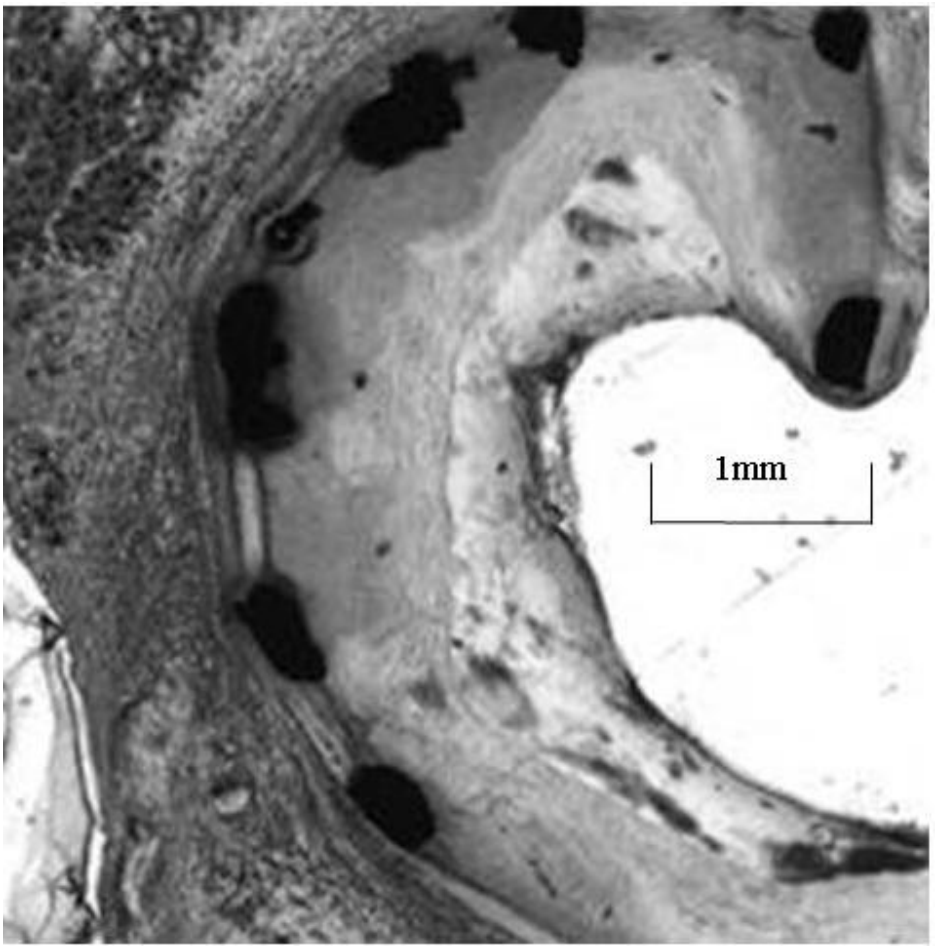


Figure 5.3 Histological cross-section of pig coronary artery. Section from an animal which died at day 2 (Group 4). There is a thick, adherent, laminated layer of thrombus lining the entire stented segment of artery.

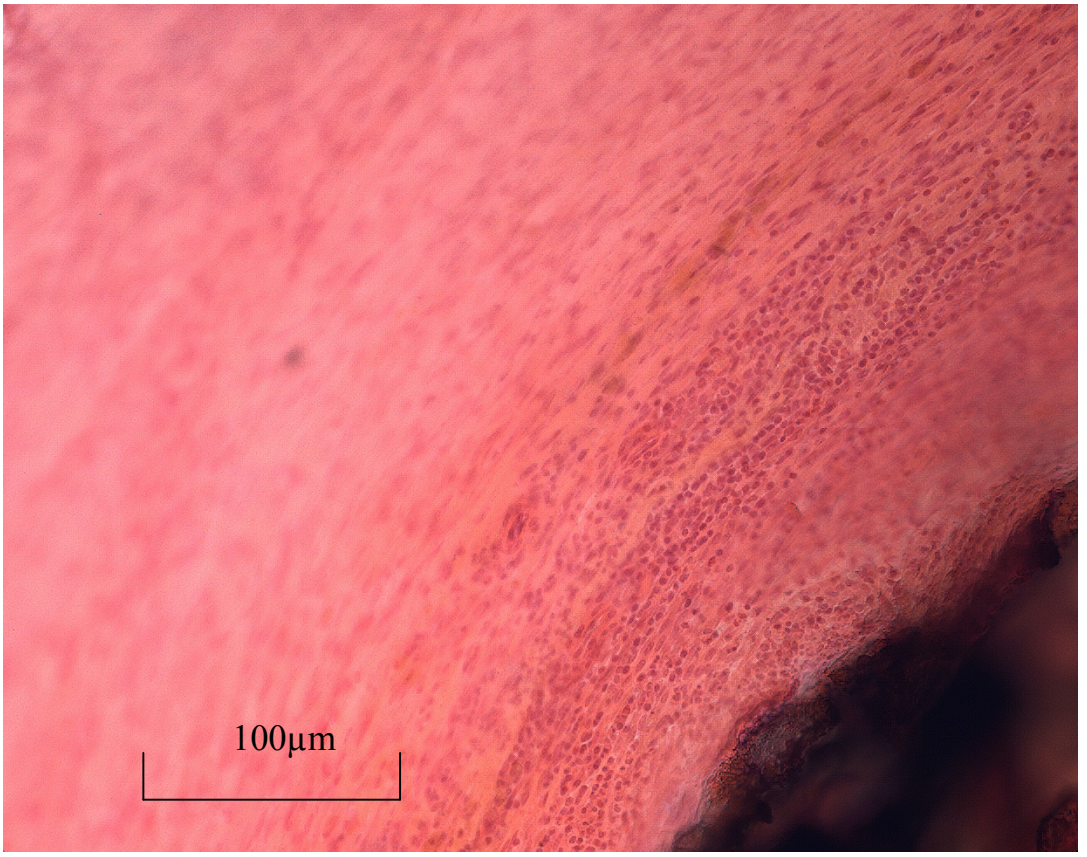


Figure 5.4 Histological cross-section of pig coronary artery. High power shows the innermost layer to be composed almost entirely of small, round, inflammatory cells.

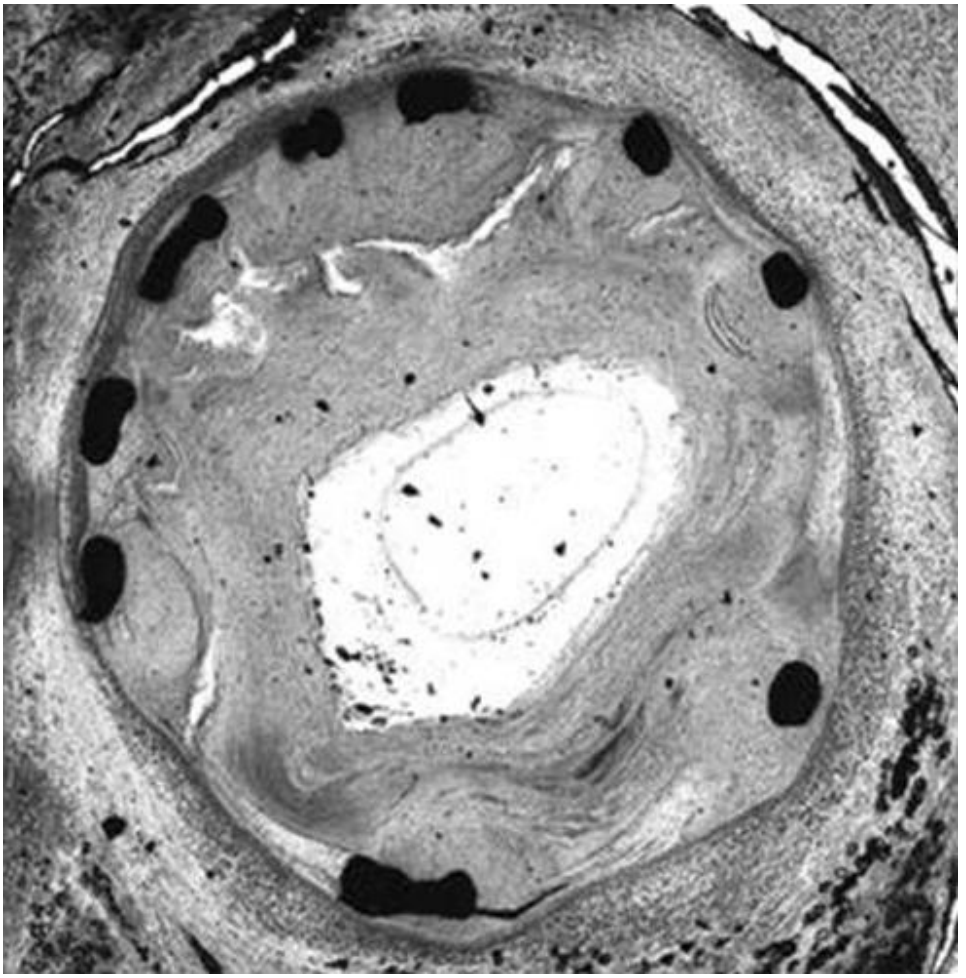


Figure 5.5 Histological cross-section of pig coronary artery. Section from a pig which died at day 4 (Group 5). There is profuse neo-intimal hyperplasia.

Figure 5.6 Histological cross-section of pig coronary artery. Section from a pig which died at day 11 (Group 5). From figures 5.3, 5,5 and 5.6 it can be deduced that the thrombus matures extremely rapidly into a thick, occlusive neointima.

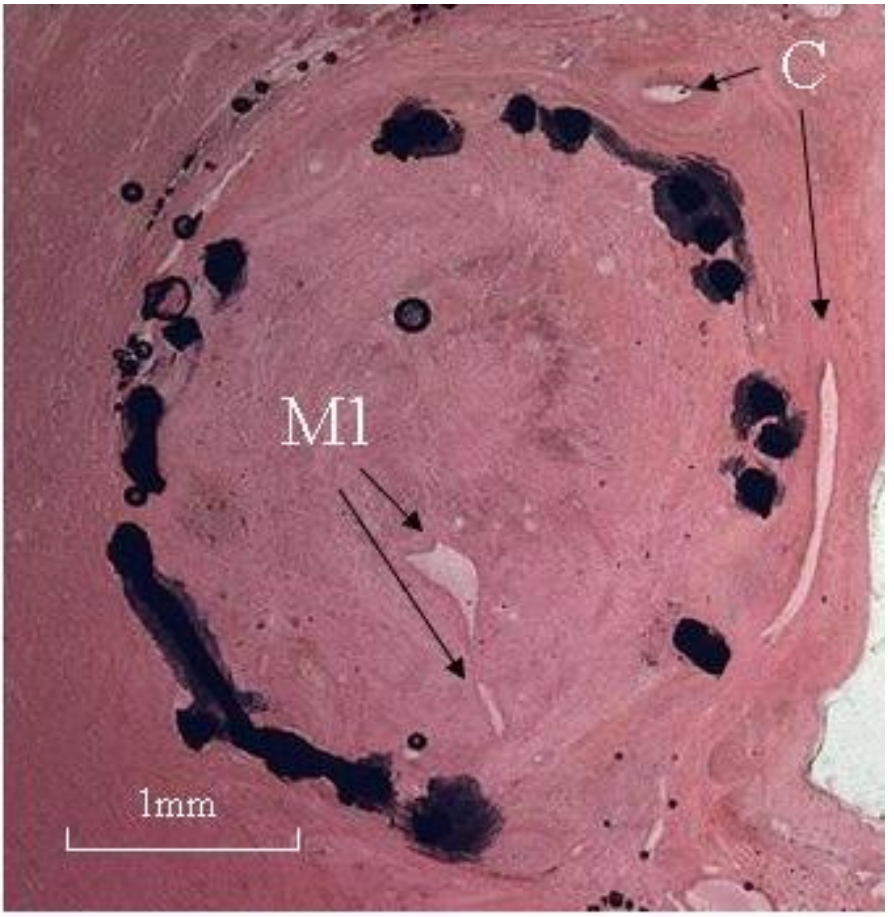


Figure 5.7 Section from a pig which survived to 28 days during the CTO model validation phase. (Hematoxylin and Eosin). The neointima is mature, and completely occludes the lumen. 'Large' microvessels (100-200μm diameter) are visible in the centre of the occlusive neointima (M1) and similar sized collateral vessels are seen in the adventitia around the stented segment (C).

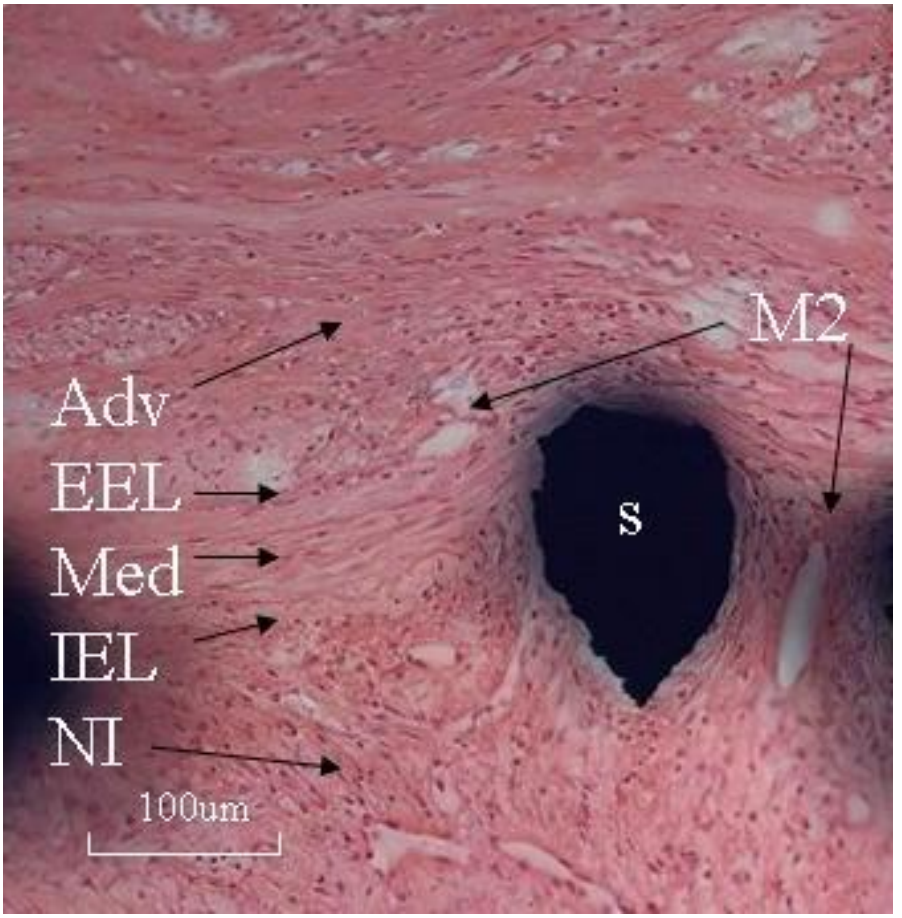


Figure 5.8 High power view of the neointima around a stent strut from another animal which survived to 28 days. The neointima (NI), internal elastic lamina (IEL), media (Med), external elastic lamina (EEL) and adventitia (Adv) are labelled. 'Small' microvessels (10-20μm diameter) are visible in the neointima around the strut (M2). The neointima of the CTO appears to be composed of the typical VSMC and matrix.

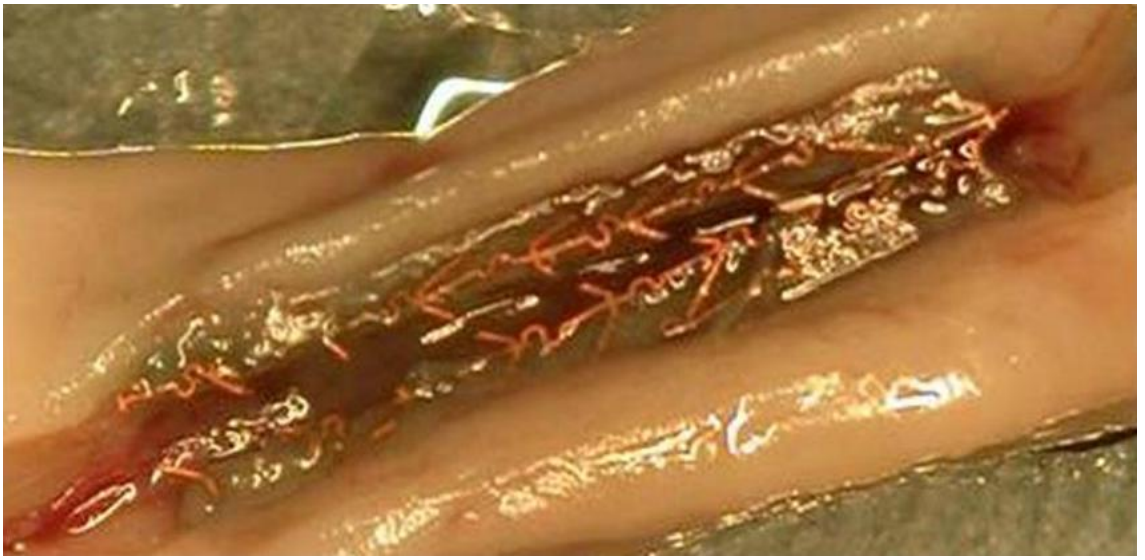


Figure 5.9 View of the opened LAD coronary artery of a copper-stented pig shortly after its sudden death 2 days after deployment (CTO model validation phase). In this case aspirin was the sole anti-platelet therapy. The lumen of the stent is filled with thrombus, which extends downstream in the artery (arrow). Subsequent animals were treated with both aspirin and a second anti-platelet drug, clopidogrel, throughout the first 28 days post implantation.

5.4 Results of a randomized, controlled and blinded study of a DMOG-eluting coronary stent in an occluded porcine coronary artery (CTO) (stage 2)

5.4.1 Outcomes of animals in the study

The methodology of the in-vivo study is described in chapter 4. 20 animals underwent the standard procedure (implantation of a copper stent) with plans for further a study procedure at day 28 (confirmation of presence of CTO and implantation of study stent) and at day 56 (terminal experiments and histology). Each animal was randomized into either treatment (DMOG stent) or control group. The randomization was performed by PolyBioMed Ltd. who supplied the DMOG or control (polymer-only) stents. Stents were supplied as randomly numbered samples. The investigators were only un-blinded to the treatment allocation after completion of final data analysis.

17 animals survived to 56 days (85%) and completed the study. There were three wound infections related to the vascular cut-down, two of which required subsequent incision and drainage. One of the latter animals had a fibrinous pericarditis at the time of final thoracotomy but had not exhibited signs of systemic illness. All adverse events were reported to the local supervising veterinary officer under the terms of the Home Office licenses. *(table 5.1)*

ANIMAL	GROUP	CTO	COMPLETED	COMMENT
Pig P/ JG958	Control	Yes	Yes	†
Pig Q/ JG959	DMOG	Yes	Yes	Tamponade-survived
Pig R/JG960	Control	Yes	Yes	Repair of RICA.
Pig S/JG961	DMOG	Yes	Yes	
Pig T/ JG962	Control	Yes	Yes	
Pig U/ JG963	n/a	No	No	† day 1- acute ST
Pig V/JG964	DMOG	Yes	Yes	
Pig W/ JG965	Control	Yes	Yes	
Pig X/ JG966	DMOG	Yes	No	† d29- atrial clot
Pig Y/ JG967	Control	Yes	Yes	
Pig KK/JG 978	Control	Yes	Yes	
Pig LL/ JG 979	DMOG	Yes	Yes	
Pig MM/ JG980	Control	Yes	Yes	
Pig NN/JG 981	Control	Yes	Yes	
Pig OO/JG982	DMOG	Yes	Yes	Wound infection
Pig PP/JG983	DMOG	Yes	Yes	
Pig QQ/JG984	DMOG	Yes	No	d30- LAD clot
Pig RR/JG985	DMOG	Yes	Yes	Wound infection
Pig SS/JG986	Control	Yes	Yes	Faulty PW d56
Pig TT/JG987	DMOG	Yes	Yes	

Table 5.1 Outcomes of study animals. ST= Stent thrombosis. RICA= right internal carotid artery. PW= Pressure wire. †= died.

5.4.2 Success rates for producing coronary occlusion (CTO)

All 19 pigs surviving to 28 days had coronary occlusions at the site of the copper stent implant demonstrated at day 28 angiography. *(fig 5.10)*

All the animals had extensive bridging antegrade collaterals visible at angiography. There was uniformly brisk coronary flow in the distal vessel (TIMI grade 3 flow present at 28 days). Review of the angiogram images suggested that the majority of these vessels arose from the adventitia surrounding the occluded vessel. *(fig. 5.11)*

There was minimal retrograde collateralization from the left coronary system present in 3 animals at 28 days.

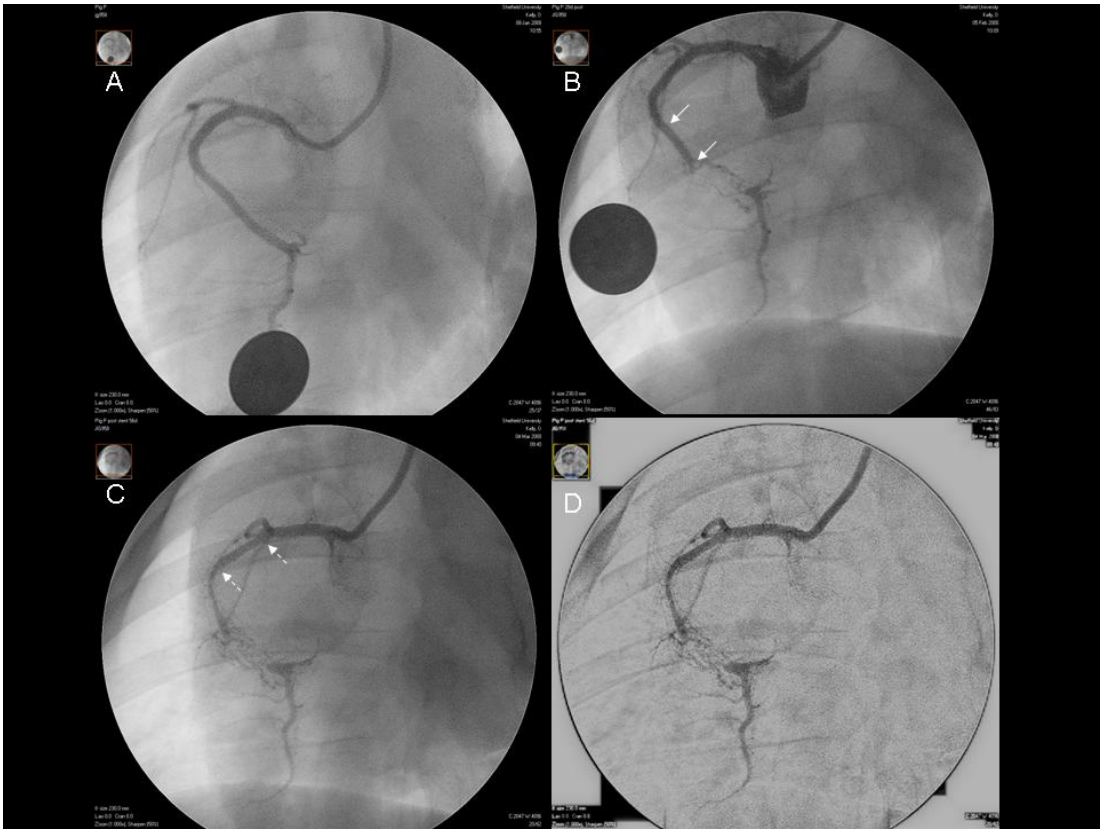


Figure 5.10 Angiographic images from Pig P. (A) Initial RAO 30° view of RCA at experiment 1, day 0 prior to implantation of copper stent. (B) Same view at experiment 2, day 28 demonstrating mid vessel CTO at distal edge of copper stent (solid arrows) with antegrade bridging collaterals. (C) Experiment 3, day 56 following implantation of study (control, PEP polymer only) stent (dashed arrows). (D) Digital subtraction image of C used to highlight microvessels for image analysis.

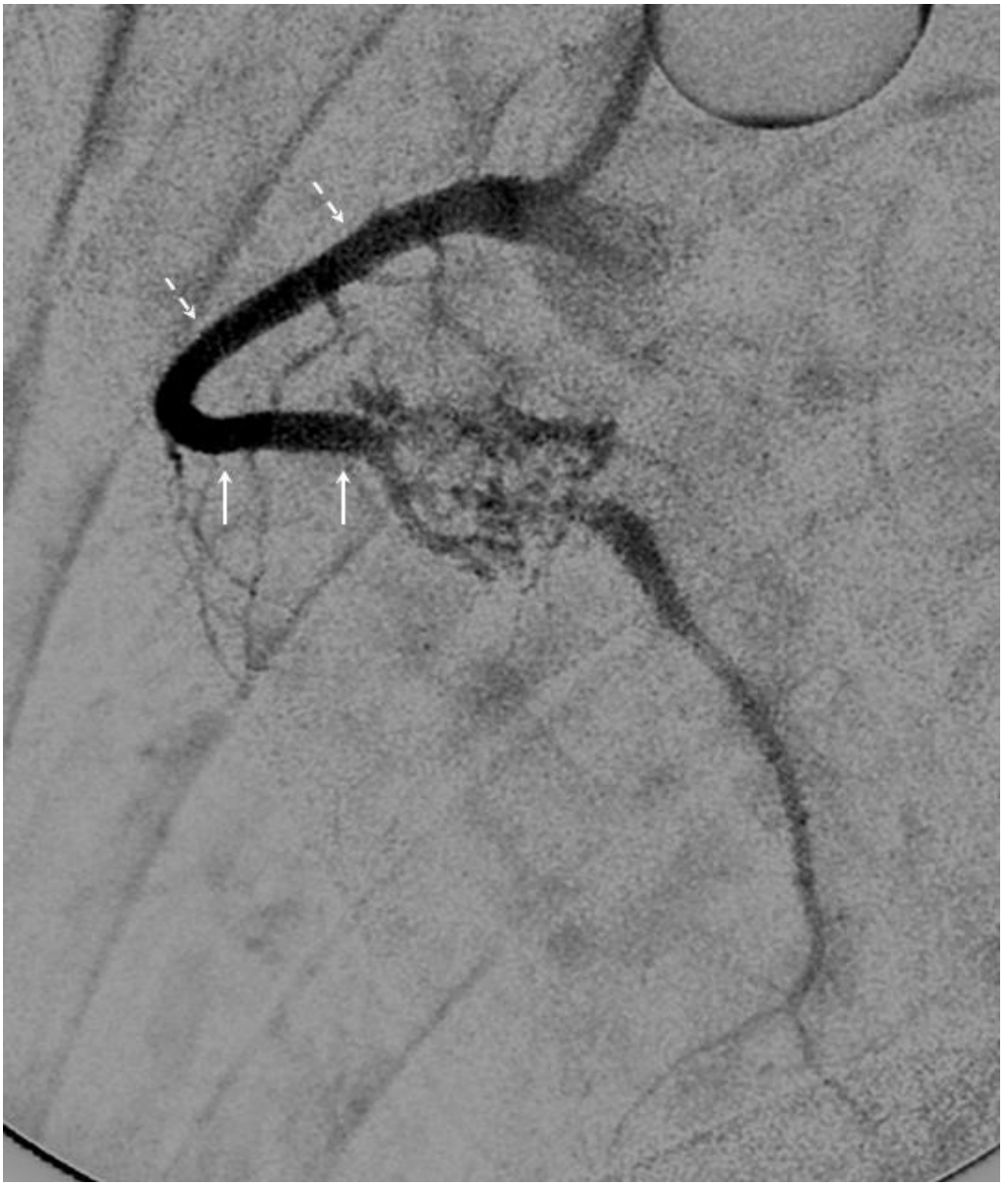


Figure 5.11 Digital subtraction image from Pig TT, day 56. Marked antegrade collateral vessel formation is seen with vessels appearing to arise from the adventitia. The solid arrows highlight the Copper stent; the dashed arrows highlight the study (DMOG) stent.

5.4.3 Angiographic collateral scores

Collaterals were graded at day 56 according to accepted measures of antegrade collateral filling. The recipient filling grade score was recorded along with a collateral washout score (frame count for disappearance of 15ml of dye after deflation of proximal balloon). Additionally the number of angiographically visible collaterals providing a continuous connection from donor to recipient vessel segment was counted. In the following sections the Rentrop classification was adjusted to be compatible with analysis by the statistical program GraphPad Prism4- this would not process data with a value of zero, therefore +1 was added to the Rentrop score such that Rentrop grade 0 (no filling) was scored as 'Adjusted Rentrop Grade 1' and Rentrop grade 3 was scored as 4. (see section 4.5.2.5, Angiographic collateral assessment for explanation of Rentrop grade).

5.4.3.1 Rentrop classification

Both the day 28 and day 56 angiograms demonstrated abrupt mid vessel occlusion with marked antegrade collateralization and either partial (Adjusted Rentrop grade 3) or complete (Adjusted Rentrop grade 4) recipient vessel filling. There were no differences between the control or DMOG groups in these indices at 28 days *(table 5.2 & fig 5.11)* The Adjusted Rentrop score lacked discriminating power due to the uniform extensive antegrade collateralization seen in both groups 28 days after copper stent implantation.

ADJUSTED RENTROP SCORE	CONTROL	DMOG	P VALUE
Day 28	3.6±0.18	3.6±0.18	ns
Day 56	3.7±0.17	3.9±0.13	0.34

Table 5.2 Adjusted Rentrop collateral score. There is no difference between the groups either before study stent implantation (day 28 post copper stent) or 28 days following study stent implantation (day 56).

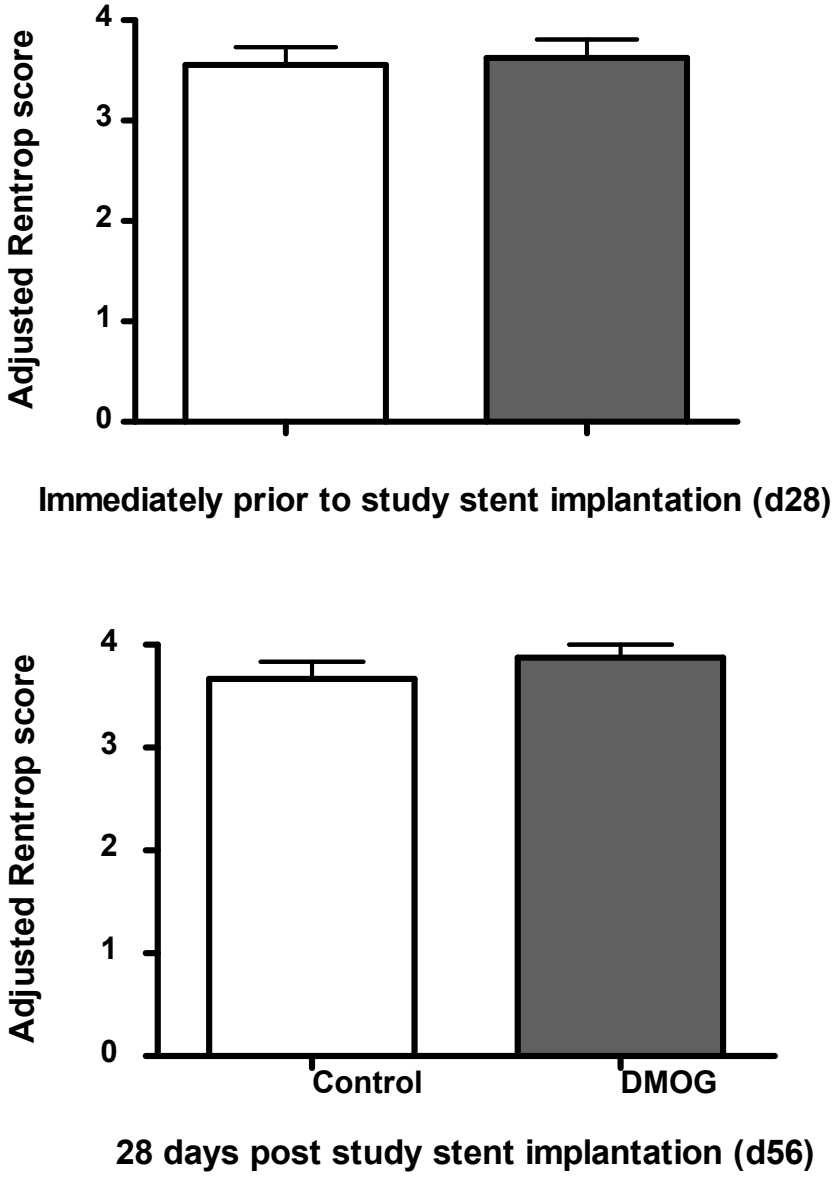


Figure 5.11 Mean Rentrop scores for control and DMOG stent group at day 28 (baseline immediately prior to study stent implantation) and day 56. The score is semi-quantitative with higher scores are indicative of greater distal vessel filling. No significant difference was seen between the groups at 56 days.

5.4.3.2 Coronary artery washout (frame count) score

Due to brisk antegrade flow and prompt filling of the distal vessel segment the collateral washout score was uniformly low, with all animals clearing 15ml of dye from the proximal vessel segment within 6 frames after deflation of a proximal occlusion balloon. There was no difference between the control or DMOG groups in this score either at baseline at 28 days (just prior to study stent implantation) (4.5 ±0.38 vs. 4.3±0.31 frames, p=0.76 or at 56 days, 28 days after study stent implantation (4.12 ±0.3 vs. 3.63±0.32 frames; p=0.27. *(table 5.3 & fig5.12)*

CORONARY WASHOUT SCORE (NO. OF FRAMES)	CONTROL	DMOG	P VALUE
Day 28	4.5±0.38	4.3±0.31	0.76
Day 56	4.12±0.3	3.63±0.32	0.27

Table 5.3 Coronary washout score. There is no difference between the groups either before (day 28) or 28 days following study stent implantation (day 56).

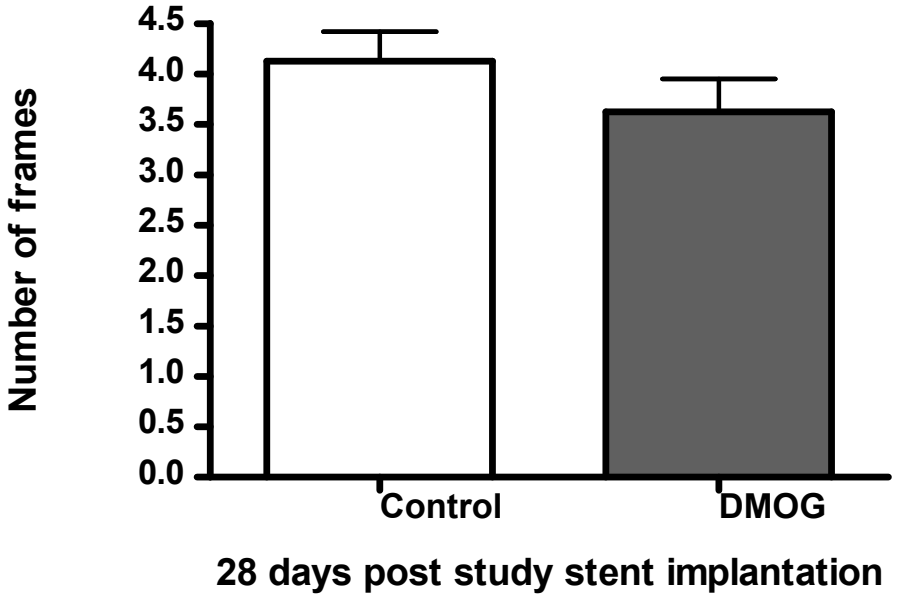


Figure 5.12 Coronary washout scores. This is the number of frames required for dissipation of 15ml dye injected into proximal artery after release of proximal balloon. A lower frame count is indicative of more rapid distal run-off. There was no significant difference between the groups with brisk antegrade flow in both control groups (4.1 ±0.3 vs. 3.6±0.3 frames, p=0.27).

5.4.3.3 Angiographic assessment of collateral vessels

Assessment of antegrade collateral vessel area was performed using methods outlined in chapter 3. Collateral volume was chosen as the primary angiographic measure as it was not possible to accurately count individual collateral vessels. Invariably the area of occlusion immediately distal to the copper stent was bridged by a fine network or 'sheath' of collateral micro-vessels. This appearance did not preclude assessment of the number of continuous collaterals but made discrimination between angiogram appearances more difficult, i.e. it was difficult to grade the relative contribution to flow as the majority of constituent vessels were below the diameter of minimum angiographic resolution (approximately 100μm). The area of occlusion was bridged by a fine network of collateral microvessels. *(fig 5.13)* Therefore measurement of the area of collateral coverage (as a surrogate for collateral volume) gave a more accurate assessment of collateralization. The most striking result was that at day 28 there was marked antegrade collateralization, prior to implantation of the study stent, with most cases showing Rentrop grade 4 collaterals (complete filling of the distal RCA).

5.4.3.4 Collateral Area

There was a difference in collateral vessel area between the groups at the day 28 baseline before implantation of the control/ DMOG stent. There was a lower collateral area in the treatment (DMOG stent) prior to implantation of the study stent (control or DMOG) at day 28 (15.8mm^2 in the DMOG group vs. 22.9mm^2 in the control group, p=0.10). *(table 5.4 & fig 5.14)*. The reason for this baseline difference is not clear but in the absence of any obvious reason for this disparity (the difference being noted prior to the treatment or control stent being implanted) was most probably a chance finding. Clearly such disparity was unfortunate in making subsequent angiographic comparison of collaterals between the groups more difficult to interpret. This is further discussed below in section 5.6.3.2.

With the disparity in baseline collateral area between the groups, there was no difference in the absolute collateral area at 56 days (26.5 mm^2± 4.1mm^2 vs. 25.7 mm^2 ± 5.2 mm^2 in the control and DMOG groups respectively, p=0.91. There was however a trend to a greater increase in visible collaterals following DMOG stent implantation. The absolute increase in collateral volume from day 28 to day 56 was (mean±SEM) 3.6mm^2 ± 1.5 mm^2 in the control group and 10mm^2 ± 4.1 mm^2 in the DMOG group, p=0.15. There was a trend towards a greater percentage increase in collateral area in DMOG group (84.5% ± 34.5%) compared with the control group, (16.5% ± 5.9%), p=0.057 *(table 5.5 & fig 5.15)* The standard error

of these results is large, reflecting considerable inter-animal variation in collateral formation.

There was a greater dispersion of individual collateral area among animals in the control group than in the DMOG group (t-test for variance between the groups =0.04) *(fig 5.16)* That there were fewer collaterals at baseline in the group subsequently receiving DMOG stents clearly impacts upon interpretation of results at 56 days and is the reason for expressing change in collateral area as a percentage of the day 28 mean.

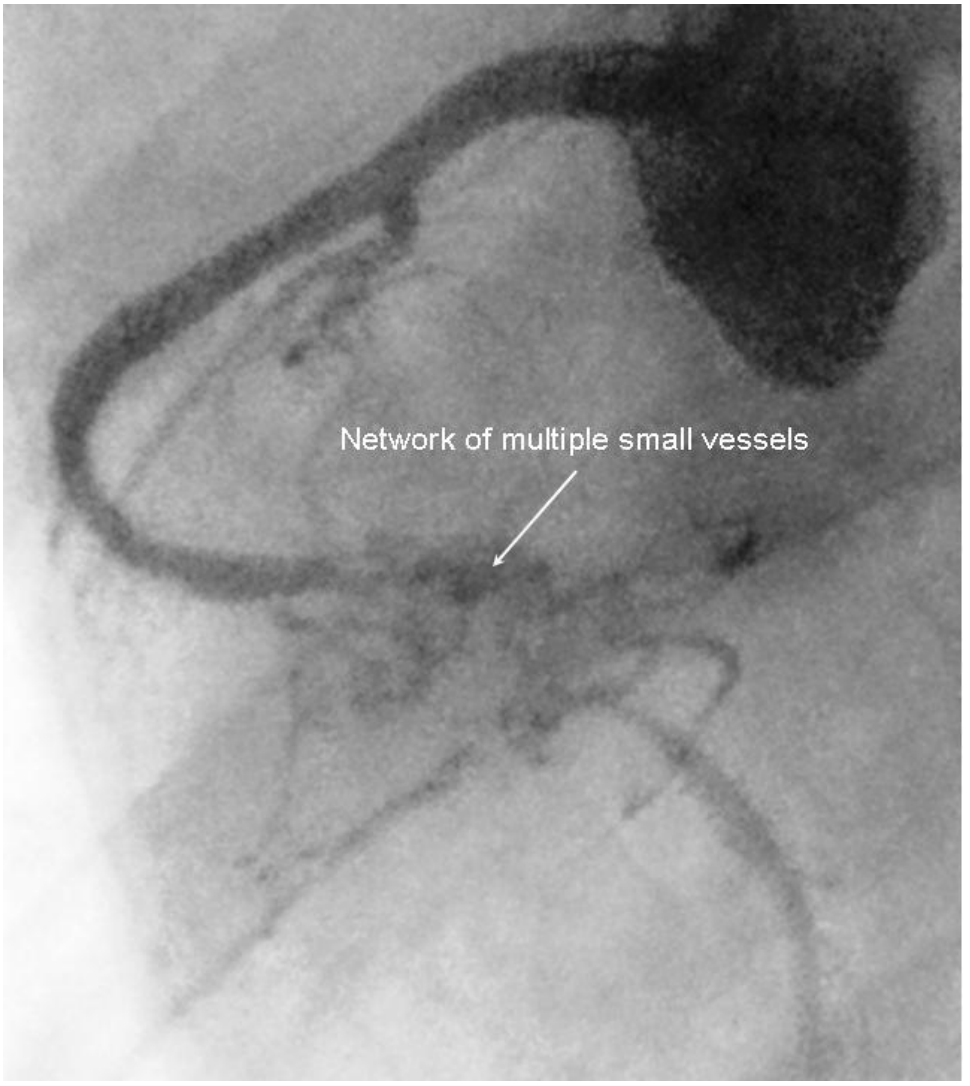


Figure 5.13. This angiogram (Pig RR at day 56) provides an illustration of the difficulties in angiographic grading of multiple antegrade microvessel collaterals where vessel diameter is below the resolving power of the angiogram. In this case a network of antegrade microvessels (arrow) is counted as a single continuous collateral.

ANGIOGRAPHIC COLLATERAL AREA (MM^2)	CONTROL	DMOG	P VALUE
Day 28	22.9± 3.4	15.8± 1.9	0.10
Day 56	26.5± 4.1	25.7± 5.2	0.91

Table 5.4 Collateral area. At baseline here is a numerical difference, not reaching statistical significance, between the two groups in terms of angiographic collateral area with more collaterals seen in the control group. There is no difference in collateral area at day 56.

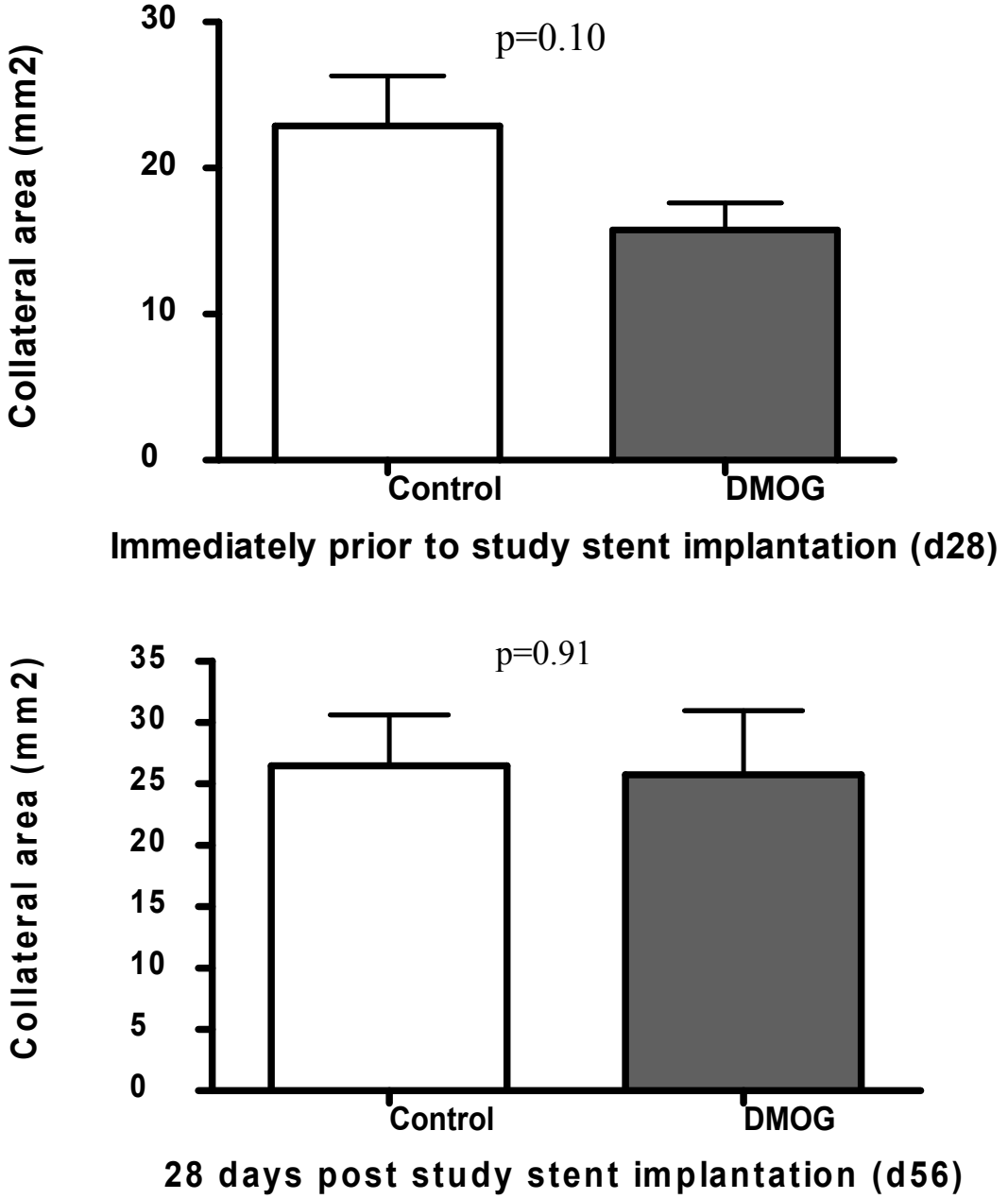


Figure 5.14 Angiographic collateral area at day 28, (baseline immediately prior to study stent implantation) and at day 56. At baseline there is a non-significant excess of collaterals in the control group. There was no difference in the absolute value of collateral area 28 days following study stent implantation.

ABSOLUTE INCREASE IN COLLATERAL AREA (MM2)	CONTROL	DMOG	P
	3.6± 1.5	10± 4.1	0.15

PERCENTAGE INCREASE IN COLLATERAL AREA (%)	CONTROL	DMOG	P
	16.5± 5.9	84.5± 34.5	0.057

Table 5.5 There is a greater increase in collateral area 28 days following implantation of the study stent in the DMOG group (ie. from day 28 to day 56). When expressed as a percentage change in collateral area there is a strong trend favouring the DMOG group (p=0.057).

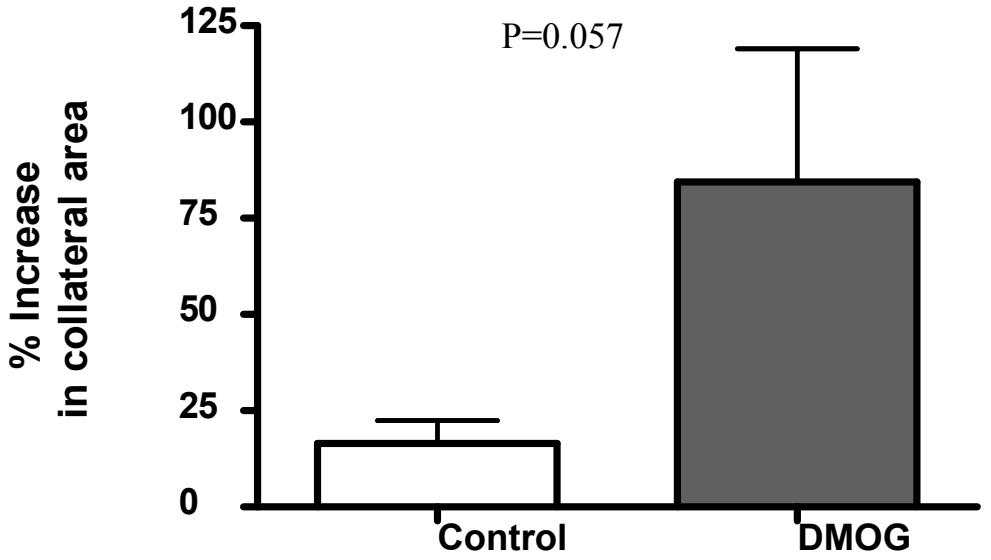


Figure 5.15 Increase in collateral area 28 days following implantation of the study stent in the DMOG group (i.e. from day 28 to day 56). Expressed as a percentage increase there is a strong statistical trend towards a greater increase in collateral area in the DMOG stent group (p=0.057)

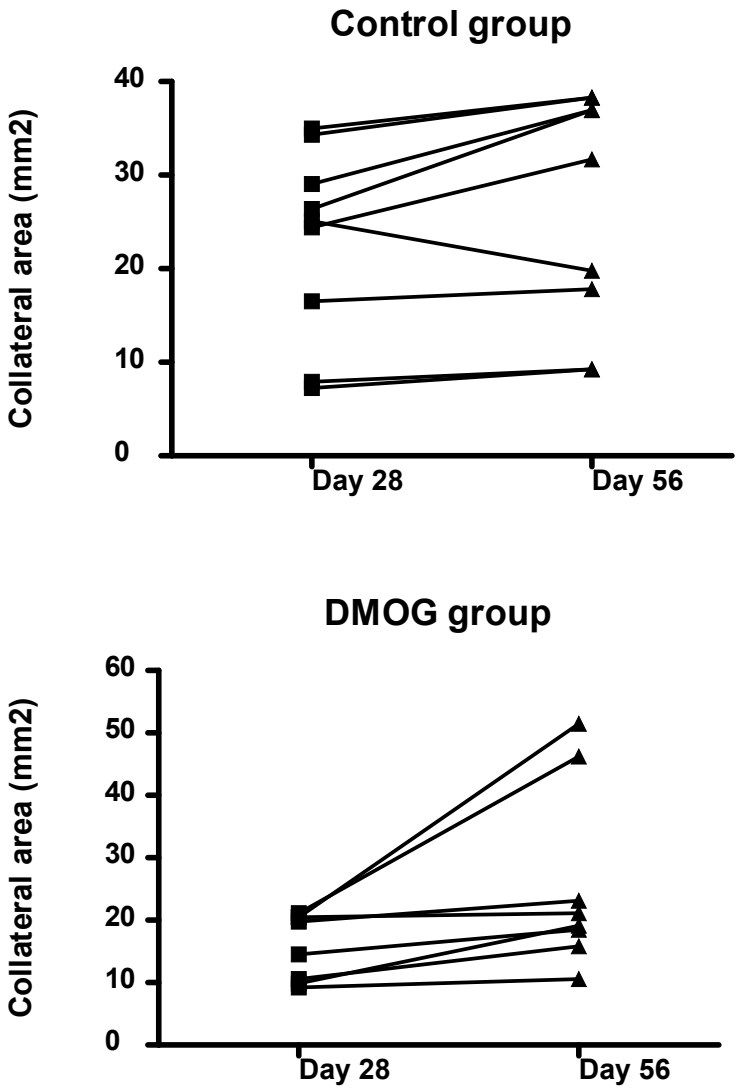


Figure 5.16 Before and after plot of individual pig collateral area. There is more baseline variation in collateral volume at day 28 in the control group. There is a significant difference in day 28 inter-animal variance between the control and DMOG groups (p=0.04).

5.4.3.5 Number of angiographically visible antegrade collaterals

The results were similar to those for collateral area. There was a non-significant trend to a greater number of antegrade right coronary artery (RCA) collaterals in the control group at baseline prior to implantation of the study stent. At 56 days there was no difference in the number of large antegrade collaterals between the groups, neither was there any significant difference in the extent of change in collateral number between day 28 baseline and day 56. *(table 5.6 & fig 5.17)*

ANGIOGRAPHIC COLLATERAL AREA (MM^2)	CONTROL	DMOG	P VALUE
Day 28	2.7± 0.29	2.0± 0.19	0.12
Day 56	3.2± 0.32	2.9± 0.23	0.40
Percentage increase (%)	20.7 ± 8.4	44± 11.2	0.35

Table 5.6 Collateral number. There were no significant differences in the number of continuous antegrade collaterals. The increase seen in the DMOG group was proportionally greater (44% vs. 20.7%) but this was not statistically significant (p=0.35).

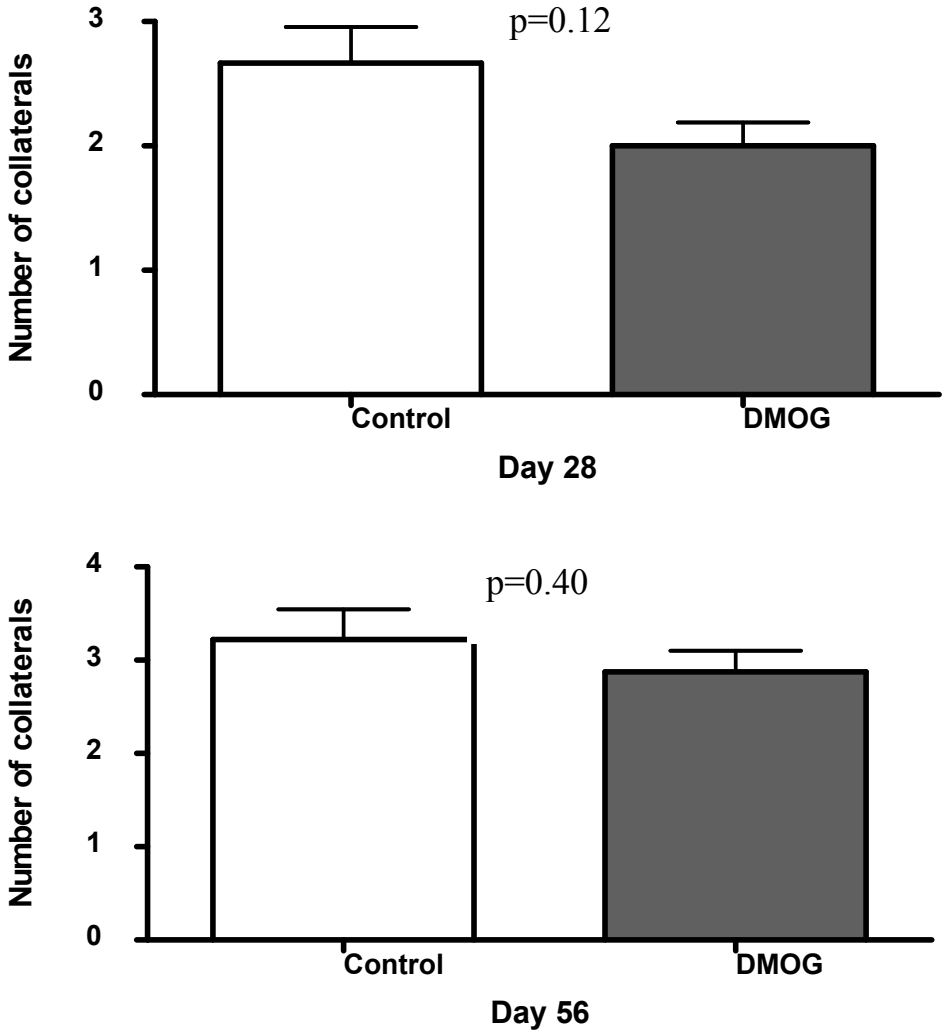


Figure 5.17 Angiographic collateral number. There were no significant differences in the number of continuous antegrade collaterals (>0.4mm diameter) at baseline (day 28, top) and day 56 between the two groups.

5.4.4 Analysis of Left ventriculograms

Left ventriculograms were analysed as described in Chapter 3. The average of two orthogonal projections was taken for end-systolic area (ESA) and calculated left ventricular ejection fraction (LVEF).

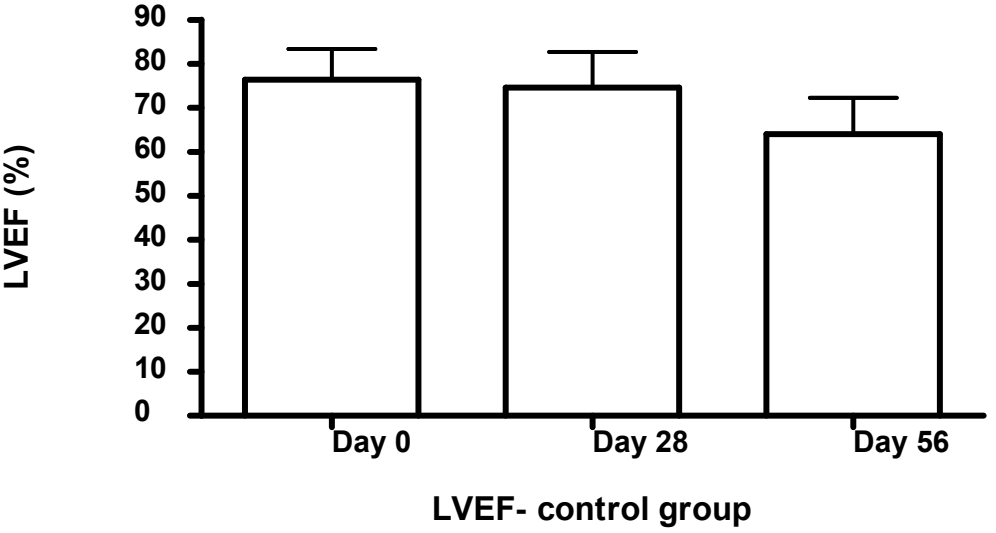
There were no differences between the groups in respect of ESA or LVEF at any time-point. *(table 5.7 & fig 5.18)* There was no difference in the change in LVEF observed between day 28 and day 56 between the two groups. There was no evidence of significant change in LVEF within the study population as a whole between day 0 and day 28 LEVF at day 0 was 71.9±4.5 and at day 28 was 74.7±5.3, p=.069. Mean LVEF at day 56 was 69.6%, p=0.49.

From day 28 to day 56, LVEF remained constant in the DMOG group at +1.2% ± 7.4%, while there was a non-significant reduction in LVEF in the control group of -11% ± 8.3%, p=0.31. Overall end-systolic area rose in the entire cohort throughout the study period following copper stent implantation but did not differ between the groups. *(table 5.8 & fig 5.19)*

There was no significant difference between the groups in left ventricular diastolic pressure, elevation of which is a marker of left ventricular dysfunction. *(table 5.9 & fig 5.20)*

LEFT VENTRICULAR EJECTION FRACTION (%)	CONTROL (MEAN ±SEM)	DMOG	P VALUE
Day 0 LVEF	75±7.8	69±4.9	0.52
Day 28 LVEF	75±8.1	75±-7	0.98
Day 56 LVEF	64±8.3	76±4.2	0.23
Δ LVEF Day 56 to Day 28	-11±8.3	1.2±7.4	0.31
Δ LVEF Day 56 to Day 0	-2.6±11	7.1±6.1	0.46

Table 5.7 Left ventricular ejection fraction. There were no significant differences in LVEF or in the change in LVEF between day 28 and day 56 between the groups.



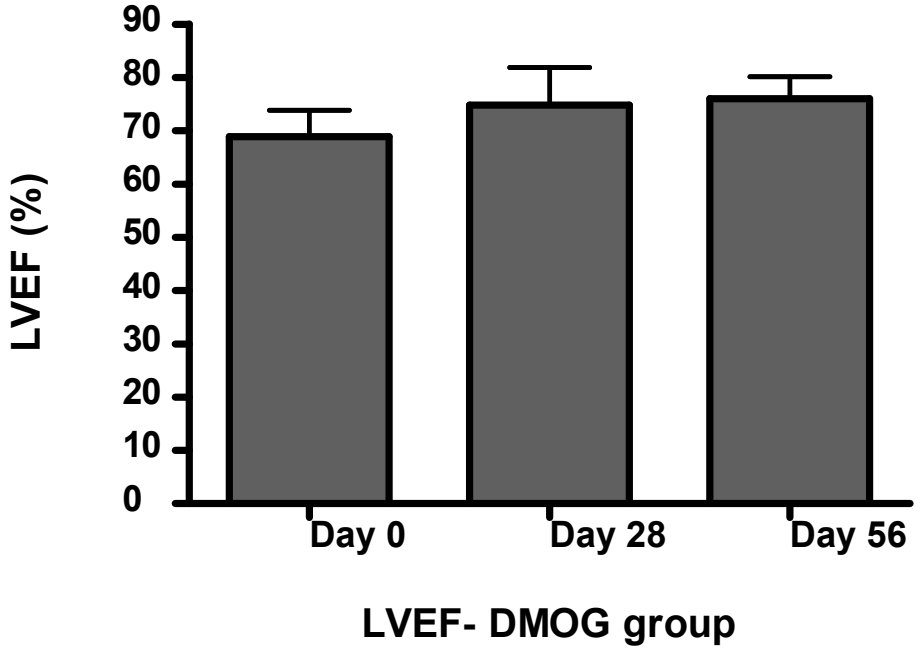


Figure 5.18 Left ventricular ejection fraction. There were no significant differences in LVEF or in the change in LVEF between day 28 and day 56 between the groups.

LEFT VENTRICULAR END-SYSTOLIC AREA (CM^2)	CONTROL (MEAN ±SEM)	DMOG	P VALUE
Day 0 LVESA	15.6±1.1	15.7±1.8	0.98
Day 28 LVESA	18.1±2.4	17.7±2.8	0.93
Day 56 LVESA	19.8±2.6	18.5±1.7	0.69
Δ LVESA Day 56 to Day 28	1.9±2.4	0.9±1.7	0.74

Table 5.8 Left ventricular end-systolic area. There were no significant differences in LVESA between the groups at any time-point.

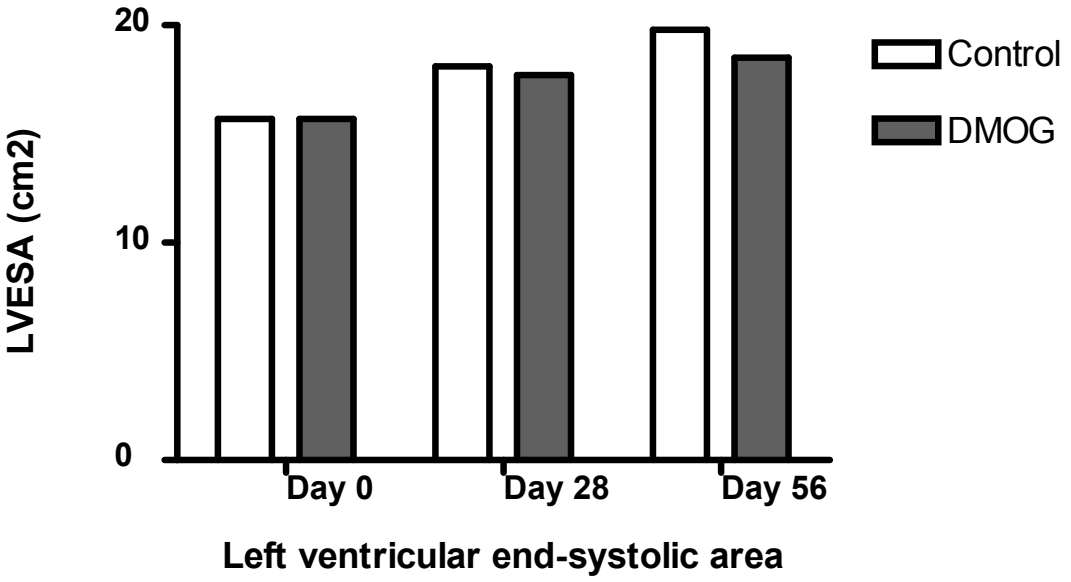


Figure 5.19 Left ventricular end-systolic area. There were no significant differences in LVESA between the groups at any time-point. Overall end-systolic area rose in the entire cohort between day 0 and day 56 from 15.73cm^2 ± 0.96 to 19.4 cm^2 ± 1.6 cm^2, p=0.05.

LEFT VENTRICULAR END-DIASTOLIC PRESSURE (MMHG)	CONTROL (MEAN ±SEM)	DMOG	P VALUE
Day 0 LVEDP	24±1.4	25±1.6	0.55
Day 28 LVEDP (mmHg)	24.2±1.5	24.7±2.7	0.87
Day 56 LVEDP (mmHg)	23.7±1.3	27.4±3.4	0.48
Δ LVESA Day 56 to Day 28 (mmHg)	-0.56±2.2	2.7±1.5	0.26

Table 5.9 Left ventricular end-diastolic pressure. There were no significant differences in LVEDP between the groups at any time-point.

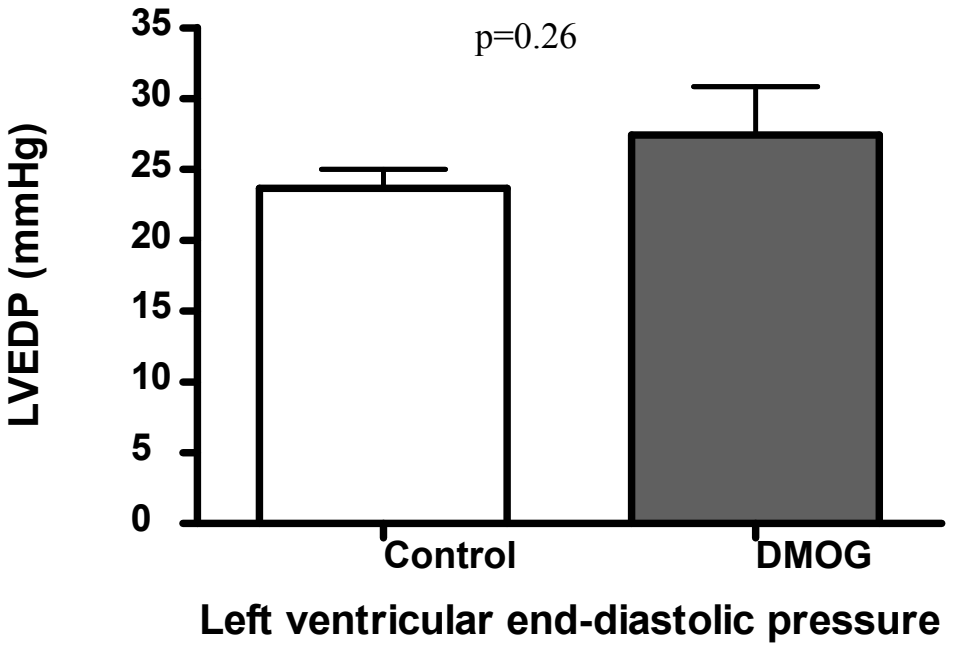


Figure 5.20 Left ventricular end-diastolic pressure. There was no significant difference in LVEDP between the groups at day 56.

5.4 5 Coronary physiological measurements

Estimation of coronary 'Distal arterial run-off index' (DRI) as a surrogate of collateral flow index (CFI) was made using the Pressure wire with the transducer placed as distal as possible in the artery but proximal to the occlusion point with proximal balloon occlusion used to measure 'Pw'. CFI was measured in the usual fashion (Pd-Pv/ Pa-Pv) in the non-occluded artery at day 0 with proximal balloon occlusion of the vessel. DRI was measured as a surrogate of CFI in the occluded artery at day 28 prior to implantation of the DMOG or control stent, and before sacrifice at day 56. Data presented represent values of DRI (Pw-Pv/Pa-Pv) with proximal balloon occlusion and after maximal vasodilatation with intra-coronary adenosine 60μg. As discussed above in section 4.5.2.6 (Coronary physiological measurements) interpretation of these results is limited by the inability to achieve steady-state hyperaemia as we were unable to establish a reliable intravenous adenosine infusion and had to resort to use of intra-coronary bolus adenosine. In the setting of poor distal collateral flow this may have resulted in inadequate delivery of adenosine to the distal coronary bed and thus sub-optimal hyperaemia. Therefore our invasive coronary physiology results must be interpreted with caution.

The baseline fractional flow reserve (Pd/Pa) in all cases following adenosine on day 0 was between 0.94 and 1.0 indicating no flow limitation.

There was a significant increase in collateral flow index as measured between day 0 and day 28 after development of a coronary occlusion with antegrade collateral flow. DRI (mean±SEM) increased from 0.12±0.02 to 0.40±0.04, $p<0.0001$.

No difference was detected between the control or treatment DMOG stent groups either at baseline before implantation of the study stent (at day 28) or at the final experiment on day 56 when DRI was 0.32±0.06 in the control group vs. 0.26±0.07, $p=0.07$. *(table 5.10 & fig 5.21)*

DISTAL RUN-OFF INDEX (DRI, NO UNIT)	CONTROL (MEAN ±SEM)	DMOG	P VALUE
Day 0 CFI	0.13±0.03	0.11±0.03	0.72
Day 28 DRI	0.41 ± 0.05	0.40 ± 0.06	0.57
Day 56 DRI	0.32 ± 0.06	0.26 ± 0.07	0.07
Δ DRI d28 to d56	-0.08 ± 0.08	-0.18 ± 0.11	0.45

FRACTIONAL FLOW RESERVE (FFR, NO UNIT)	CONTROL (MEAN ±SEM)	DMOG	P VALUE
Day 0 FFR Pre-adenosine	0.96±0.006	0.95±0.008	0.52
Day 0 FFR Post-adenosine	0.99±0.004	0.97±0.014	0.36

Table 5.10 Distal arterial Run-off Index (DRI). There were no differences in DRI between the groups at any time-point during the study. There was a significant increase in collateral flow among all the animals from baseline to day 28 (following the development of coronary occlusion with antegrade collaterals). There were no baseline differences in fractional flow reserve, as there was no significant underlying coronary disease.

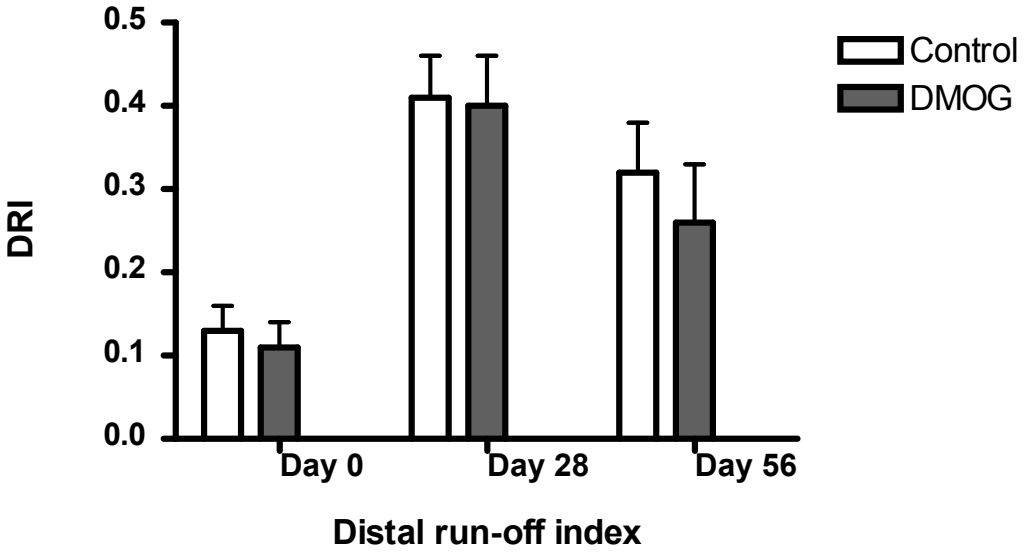


Figure 5.21 Distal run-off index (DRI) as a surrogate of Collateral Flow Index (CFI). CFI was measured at day 0 and DRI at day 28 and day 56. There were no differences in CFI/ DRI between the groups at any time-point during the study.

5.4.6 Histology

Analysis was performed on haematoxylin and eosin-stained sections from the right coronary artery. Sample preparation methods are described in detail in chapter 4.

Samples were collected from three areas of the right coronary artery: (a) from within the study stent (which was the proximal of the two stents); (b) more distally within the region of the copper stent (distal stent); and (c) in the distal vessel, downstream from the copper stent and point of occlusion. Where data from two samples in each area were available an average vessel count was obtained.

Macroscopic examination was undertaken at the time of sacrifice (day 56). Typically areas of myocardial infarction were seen in the inferoposterior wall of the left ventricle, corresponding with the territory subtended by the right coronary artery. *(fig.5.22)* Macroscopic tansverse sections typically revealed limited basal septal infarction consistent with mid-RCA occlusion. *(fig 5.23)*

Analysis was on haematoxylin and eosin-stained sections from the right coronary artery. For each sample the number of small (<40μm) and large (≥40 μm) vessels were counted in 40 high power (60x) fields which included the vessel lumen and

surrounding adventitia. The results are presented as the number of large collaterals and the total number of collateral vessels. *(Table 5.11 & figs 5.24-5.26)*

There were significantly more collateral micro-vessels seen around the copper stent (the area of occlusion) in the DMOG stent group (29.9±2.6 vs. 18.4±3.1 control), p=0.01. Numbers of collaterals were similar more proximally around the study stent implantation site and distal to the copper stent. There was no difference in the number of adventitial micro-vessels seen in segments of mid left anterior descending artery from animals in either group. Immunohistochemistry confirmed neovascularization within the RCA sections around the copper stent predominately arising from within the adventitia, and positive staining of endothelial cells within these vessels with Factor-VII related antigen.

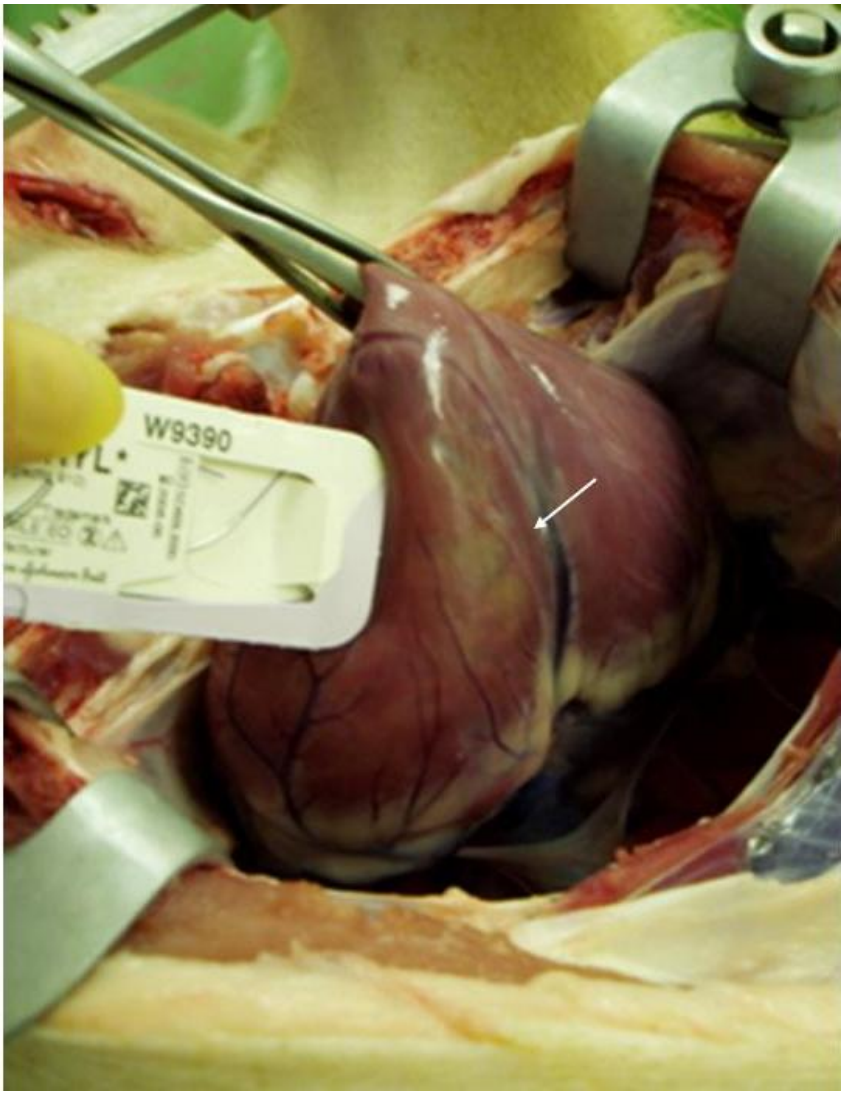


Figure 5.22 Day 56. The pig heart is retracted prior to ligation of the great vessels and explantation. An area of infarction (arrow) is seen in the infero-posterior wall of the left ventricle consistent with RCA occlusion.

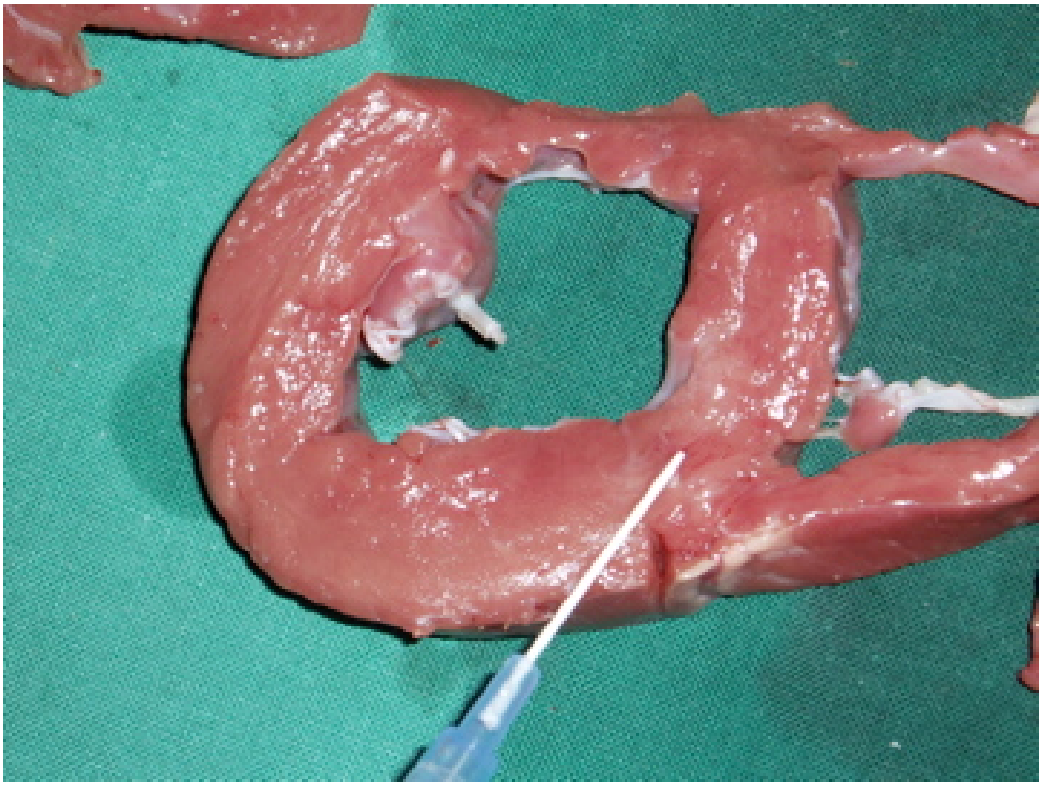


Figure 5.23 Transverse section of explanted pig heart showing area of infarction in the basal septal region of the left ventricle in a territiory subtended by the occluded RCA.

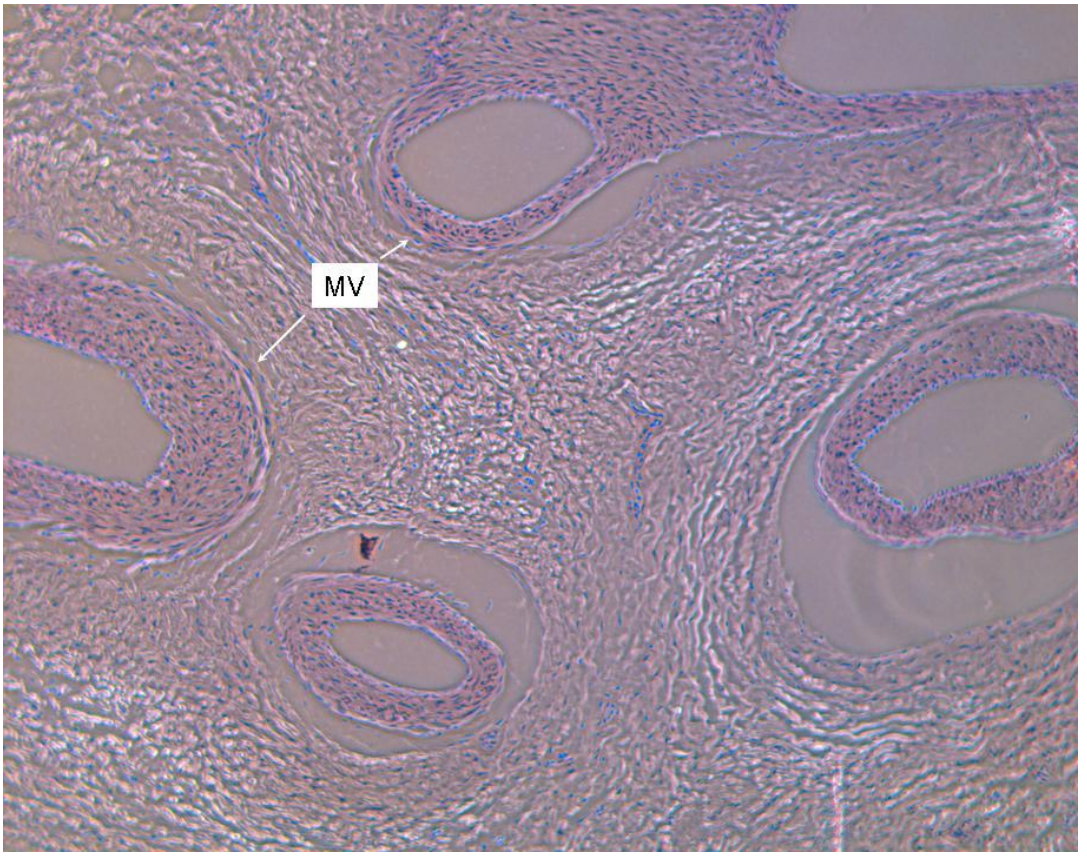


Figure 5.24 H&E section from RCA mid DMOG stent (i.e. proximal stent). Section from HPF (60x) of Pig Q/JG959 RCA demonstrating large adventitial microvessels (MV).

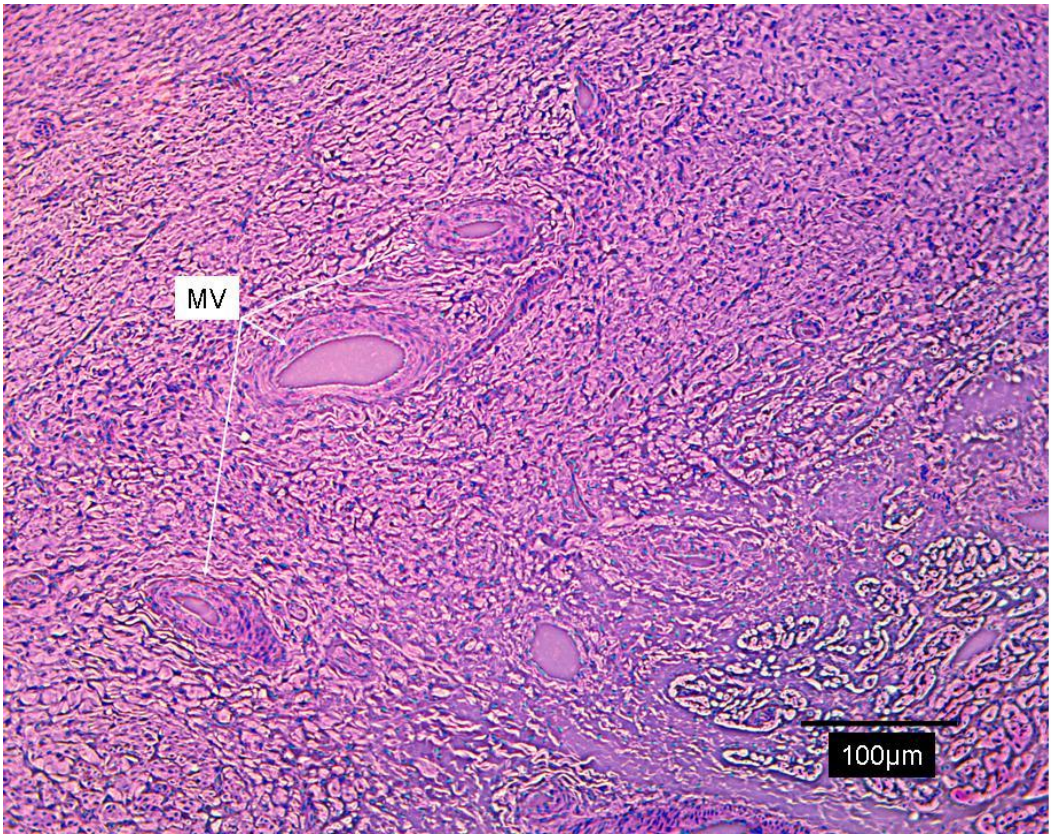


Figure 5.25 Representative H&E section from RCA mid Copper stent (ie. distal stent). Section from single high-power field (40x) of Pig OO/JG982 RCA demonstrating adventitial microvessels (MV).

RCA COLLATERAL VESSELS	CONTROL	DMOG	P VALUE
Proximal stent Large collaterals	9.6±2.2	7.6±1.5	0.49
Proximal stent Total collaterals	10.5±2.4	7.8±1.5	0.38
Distal stent Large collaterals	18.3±2.7	26.6±2.1	0.0293*
Distal stent Total collaterals	18.4±3.1	29.9±2.6	0.0125*
Distal vessel Large collaterals	10.8±2.5	11.0±2.3	0.96
Distal vessel Total collaterals	11.1±2.4	11.8±2.3	0.85
Mid LAD total collateral vessels	1.9±0.4	2.3±0.3	0.24

Table 5.11Analysis of coronary artery collaterals. There were significantly more collateral microvessels seen around the copper stent (the area of RCA occlusion) in the DMOG stent group (mean±SEM) 29.9±2.6 vs 18.4±3.1, p=0.01.

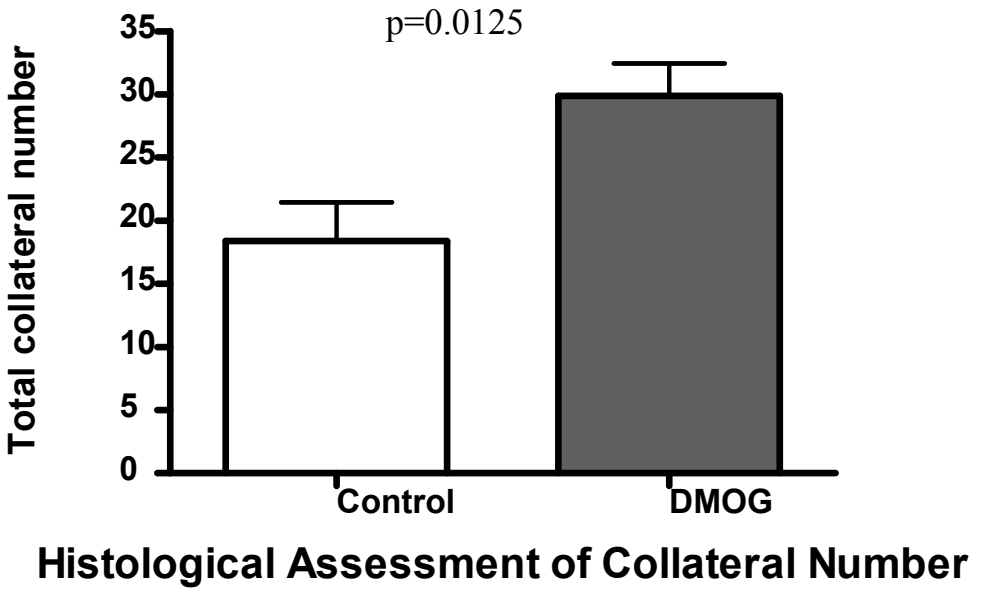


Figure 5.26 Total number of antegrade collateral vessels at copper stent site, as assessed histologically.. There were significantly more collateral microvessels around the distal (copper) stent, corresponding to the area of vessel occlusion in the DMOG stent group (mean±SEM) 29.9±2.6 vs. 18.4±3.1, p=0.0125.

5.4.7 Ex-vivo coronary flow

Ex-vivo coronary artery flow was assessed at the terminal day 56 experiment as described in Chapter 3. This test was a measure of coronary artery run-off and therefore a surrogate of distal myocardial perfusion. Flow rates in the left anterior descending artery (LAD) were higher than in the right coronary artery (RCA). All RCAs showed complete luminal occlusion. There was no difference in either RCA or LAD flow between the control and treatment (DMOG) groups. *(table 5.12)*

EX-VIVO CORONARY FLOW	**CONTROL (MEAN ±SEM)**	**DMOG**	**P VALUE**
RCA flow (ml/min)	58.2±6.5	58.4±6.6	0.98
LAD flow (ml/min)	95.1±3.7	91.1±3.6	0.46

Table 5.12 Ex-vivo coronary flow. There was no difference in coronary flow between the two groups.

5.5 Positive findings from the in-vivo study

The positive results from the in-vivo study were as follows:

- We confirmed the ability to perform three serial complex interventions on pigs via internal carotid artery cut-down.
- We used a reliable, reproducible chronic arterial occlusion model with survivable occlusions to 56 days in 85% of animals in this study
- We demonstrated successful use of the coronary pressure wire to detect consistent rise in collateral flow index in both the control and treatment groups following vessel occlusion.
- We showed extensive antegrade collateralization following vessel occlusion at day 28 after copper stent implantation with limited retrograde collateralization.
- We produced a statistically significant excess of coronary microvessels around the copper stent in the DMOG group.
- We produced no evidence, angiographically or physiologically, of increased myocardial perfusion in either the control or treatment groups.

5.6 Discussion and in-vivo study limitations

The programme of work in this section of the thesis built upon prior experiments to develop a novel entirely endovascular and percutaneous model of chronic total coronary occlusion (CTO).

5.6.1 CTO model

This work rejected constricted stent grafts, predominantly for technical (deployment) reasons, and showed that a copper-plated, balloon-expandable stent, with strict rules (sizing 1:1; one stent only in mid RCA; and appropriate anti-platelet therapy), is simple to use, and capable of producing a reliable model, without complications. We acknowledge that this model is a variant of that described by Song *et al* in 2005, whose work was published while ours was ongoing.[336] Our paper is a thorough validation of their model, as our work was conducted independently. We deployed the stent in mid RCA; they, however, used the circumflex artery. In other respects, our methods were similar. With 20 consecutive animals surviving to planned sacrifice at 28 days, all having CTOS, and all having antegrade collateral vessels, we can conclude that this is indeed a robust and reproducible model of coronary artery CTO.

The advantages of this model include its simplicity, because it employs standard PCI techniques which are utilized worldwide. It does not require a high level of

surgical expertise, as unlike external constrictors no thoracotomy is required.[356] The CTO produced does not involve occlusion of the lumen of the artery with extraneous or non-biological material. The histological features are those of a clinical CTO. It is, potentially, therefore, 'crossable' with appropriate PCI equipment if needed in a study, and will present the technical problems of a clinical CTO as far as PCI technology is concerned. Initial reports on the use of copper stents to produce a CTO have varied in techniques and outcomes. The Stanford group employed a copper stent to induce chronic myocardial ischemia and to test atherectomy devices in the porcine model.[357, 358] The Cornell group have refined the use of copper stents to induce occlusion in the recessive circumflex arteries of Yorkshire pigs, with <100% success and survival.[336] Similarly the use of copper stents to provoke myocardial infarction in pigs is associated with around 50% early mortality.[359] Introducing debris such as bone chips into the arterial lumen can be successful in producing arterial occlusion, but also carries a high early mortality.[332]

The reason why copper coating stimulates an intense vascular reaction is not clear. Copper is an essential trace element, but in large dose is toxic. Copper-mediated catalysis leads to the generation of reactive oxygen species, with oxidative damage to membranes and molecules and apoptosis.[360] In our early experiments with inadequate anti-platelet therapy, excessive vascular injury or implantation in too proximal a site in the coronary artery, there was a high rate of early attrition, associated with an intense inflammatory reaction with

superimposed thrombosis within the copper stent.[360]

5.6.2 Patterns of porcine collaterals

The presence of collateral vessels in a CTO in the pig is of significant interest. Prior to Song's description,[336] this has not been noted previously in pigs. In each of the 20 CTOs produced with the copper stent in this series, antegrade collaterals were observed angiographically. In that group, there were no contra-lateral (left-to-right) collateral vessels from the left coronary artery. Magnified angiographic views showed the antegrade collaterals to be micro-vessels in the adventitial space around the occluded stent *(fig5.10B)* They were visible histologically, together with micro-channels through the neointima within the stent *(figs. 5.7 & 5.8)* In contrast, the Symbiot™ constricted stent graft, albeit in the few animals that survived to the late time-point, was associated with retrograde (left to right) collateral vessel formation.

In conclusion, we have eliminated several potential models of CTO as unworkable, and described in detail and validated a reliable, simple, endovascular model of coronary artery CTO and collateral vessel formation, created with a copper-plated stent, which may be used to test potential mechanical and biological therapeutic strategies in the porcine coronary artery model.

5.6.3 In-vivo DMOG-stent or control-stent study

After positive results in cell culture testing we planned in-vivo experiments in the pig to test a 'proof-of-concept' hypothesis that hypoxia-inducible factor up-regulation would result in measurable improvements in myocardial perfusion following coronary occlusion. Di-methyl oxalyl glycine (DMOG) was selected as an available HIF hydroxylase inhibitor with prior data on its use in pre-clinical angiogenesis studies. This was planned to be the first application of DMOG in coronary angiogenesis. The in-vivo study programme was ambitious and intensive in terms of veterinary care requiring 60 catheterization procedures over a 9 month period (not including preliminary studies) with complex invasive physiological measurement.

5.6.3.1 Physiological measurement of collateral function

The methods of assessment of myocardial perfusion were chosen as those that were feasible within the setting of the field laboratory and were analogous to methods used in clinical interventional cardiology. The collateral flow index (CFI) is a well validated measure of collateral vessel function and readily measured using the intra-coronary pressure wire. CFI in the territory of a CTO is influenced not only by the anatomy of the donor atery but also microvascular function, duration of vessel occlusion and left ventricular function.[361-364]

As outlined in chapter 4, I did not attempt to cross the occlusion at day 56 to record collateral flow index, but rather relied instead on a surrogate method employing a proximal occlusion balloon with the pressure-wire transducer proximal to the occlusion point. This method of assessing distal run-off index (DRI) as a surrogate of CFI was clearly inferior to CFI calculated using Pw measured distal to the occlusion. Within the constraints of what we found possible using this model, the method used was consistent across all three experiments, had an 'internal control' when comparing the same animal at different time-points, and had the benefit of not disrupting the artery and affecting subsequent histological analysis. Maximal hyperaemia was achieved with intra-coronary adenosine.[365] Previous experiments had showed guide-wire crossing of the induced CTO to be very difficult and poorly tolerated by the animals.

5.6.3.2 Angiographic analysis

In our study I had access to modern angiographic equipment and digital archiving, nonetheless the maximum resolution of microvessels even with contemporary equipment is for vessels with luminal diameter in the region of 200-400μm. As the majority of collateral microvessels detected histologically range between 40-400μm many fell below the minimum resolution of the angiography equipment. In practice this meant it was difficult to discriminate quantitatively between areas of bridging collateralization that were composed of a 'sheath' of fine microvessels. I took the approach of counting large collateral vessels (ie >100μm

based on calibration against a fixed reference). This was probably not a sensitive enough measure to accurately interrogate collateral flow. Histological analysis was based on simple counting of collateral micro-vessels within 40 high power (60x) fields including the vessel lumen and surrounding adventitia. Although this approach was unable to discriminate between pre-existing adventitial micro-vessels and new collaterals it had the advantage of being simple, reproducible and constant between the groups. Treatment allocations (and the slide identities with respect to individual animals) were blinded to the investigators until analysis was completed.

Notably there was extensive antegrade collateralization at the point of vessel occlusion in all pigs at 28 days, demonstrated both histologically and by increased collateral flow index following copper stent implantation on day 0. This degree of collateralization was previously thought not to occur in pigs. Commonly used methods of angiographic grading of collateral vessels (eg. the Rentrop grade) were unhelpful because of the large number of antegrade collateral vessels formed by the copper stent occlusion. Almost all the angiograms demonstrated Rentrop maximal grade 4 collateral vessels with rapid filling of the distal coronary.

As discussed in section 5.4.3.4, there was a disparity in the observed collateral vessel area between the groups at the day 28 pre-treatment baseline with a lower collateral area in the group which would subsequently receive a DMOG stent ($15.8mm^2$ in the DMOG group vs. $22.9mm^2$ in the control group). It is interesting

to speculate that this difference may have lead to subsequent over-estimation of the effect of DMOG on angiographic collateral volume, if one assumes that the natural history of the DMOG group might be one of gradual 'catch-up' development of angiographic collaterals to a similar extent to that seen in the control group at baseline. In any event this baseline disparity undoubtedly complicates interpretation of subsequent results. Equally it might also be speculated that the well-developed extent of antegrade collateralization evident at day 28 (prior to implantation of the study stent) seen in both groups may have led to subtle angiogenic effects of DMOG being masked.

There did appear to be a greater increase in angiographic collateral volume around the point of arterial occlusion (at the site of the copper stent) in the DMOG group between day 28 and day 56. This result must be interpreted with caution given that this relative increase in the DMOG group is against the background of unequal baseline angiographic collaterals. It should also be noted that angiography is insensitive in determining the total number of collateral vessels present, limited by a minimum diameter resolution of approximately 200μm.

5.6.3.3 Histological and ex-vivo analysis

Histological examination revealed increased numbers of collateral vessels around the copper stent in the DMOG group at day 56, but the numbers of collateral vessels seen more distally did not differ between the groups. The findings of the

ex-vivo coronary flow experiments are consistent with those of the pressure wire-derived collateral flow index in showing no difference in coronary perfusion.

The histological analysis was based on previously published techniques of scoring a mean vessel density across a set number of high power fields.[208, 222] I counted micro-vessels both smaller and larger than 100μm in diameter, but the main difference in collateral density at the site of the copper stent was in the number of larger collaterals.

The wider significance of the results and the potential role of HIF hydroxylase inhibitors is discussed in Chapter 6.

5.7 Study limitations

5.7.1 Delivery of DMOG

As described in Chapter 2, an indirect analysis of DMOG stent elution was performed using a bioassay. This demonstrated biological activity of DMOG in cell culture after simulated elution for 21 days. The polymer into which the DMOG was loaded was programmed to allow gradual elution of the entire amount of DMOG (approximately 400μg) from the stent within 28 days. As with any in-vitro elution test, the precise nature of DMOG release cannot be

determined with certainty, nor can the pharmacokinetics of DMOG release or endothelial absorption in-vivo. It is therefore presumed but cannot be confirmed that a biologically active concentration of DMOG (equivalent to 100-250μM direct to endothelial cells in culture) was delivered to endothelial cells around or distal to the DMOG stent. Whether this had effects distal to the immediate area of implantation, for example by virtue of up-regulation of transcription, is unknown. The only detected difference in the study group appears to have occurred immediately downstream to the site of DMOG release and had no impact of myocardial perfusion as assessed by our physiological techniques.

5.7.2 Physiological measurements

Our 'proximal balloon occlusion' method of measuring collateral flow index, while supported in the literature and chosen to avoid disruption of the CTO by wire passage, can be criticized as only giving an indirect measure of CFI in the distal coronary bed as the CTO was not crossed.

Angiographic assessment of collateral vessels was limited by the resolving power of the angiogram and the diffuse 'sheaths' of microvessels that developed made angiographic quantification difficult and ultimately insensitive. More sensitive techniques such as magnetic resonance perfusion imaging and wall motion assessment were impractical and prohibitive in terms of cost.

Finally the time-course of events, namely prompt development of a coronary occlusion with very extensive antegrade collateral vessels, made detection of incremental increases in antegrade collateral flow difficult. Antegrade collateralization was already well advanced in both groups at day 28 prior to implantation of the study stent as a result of the intense inflammatory reaction provoked by the copper stent. Whilst this model of CTO is far preferable to external compression as an analogue of spontaneous coronary occlusion, the extent to which the intense fibrotic inflammatory reaction affects the subsequent response to growth factors is unknown

Chapter 6.

Discussion and Conclusions.

6.1 Introduction

In this chapter the results of the work with locally delivered di-methyl oxalyl glycine (DMOG) will be summarized. The scope for further study of DMOG-eluting stents will be discussed. Finally the potential clinical application of this work will be considered, including potential limitations on the use of DMOG-eluting stents in humans.

6.2 Stent loading and elution

Approximately 400μg of DMOG was loaded onto each study stent, with the total amount determined primarily by the total thickness of polymer that could be applied without evidence on electron microscopy of polymer cracking. While experimental pH, temperature and plasma proteins were similar to blood, simple agitation was used rather than a perfusion circuit to mimic the relatively static conditions of a chronic total occlusion (CTO) with limited antegrade flow via collateral vessels.

The inability, despite repeated attempts with mass spectroscopy, to obtain a reproducible measure of DMOG concentration in solution undoubtedly reduced the accuracy of our elution measurements. This problem was compounded by inability, due to small molecular size, to radiolabel DMOG and the problem of rapid esterification in the solutions required to run mass spectroscopy. We

therefore used a bioassay which gave a semi-quantitative measure of the presence of DMOG.

The alternative approach of a bio-assay was novel, based in part on previous work by Professor Gershlick's group where a similar bio-assay was used to confirm biological activity of vascular endothelial growth factor (VEGF) eluted from a polymer-coated stent.[345] In blinded experiments with stent wires the bio-assay was highly discriminative for the presence of DMOG, and DMOG-coated stent wires produced a clear tubule-forming effect equivalent to a concentration of DMOG of 100-150μM. This allowed us to be confident that DMOG continued to be eluted from the stent for up to 21 days of elution in albumin solution. The consequence of this approach was that a precise elution curve could not be constructed, rather only a binary measure of whether DMOG was eluting at any time-point. One advantage of the bio-assay was however that it gave further confirmation that stent-loaded DMOG (in addition to DMOG in solution) was biologically active. A similar concentration of DMOG to that eluted from the stents (100-150μM) has been shown *in vitro* to enhance HIF-1α and -2α as well as VEGF and its tyrosine kinase receptor (Flt-1), along with platelet-endothelial cell adhesion molecule-1 expression under conditions of hyperoxia.[206, 207, 366] The fact that HIF up-regulation has such pleiotropic potential underscores the theoretical benefit of a 'master-switch' therapy for promotion of angiogenesis. The elution profile of DMOG in our experiments was limited to presence or absence of biological effect at individual time-points. The sustained DMOG release over

three weeks seen with DMOG-eluting stents may be contrasted with other forms of local delivery, especially local drug delivery balloons. In these forms of delivery, drug delivery is only temporary, occurring while the device is left in situ. Currently available drug-eluting stents elute drug over between 30 days (Cypher, J&J, Cordis) and 90 days (Taxus, Boston Scientific).

6.3 Cell culture

6.3.1. Tissue-culture angiogenesis assay

The tissue culture (micro-vessel outgrowth) model was used first to determine whether there were pro-angiogenic effects with DMOG *in-vitro.* We felt this was an appropriate initial step as tissue culture has theoretical advantages over cell culture in more closely representing in-vivo physiology. Previous work with similar assays has primarily involved investigation of anti-angiogenic compounds, however the rat aortic model has been used to confirm the pro-angiogenic effect of VEGF.[266, 367] We found no evidence of increased micro-vessel outgrowth in an ex-vivo porcine carotid model after administration of either the non-selective prolyl-hydroxlase inhibitors DMOG, N-oxalyl glycine (NOG), or cobalt chloride or the selective PHD inhibitor, FG-2216. Similarly there was no effect with VEGF. The model however showed linear response to increasing concentrations of fetal bovine serum (FBS). FBS is known to contain numerous growth factors. Microvessel outgrowth appeared to be maximal even at low levels of serum in this model, and that any incremental stimulation which may be the result of

administration of exogenous agents was therefore difficult to detect. As tissue explants required relatively high levels of basal serum in order to remain viable and prolonged culture was associated with a high incidence of culture infection we feel tissue culture is unlikely to be suitable as a screening test for potential pro-angiogenic compounds, but is ideal for rapid screening of anti-angiogenic compounds.

6.3.2 Cell culture tubule-formation angiogenesis assay

There was a significant increase in tubule-formation with addition of both VEGF and DMOG to a HUVEC-based matrigel cell culture angiogenesis model. There is general agreement that tubules in this model represent the early stages of endothelial re-organization into immature blood vessels.[269] The observation that both DMOG-induced and VEGF-induced tubule formation was inhibited by addition of a VEGF2 receptor inhibitor suggests that DMOG is in part acting via upregulation of VEGF. This finding is consistent with previous published work on cells derived from tissue explants of primate and human lungs[206, 207], but the use of HUVEC tubule-formation to detect a physiological effect of a PHD inhibitor such as DMOG is novel. We used this assay to determine that maximal tubule formation occurred with a local DMOG concentration of 125μM, and that this was similar to the effect seen with VEGF at dose 250μg. There was an inhibitory effect on cell proliferation and migration with DMOG concentrations greater than 500μM which was also seen with very high concentrations (>2mM DMOG) in the

tissue culture experiments. DMOG therefore has a relatively narrow therapeutic window in cell culture. Systemic DMOG has however been used in large dose (up to 20mg/kg) in animal studies with no immediate deleterious effects.[208, 222]

6.4 In-vivo experiments.

6.4.1 The percutaneous CTO model

The model of coronary occlusion via entirely percutaneous means is original and has widespread future potential in the field of CTO research for testing new mechanical and biological treatments. While another group published limited experience using a copper stent model during the period of our research,[336] we had already made serial refinements to the technique. There was 100% success in producing a CTO with >85% survival of animals to 56 days. To achieve this we implanted stents with a balloon: artery diameter ratio of 1:1 and used abluminal-only copper coating. The early problems of acute stent thrombosis and associated mortality appears to have been related to arterial injury due to balloon oversize, use of the left anterior descending artery and intimal coating of the stent with copper.

One potential disadvantage of our model is that it used healthy juvenile pigs with normal coronary arteries. Diseased endothelium may be less responsive to stimulation by exogenous growth factors, and a number of hyperlipidaemic

atherosclerotic animals models do exists, albeit at significantly increased overall cost.[368]

6.4.2 Randomized study of DMOG vs. control stents in CTO

The in-vivo trial of DMOG stents was the first study of PHD inhibition in the heart, and the first study using DMOG in the pig. It showed an increase in collateral formation as assessed by histological measures at the site of the copper stent. There was however no increase in distal collateral density or in physiological indices of myocardial perfusion, nor could a difference in absolute numbers of angiographic collateral density be detected.

6.4.2.2 Histology

It was envisaged during the design of the study that histology would be the primary tool for discrimination of any pro-angiogenic effect given the limited resolving power of conventional angiography. Even with the relatively modern equipment available during this study, the maximum resolution of an expanded and digitally-subtracted angiogram (at around 200μm diameter) is below that of around 50% of the adventitial micro-vessels seen at histology. Subsequent to completion of the study we have learnt of the emerging technique of ex-vivo or post-mortem micro computed tomography which can generate detailed images of microvessels (personal communication, B.Strauss). This technique requires

specialized equipment but produces digital images similar to vascular latex-filled models and could have improved the accuracy of collateral quantification.[369]

6.4.2.3 Distal perfusion

Similarly our methods for assessing distal myocardial perfusion were relatively insensitive. Histological examination of the copper stent CTO model demonstrated a dense fibrotic occlusion. We chose not to cross the CTO- our concern was that repeated attempts at passing an angioplasty guide wire across a dense fibrotic CTO would be (a) highly disruptive to intra-luminal histological appearances and (b) unsuccessful in a significant number of cases. As such we resorted to proximal balloon occlusion of the artery, with adenosine-induced hyperaemia and correction for right atrial pressure, with 'distal arterial run-off' (DRI) as a surrogate marker of collateral flow. This measure would be influenced by the presence of side-branches. Fortunately the right coronary arteries of Yorkshire pigs have few very small side-branches, but this limitation remains. Comparison of DRI was made between serial time-points in the same animal, and there was therefore an 'internal control' for the limitations of this technique.

While it would have been possible to place a pressure wire into the distal artery under direct vision via thoracotomy to obtain a collateral flow index (CFI) reading during the terminal experiment at 56 days, we did not feel this would give a representative physiological measurement.

6.4.2.4 Interpretation of results

Perhaps most relevant to interpretation of the physiological data is that by 28 days (the time of implantation of the DMOG or control stents), all animals had CTOs with very extensive antegrade collaterals producing brisk distal arterial flow. This may have made detection of an incremental increase in myocardial perfusion difficult.

The histological change seen in the DMOG group was limited to the area around the DMOG stent. This may be related to higher local concentration of DMOG at this point than more distally in the artery. Alternatively, given the extensive baseline collateralization seen at day 28, it may be that this area was the only significantly ischaemic area of myocardium. The copper stent itself may have promoted angiogenesis due to an inflammatory stimulus. Copper is known to accelerate oxygen-mediated oxidation of thiols in aqueous solution and facilitate generation of reactive oxygen species.[370]

The limited in-vivo pre-clinical studies performed to date in small animals suggest that any pro-angiogenic effect of DMOG may be limited to hypoxic tissue.[222] Furthermore in a rabbit hind-limb model the combination of ischaemia and DMOG increased capillary: fibre density in skeletal muscle along with expression of the VEGF-receptor Flk-1 whereas DMOG treatment without arterial

ligation did not.[208] Our observation of DMOG-effect limited to the ischaemic region of myocardium is consistent with these findings.

The increased collateral density seen around the copper stent was not accompanied by a detectable difference in collateral run-off between the groups. This may have been in part due to the brisk distal flow observed at day 28 in both groups. We have demonstrated that extensive antegrade bridging collaterals are a feature of the copper CTO model. Additionally the shortcomings in our method of measuring distal perfusion, as outlined above, may have reduced our ability to discriminate small differences in distal myocardial perfusion. Future studies may use conventional CFI with crossing of the CTO once further experience has been gained with the copper stent model. Non-invasive imaging such as MR perfusion imaging would have yielded information on distal myocardial perfusion but was not available during this study.

6.5 Summary of results

6.5.1 Cell and tissue culture

In-vitro tissue culture assay

No additional stimulation with addition of DMOG or VEGF

Cell culture tubule-formation assay

Statistically significant excess of tubule-formation with DMOG, which was inhibited by VEGFR-2 inhibitor.

6.5.2 Zebrafish embryo models

Gridlock-mutant model

No difference in collateral vessel recruitment

Fli-1 green fluorescent protein model

Dose-dependent effect on vascular angiogenesis

6.5.3 Stent loading and elution

Stent fabrication

DMOG (400μg approx.) loaded into programmed elution polymed (PEP) by PolyBioMed Ltd. Polymer integrity confirmed by SEM. Unable to directly measure DMOG in solution by mass spectroscopy (at University of Oxford Chemistry research laboratory).

Elution testing

Identification of 12 blinded stent wires (DMOG or control) using cell-culture bio-assay.

Bio-assay used to detect elution of DMOG from stent after 21 days elution.

6.5.4 CTO model

Copper-stent model

Abluminal-only copper loaded onto bare-metal stents by Brivant, Ltd.
Novel entirely endovascular occlusion model developed with 100% success rate for induction of CTO and 85% survival to 56 days. Extensive antegrade collateralization was seen after 28 days. Histology similar to man with luminal and adventitial microvessels, and dense inflammatory infiltrate around stent struts.

6.5.5 In-vivo study

No consistent differences seen between the DMOG and control stent groups

Although there were excess adventitial collaterals seen (29.9±2.6 vs. 18.4±3.1, p=0.0125) in the DMOG group this was the only positive finding supportive of excess angiogenesis in the DMOG group. Immunohistochemistry demonstrated that the micro-vessels were lined with endothelium. There was no difference in distal myocardial perfusion.

Most measures of collaterals showed no difference between the DMOG and control groups. The relative insensitivity of coronary angiography (limit of resolution 200μm) in detecting collateral microvessels has already been

discussed. The observed increase in microvessels was observed only on histology at the level of the copper stent, with no difference in microvessels more distally in the artery or in physiological measures of distal coronary perfusion.

There are several possible reasons for the above 'negative' findings. Performing this study has been valuable in confirming the limitations of coronary angiography for collateral quantification. Our sample size was based on practical restraints of time and resource, but may have been under-powered to detect subtle differences in histology and coronary perfusion. Of equal importance was the finding of unexpectedly marked (Rentrop grade 3) antegrade collaterals present in all animals at 28 days after copper stent implantation. This is likely to have limited the ability of the model to detect subtle increases in microvessel density or differences in (the already brisk) coronary perfusion. The baseline difference in angiographic collaterals between the groups complicated interpretation of the angiographic results and as discussed above may have led to an over-estimation of the angiographic DMOG effect.

6.6 Future studies with a DMOG-eluting stent

Further pre-clinical studies with DMOG are indicated to investigate whether the positive biological effect seen in cell culture experiments with DMOG extends to an in-vivo model. The newly developed copper stent CTO model appears to be appropriate for this purpose. These experiments could be performed in a different species to the pig. Additional methods should be used to assess collateral number,

including micro-CT, along with improved measures of perfusion such as collateral flow index (after crossing the occlusion). Further histological examination of the heart, liver and lymphatics of the animals should be performed to exclude metaplasia. Measurement of inflammatory markers and careful histological examination of the coronary arteries should be performed at the start and end of the study to exclude accelerated atherosclerosis. Pre and post-study organ samples should be assayed for HIF levels to determine whether there is any unwanted extra-cardiac effect upon gene transcription.

The *in vivo* experiments support the concept of local delivery of DMOG as a potential treatment to promote neo-angiogenesis in occluded coronary arteries. The DMOG-eluting stent could be tested for a neo-angiogenic response in ischaemic tissues. An initial approach might be to implant DMOG–eluting stents in patients with critical limb ischaemia. Work discussed in chapter 1 (1.13) used VEGF in patients with critical ischaemia to delay amputation. A similar clinical trial would gather data on the safety/side-effect profile of the stent/DMOG system and establish the efficacy of local DMOG delivery in humans.

Stents loaded with DMOG could be placed percutaneously into the arterial tree of the affected leg. The positioning of the stent would be immediately proximal to the occluded section(s) of artery. The endpoints of such a study would be:

- Progression of disease sufficient to warrant amputation.
- Withdrawal due to side-effects/complications of DMOG or of stent implantation.

- At three months, angiographic reassessment of collateral density.
- Symptoms, Ankle-brachial artery pressure index (ABPI), exercise tolerance and serum levels of DMOG would be assessed at 7, 14, 28 days and at three months. If successful and safe, then a similar trial could be attempted in patients with coronary CTO and evidence of both a high ischaemic burden and myocardial viability in the relevant territory who were judged to be unsuitable for traditional revascularisation techniques. The end-points for such a study would include quality of life measures and physiological measurement of myocardial perfusion. The ideal modality for measuring subtle change in myocardial perfusion would be pre- and post treatment perfusion magnetic resonance imaging with accurate comparisons of the extent of perfusion based on a 16 myocardial-segment model or similar.

6.7 Long-term future of locally-delivered DMOG

The work of this MD thesis has been to investigate an initial 'proof-of-concept' for stent-loaded DMOG as a treatment for coronary occlusion. While the results of this programme of work and other similar pre-clinical studies are encouraging, there are further obstacles to be overcome before the DMOG-eluting stent could be used in mainstream clinical practice. These obstacles include concerns over the safety of DMOG administration in humans which are common concerns to the use of many growth factors.

6.7.2 Mortality risk

The aspect of most concern to any future use of DMOG in clinical trials is the risk of death consequent to the therapy. Use of VEGF in clinical trials was temporarily halted in 2000 after the death of a young man in an angiogenesis trial. In this case large amounts of the adenoviral vector used to deliver DNA coding for VEGF was accidentally injected into the patient's hepatic artery with fatal consequences[371]. Ultimately it was felt that this case was not directly due to the use of VEGF, but rather to the viral vector. No other deaths have yet been associated with VEGF use in humans, but the total number of patient that have been treated with VEGF in any form still is significantly less than 1000.

6.7.3 Risk of neoplasm

DMOG is a potential carcinogen. Use of DMOG, via upregulation of VEGF, may have unwanted neoplastic effects either in the heart or at distal sites, although this has not yet been demonstrated in human subjects. Myoblasts genetically engineered to synthesize VEGF have been implanted into the heart in a mouse model. In one study, 2 of 19 animals treated with VEGF developed highly vascular tumours.[135] In cell culture, VEGF receptors have been demonstrated in uterine smooth muscle cells, which proliferate in response to VEGF.[372] VEGF receptors have also been demonstrated in human cell tumour lines including glioma, melanoma and squamous cell carcinoma of neck.[373] These represent the

recognized role of VEGF and similar growth factors in preservation of tumour cell viability. While anti-angiogenic compounds have potential as cancer treatments, no causal link has been made between exogenous growth factors and initiation of cancer.

6.7.4 Applicability of pre-clinical testing

Marked inter-species differences are seen in response to growth factors, and regardless of encouraging initial studies in animals there is no guarantee that DMOG will exert a significant angiogenic effect in humans. It is striking that, despite various positive studies in animals of locally delivered VEGF, there was no effect in the VIVA trial (Vascular Endothelial Growth Factor in Ischaemia for Vascular Angiogenesis). In this study, VEGF165 was given to patients with untreatable severe ischaemia, most of it by systemic delivery, with an intra-coronary bolus. Despite some signs of improved perfusion, no increase of exercise time or reduction of angina was seen with VEGF compared to placebo. A strong, sustained placebo effect was seen.[153] In a separate study, no ill-effects were seen in a series of ten patients who received VEGF plasmid infusion into the coronaries after angioplasty.[374]

6.7.5 Potential atherosclerotic effects

DMOG acts via VEGF, which has widespread effects on multiple cell types. One group has reported the detection of KDR (VEGR-2) receptors on human smooth muscle cells in specimens of atherosclerotic arteries.[375] It may be that the atherosclerotic process itself is the stimulus for the production of VEGF receptors in the smooth muscle cells. It is possible that in the presence of atherosclerosis, VEGF promotes smooth muscle cell replication and might in fact exacerbate the growth of neointima. VEGF mRNA and VEGF receptors have been demonstrated in atherosclerotic, but not normal, human coronary arteries.[107] DMOG may therefore be an important promoter of the atherosclerotic process and exogenous delivery of DMOG to diseased coronary vessels may accelerate the growth of these plaques. In our experiments no significant angiographic restenosis was noted in any of the DMOG stents at the terminal experiment on day 56.

The additional effects of VEGF (vascular permeability, chemo-attraction for macrophages) may destabilise existing plaques leading to increased risk of plaque rupture and resulting acute coronary syndromes. No evidence of atherosclerotic change has been seen in pre-clinical trials although follow-up is short and the majority of studies used juvenile non-atherosclerotic animals. No accelerated atherosclerosis was seen in the existing, small-scale, human trials of VEGF in ischaemic heart disease. It may be that any potential for atherosclerotic acceleration is of limited clinical consequence in the ‘no option’ target patient

group in whom these treatments would be used. The starting point for clinical investigation is likely to be a 'first-in-man' study in patients with end-stage peripheral vascular disease and critical limb ischaemia. Progression of atherosclerosis would be an important safety end-point of any clinical study and may require mandated surveillance angiography, although any adverse effects due to atherosclerosis progression would impact upon clinical safety and efficacy end-points.

6.8 Final conclusions

Successful drug absorption and elution of a 'master-switch' gene transcriptional agent has been demonstrated using DMOG. This was important, as one of the key advantages to the use of stents to deliver drug is that the release of the drug is sustained. This has been demonstrated clearly with the use of a bio-assay. The effectiveness of DMOG as a stent delivered pro-angiogenic compound has been confirmed in cell culture. To demonstrate the direct effect of a DMOG-coated stent on endothelial cells is an original finding. This work extends previous cell culture experiments by introducing the stent itself into the culture dish. This may be important since *in vivo* the drug is released by passive diffusion into nearby tissues, producing a gradient of concentration of drug rather than a uniform concentration as seen in conventional cell culture. This model also allows for any direct effect on cell growth of the stent or the polymer coating on the stent. The cell culture results were obtained from carefully designed experiments that

predominantly used well-established methods that have been the subject of published work in the past. A bio-assay was used to confirm elution of DMOG from the stents up to 21 days as initial attempts to directly measure DMOG in solution were unsuccessful due to small molecular size. The elution characteristics of DMOG are therefore approximate, but importantly the biological effect of DMOG eluting from the stent has been directly confirmed in the bio-assay.

The in-vivo work is completely original since DMOG has never been delivered bound onto a stent, used in the heart of any animal, or delivered in a sustained fashion in an animal model. The animal model has been developed entirely by our group, with novel use of a copper-coated stent to achieve an entirely endovascularly produced CTO. The model produces dense fibrotic inflammatory CTOs with extensive antegrade collateral formation by 28 days. While the CTO is histologically similar to human CTO, how the intense inflammation present affects the degree of antegrade collateralization seen or how it affects the response of viable endothelium to further angiogenic stimulation is unknown.

Some interesting results have been produced in the course of the studies. These results provide a starting point for further research into DMOG in the future. The possible future uses of the DMOG-eluting stent have been discussed together with caveats regarding the widespread use of DMOG in humans.

Chronic total coronary occlusion is a common and disabling condition, estimated to affect around a third of patients with angina, or up to one million patients in the United Kingdom alone. Many patients identified as having CTO are not currently considered for revascularization. Of those undergoing PCI, success rates overall are around 70%, or up to 90% in expert hands. This leaves a significant minority who suffer with angina but are unsuitable for, or unsuccessful in attempts at conventional PCI. Many more patients are not fit for, nor do not wish coronary bypass surgery. The availability of a safe and effective alternative treatment for those who remain symptomatic despite optimal medical therapy is therefore an important unmet need.

Drug-eluting stent research has been characterised by successful translational research programmes. Unlike the situation with anti-restenotic therapies, angiogenesis research remains at an early stage. The situation is analogous in many ways to that seen before early clinical trials of anti-restenotic drugs, with a consistent signal of benefit appearing in pre-clinical studies. In general, cardiovascular angiogenesis research has the aim of relieving ischaemic symptoms in patients unsuitable for conventional therapies. We hope that the work presented in this thesis contributes to this goal, and look forward to further clinical trials of angiogenic therapies for severe occlusive coronary disease.

Appendix 1- Publications to date from this thesis

Abstracts:

Increased Endothelial Cell Mobility Following Stabilization of Hypoxia-Inducible Factor-Steps Towards Therapeutic Angiogenesis.
D J Kelly, J Mecinovic, K Chitkara, C J Schofield, J Gunn, A H Gershlick. SCAI/ACCi2 Interventional abstracts *J. Am. Coll. Cardiol.,* March 11, 2008; 51: B23 - B98.

Evidence for a potential new method of therapeutic angiogenesis: increased endothelial cell mobility and tubule formation in cell culture after stabilization of hypoxia-inducible factor. D J Kelly, J Mecinovic, C J Schofield, J Gunn, A H Gershlick. *Heart* 2008;94;A86

In-Vivo Observations On Angiogenesis: Dose-Dependent Effects Of The Prolyl-4-Hydroxylase Inhibitor, Di-Methyl Oxalyl Glycine, On Arterial Development In Embryonic Zebrafish. D J Kelly, A H Gershlick, J Mecinovic, C J Schofield, D Crossman, J Gunn, T Chico. *Heart* 2008;94;A87

A Porcine Endovascular Model Of A Chronic Total Coronary Artery Occlusion. D J Kelly, A C Morton, T Raina, H Lupton, J Gunn. *Heart* 2008;94;A85

Upregulation of Hypoxia-Inducible factor by Di-methyl Oxalyl Glycine Increases Neovascularization within Ischaemic Myocardium in a Porcine Coronary Occlusion Model. D J Kelly, J Mecinovic, C J Schofield, J Gunn, A H Gershlick. Accepted American College of Cardiology 59th Annual Scientific Session March 2010. *J. Am .Coll. Cardiol. 2010;55:A216.E2047*

Upregulation of Hypoxia-Inducible Factor by Di-Methyl Oxalyl Glycine (DMOG) Increases Neovascularization Within Ischaemic Myocardium in a Porcine Coronary Occlusion Model. Damian J. Kelly, Jasmin Mecinovic, N Arnold, Christopher J. Schofield, Julian Gunn, Anthony H. Gershlick1. British Cardiovascular Society Annual Scientific Conference 2001. *Heart* In Press.

Word count

Thesis word count (excluding references, tables, and figures= c.30,400 words)

UPREGULATION OF HYPOXIA-INDUCIBLE FACTOR BY DI-METHYL OXALYL GLYCINE (DMOG) INCREASES NEOVASCULARIZATION WITHIN ISCHAEMIC MYOCARDIUM IN A PORCINE CORONARY OCCLUSION MODEL

Damian John Kelly, Jasmin Mecinovic, Nadine Arnold, Kim Suvarna, Christopher J. Schofield, Julian Gunn, and Anthony H. Gershlick

J. Am. Coll. Cardiol. 2010;55;A216.E2047
doi:10.1016/S0735-1097(10)62048-7

Background: Chronic total coronary artery occlusion (CTO) remains a significant challenge for percutaneous coronary intervention (PCI), and a common reason for referral for coronary bypass surgery. This study was designed to investigate a potential alternative treatment strategy for CTO through promotion of antegrade collaterals around the CTO. We investigated the effect of the prolyl-4-hydroxylase inhibitor di-methyl oxalyl glycine (DMOG) and thus a HIF 1α promoter on collateral vessel formation in a novel endovascular porcine model of coronary occlusion.

Method: DMOG was loaded onto a polymer-coated coronary stent. A percutanous model employing copper-coated stents was used to produce CTO lesions in 20 Yorkshire white pigs. DMOG stents were implanted at day 28 and angiographic and physiological data collected on distal coronary and collateral flow. At day 56 the animals were sacrificed and histological analysis performed.

Results: A complete total coronary occlusion was present in all animals at day 28 following implantation of a copper stent. At 56 days there was a larger increase in angiographic collateral area in the DMOG group vs. controls (10±4.1 mm2 increase vs. 3.6±1.5mm2; 84.5% vs. 16.5% increase, p=0.057). Collateral flow index increased in the study group as a whole from day 0 to day 28 post-copper stent (0.12±0.02 to 0.4±0.04, p<0.001) but there was similar distal perfusion in both groups at day 56. Histology revealed an increase in collateral number (vessels >100μm diameter) seen around the site of occlusion in the DMOG group (29.9±2.6 vs. 18.4±3.1, p=0.01).

Conclusion: DMOG increases the number of collateral vessels seen at the site of vessel occlusion but did not increase neovascularization in distal tissue. At baseline distal myocardium was subtended by extensive antegrade collaterals. The effect of DMOG in increasing neovascularization appeared to be resticted to ischaemic tissue. Implantation of a copper stent provides a reliable entirely endovascular method of producing a CTO with marked antegrade collateral formation at 28 days. Proximal placement of stents delivering angiogenic compounds may provide a clinical management option in resistant CTO lesions.

initial 3-hour hypoxia exposure reflecting an increase in pulmonary vascular resistance (pre-hypoxia 252 ms; pre-infusion 192 ms; $p<0.05$). There was a significant reversal of this trend after the infusion of nitrite (peak 211 ms; $p<0.05$). This effect was reduced at 1 hour post-infusion (see table and fig).
Discussion: Our in-vivo model demonstrates that the vasodilator effects of nitrite are enhanced by hypoxia and that this difference is observed at low levels of supplementation. This feature of nitrite pharmacokinetics could have therapeutic implications in the fields of coronary ischaemia and pulmonary hypertension.

084 INDICES OF APOPTOSIS IN PATIENTS WITH IMPAIRED GLUCOSE TOLERANCE AFTER ACUTE MYOCARDIAL INFARCTION

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Background: Impaired glucose tolerance (IGT) after acute myocardial infarction (AMI) is largely ignored despite evidence of an increased cardiac event rate in such patients. We hypothesised that apoptotic cell death following AMI measured by an increase in soluble Fas and Fas ligand, two transmembrane glycoproteins involved in apoptosis, would be more pronounced in IGT patients compared with those with normal glucose tolerance (NGT).
Method: Consecutive non-diabetic patients presenting with AMI underwent standard oral glucose tolerance testing 3–5 days after admission. Apoptosis was assessed by measuring soluble Fas and Fas ligand levels in the fasting state and after 75 g glucose load. Soluble Fas and Fas ligand levels were measured by ELISA.
Results: 125 patients (mean age 59 years (SD 12.5); 107 (86%) men) were studied. Baseline levels of soluble Fas were higher in IGT patients compared with NGT patients ($p<0.01$), with a significant increase in soluble Fas levels in response to glucose challenge in the IGT and diabetes mellitus groups (table). There was no significant difference in soluble Fas ligand levels between the groups both pre and post-glucose challenge.
Conclusion: IGT post-AMI is associated with significantly higher levels of soluble Fas when compared with NGT. Interestingly, the levels of soluble Fas in the IGT group are comparable with those with frank diabetes. A further increase in soluble Fas appears to occur in response to a rise in plasma glucose levels. The increase in soluble Fas may play an important role in the pathophysiological mechanisms of IGT post-AMI and its associated poor clinical outcome in such patients. Is it time actively to seek and manage IGT post-AMI?

085 A PORCINE ENDOVASCULAR MODEL OF A CHRONIC TOTAL CORONARY ARTERY OCCLUSION

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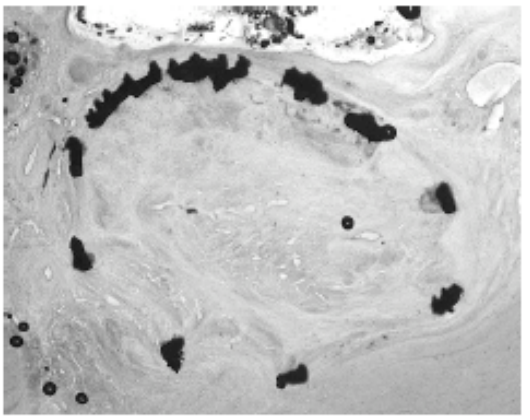
Background: Chronic total coronary artery occlusion (CTO) remains a significant clinical challenge for percutaneous coronary intervention. There is a need for a reliable, simple, endovascularly produced, large animal model of CTO to test new therapies.
Aim: To produce a survivable CTO in a normal pig coronary artery using endovascular means.
Methods: 34 pigs (38 vessels) underwent endovascular intervention to their coronary arteries in a sequential, "block", evolutionary, experimental design of three to four animals in each group, in which different potential methods were tested in each group. Three devices were studied: a suture-constricted, balloon-expandable, PTFE-coated, stainless steel stent graft; a suture-constricted, self-expanding, nitinol, PTFE-coated stent graft; and a copper-plated, stainless steel, balloon-expandable stent (fig). Two pig sizes were tested: 18 and 50 kg. Four deployment sites were tested: proximal and mid-left anterior descending artery and right coronary artery. Two stent–artery ratios were tested: 1 : 1 and 1.25 : 1. Two antiplatelet strategies were tested: aspirin alone for 5 days and aspirin and clopidogrel for 5 weeks. The endpoint was animal survival with angiographic and histological evidence of a CTO at 28 days.
Results: The best results (100% survival with angiographic and histological evidence of a CTO) was obtained in 50 kg pigs, with deployment of a copper-plated, balloon-expandable stent in mid-right coronary artery, at a stent–artery ratio of 1 : 1, with 5 weeks dual antiplatelet therapy.
Conclusions: A reliable, simple, endovascular model of CTO is possible in the pig coronary artery using a copper-plated stent. It would lend itself to the testing of new recanalisation and revascularisation strategies.

Abstract 084 Soluble Fas levels by baseline glycaemic status post-acute myocardial infarction

sFas levels median (IQR)	NGT (n = 52)	IGT (n = 48)	DM (n = 25)
Fasting	4 (3–6)	6 (4–9)*	6 (5–8)*
2 Hours post-glucose challenge	4 (3–6)	7 (4–10)*†‡	6.5 (4–10)*†‡

DM, diabetes mellitus; IGT, impaired glucose tolerance; IQR, interquartile range; normal glucose tolerance; sFas, soluble Fas.
*Significantly higher ($p<0.01$) compared with NGT.
†Significantly higher soluble Fas levels at 2 hours post-glucose challenge ($p<0.01$).
‡Significantly higher soluble Fas levels compared with NGT group ($p<0.05$).
Between-group analyses by Kruskal–Wallis with Dunn's post-hoc test. Pre and post-glucose challenge analysis by Wilcoxon signed ranks test.



Abstract 085

086 EVIDENCE FOR A POTENTIAL NEW METHOD OF THERAPEUTIC ANGIOGENESIS: INCREASED ENDOTHELIAL CELL MOBILITY AND TUBULE FORMATION IN CELL CULTURE AFTER STABILISATION OF HYPOXIA-INDUCIBLE FACTOR

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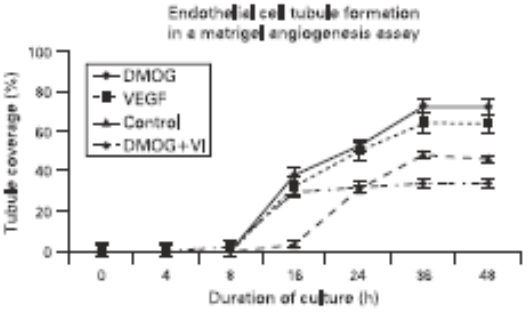
Introduction: Chronic total coronary occlusions are often resistant to treatment by percutaneous intervention. Clinical trials of therapeutic angiogenesis with the systemic administration of single-protein growth factors such as vascular endothelial growth factor (VEGF) have been negative. Hypoxia-inducible factor (HIF) binds DNA sequences within the hypoxia response elements of several target genes involved in angiogenesis, and thus the



Abstract 086 Promotion of endothelial tubule formation in an in-vitro matrigel-based angiogenesis assay.
DMOG, di-methyl oxalyl glycine; VEGF, vascular endothelial growth factor.

upregulation of HIF is potentially a more physiological pro-angiogenic therapy than the addition of growth factors such as VEGF. In humans the proteolytic stability of HIF is regulated via oxygen-dependent hydroxylases. Endothelial cells coalesce into tubule structures when cultured on the basement membrane extract, matrigel. We tested whether a prolyl-4-hydroxylase inhibitor, di-methyl oxalyl glycine (DMOG), could accelerate endothelial cell mobility and tubule formation in an in-vitro angiogenesis model.

Method: EHy926 human umbilical vein endothelial cells were cultured in DMEM with 2% HAT and 2% FBS. 72 wells of a 96-well plate were inoculated with 40 000 cells per well, passage 4/5, over 50 µl of reduced growth-factor matrigel. 100 µl of culture medium was added with 125 µmol DMOG, 250 µg VEGF or 125 µmol glycine (control). 160 µmol of a specific VEGF receptor inhibitor was added to alternate wells. Wells were photographed at 4, 8, 16, 24, 36 and 48 hours for evidence of cell migration. Blinded off-line digital image analysis using a 10 × 10 overlay-grid was performed to measure the percentage proportion of grid squares containing one or more branching tubule structure (tubule coverage). Statistical analysis was by paired t-test.

Results: Minimal tube formation was present at 8 hours. By 16 hours DMOG culture produced similar tubule coverage to VEGF but more than control, mean (SD) DMOG 38.7 (3.6), VEGF 33.4 (4.6), p = 0.60; control 4 (1.7), p = 0.012. The addition of a VEGF receptor inhibitor reduced mean tubule coverage at 16 hours in DMOG culture to 30.3 (2.5), p = 0.038 and essentially abolished migration under VEGF culture, tubule coverage 0.5 (0.7). Cell migration was complete in all cultures by 36 hours (fig).

Conclusions: The addition of DMOG to endothelial cells in matrigel culture accelerates endothelial cell migration and tubule formation. This effect is partly mediated via VEGF. HIF-stabilising compounds may promote collateral vessel formation in-vivo. We are currently investigating their use as stent-based therapy in a novel porcine percutaneous model of coronary occlusion.

087 IN-VIVO OBSERVATIONS ON ANGIOGENESIS: DOSE-DEPENDENT EFFECTS OF THE PROLYL-4-HYDROXYLASE INHIBITOR, DI-METHYL OXALYL-GLYCINE, ON ARTERIAL DEVELOPMENT IN EMBRYONIC ZEBRAFISH

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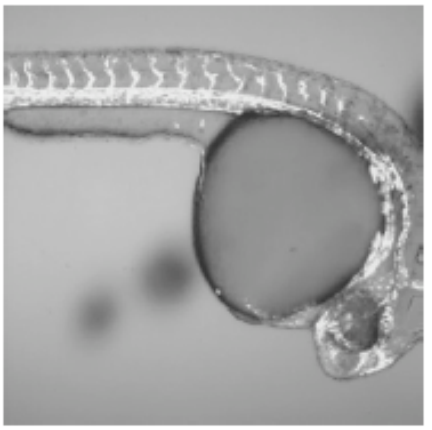
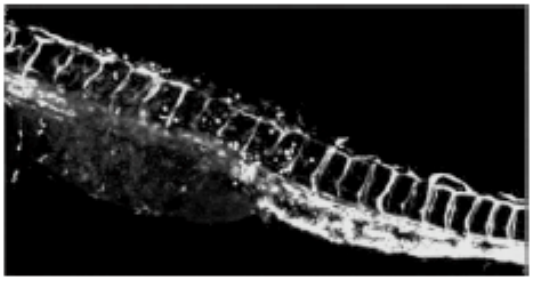
Introduction: Hypoxia-inducible factor (HIF) binds DNA sequences within the hypoxia response elements of several target genes involved in angiogenesis. The manipulation of HIF is a potential target for therapeutic angiogenesis. In humans the proteolytic stability of HIF is regulated via oxygen-dependent hydroxylases. We tested whether a prolyl-4-hydroxylase inhibitor, di-methyl oxalyl glycine (DMOG) was active in-vivo using a zebrafish model of arteriogenesis and collateral vessel formation.



Abstract 087 Figure 1 Control Fly-1 mutant zebrafish 29 hours post-fertilisation.

Method: The zebrafish (*Danio rerio*) embryo, by virtue of its translucency, is used as a model of arteriogenesis. Gridlock mutant embryos (GM), n = 320, were produced by insertional mutagenesis and expressed a phenotype with occluded proximal aorta in association with a variable degree of distal collateral formation via intersegmental vessels. GM embryos, 3 days post-fertilisation were exposed to a range of DMOG concentrations in E3 medium and the proportion of embryos displaying distal aortic flow via collateral vessels was quantified by light microscopy. Transgenic zebrafish embryos (Fly 1 mutants, fig 1) expressing green fluorescent protein as an endothelial cell marker (n = 956), were collected 3 hours post-fertilisation and exposed to increasing doses of DMOG up to 100 µmol. The effect upon arteriogenesis was observed with fluorescent confocal microscopy.

Results: DMOG did not affect the rate of collateral recruitment among GM embryos. At day 5 post-fertilisation, there was no difference in the rate of collateral recruitment between the embryos exposed to DMOG 100 µmol and control GM embryos: distal aortic flow was seen in 38.5% of those in DMOG and 49.5% of embryos in control medium, p = 0.38. A dose-dependent relationship was observed between DMOG concentration and altered arteriogenesis in the Fly-1 mutant embryos. At 27 hours post-fertilisation 5.7% of control embryos displayed evidence of alteration in development of



Abstract 087 Figure 2 Mid-fish segment at 80 hours post-fertilisation showing alterations in vascular development after exposure to di-methyl oxalyl glycine 100 µmol.

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