Inter-Individual Variation in Saliva Antioxidant Status in Relation to Periodontal Disease

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By

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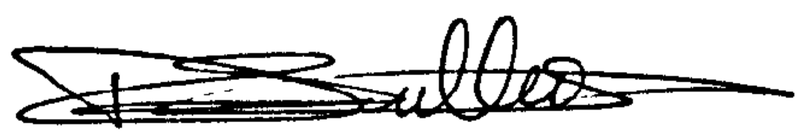
Declaration

All the work in this thesis was completed by the author with the exception of the dental examinations which were carried out by Mr Mike Busby.



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ABSTRACT

Dean Vincent Sculley

Inter-Individual Variation in Saliva Antioxidant Status in Relation to Periodontal Disease.

Free radicals have been implicated in the aetiology of many human disease states, including periodontal disease (PD). Saliva contains an array of antioxidants that neutralise the harmful effects of free radical formation within the oral cavity. Increased salivary antioxidant capacity may limit oxidative tissue damage and improve the health of the gingival tissues. In this thesis, the antioxidant profile of saliva was assessed in relation to PD severity. Salivary protein carbonyl concentration was assessed as a biomarker of oxidative tissue damage.

Urate, ascorbate and albumin contributed 85% of the total scavenging antioxidant capacity (TAA) which followed a diurnal rhythm (peak 5.00pm, nadir 3.00am). Salivary antioxidant flow rate was significantly lower in individuals with severe PD (TAA 0.210 ± 0.02 μM/ml/min) than those with healthy gingivae (TAA 0.270 ± 0.02 μM/ml/min) ($p<0.05$). Oxidative injury was greater in the group with severe PD (protein carbonyls = 25.43 ± 11.09 fmoles/g protein) compared to the healthy group (7.77 ± 2.38 fmoles/g protein) ($p<0.05$). Individuals with low salivary TAA were 4.5 times more likely to suffer severe PD. TAA in males (654 ± 25 μM/ml/min) was significantly higher than in females (545 ± 23 μM/ml/min) ($p=0.002$), but no relationship was noted between sex and periodontal health. TAA and urate flow rates followed the same monthly cyclical pattern as progesterone and β-estradiol in women. Nutritional antioxidant supplementation was found to have little effect on salivary antioxidant status with only a transient increase in ascorbate evident.

These data suggest that severe PD is associated with a decreased salivary antioxidant flow rate and increased oxidative injury. In addition, dietary antioxidant supplementation does not appear to increase salivary antioxidant status as it does in plasma. Toothpastes and mouthrinses containing antioxidants may reduce the extent of oxidative injury *in vivo*, with more research required in this area.

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ABBREVIATIONS

4-AAP	4-aminoantipyrine
ABAP	2-azobis (2-amidinopropane) hydrochloride
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline sulphonate)
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency disorder
ANOVA	Analysis of variance
AO	Antioxidant
ATP	Adenosine triphosphate
BCG	Bromcressol green
BSA	Bovine serum albumin
CHD	Coronary heart disease
CHO	Carbohydrate
CPITN	Community Periodontal Index of Treatment Needs
CSF	Colony stimulating factor
CuZnSOD	Copper zinc superoxide dismutase
CVA	Cerebrovascular accident
CVD	Cerebrovascular disease
DHBS	3,5-dichloro-2-hydroxybenzine-sulphonate
DNA	Deoxyribosenucleic acid
DNP	Dinitrophenylhydrazine
e^-	Electron
ECL	Enhanced Chemiluminescent

EC-SOD	Extracellular superoxide dismutase
ELF	Epithelial lining fluid
FRAP	Ferric reducing ability of plasma
GCF	Gingival crevicular fluid
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidised glutathione
HPBMC	Human peripheral blood mononuclear cells
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule
IgA	Immunoglobulin A
IL	Interleukin
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MG	Mucous glycoprotein
MnSOD	Manganese superoxide dismutase
f-MLF	N-formyl-methionine-leucine-phenylalanine
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NFκB	Transcription factor
ODFR	Oxygen derived free radicals
PAF	Platelet activating factor

PGE_2	Prostaglandin E2
PMN	Polymorphonucleocytes
PRP	Protein rich glycoprotein
PUFA	Polyunsaturated fatty acid
RNA	Ribosenucleic acid
ROS	Reactive oxygen species
SEM	Standard error of mean
SMP	Submitochondrial particles
SOD	Superoxide dismutase
TAA	Total antioxidant activity
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TPTZ	2,4,6-tripyridyl-s-triazinine
TRAP	Total radical trapping antioxidant potential
(Trolox)	(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid)
VCAM	Vascular cell adhesion molecule
VEC	Vascular endothelial cell

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CHAPTER ONE

Introduction

1.1 General introduction

There is growing evidence that reactive oxygen species (ROS) and free radicals play a significant role in the pathogenesis of over 50 disease conditions, such as AIDS, atherosclerosis and associated vascular diseases, chronic inflammatory conditions, mutagenesis and cancer, neurodegeneration, immunologic disorders, cataracts and aging (Halliwell and Gutteridge, 1984, 1989; Merry *et al.*, 1989; Halliwell and Cross, 1991; Gutteridge, 1992; Ames and Shigenaga, 1993; Gutteridge and Swain, 1993; Castro *et al.*, 2001; Mayne, 2003).

Free radicals can be defined as "any species capable of independent existence, containing one or more un-paired electrons" (Halliwell, 1991). These can be ROS, also known as oxygen derived free radicals (ODFR), such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and nitric oxide radicals ($NO^{\cdot}$), or non-radical by-products of O_2 such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL) (Waddington *et al.*, 2000). Of these, $OH^{\cdot}$ is the most reactive and damaging (Halliwell *et al.*, 1992).

Free radicals can be classed as oxidising or reducing, that is, they either take an electron from another molecule, or donate an electron (Halliwell, 1991). Free radicals, by virtue of their extreme reactivity, are well known for damaging effects within biological systems and *ex vivo*. Examples of free radical reactions are the development of rancidity in unsaturated fatty acid content of foodstuffs and the degradation of natural rubber (Dadvand *et al.*, 2000).

Most ROS and related radicals have an extremely short life in biological systems. The half-life for OH· at a concentration of 100mM is only 10^{-9} seconds, whilst for the peroxyl radical (ROO·) at 1mM the half-life is 7 seconds (Gutteridge and Halliwell, 1994). Radicals such as $O_2^{\cdot -}$ and derived agents such as H_2O_2 are removed quickly by the enzymatic actions of superoxide dismutase and catalase respectively. However, these radicals are also actively manufactured by host cells for use against pathogens (Guarnieri *et al.*, 1991). The existence of ROS and free radicals is generally detectable only by their effects on other molecules. *In vivo*, the effects of oxidative damage to tissues can be quantitively determined.

1.2 Free radical formation

Sources of free radicals can be both exogenous and endogenous. Exogenous factors include certain drugs, radiation, tobacco, pesticides, air pollutants, solvents, aromatic hydrocarbons and ozone. Generally, the exogenous sources of free radicals require metabolic processing in order to produce free radicals within biological systems. Drugs whose metabolism is dependent on quinoid groups or bound metals are capable of generating free radicals. Electromagnetic and particulate radiation can result in the formation of free radicals by the transfer of energy into cells (Freeman and Crapo, 1982). This energy is usually absorbed by the water within the cell and can result in the breaking of hydrogen-oxygen covalent bonds, producing hydroxyl radicals (Halliwell, 1991). Tobacco smoke contains aldehydes, epoxides, peroxides, nitric oxide and carbon-centred radicals. Ozone, whilst not a free radical, is a potent oxidising agent as it has two unpaired electrons (Gutteridge and Halliwell, 1994). Singlet oxygen is also not a true free radical due to the absence of an unpaired electron. Singlet oxygen is formed when the spin direction of the electron in the outer orbit is reversed, causing instability (Chapple, 1997).

This makes singlet oxygen more reactive than triplet oxygen (the low energy state of atmospheric oxygen), particularly with lipids (Gutteridge and Halliwell, 1994).

1.3 Reactive oxygen species

One of the principle sources of ROS is the formation of superoxide as a by-product of aerobic metabolism and as such, a primary source is within the mitochondria (Chapple, 1997b; Esposti, 2002). Superoxide production can be mediated by semi-ubiquinone, as seen in the electron transport chain within mitochondria, or by enzymes such as NADPH oxidase or xanthine oxidase (Droge, 2002). Within the mitochondrial inner membrane, superoxide production occurs in the locality of NADH dehydrogenase and ubiquinone-cytochrome *b* (Freeman and Crapo, 1982). Superoxide is also produced by phagocytes such as polymorphonucleocytes (PMN). The reduction of oxygen is catalysed by the enzyme NADPH oxidase, using hexose monophosphate as a substrate, and can be seen in the equation below.

$$2\,NADPH + 2O_2 \rightarrow NADPH\ oxidase \rightarrow 2\,NADP + 2H + 2O_2^{\cdot-}$$

The reactive oxygen species have a capacity to inter-convert and in biological systems, the commonplace formation of superoxide from oxygen may result in the generation of more potent ROS and their derivatives. These inter-conversions from oxygen are shown below.

$$O_2 + e^- \rightarrow O_2^{\cdot-} + 2H^+ + e^-\ (SOD) \rightarrow H_2O_2 + e^- \rightarrow OH\cdot + OH^-$$

1.3.1 Superoxide

The superoxide radical is formed by the univalent reduction of the triplet oxygen molecule. Triplet oxygen ($^3O_2$) is the form of oxygen present in the atmosphere. The uptake of an electron forms a pair with one of the two unpaired electrons of the oxygen molecule, creating one unpaired electron. Superoxide is produced *in vivo* in many ways (Halliwell, 1991). Leakage of electrons from carriers in the transport chain within the mitochondrial membrane allows them to combine with oxygen to produce superoxide (Freeman and Crapo, 1982). The interaction of sugars such as glucose with proteins can also form superoxide, as can the auto-oxidation of thiols, adrenaline and flavine nucleotides. Transition metals such as iron and copper speed this process up considerably (Young and Woodside, 2001). Superoxide is also produced by activated phagocytes during the respiratory burst and is used as a destructive agent against bacterial infection (Halliwell, 1991).

1.3.2 Hydroxyl radical

The hydroxyl radical is the most reactive and destructive of free radicals (Halliwell, 1996). It instantly causes damage to all biological molecules, typically inducing a chain reaction as the affected molecules try to balance their unpaired electrons. Hydroxyl radicals are the most destructive end product of most of the free radicals occurring *in vivo* (Young and Woodside, 2001). The hydroxyl radical is formed directly when cells are exposed to high-energy radiation such as gamma rays. The energy is absorbed by the water within the cell, causing the separation of the oxygen-hydrogen covalent bond. This creates a hydrogen atom and the hydroxyl radical (Gutteridge and Halliwell, 1994). However, the majority of hydroxyl radical production occurs when superoxide and hydrogen peroxide are broken down by catalytic action in the presence of transition metals such as iron and copper

(Young and Woodside, 2001). Iron II or copper I can react with hydrogen peroxide to form hydroxyl radicals in a reaction defined by Fenton in 1894:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH\cdot + OH^-$$

Hydroxyl radicals can also be formed by the interaction of superoxide with hydrogen peroxide. This reaction has an extremely low rate constant in aqueous solution however, and so production of hydroxyl radicals is negligible (Gutteridge and Halliwell, 1994). Nevertheless, in the presence of transition metal catalysts, this reaction can occur at a much faster rate. In the Haber-Weiss reaction, superoxide causes the reduction of iron. The iron then reduces hydrogen peroxide to form the hydroxyl radical (Freeman and Crapo, 1982):

$$Fe(III) + O_2\cdot^- \rightarrow Fe(II) + O_2$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH\cdot$$

$$O_2\cdot^- + H_2O_2 \rightarrow OH^- + OH\cdot + O_2$$

The hydroxyl radical is so potent a threat in terms of tissue damage because it reacts with virtually all biological molecules including amino acids, lipids, sugars and nucleotides (Young and Woodside, 2001).

1.3.3 Nitric oxide and hydrogen peroxide

Nitric oxide ($NO\cdot$) is classed as a free radical and has a slight reducing action. $NO\cdot$ is produced by macrophages and by the vascular endothelium *in vivo* from L-arginine and is a biologically active species that is involved in the normal regulation of blood pressure and cardiovascular function (Stuehr *et al.*, 1990; Chapple, 1997). It can combine with oxygen

to produce the nitrogen dioxide radical ($NO_2\cdot$), which has a strong oxidising action. Nitrogen dioxide is a free radical that can initiate lipid peroxidation and also damage proteins (Halliwell *et al.*, 1992). NO· also has the ability to produce hydroxyl radicals in a reaction involving superoxide via the peroxynitrite ion ($ONOO^-$) (Chapple, 1997):

$$NO\cdot + O_2\cdot^- \rightarrow ONOO^- + H \rightarrow OH\cdot + NO_2\cdot$$

The peroxynitrite ion can also induce lipid peroxidation in its own right (Darley-Usmar *et al.*, 1995).

Hydrogen peroxide is not itself a free radical, but is usually referred to as a reactive oxygen species (ROS). Hydrogen peroxide is produced wherever superoxide is present as it is a product of the dismutation reaction of superoxide, which may be spontaneous or enzyme catalysed (Castro and Freeman, 2001). One superoxide radical releases its electron and becomes oxygen whilst a second takes up the electron and is reduced to form hydrogen peroxide:

$$2O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide can also be product of the combination of two hydroxyl radicals. Their two unpaired electrons join to form an oxygen-oxygen covalent bond (Gutteridge and Halliwell, 1994; Young and Woodside, 2001):

$$OH\cdot + OH\cdot \rightarrow H_2O_2$$

One important property of hydrogen peroxide is its capacity to penetrate cell membranes – something superoxide cannot do – and therefore travel some distance from its manufacture (Chapple, 1997b). This becomes a major problem when it is considered that hydrogen peroxide is a precursor of the hydroxyl radical (Young and Woodside, 2001). With the simple addition of an electron, hydrogen peroxide produces the hydroxyl radical (Gutteridge and Halliwell, 1994):

$$H_2O_2 + e^- \rightarrow OH\cdot + OH^-$$

1.3.4 Transition metals and reactive oxygen species

As mentioned previously with regard to the Haber-Weiss reaction, transition metals play an important role in the formation of free radicals. Metals such as iron and copper act as catalysts in oxidation reactions by donating or receiving electrons, effectively altering their valency in the process. For example, Fe(III) can be reduced to Fe(II) with the addition of one electron. Similarly, an electron added to Cu(II) causes a reduction to Cu (I). Both of these actions are reversible, with Fe(II) reducing oxygen to form Fe(III) and superoxide ($O_2\cdot^-$) (Halliwell, 1991). Obviously, transition metals pose a great threat to a living organism if left unbound to carrier metalloproteins such as ferritin, transferrin or ceruloplasmin (Halliwell, 1996; Chapple, 1997b). The most dangerous property of these transition metals is their ability to produce the hydroxyl radical (OH·), which occurs when both iron and copper combine with hydrogen peroxide (H_2O_2).

1.4 Free radical mediated damage to biological systems

1.4.1 Lipid peroxidation

The phospholipid bilayer of human cell membranes has a 'fluid' consistency which allows the various enzymes and transport proteins to function normally. This fluid property originates from the polyunsaturated fatty acid (PUFA) side chains in the membrane and is an important factor that modulates the cell-signalling functions of the cell (Castro and Freeman, 2001). These PUFA side chains are susceptible to oxidative damage. The free radicals attack the side chains by extracting the hydrogen atoms from carbon in the fatty acid backbone. This leaves the carbon atom with an unpaired electron as the carbon and hydrogen share electrons in a covalent bond (Niki, 1997). This carbon radical now seeks to pair its electron and usually does so by combining with oxygen present in a dissolved state in the membrane. The product of this reaction is a highly reactive peroxyl radical that can cause oxidative damage to nearby PUFA side chains and proteins (Gutteridge and Halliwell, 1994).

The peroxyl radical has the capacity to take further hydrogen atoms from PUFA side chains, thereby forming hydroperoxide and alkyl radicals. The alkyl radicals then bind with oxygen to form further peroxyl radicals, continuing the oxidative chain reaction that can also occur in plasma lipoproteins (Castro and Freeman, 2001). Lipid peroxidation may be halted by chiefly by the chain breaking action of α-tocopherol (Castro and Freeman, 2001). The damaging peroxyl radical is converted to a lipid peroxide by the donation of an electron by α-tocopherol, which in turn is converted to a tocopherol radical (Gutteridge and Halliwell, 1994). The tocopherol radical can be converted back to α-tocopherol by ascorbate (Halliwell, 1996). Lipid peroxidation can be determined by the measurement of the hydrocarbons ethane and pentane in the exhaled breath (Peredi *et al.*, 2002). These

simple alkanes are end products of lipid peroxidation, as is malondialdehyde which is determined in the TBARS (Thiobarbituric acid reactive substances) test, a commonly used assay of free radical mediated damage (Vannucchi *et al.*, 2003).

1.4.2 Protein oxidation

In addition to proteins in the cell membrane being damaged as a consequence of lipid peroxidation, proteins can also be attacked directly by free radicals (Gutteridge and Halliwell, 1994). The hydroxyl radical, produced via ionising radiation, will cause damage to all amino acid residues, with the sulfhydryl group on cysteine residues a particular target. Other common targets include methionine, phenylalanine, hystidine, proline, arginine, tyrosine and tryptophan (Castro and Freeman, 2001). The protein radicals that are produced as a consequence of free radical attack can cause scission in the polypeptide chains, crosslinking and modification of amino acids. This results in a compromised functional ability and enhanced liability to proteolysis and denaturation (Niki, 1997).

A useful biomarker of protein oxidation is the formation of protein carbonyls. These are produced when the side chains of lysine, proline, arginine and theonine residues are oxidised. These carbonyls react with 2,4-dinitrophenylhydrazine and can be measured colourmetrically (Berlett *et al.*, 1998).

1.4.3 DNA oxidation

Oxidative damage to DNA occurs in all aerobic cells, resulting in DNA fragmentation; (Dizdaroglu *et al.*, 2002). DNA is especially susceptible to damage by the hydroxyl radical as DNA and proteins nearby often bind iron and copper ions, which can catalyse the formation of hydroxyl radicals when hydrogen peroxide is present (Gutteridge and

Halliwell, 1994). Free radicals can attack the DNA backbone, inflicting single and double strand breakages. Purine and pyrimidine bases can also be damaged by oxidation leading to potentially dangerous adducts, as can the deoxyribose sugar (Wallace, 1998). The resultant DNA fragmentation activates the enzyme poly (ADP-ribose) synthetase, which uses nicotinamide adenine dinucleotide (NAD) within the cell to help repair the damage. As NAD is involved in the transport of electrons and, therefore cell metabolism, it is possible that the cell may die if the supply of NAD is exhausted due to repair of the oxidative damage (Gutteridge and Halliwell, 1994). Oxidative damage to DNA is associated with mutagenesis, carcinogenesis and aging (Dizdaroglu *et al.*, 2002). Measurement of DNA oxidation often centres on the determination of oxidation by-products of guanine bases. Oxidation of C-8 guanine results in mutation and usually a G-to-T transversion (Mayne, 2003).

1.5 Antioxidants

Antioxidants are present in all body fluids and tissues and protect against environmental and endogenously formed free radicals (Halliwell, 1991). Antioxidants can be defined as "those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay or inhibit oxidation of that substrate" (Gutteridge, 1994). Antioxidants in the human body can be categorized into three main groups. These are antioxidant enzymes, chain-breaking antioxidants and metal binding proteins (Cao and Prior, 1998).

1.5.1 Antioxidant enzymes

1.5.1.1 Superoxide dismutase

Most antioxidant enzymes are located within cells. Superoxide dismutase (SOD) catalyses the dismutation of superoxide to hydrogen peroxide and oxygen. SOD exists in two isoforms. Manganese SOD (MnSOD) is found in mitochondria whilst copper-zinc SOD (CuZnSOD) is present in cell cytoplasm and organelles (Halliwell, 1999a; Young and Woodside, 2001). There is also evidence of an extracellular SOD activity (EC-SOD), which contains both copper and zinc but is distinct from cellular CuZnSOD (Karlsson and Marklund, 1988). It is believed that this secretory EC-SOD is manufactured by fibroblasts and endothelial cells and expressed on the surface of the cells. It is postulated that EC-SOD helps to protect nitric oxide - a vascular relaxing factor – from reactions with superoxide, which would form peroxynitrite (Young and Woodside, 2001). SOD is the most abundant antioxidant enzyme in humans (Fridovich, 1986).

1.5.1.2 Catalase

Catalase is another antioxidant enzyme largely present in the intracellular compartment. This is usually contained within peroxisomes (Halliwell, 1996). The main function of catalase is to rapidly remove the hydrogen peroxide produced by SOD. Catalase catalyses the conversion of hydrogen peroxide to water and oxygen and has a large capacity to breakdown hydrogen peroxide at a very fast rate (Young and Woodside, 2001).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

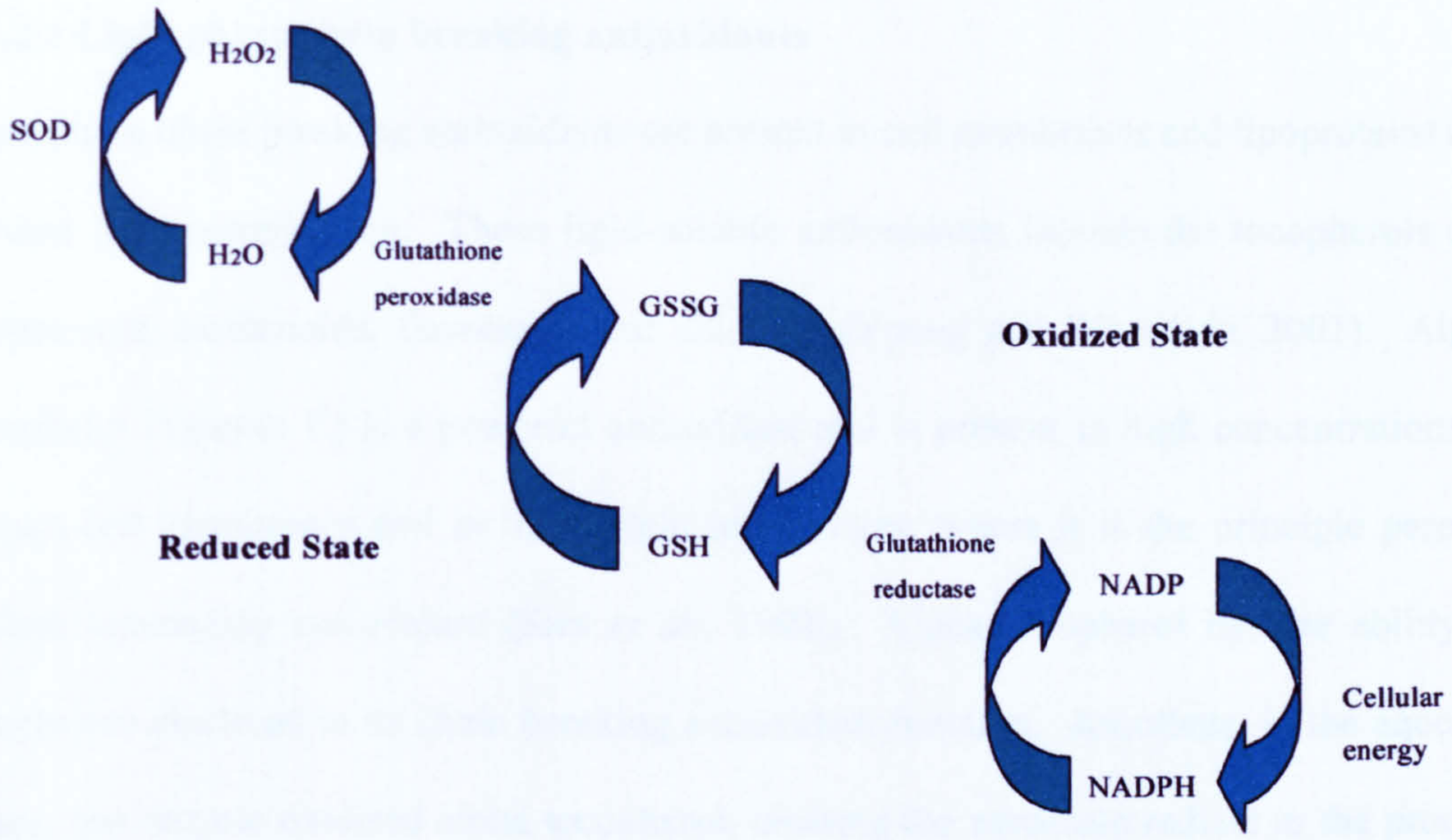
However, catalase has a low affinity for hydrogen peroxide and so the concentration of hydrogen peroxide needs to be high in order for catalase to act effectively (Gutteridge and

Halliwell, 1994). Catalase is found in high concentrations in the liver and erythrocytes and in reduced amounts in all other tissues (Young and Woodside, 2001).

1.5.1.3 Glutathione peroxidase

Glutathione peroxidase (GPx) is a selenoprotein found in almost all cells, and is particularly localized within the mitochondria (Rahman and MacNee, 1999). As a major scavenger of hydrogen peroxide, glutathione peroxidase reduces the high concentration found here after the dismutation of superoxide formed during respiration (Burke *et al.*, 2003). Glutathione peroxidase acts as a catalyst in the oxidation of glutathione (GSH), a process that involves the use of a hydroperoxide such as hydrogen peroxide or lipid hydroperoxide. These interactions can be seen below in Figure 1.1. However, this reaction only possible with a ready supply of reduced glutathione, which is usually maintained at a high concentration due to the action of glutathione reductase (Young and Woodside, 2001). As glutathione peroxidase contains selenium at its active centre, this element is intrinsic to its functional efficacy (Hu and Diamond, 2003).

Figure 1.1. Glutathione redox cycle (Urso and Clarkson, 2003).



1.5.2 Chain breaking antioxidants

Chain breaking antioxidants differ from the antioxidant enzymes in that they are consumed by the action of scavenging the free radical (Halliwell, 1991). When free radicals react with molecules, the production of secondary radicals often occurs. This is because in the process of receiving or donating an unpaired electron from or to another molecule, that molecule becomes reactive as it now has an unpaired electron (Young and Woodside, 2001). This reaction often continues until either two radicals bond to form a stable molecule or the chain reaction is quenched by a chain breaking antioxidant (De Zwart *et al.*, 1999). Chain breaking antioxidants have the capacity to donate an electron to or accept an electron from a free radical. This reaction results in the formation of a stable molecule that does not initiate further free radical reactions (Halliwell, 1995). Chain breaking antioxidants can be classed as active in either the aqueous phase or the lipid phase. The

major chain breaking antioxidants are derived from the diet and as such, the supply of these free radical scavengers may be limited.

1.5.2.1 Lipid phase chain breaking antioxidants

Lipid phase chain breaking antioxidants are present in cell membranes and lipoproteins and prevent lipid peroxidation. These lipid-soluble antioxidants include the tocopherols and tocatrienols, carotenoids, flavonoids and ubiqunol (Young and Woodside, 2001). Alpha tocopherol (vitamin E) is a powerful antioxidant and is present in high concentrations in human cell membranes and in lipoprotein transporters, where it is the principle peroxyl radical scavenging antioxidant (Sies *et al.*, 1992). Alpha tocopherol has the ability to donate two electrons in its chain breaking antioxidant function. Ascorbate, in the aqueous phase, can recycle oxidised alpha tocopherol, creating the ascorbate radical in the process (Niki, 1987). Although ascorbate is an aqueous phase antioxidant, the alpha tocopherol present in a cell is at the boundary of the membrane and therefore accessible to the water-soluble ascorbate (Buettner, 1993).

Beta carotene is the most abundant carotenoid and is an effective scavenger of singlet oxygen (Fukuzawa *et al.*, 1998). It has the ability to trap more lipid free radicals than alpha tocopherol, but is only effective at low oxygen tensions (Buettner, 1993; Young and Lowe, 2001). A novel feature of beta-carotene its ability to release the energy, created during the trapping of singlet oxygen, as heat rather than having to undergo resynthesis (Sies *et al.*, 1992).

Flavonoids are polyphenolic antioxidants that are found in fruits, vegetables, tea and wine and are the largest group of phenolic compounds (Rice-Evans *et al.*, 1996). Flavonoids

may help to prevent against coronary heart disease (CHD), with research indicating a relationship between higher intakes and reduced incidence of CHD, cardiovasculer disease (CVD) and cancer (Hertog *et al.*, 1993; Kris-Etherton *et al*, 2002).

Ubiquinol-10 is the reduced form of coenzyme Q10, also known as ubiquinone, and is present on the mitochondrial inner membrane where it functions as a carrier of electrons and protons during respiration and production of adenosine triphosphate (ATP) (Alleva *et al.*, 2001; Hughes *et al.*, 2002). Ubiquinol-10 can also scavenge peroxyl radicals at a higher rate than the tocopherols or carotenoids, and plays an important antioxidant role in the prevention of lipid peroxidation within the mitochondria and in other areas of lipid oxidation, such as those leading to vascular diseases (Forsmark *et al.*, 1991; Alleva *et al.*, 2001; Choy *et al.*, 2003). In studies involving the interaction of ubiquinol-10 with α-tocopherol in submitochondrial particles (SMP), ubiquinol-10 was believed to be responsible for the regeneration of α-tocopherol (Lass & Sohal, 1998; 2000).

1.5.2.2 Aqueous phase chain breaking antioxidants

The aqueous phase chain breaking antioxidants scavenge free radicals in plasma, saliva and other biological fluids, as well as within the cytosol. Ascorbate is a major plasma antioxidant that can scavenge superoxide, hydrogen peroxide, hydroxyl radicals, hypochlorous acid, peroxyl radicals and singlet oxygen (Jialal *et al.*, 1990; Frei, 1991; Young and Woodside, 2001). Ascorbate donates two electrons in two stages. The loss of the first electron results in the formation of the semidehydroascorbate radical. The donation of a second electron converts this to dehydroascorbate (Halliwell, 2001). This is hydrolysed to diketogulonic acid that is broken down to oxalic acid. Dehydroascorbate can also be converted back to ascorbate by the enzymatic action of thioredoxin reductase or by

a non-enzymatic process using reduced glutathione (May *et al.*, 1996,1998; Halliwell, 1999).

Uric acid is a product of purine metabolism, being specifically generated from adenine and guanine bases. In plasma, 99% of uric acid is made up of its monovalent anion, urate (Becker, 1993). In man, urate is the final product of purine metabolism. Humans do not express the gene for uricase, which breaks urate down to allantoin (Yeldandi *et al.*, 1992). It has been proposed that this factor is one reason as to why humans and higher primates have a longer lifespan compared to other species. Indeed, a positive correlation has been observed between plasma urate concentration and lifespan (Cutler, 1984).

Urate scavenges ROS, particularly oxidising substances such as ozone. When urate undergoes a one-electron oxidation, a urate radical is produced. Ascorbate can regenerate urate with the donation of an electron, producing the relatively unreactive ascorbate radical. A two-electron oxidation results in the formation of allantoin, a process that can be initiated by hypochlorous acid (Becker, 1993). Urate is responsible for 30-65% of peroxyl radical scavenging capacity in plasma and is the principal antioxidant present in saliva (Wayner, 1987; Moore *et al.*, 1994; Chapple *et al.*, 1997).

Albumin can act as a chain breaking antioxidant with the ability to scavenge peroxyl radicals and hypochlorous acid (Stocker *et al.*, 1991; Hu *et al.*, 1993). Albumin can also bind copper ions and so prevent the formation of the hydroxyl radical (Neuzil and Stocker, 1994). A reduction in serum albumin concentration, and therefore a decrease in antioxidant capacity, has been associated with an increase in risk of mortality (Bourdon *et al.*, 1999).

Reduced glutathione is an aqueous phase antioxidant present in the intracellular space. It acts as a chain breaking antioxidant, in addition to being essential in the function of glutathione peroxidase (Beutler, 1979; Halliwell, 1991; Sastre *et al.*, 1996).

1.5.2.3 Transition metal binding proteins

As previously mentioned, metals such as iron and copper can acts as catalysts for the formation of the potent hydroxyl radical. The binding of these metals is vital to prevent these reactions. These transition metals are effectively sequestered by proteins such as ferritin, transferrin, lactoferrin and ceruloplasmin (Halliwell and Gutteridge, 1989; Chapple *et al.*, 1997; Halliwell, 1999a). Therefore, these proteins effectively serve an antioxidant role by preventing the metal catalysts performing their catalytic role in free radical production. The importance of these proteins as antioxidants has been illustrated by studies of knock-out mice. For example, mice lacking caeruloplasmin accumulate iron and copper and suffer free radical-mediated tissue injury, whilst in humans, caeruloplasmin deficiency leads to diabetes, neurodegeneration and retinal damage (Shim and Harris, 2003).

1.5.3. Interaction of antioxidants

In addition to the individual actions of the above antioxidants, many also interact with each other *in vivo* (Young and Woodside, 2001). An example of this reciprocal arrangement is the re-formation of α-tocopherol by ascorbate. After performing its role as a chain breaking antioxidant to halt lipid peroxidation, α-tocopherol is converted to a tocopherol radical. Ascorbate is believed to reduce the tocopherol radical close to the lipid membrane (May *et al.*, 1998). This produces the ascorbate radical, a relatively inactive molecule that can be converted back to ascorbate and dehydroascorbate by the simple act of two

ascorbate radicals combining. Reduced glutathione can then regenerate dehydroascorbate to ascorbate (Gutteridge and Halliwell, 1994). There is also evidence that urate also has the capacity to protect ascorbate against iron-induced oxidation whilst remaining largely undamaged itself (Sevanian *et al.*, 1985).

1.6. Oral Diseases

The oral cavity is constantly subject to the influx of potentially pathogenic agents, whether they be nutritionally based, environmental pollutants, tobacco or alcohol. These all have an effect on the health of the epithelial surface of the mouth (Slaughter *et al.*, 1953). The major diseases of the mouth are cancer, periodontitis and dental caries.

1.6.1 Oral cancers

Over 300,000 new cases of oral cancer arise each year (Sudbø *et al.*, 2003). Squamous cell carcinoma is the 6th most common form of cancer in the world, with 30,000 new cases per year in the USA (Nagler, 2002). The two primary risk factors for oral cancer are tobacco use and alcohol consumption (Adhvaryu *et al.*, 1991; Popp *et al.*, 1994), with these two products acconting for 75% of oral cancers in the USA (Blot *et al.*, 1988). Genetic factors involved in the aetiology of oral cancers include the activity and action of oncogenes and tumor suppressor genes. Proto-oncogenes, present in every cell, are implicated in the growth of malignant tumors. Tumor suppressor genes have the capability to inhibit the growth of tumors (Burkhardt, 1996).

1.6.2 Dental caries

Dental caries is a widespread disease condition that results in the gradual demineralisation of the tooth. Organic acids, principally lactic acid, are produced by bacterial glycolysis on the surface of the tooth and are the cause of the tooth erosion (Featherstone, 1996). *Streptococcus mutans* is the dominant bacterial species responsible for dental caries, although a further four species of *Mutans streptococci* are also implicated in this disease (Bowden, 1990). *Mutans streptococci* are highly successful colonisers of the mouth for three reasons. Firstly, they have the capability to produce 3 moles of adenosine triphosphate (ATP) per mole of glucose in contrast to only 2 moles of ATP by other bacterial species (Simmonds *et al.*, 2000). They can also maintain normal metabolism in an acidic environment, typically as low as pH 5.0. Other bacteria, with the exception of *Lactobacilli*, cannot sustain their glycolytic function under these conditions. Lastly, *Mutans streptococci* have the ability to store carbohydrate for use later, when the supply may be low (Simmonds *et al.*, 2000). The principle risk factors for dental caries include a high sucrose intake, particularly when frequent intake occurs, and poor fluoride status (Beighton *et al.*, 1996).

1.6.3 Periodontal disease

Periodontal disease is one of the most commonly reported chronic adult conditions (Brown and Loe, 1993). The disease state ranges from gingivitis through to periodontitis and advanced periodontitis. Gingivitis is characterised by inflammation of the gum caused by plaque deposits, with possible bleeding when brushed or probed. Gingivitis has a high prevalence rate, affecting up to 50% of the population (Ridgeway, 2000). Periodontitis can be identified by the hardening of the plaque deposits found in gingivitis, leading to calculus formation and gum recession. This results in the formation of pockets of between

3.5-5.5mm depth between the tooth surface and the gum. Periodontitis will occur in 80% of the population at some point during their lifetime. Risk factors for periodontitis include increasing age, gender, socio-economic status, race and place of residence (Ridgeway, 2000). Advanced periodontitis is distinguished by excessive loss of gingival tissue and alveolar bone and pockets greater than 5.5mm in depth. This condition often leads to tooth exfoliation due to the destruction of the tooth connective ligaments (Chapple *et al.*, 1997).

1.6.3.1 Microbiology of periodontal disease

The primary cause of periodontal disease is believed to be an imbalance in the bacterial species that colonise the oral cavity, and the host immunological response to these bacterial pathogens (Lamont and Jenkinson, 1998). There are more than 300 distinct species of bacteria present in the gingival area of the mouth, most of which exist in a commensal relationship with the host. However, three potentially harmful species have been identified as being ubiquitous in periodontal plaque formations, and are distinct from the bacterial species which mediate the formation of caries, where *Streptococcus mutans* is the major pathogen. These bacteria, known as periodontal pathogens, are *Actinobacillus actinomycetemcomitans, Bacteroides forsythus and Porphyromonas gingivalis* (Jenkinson and Dymock, 1999). Of these, *P. gingivalis* shows extensive proliferation in most diseased individuals (Haffajee and Socransky, 1994; Chapple *et al.*, 1997; Lamont and Jenkinson, 1998).

1.6.3.2 Porphyromonas gingivalis and periodontal disease

P. gingivalis is a gram-negative, non-motile, coccobacillus anaerobe that is transmitted via saliva or other vectors from infected individuals (Greenstein and Lamster; 1997). Interfamilial transmission is common, although bacteria from affected family members do

not always possess the same genotype (Lamont and Jenkinson, 2000). Antecedent bacteria such as oral *Streptococci* are usually required to provide the ideal conditions for *P. gingivalis* colonisation. These include the availability of growth substrates, reduced oxygen tension and the provision of an attachment site. Reduced oxygen tension is also found in the sub-gingival area favoured as the site of attachment and proliferation. For these reasons, *P. gingivalis* is often involved in secondary colonisation. Major colonisation sites include the teeth, gingiva, cheek and tongue and wherever antecedent bacteria may be present (Theilade, 1990).

1.6.3.3 Mechanisms for bacterial binding

The mechanisms used for *P. gingivalis* binding include the utilisation of fimbriae and membrane proteins (Amano, 2003; Lamont and Jenkinson, 1998). Fimbriae have a rod-like structure and are constructed from a hydrophobic protein known as pilin. These have specific adhesion tips that enable the bacteria to bond with host cells or other bacteria (Sojar *et al.*, 2002; Yilmaz *et al.*, 2002). *P. gingivalis* fimbriae have been demonstrated to stimulate the synthesis of the pro-inflammatory cytokine interleukin 1β (IL-1β) in human gingival fibroblasts and hence initiate host responses (Ogawa *et al.*, 1991). *P. gingivalis* also possesses at least eight haemagglutinins that mediate its binding to host cell receptors, most of which are oligosaccharides (Lamont and Jenkinson, 2000). The binding to erythrocytes may also provide haem which is required for growth, though *P. gingivalis* also produces proteolytic enzymes that degrade substrates such as collagen, fibronectin, fibrinogen, laminin and keratin, providing molecules necessary for metabolism (Travis *et al.*, 1997; Lamont and Jenkinson, 2000).

1.6.3.4 Bacterial lipopolysaccharide

Bacterial polysaccharides act to counter phagocytosis by host immune cells whilst liposaccharides induce the production of the reactive oxygen species superoxide ($O_2^{\cdot-}$) by PMN, resulting in increased inflammation (Jenkinson and Dymock, 1999). Bacterial polysaccharide antigens also induce the release of IL-1β from human peripheral blood mononuclear cells (HPBMC's) (Naito *et al.*, 1992). Fimbriae and proteins assist in the binding to epithelial cells, with the bacterium being taken into the cell cytoplasm but not bound in a vesicle. This is a result of proteins secreted by *P. gingivalis* which interfere with normal intracellular function (Park and Lamont, 1998).

1.6.3.5 Bacterial insult to host tissues

Once in the cytoplasm, *P. gingivalis* replicates in relative safety away from the host immune cells due to its location within the cell. Ingression deeper into connective tissue structures occurs via degradation of the network of cell junctions, leading to a proliferation into the host tissues (Katz *et al.*, 2000). *P. gingivalis* possesses an array of agents that can damage host tissue, modulate host cell functions and evade detection. Large amounts of the cysteine proteinases Arg-gingipain (Rgp) and Lys-gingipain (Kgp) are produced to facilitate the extraction of peptides and amino acids from the surrounding tissues, utilizing these substrates in the construction of fimbriae, haemagglutinins and haemoglobin receptor sites (Kadowaki *et al.*, 2000). These proteinases therefore provide a direct insult to the surrounding host tissue integrity (Lamont and Jenkinson, 2000).

In addition, phospholipase A production by *P. gingivalis* induces precursors of prostaglandins such as cyclo-oxygenase II that may cause prostaglandin-mediated bone resorbtion, whilst alkaline and acid phosphatases contribute to the degradation of alveolar

bone (Frank and Voegel, 1978). Bacterial lipoprotein lipase activates osteoclasts and induces the release of prostaglandin E2 (PGE_2), IL-1β and tumor necrosis factor α (TNF-α), all of which mediate bone resorbtion and reduce host osteoblast activity (Havemose-Poulsen and Holmstrop, 1997). *P. gingivalis* also alters the cellular control of matrix metalloproteinases, thereby disrupting the status of the extracellular matrix and preventing cell repair (Fravalo *et al.*, 1996). A reduction of IL-8 secretion by epithelial cells, mediated by the bacterium, inhibits the recruitment of PMN to the infected area. Their absence results in a build-up of bacteria and an elevation in disease activity (Lamont and Jenkinson, 2000).

P. gingivalis also inhibits PMN chemotaxis by secreting low-molecular weight molecules such as succinic acid, which is thought to reduce the intracellular pH of the PMN (Rotstein *et al.*, 1985) and depolarize the PMN membrane causing immobilization (Novak and Cohen, 1991). Thus, any PMN recruited early in the infection process become immobilized but active at the infection site and will contribute to host tissue damage.

1.7 Cytokines implicated in inflammatory conditions

Cytokines are molecular messengers, usually peptides, proteins or glycoproteins, which have the ability to induce alterations to the function of other cells (Okada and Murakami, 1998). However, cells can only respond to particular cytokines if they express the appropriate receptor. Unlike the endocrine hormones, cytokines are not stored in glands but are synthesised when the cytokine-producing cells are exposed to the relevant stimuli (Henderson *et al.*, 1996; Hasko and Szabo, 1998). Cytokines can be divided into two main groups. These are pro-inflammatory and anti-inflammatory, with the latter often involved in stimulating an allergic response.

1.7.1 Cytokine characterisation

T lymphocytes, in particular the CD4-expressing T lymphocytes, are a principle source of cytokines. The cytokines produced are further categorised as either Th1 or Th2 cytokines (Berger, 2000). Th1 cytokines are responsible for inducing inflammation and neutrophil recruitment following a bacterial insult. These include interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor (TNF) and colony stimulating factor (CSF). IL-8 also induces chemotactic changes in phagocytes. Th2 cytokines include interleukins 4, 5, 10 and 13. Interleukin 10 serves as an anti-inflammatory agent that can help to balance the effects of the Th1 cytokines (Berger, 2000). When cytokines bind with their specific receptors, a system of intracellular messages produces modulation of specific genes. The result can be the synthesis of proteins such as other cytokines or proteases and other inflammatory mediators such as nitric oxide or lipid-derived prostanoids and leukotrienes (Henderson *et al.*, 1997).

1.7.2 Modulators of cytokine production

Cytokine production and behaviour determine the outcome of any insult to a living organism, with both endogenous and exogenous factors capable of cytokine modulation (Grimble, 1996). The importance of cytokine production and function is highlighted in those suffering from malnutrition, where resistance to disease is reduced as a result of insufficient cytokine action (Keenan *et al.*, 1982; Kauffmann *et al.*, 1988).

1.7.2.1 Endogenous cytokine modulators

Organs such as the liver and brain, together with the adrenal cortex and immune system as a whole exert influence over cytokines during inflammation. Principle pro-inflammatory cytokines such as IL-1 and TNF-α have antagonists in the form of interleukin-1 receptor

antagonist and domains of TNF receptors respectively. These are present in plasma and compete with IL-1 and TNF-α for receptor sites on target cells (Grimble, 1996). The production of these antagonists is promoted by IL-4 and IL-10, which also act to inhibit pro-inflammatory cytokines (Burger and Dayer, 1995).

A further endogenous source of cytokine modulation is mediated by the hypothalamo-pituitary-adrenal axis, whereby glutocorticoids secreted into the circulation stimulate the cytokine-suppressing protein lipocortin (Del Rey *et al.*, 1987). Glucocorticoids also stimulate the production of acute phase proteins, with help from IL-1 and IL-6, from amino acids sourced from peripheral tissue (Perlmutter *et al.*, 1986). These proteins reduce the extent of neutrophil recruitment and production of superoxide and TNF-α (Costello *et al.*, 1984).

1.7.2.2 Nutritional modulation of cytokine action

Cytokine functions during inflammation are often adverse, unlike during infection where they are of benefit. The capability of dietary intervention to modulate cytokine production and function is, therefore, of great interest (Grimble, 1992). Fats can influence cytokine function and may be able to exert some control over cytokine production (Grimble, 1996). Those fats rich in n-3 polyunsaturated fatty acid (PUFA), n-9 monounsaturated fatty acid (MUFA) and low in n-6 PUFA have been found to induce a decreased sensitivity to cytokine action.

Fats high in n-6 PUFA increase cytokine responsiveness (Grimble, 1994; Besler and Grimble, 1995), with the exception of evening primrose oil (EPO) (Grimble, 1998). The influence of n-6 PUFA on pro-inflammatory cytokine production has been observed in

humans in a trial involving the reduction of saturated fat and an increase in n-6 PUFA over a 24 week period. IL-1 and TNF-α production were both found to be elevated after stimulation by endotoxin (Meydani *et al.*, 1993). In contrast, n-3 PUFA's and fish oil have been found to reduce the cytokine-mediated response to inflammatory conditions such as rheumatoid arthritis, psoriasis, asthma, multiple sclerosis, Crohn's disease and ulcerative colitis (Grimble, 1994).

Under inflammatory conditions, cytokines can also mediate the metabolism of proteins and amino acids. Most of the additional demand for protein by the immune cells and acute phase proteins is met by the catabolism of peripheral tissues (Grimble, 1996). In addition, an elevated demand for sulphur and associated amino acids occurs that may be caused by the increased use of glycine, serine, methionine and cysteine to be used in the construction of glutathione, metallothionein and acute phase proteins; all of which are seen in increased amounts following infection or trauma (Farante *et al.*, 1988; Grimble, 1994). When a diet low in sulphur amino acids is encountered, production of glutathione may be compromised due to the available cysteine being used for protein production in preference to glutathione (Grimble, 1994; Hunter and Grimble, 1994). This could result in a decrease in glutathione concentration and therefore a reduction in antioxidant capacity (Grimble, 1996). Studies using rat models have demonstrated that when a low-protein diet was administered and an inflammatory response initiated, higher lung glutathione concentrations were observed in those where methionine and cysteine were included in the diet (Hunter and Grimble, 1994).

A diet deficient in certain micronutrients could have a direct effect on the production of some antioxidants. Metallothionein, caeruloplasmin, superoxide dismutase and glutathione

peroxidase contain either copper, zinc or selenium. An inadequate intake of these micronutrients may prevent the formation of these antioxidants, thereby reducing antioxidant capacity (Grimble, 1996). Studies of rat models show that a dietary deficiency in copper reduces the ability to raise plasma caeruloplasmin and copper-zinc superoxide dismutase in the lung after administration of both endotoxin and oxidative stress (Grimble,1996). Therefore, micronutrients can influence the production of certain antioxidants. In so doing, a reduction in pro-inflammatory cytokines is observed which decreases the activation of transcription factors such as NFκB and NFIL-6, leading to suppression of the synthesis of acute phase proteins and cytokine production (Grimble, 1998).

1.8 Host response to *P. gingivalis* infection.

When stimulated by bacterial pathogens, most notably by bacterial lipopolysaccharides, the host undergoes a series of local and systemic effects that are collectively known as the acute-phase response (Koj, 1996). Host cells release pro-inflammatory cytokines as part of the immune response. These include IL-1α and β, IL-6, IL-8 and TNF-α. IL-1 and TNF-α are the first cytokines to be produced by injured host cells, such as endothelial cells, and by PMN and can induce the synthesis of IL-6 and IL-8 (Ishihara *et al.*, 1997; Koj, 1996; Imatani *et al.*, 2001). Once in the circulation these cytokines mediate the synthesis of intercellular adhesion molecules (ICAM's) and vascular cell adhesion molecules (VCAM's) and the expression of P and E-selectin on vascular endothelial cells (VEC's). These adhesion proteins on the surface of the vascular endothelium, together with L-selectin on the neutrophil cell surface, recruit PMN to the site of infection by binding via calcium-dependant mechanisms to oligosaccharides such as sialyl Lewis x on the cell

surface (Taubman and Kawai, 2001; Tonetti, 1997; Lappin *et al.*, 2003; Gainet *et al.*, 1999).

Leukocyte adhesion is facilitated by sialmucins and β_2-integrins present on neutrophils (Henderson *et al.*, 1998; Lamont and Jenkinson, 1998). Carbohydrates present on the neutrophil surface bind to P and E-selectin on the endothelial cell surface and together act to reduce the speed of the neutrophil in circulation and prepare for the next stage of recruitment (Darveau *et al.*, 1995). As the neutrophil moves slowly across the endothelial surface, IL-8 and platelet activating factor (PAF) mediate the binding of the PMN to the endothelial surface via phosphorylation of β_2-integrins, thus allowing binding to ICAM's. Once attached, diapedesis occurs if the gradient of the chemical attractant, such as IL-8, caused by the invading bacteria is high enough, resulting in tissue entry by the PMN (Konstantopoulos and McIntire, 1996).

1.8.1 The role of PMN in inflammatory conditions

PMN play a major role in the aetiology of periodontal disease, as they are the predominant host immune response to oral bacterial infection. Plasma levels of IL-8 have been found to be significantly higher in periodontitis patients when compared with healthy controls, indicating major PMN recruitment into the site of inflammation (Gainet *et al.*, 1998). In addition, patients suffering from an aggressive form of periodontitis possessed PMN with an increased output of H_2O_2 (Gainet *et al.*, 1999). Once at the site of infection and inflammation, PMN can phagocytose the pathogen and destroy it with the use of proteolytic enzymes, such as elastase, stored in granules and by the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hypochlorous acid.

PMN are not the only cells to produce ROS. Mitochondria commonly 'leak' electrons from carriers in the respiratory chain and combine with oxygen, with around two percent of oxygen consumption reduced in this way (Halliwell, 1991). PMN achieve the production of ROS using a cyanide-insensitive, non-mitochondrial increase in oxygen consumption that is around 10-20 times that of resting consumption, and is catalysed by NADPH oxidase (Battino *et al.*, 1999). Superoxide can then be used to generate further ROS. For example, the dismutation of superoxide, catalysed by superoxide dismutase, produces hydrogen peroxide (H_2O_2). Hydrogen peroxide can combine with chlorine ions to form hypochlorous acid (HOCL) in a reaction catalysed by neutrophil myeloperoxidase (Davies, 1995).

Possibly the most reactive of ROS, the hydroxyl radical (OH·) can also be produced from hydrogen peroxide and hypochlorous acid in the presence of a transition metal such as iron or copper (Battino *et al.*, 1999). Once produced, these enzymes and ROS degrade and destroy the bacteria held in the phagosomal space. However, these potent compounds can also be discharged into the extracellular space surrounding the PMN (Guarnieri *et al.*, 1991; Swain *et al.*, 2002). As the ROS released is not target-specific, damage to host tissues can also occur.

1.8.2 PMN response and 'priming'

PMN can induce an auto-amplification effect as they can produce IL-8, therefore attracting more PMN into the infection site. PMN in periodontal patients are present in increased numbers, and have increased adhesion and oxidative activity (Asman, 1987). The response of PMN to bacterial insult is usually finely balanced to prevent over-exposure of damaging enzymes and ROS into the area of infection. To achieve this, the neutrophils undergo

'priming' which is a two-stage process in which the PMN are initially exposed to the 'priming agent' such as TNF-α. At this point, the PMN do not become active but on exposure to a second stimulus they become fully activated (Lamster and Novak, 1992). Therefore, priming can be defined as an amplification of the functional response by previous exposure to a stimulus (Swain *et al.*, 2002).

The level of PMN priming is associated with the severity of the infection and inflammation. Neutrophils displaying a primed phenotype have been found in the blood of subjects where an activated defence system would be appropriate, such as trauma victims, but also in those suffering from chronic inflammatory diseases such as rheumatoid arthritis and periodontal disease (Gainet *et al.*, 1999; Eggleton *et al.*, 1995). These neutrophils have been observed to possess an enhanced oxidative burst response to the chemotactic peptide N-formyl-methionine-leucine-phenylalanine (fMLF), increased expression of the LPS binding receptors CD11b and CD18 and a reduction in surface L-selectin, thought to be a result of degranulation (Zimmerli *et al.*, 1986; Kuhns *et al.*, 1995; Sengelov *et al.*, 1995; Gainet *et al.*, 1999). Additional effects of neutrophil priming include the synthesis and release of bioactive lipids, increased phagocytotic activity and a modification to the rate of neutrophil apoptosis. This apparent disturbance in the regulatory mechanisms of PMN in such cases results in higher levels of ROS production and an increase in host tissue damage once these are released into the extracellular space (Guarnieri *et al.*, 1991; Swain *et al.*, 2002).

1.9 Periodontal disease and other inflammatory disorders

One aspect of periodontal disease that is currently attracting interest is its possible association with atherosclerosis and the development of coronary heart disease (CHD) and

cardiovascular disease (CVD). Atherosclerosis is a progressive condition characterised by arterial lesions on the intimal surface. These contain a centre of necrotic lysed cells, foam cells, cholesterol ester crystals and proteins such as fibrin and fibrinogen. As the lesion grows, the cross-sectional area of the artery is reduced, increasing the risk of thrombus formation via the aggregation of platelets in the circulation (Beck *et al.*, 1996). Damage to the arterial intima, either through infection, inflammation processes or mechanical action is a prerequisite for atherosclerotic plaque formation.

The link between periodontal disease and CHD has been partly attributed to socio-economic factors such as diet, smoking and chronic infections. These are risk factors that are shared by sufferers of both disease states (Katz *et al.*, 2001). Patients suffering from cardiovascular and cerebrovascular disease have been shown to have a higher incidence of poor oral hygiene (Beck *et al.*, 1996). Indeed, the number of missing teeth has been related to the incidence of ischemic heart disease even after adjustment for age, hypertension, geographic area, education and smoking (Paunio *et al.*, 1993). In a study identifying 17 personal characteristics as risk factors for periodontal disease, Beck (1999) found that 9 of these were also risk factors for CHD. There was a positive association found with age, tobacco and alcohol consumption, hypertension, stress and social isolation, a negative association with education and financial position and an increase in cases in males compared to females.

Recent research has found that periodontitis and CHD possess similar pathogenic factors and mechanisms. Both viral and bacterial pathogens have been proposed as instigators of atherosclerosis (Saikku *et al.*, 1998). The inflammatory process is an important risk factor associated with both periodontal disease and cardiovascular and cerebrovascular disease

(Danesh *et al.*, 1997). Patients with periodontal disease display increases in the plasma clotting factors fibrin and fibrinogen and white blood cell count, which are not associated to smoking or social class (Lowe, 1992). This may cause hyperfibrinogeneamia, resulting in an elevated risk of thrombosis development and heart disease (Kinane and Lowe, 2000).

Atherosclerosis and periodontal disease also share some markers of chronic inflammatory disease found in the circulation (Katz *et al.*, 2001). The development of atherosclerosis may be influenced by cytokines produced as a result of oral bacterial infection. Pro-inflammatory cytokines such as IL-1, Il-6 and TNFα promote an increase in adhesion molecule expression on vascular endothelial cells, causing a proliferation of monocyte migration into the subintimal region of arteries and the subsequent development of atherosclerotic plaques (Gamble *et al.*, 1985). These cytokines may also inhibit lipoprotein lipase leading to lipaemia and increase fibrinogen production (Beutler and Cerami, 1989).

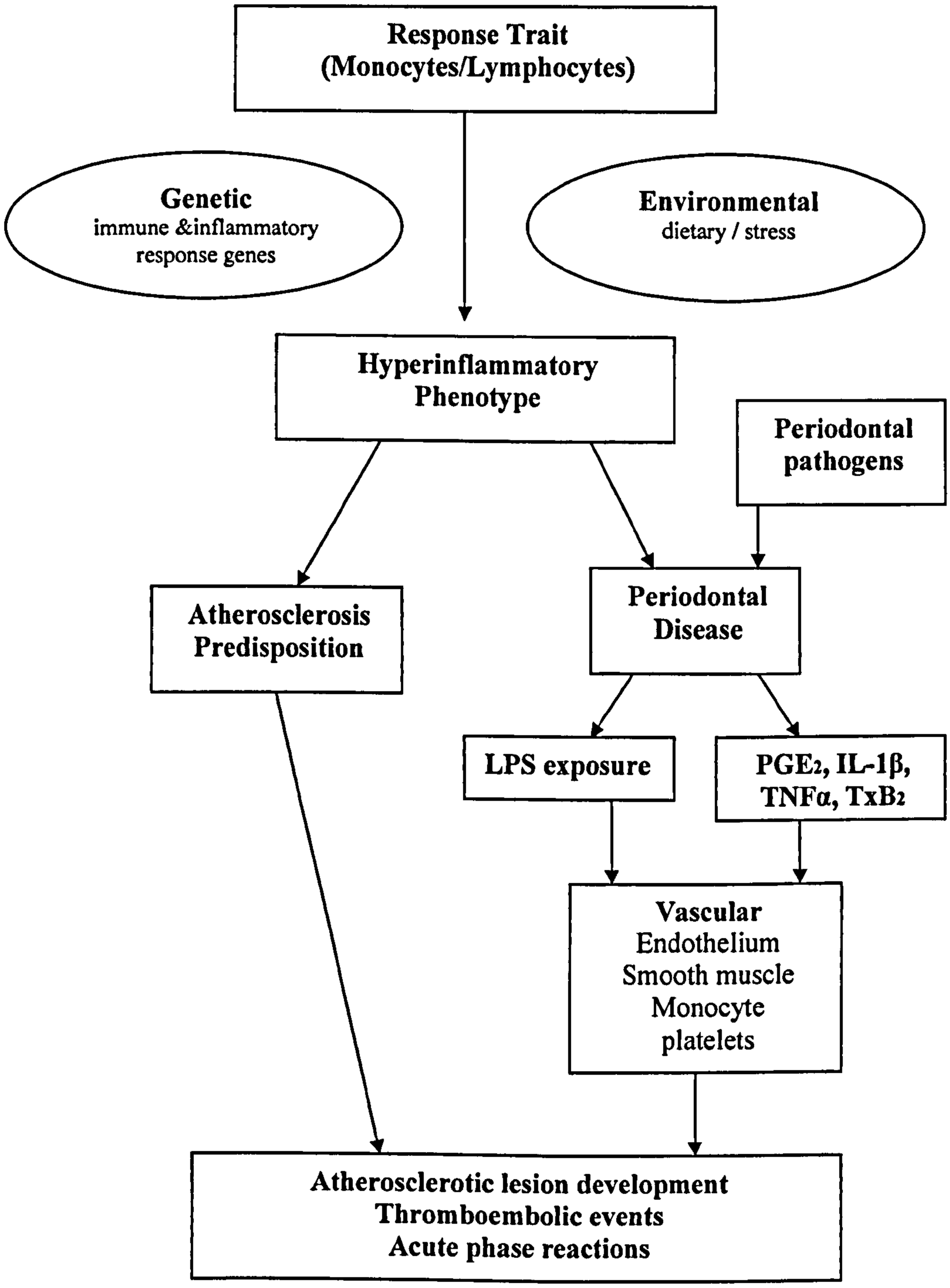
Bacterial lipopolysaccharide (LPS) from the oral cavity may also be implicated in the pathogenesis of CHD. Under normal conditions, the presence of bacterial LPS in plasma is improbable as it is usually bound to plasma proteins. However, in cases of trauma to the gingiva either by the direct mechanical action during a dental scale, or by the chronic presence of an abscess, bacteria may enter the circulation and lead to bacteremia. Free LPS may cause activation of leukocytes, platelets or the endothelium (Kinane and Lowe, 2000).

The binding of LPS to lipopolysaccharide-binding protein will enable binding to CD14 receptors present on monocytes, macrophages and endothelial surfaces, inducing cellular activation. The resultant increase in adhesion molecule expression by cytokines and

chemokines would facilitate leukocyte attachment and migration into the subintimal arterial region, promoting an increase in the number of foam cells and smooth muscle cells and thereby aiding the formation of an atherosclerotic plaque (Tobias *et al.*, 1997). Infection by gram-negative bacteria has been associated with an increase in LPS and endotoxin in the circulation, leading to the initiation of the inflammatory response and cell infiltration into major blood vessels, along with an increase in vascular smooth muscle cell inflammation, vascular fat degeneration and increased intravascular coagulation (Marcus and Hajjar, 1993; Beck, 1992). A proposed mechanism for the biological basis of association between periodontal disease and cardiovascular disease is shown in Figure 1.2.

The relationship between periodontal disease and cerebrovascular disease has not been extensively researched. In a recent study, periodontitis was found to be a significant risk factor for cerebrovascular accidents, with a particular impact on the risk of non-hemorrhage stroke (Wu *et al.*, 2000). This is believed to be mediated by a common PMN-mediated pro-inflammatory cytokine response to the bacteria (Wu *et al.*, 2000). Indeed, bacteria responsible for periodontitis have been found in atherosclerotic plaque deposits collected from CVA sufferers (Haraszthy *et al.*, 1998).

Figure 1.2. Theoretical mechanism for association between periodontal disease and cardiovascular disease (Beck *et al.*, 1996).



To avoid oxidative destruction by superoxide radicals released by PMN, *P. gingivalis* produces a form of superoxide dismutase, converting the superoxide to hydrogen peroxide (Shelburne *et al.*, 2002). As previously stated, *P. gingivalis* possesses haem, the dominant species of which is u-oxo bishaem of iron protoporphyrin IX produced via iron II obtained from host haemoglobin (Smalley *et al.*, 1998). This may act as a buffer to protect the bacteria against hydrogen peroxide, negating the need of an enzymatic degradation system (Smalley *et al.*, 2000).

However, whilst the bacteria may be relatively well protected, superoxide produced by the host response, and its by-products, especially the hydroxyl radical can cause extensive damage to host tissues. Small organic biomolecules that are targeted by ROS include vitamins A, C and E, carbohydrates such as glucose and ribose, amino acids such as hystidine, trytophase, cysteine and methionine, uric acid, cholesterol and smaller peptides including glutathione. Larger proteins can undergo alterations in form and function leading to impaired function or damage to cell membrane proteins Gutteridge and Halliwell, 1994). Both DNA and RNA are susceptible to ROS mediated damage with the pyrimidine and purine bases being targets (Dizdaroglu *et al.*, 2002). Alteration of the expression of transcription factors such as NF-κB may cause modulation of cell growth, differentation and can even cause apoptosis. Lipids can undergo peroxidation by the removal of hydrogen atoms from polyunsaturated fatty acid side chains (Niki, 1997).

In terms of cardiovascular disease, the latter is an important link to periodontitis. Lipid peroxides are thought to react with apolipoprotein B, the protein constituent of low density lipoprotein (LDL) and promote its oxidation. This oxidised LDL initiates foam cell

development and atherosclerotic lesions, leading to increased risk of CHD or CVA (Greenwold and Moy, 1979; Halliwell, 1991; Battino *et al.*, 1999).

Inflammatory processes related to periodontal disease also appear to promote preterm delivery in human pregnancy (Jeffcoat *et al.*, 2001). Sheep studies indicate that this increased risk is in part a consequence of lipopolysaccharide action within the uterus, but is also mediated by pro-inflammatory cytokines and PGE2 (Newnham *et al.*, 2004).

1.10 Saliva formation and composition

Saliva is the product of three major salivary glands, the parotid, the submandibular and the sublingual and also by smaller minor or accessory salivary glands. In addition to these major forms of saliva, gingival crevicular fluid (GCF) occupies the space between teeth and gums (Navazesh, 1993; Battino *et al.*, 2002). The production of saliva is controlled via the sympathetic and parasympathetic divisions of the autonomic nervous system. Stimulation, caused by factors such as the sight or smell of foodstuffs or a masticatory action, initiates the active transport of sodium and chloride ions into the lumen of salivary gland acini (Edgar, 1992; Navazesh, 1993). The resultant osmotic gradient leads to passive diffusion of water into the lumen. As the fluid passes through the salivary ducts, sodium ions are actively reabsorbed. The chloride ions are absorbed passively in order to sustain electrical balance, whilst potassium and bicarbonate ions are secreted into the fluid, together with an array of macromolecules to produce the saliva that is released into the mouth (Edgar, 1992).

Unstimulated saliva flow rate ranges from between 0.29 to 0.41ml/min. When stimulated, this rises to between 1.6 to 2.0ml/min (Sreebny, 2000).

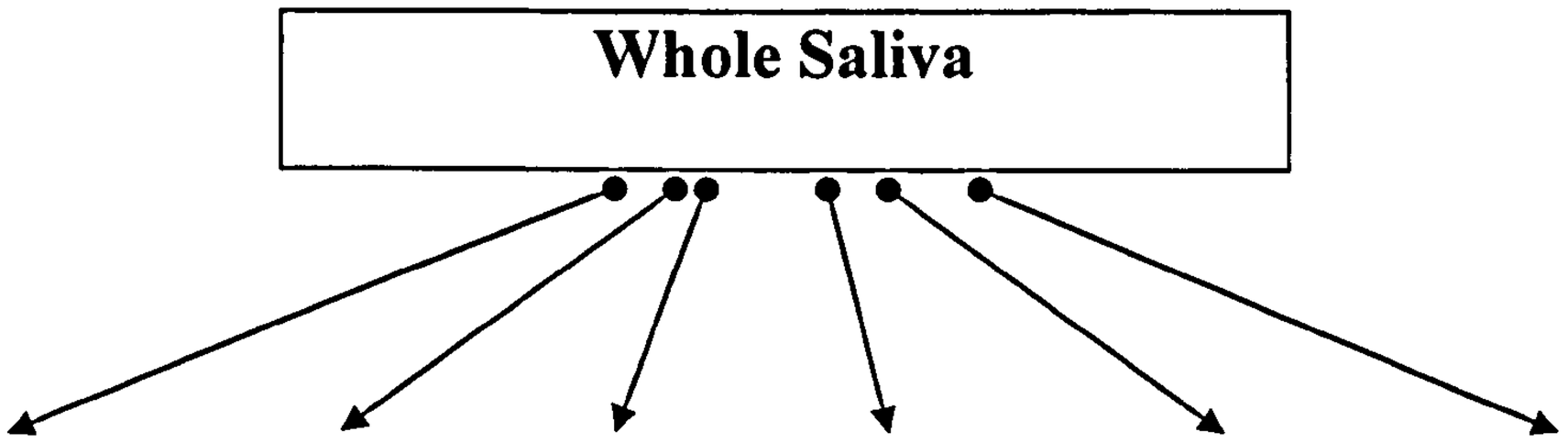
Whole saliva is a combination of gingival crevicular fluid (GCF), which has a composition similar to serum, and fluid released from salivary glands (Navazesh, 1993). Saliva has been found to be important in the maintenance of oral health, particularly the reduction of bacterial infection. Reduced saliva flow, known as xerostomia, has been shown to lead to an increase in gram-negative bacterial infection (Nieuw Amerongen and Veerman, 2002).

Proteins are present in saliva at a concentration of around 200mg/100ml. This is only approximately 3% of the protein concentration of plasma and includes enzymes, immunoglobulins, mucous glycoproteins and albumin (Edgar, 1992). Amylase, in the form of α-amylase, is secreted at a concentration of 60-120mg/100ml from the parotid salivary gland and 25mg/100ml from the submandibular. This enzyme is involved in the hydrolysis of starch polysaccharide chains. Three isoforms of immunoglobulins are present in saliva. These are IgA, at 20mg/100ml, IgG at 1.5mg/100ml and IgM at 0.2mg/100ml. Antibacterial proteins in saliva include lysozyme, lactoferrin and sialoperoxidase. Lysozyme has a direct antibacterial action by attacking the cell wall of certain bacteria (Edgar, 1992).

Lactoferrin binds the iron necessary for bacterial growth whilst sialoperoxidase oxidises salivary thiocyanate to produce antibacterial hypothiocyanate via hydrogen peroxide derived from oral bacteria (Nieuw Amerongen and Veerman, 2002). Mucous glycoproteins present in saliva include MG1 and MG2, both of which are found in sublingual and submandibular saliva and proline rich glycoproteins (PRP) are found in parotid saliva (Edgar, 1992). Other organic compounds present in saliva include free amino acids (<0.1mg/100ml), urea (12-20mg/100ml) and glucose (0.5 – 1.0mg/100ml. In addition to sodium and chloride ions, other inorganic components of saliva are potassium,

bicarbonate, fluoride and thiocyanate (Edgar, 1992). Whole saliva also contains traces of food, toothpaste, bronchial and nasal secretions and epithelial cells. The components of whole saliva can be seen in Fig. 1.3.

Fig. 1.3. Components of whole saliva (adapted from Kaufman and Lamster, 2000).



Salivary glands	Blood	Microbiota	Lining cells	Extrinsic factors	Additional fluids
• Water • Proteins • Electrolytes • Organic molecules	• Intraoral bleeding • GCF • Serum & cells	• Oral bacteria • Viruses • Fungi	• Epithelial keratins	• Food • Toothpaste • Mouthrinse	• Bronchial • Nasal

1.10.1 Salivary antioxidants

Saliva contains a number of antioxidants that protect the oral cavity from free radical-mediated damage. Urate is the primary antioxidant of saliva, accounting for over 70% of the total antioxidant activity (Moore *et al.*, 1994). Urate acts as a scavenger of free radicals and has been shown to conserve ascorbate by reducing ascorbate oxidation caused

by iron (Sevanian *et al.*, 1985). The concentration of urate in saliva is approximately 219μM, with values ranging from 87μM to 233μM (Moore *et al.*, 1994) and this is possibly associated with plasma urate. Previous research has indicated a correlation between plasma and salivary urate concentrations (Kondakova *et al.*, 1999). Ascorbate is present in unstimulated saliva at a concentration of around 9μM, whilst albumin concentration in saliva is in the region of 12μM (Moore *et al.*, 1994). Recent research has also suggested the presence of the enzymatic salivary antioxidants, salivary peroxidase and myeloperoxidase (Nagler *et al.*, 2002). This may indicate an active secretion system for salivary antioxidants rather than passive diffusion from the circulation. This becomes further evident when antioxidant concentrations are compared in unstimulated and stimulated saliva.

Stimulated saliva contains a lower absolute concentration of antioxidants, but when flow rates are taken into account, antioxidant capacity is higher than unstimulated (Moore *et al.*, 1994). Flow rate is calculated by taking a timed saliva sample, typically five minutes, and calculating the antioxidant capacity per minute of saliva flow. This provides a more realistic measurement of antioxidant delivery to the oral area using saliva as the vector, rather than taking a one-off sample (Moore *et al.*, 1994). This is important as the environment within the mouth is subject to constant change and is not static.

Very low concentrations of other antioxidant agents have also been found in saliva. Superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase have been detected (Pereslegina, 1989; Tan and Teng, 1978). It is not clear, however, whether these enzymes are actively secreted in saliva, or are a result of gingival crevicular fluid leakage. It is far more likely that these activities would be associated with shedding of

cellular material from the gums and cheeks into saliva, as with the exception of an extracellular isoforms of SOD, these enzymes are intracellular in nature.

1.10.2 Saliva collection methods.

There are numerous methods available for saliva collection. These include the collection of whole unstimulated saliva, saliva stimulated by using materials such as paraffin wax, gum base or citric acid, or collection of saliva from specific glands. When conducting analysis of saliva for antioxidants, whole saliva is more relevant as it contains GCF, immune cells and tissue metabolites (Navazesh, 1993; Kaufman and Lamster, 2000). Stimulated saliva has however been used in the analysis of antioxidants (Moore *et al.*, 1994). However, as unstimulated flow represents the major intra-oral condition, this would provide a more accurate account of the prevailing oral environment and saliva antioxidant composition for analysis (Edgar, 1992). In addition, stimulation may increase the expulsion of GCF from the periodontal pocket through the mastication process. This may artificially increase the concentration of antioxidants in the saliva (Chapple *et al.*, 1997).

1.11 Salivary antioxidants and periodontal disease.

Previous research considering salivary antioxidant status in periodontal disease is sparse and has yielded conflicting data. This may be a result of the different methodologies employed by the authors, though there are other factors that may cause discrepancies. Guarnieri *et al.* (1991) observed that PMN were found in greater concentrations at sites of gingival inflammation. They postulated that $O_2^{\cdot-}$ produced by PMN as part of the host immune response could cause host tissue oxidative damage if it was not matched by an increase in antioxidant concentration. However, they also speculated that an apparent

inhibitory mechanism observed in PMN from periodontal patients may prevent excessive damage to host tissues. Certainly, ROS produced within the oral cavity would have the capacity to damage the gums. Bartold *et al.* (1984) demonstrated that hydroxyl radicals generated *in vitro* induced major oxidative damage to isolated porcine gingivae. This injury included depolymerisation of hyaluronic acid and proteoglycans in the extracellular matrix.

Moore *et al.* (1994) measured the antioxidant capacity of saliva in diseased and healthy individuals using the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Measurements were made using both stimulated and unstimulated saliva samples. No difference in the antioxidant capacity of saliva was found between diseased and healthy patients. However, the diseased sample size was small (7 subjects) and their disease status was not well defined, having been described only as needing dental treatment. Similarly, definition of the healthy cohort was ambiguous, being described as 'apparently healthy'. Given that 80% of the population (Ridgeway, 2000) will suffer some degree of gingival disease, it is likely that these controls included some affected individuals. In addition, saliva samples were stored at -20°C and this may have allowed degradation of antioxidant capacity. Chapple *et al.*, (1997) found that antioxidant capacity in serum, assessed using TEAC, was 30% lower in samples stored at -20°C as opposed to under nitrogen.

In a similar study, Chapple *et al.* (1997) studied serum and saliva samples in diseased and control groups. Both groups were well defined, with 18 periodontal patients and 16 controls. Serum antioxidant capacity in the two groups was found to be similar. However, saliva antioxidant capacity was significantly lower in diseased patients compared to controls. This would indicate that reduced antioxidant activity in saliva is either a result of

increased free radical concentration, where the scavenging antioxidants are consumed in the quenching of the free radical chain reaction, or that the increase in free radical activity is a result of lower levels of salivary antioxidants. In addition, the ratio of saliva/serum antioxidants was also significantly lower in the diseased patients. It was proposed that the reduction in antioxidant capacity was either a direct causal factor in the development of periodontal disease, or that the decrease was due to a decline in scavenging antioxidants mediated through an increase in oxidative stress.

1.11.1 Periodontal disease assessment techniques.

Clearly, there is a need for standardisation of methods for defining periodontal disease severity and saliva sampling and testing protocols. One method of assessing the extent of periodontal disease that is common amongst British dental practices is the procedure of measuring the degradation of connective tissue around the root area of the tooth, known as the Community Periodontal Index of Treatment Needs (CPITN) (Ainamo *et al.*, 1982). This involves the examination of the gingiva with the use of a periodontal probe. This graduated probe is inserted between the tooth and the gingiva to determine the depth of any pocket formation. The dental structure is divided into six areas or sextants. Each sextant is given a score of between 0-4 depending on the extent of damage to the gingiva. Once the scores for all sextants are collected, the sum of these scores is subtracted from a theoretical perfect score of 24 to give a rating of periodontal disease. A score of 0 would indicate extensive disease in all sextants whilst healthy gingiva with no evidence of disease would score 24. A full description of the classification of this scoring technique can be seen in Table 1.1. This system provides a quick and convenient method of assessing periodontal disease severity in individuals, whilst indicating the location in the dental structure of the disease.

Table 1.1. Examination classification using periodontal probe (Ainamo *et al.*, 1982).

Score allocated to sextant area (CPITN)	Extent of gingival damage
0	No bleeding, plaque retention or pocketing > 3.5mm
1	Bleeding on probing but no plaque retention or pocketing > 3.5mm
2	Plaque retention present but no pocketing > 3.5mm
3	Pocketing >3.5mm but < 5.5mm
4	Pocketing > 5.5mm

Periodontal disease is clearly an important and possibly life-threatening condition, often underestimated by health professionals and the general population. The available evidence implicating inflammatory mediators and cells in the disease process suggests that local antioxidant status may be of some importance in determining susceptibility to the disease and its progression following initial bacterial colonisation. It is now of importance to determine the possible contribution of diet and other factors to salivary antioxidant status. In the future, antioxidant supplementation may be used in the treatment or prevention of these chronic diseases of the oral cavity.

1.12 Aims of the study

Periodontal disease is a wide-spread chronic condition that not only results in poor dental health but may have implications in the aetiology of other inflammatory diseases such as coronary heart disease. Despite these facts, very little research has been conducted in this area. The underlying factors involved in the development of periodontal disease have now been established, with strong evidence of free radical-mediated oxidative tissue damage being a primary cause of this condition. However, research in this area has been sparse and the study cohorts small and ill-defined. As yet, there has been little research investigating the possible modulation of antioxidant status within the oral cavity that may help to reduce oxidative injury. The primary aim of this thesis is to determine the antioxidant profile of saliva in a large population of dental patients and compare this to periodontal health using a standardised assessment technique. The secondary aim is to investigate the effects of nutritional modulation on salivary antioxidant status in an attempt to increase the antioxidant capacity of saliva and so decrease oxidative tissue damage.

CHAPTER 2

Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma (Poole, UK) unless stated otherwise in the text. PGE2 kits and chemiluminescent reagent were purchased from Amersham International (Aylesbury, UK). Estradiol and progesterone kits were purchased from DRG Diagnostics, Germany.

2.2 Ethical Approval

Where ethical approval was required for investigations and procedures employed in this study it was obtained from the Milton Keynes Ethical Approval Committee.

2.3 Recruitment of subjects

2.3.1 Periodontitis study

2.3.1.1 Study cohort

A group of 129 subjects (64 male and 65 female), mean age 58.5 ± 7.57 attending for routine check-up examination were recruited from Wilson House Dental Practice, Newport Pagnell, Buckinghamshire. Patients using nutritional supplements were excluded from the study to remove this potential confounding factor, as were those having just received any treatment that may have interfered with saliva flow or contaminated the sample. Saliva was collected from these patients as described in section 2.4, after examination by the dentist. Samples were stored in dry ice immediately and at -80°C on return to the

laboratory for a maximum of two months before analysis. After processing, the saliva samples were analysed for total antioxidant activity, uric acid, ascorbate, albumin and protein oxidation.

2.3.1.2 Disease assessment

During dental examination, the patients were assigned a score for periodontal disease using the Community Periodontal Index of Treatment Needs (CPITN) as an indicator of disease state (Ainamo *et al.*, 1982). This system uses a graduated periodontal probe that is inserted between the tooth surface and the gum at a standard force to measure the extent of pocket depth. In addition, a visual examination of the build-up of plaque and calculus and the extent of bleeding was made. A score of 0 indicated no inflammation whilst a score of 4 described gum tissue in an advanced state of disease. The score was allocated to sextants of the dental structure. The sum of these six scores were then subtracted from a maximum score of 24. This score was used to categorise the severity of periodontal disease in this study.

Table 2.1 CPITN scoring criteria (from Ainamo *et al.* (1982).

Sextant score	Condition of tooth and gingiva on examination
0	No bleeding, plaque or pockets evident
1	Bleeding on probing but no plaque or pockets
2	Plaque evident but pockets <3.5mm in depth
3	Pockets >3.5mm but <5.5mm
4	Pockets >5.5mm

2.3.2 Acute effect of ascorbic acid ingestion on salivary antioxidant capacity

Six healthy adult volunteers (4 male/2 female; mean age 28.17 ± 4.92) were recruited from University College Northampton. Baseline saliva samples were taken as described in section 2.4. On four separate days, a 50ml solution containing 10g glucose and either no ascorbate, 80mg, 500mg or 1000mg was given. These tests took place at the same time on each day in order to prevent any impact of diurnal variation upon the results. Further saliva samples were taken after 30, 60 and 120 minutes and processed as previously described. These were analysed for total antioxidant capacity using the FRAP method (section 2.5.1.1) and for ascorbic acid (section 2.5.2.1).

2.3.3 Diurnal saliva and antioxidant flow rate

Diurnal variation in saliva flow rate and antioxidant composition were assessed by collecting saliva samples as previously described from six healthy employees of the University of Nottingham (3 male/3 female age 29.17 ± 7.03) at 0300, 0700, 1200, 1700 and 2200 hours for three consecutive days. The samples were frozen at -20°C immediately and then stored at -80°C for a maximum of two weeks before analysis. The samples were tested for total antioxidant activity (TAA), ascorbate, urate and albumin concentration.

2.3.4 High Vs Low antioxidant Breakfast

Antioxidant flow rate and composition after the consumption of meals of high or low antioxidant content were determined. Six healthy individuals (3 male, 3 female/ aged 27.83 ± 6.15) took part in the study, all of whom were employees of the University of

Nottingham. Baseline fasted saliva samples were taken before a standard breakfast low in antioxidants was provided at 0800 hours. Further saliva samples were taken at 30 minutes, 60 minutes, 120 minutes and 240 minutes. The same six volunteers consumed a standard antioxidant-rich breakfast on the following day and an identical sampling procedure was adopted. Nutritional content of the two meals can be seen in chapter 5, Table 5.1. The samples were measured for volume before processing in salivette tubes as previously described and stored at -80°C until assayed. The samples were tested for TAA, ascorbate, urate and albumin.

2.3.5 Saliva Antioxidant Status during the Menstrual Cycle

Saliva flow and salivary antioxidant delivery rate were investigated throughout the menstrual cycle to determine any fluctuation that may be due to hormonal variation. Five healthy female volunteers were recruited from the University of Nottingham. (27 ± 1.64). Saliva was collected as in section 2.4 at the same time every 4 days throughout the menstrual cycle, beginning on the first day. Saliva was analysed for TAA, ascorbate, urate and albumin and expressed as in section 2.5.1.1.

2.4 Saliva Collection Protocol

Unstimulated whole saliva was collected over a five-minute period to allow for calculation of salivary delivery rate and antioxidant secretion in accordance with Moore *et al.* (1994). The patient was seated without exposure to salivary stimuli such as food images or smells and asked to allow saliva to pool in the mouth and discharge gravimetrically into a collecting tube using the methods of Navazesh (1993). After determining volume, saliva samples were then placed into 'salivette' tubes (Sarstedt, UK) with plain cotton inserts and

centrifuged at 3000 rpm for ten minutes at 4°C. The supernatant was then aliquotted into vials and stored at -80°C. Preliminary work showed that this method produced similar results to unprocessed saliva when measuring for total antioxidant activity, but generated a less viscous fluid allowing for more accurate pipetting.

2.5 Biochemical Assays

2.5.1 Determination of total antioxidant activity

A variety of methods were used to determine total antioxidant activity in order to assess the most appropriate assay for use with saliva. Each method possesses it's own strengths and shortcomings which depend upon the overall composition of the fluid to be tested. As saliva is poorly characterised in terms of antioxidant capacity it was necessary to make preliminary assessments of four assays, considering time taken, simplicity and associated costs.

2.5.1.1 Antioxidant delivery rate

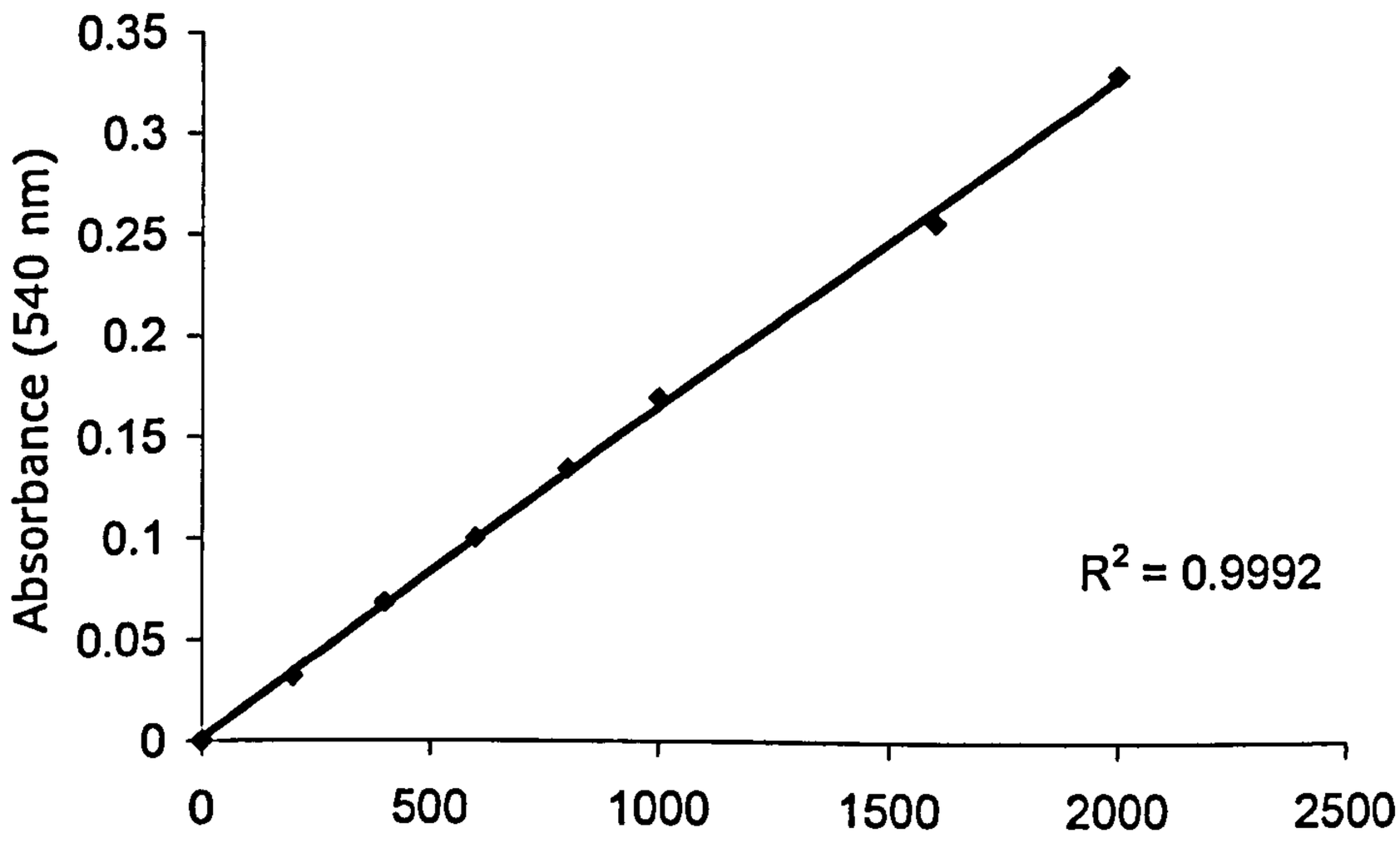
Values for antioxidant capacity in saliva are expressed as a delivery rate of μmoles/ml/min. Unlike simple calculations of antioxidant concentration in saliva (μM), delivery rate takes into account the ability of the antioxidant to be administered to the gingiva via salivary flow. This allows for a more meaningful assessment of the antioxidant status within the oral cavity. The calculations for this are as follows:

$$\text{Delivery rate} = \frac{\text{TAA (μM) x saliva volume x 5 (minutes of collection)}}{1000}$$

2.5.1.2. Ferric Reducing Ability of Plasma (FRAP)

Total antioxidant activity (TAA) of saliva and serum was calculated using an adaptation of the Ferric Reducing Ability of Plasma (FRAP) method (Benzie and Strain, 1996). 15µl of water was added to 5µl of sample in a microtitre plate. 150µl of FRAP reagent consisting of 125µl 300mM acetate buffer, 12.5 µl 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) dissolved in 40 mM HCL and 12.5 µl 20 mM Iron (III) chloride hexahydrate was then added and the samples incubated at 37°C for 4 mins. The absorbance at 540nm was determined on a plate reader (Bio-Rad, UK) and compared to a standard curve of 0-2000 µM Iron (II) sulphate. Salivary FRAP values were calculated to give a delivery rate as explained in section 2.5.1.1. Serum FRAP values were expressed as µmoles/L. An example of a FRAP standard curve can be seen in Figure 2.1.

Figure 2.1. FRAP standard curve



2.5.1.3. Trolox Equivalent Antioxidant Capacity (TEAC)

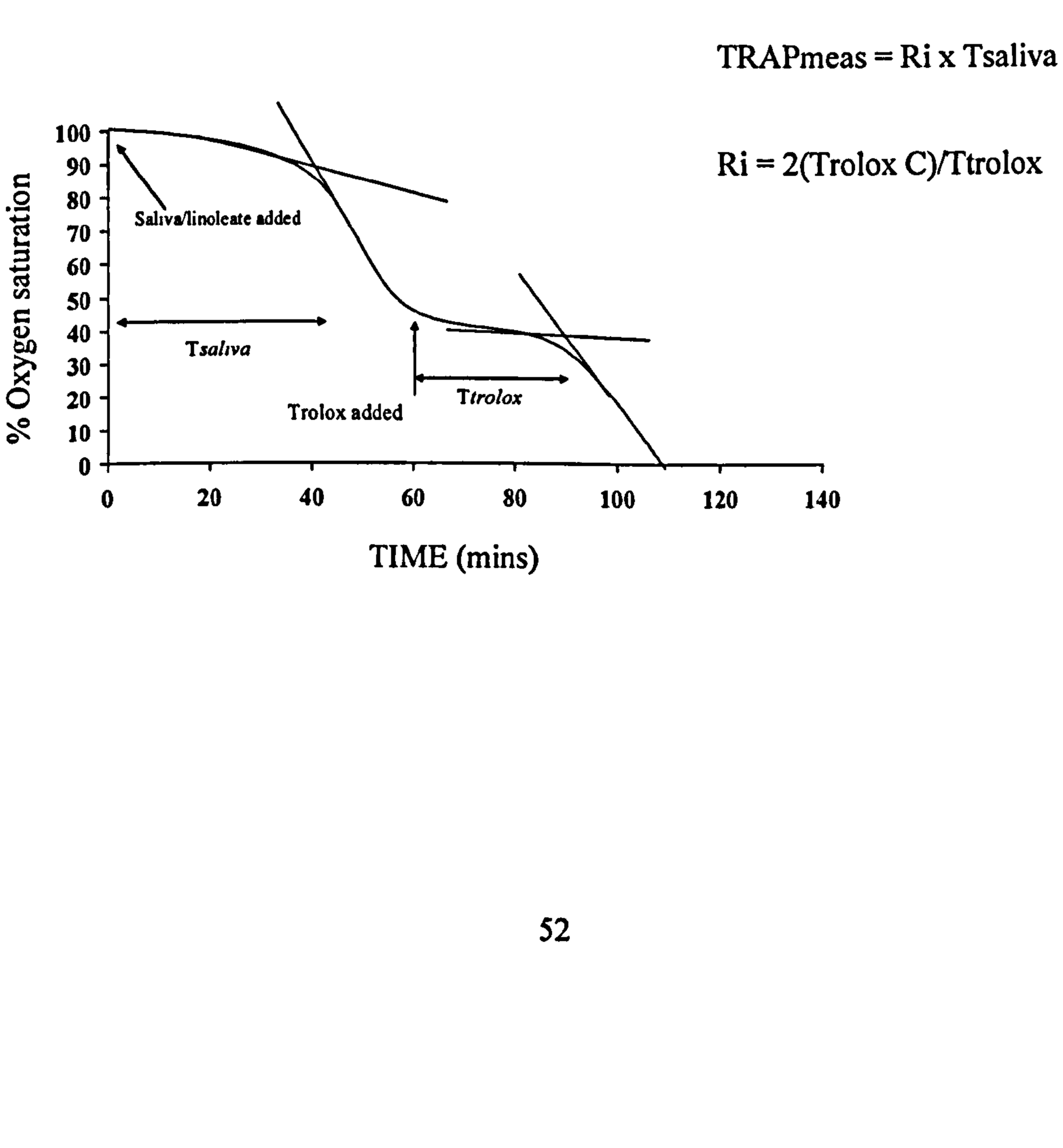
Total antioxidant capacity was also determined by using the Trolox Equivalent Antioxidant Capacity (TEAC) kit (Randox Laboratories Ltd), based on the method of Miller *et al.* (1993). This method relies on the antioxidant capacity of the sample to inhibit the production of the ABTS radical cation produced by mixing ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and hydrogen peroxide. The ABTS cation produces a blue-green colour and is read at 600nm. The suppression in colour production is proportional to the antioxidant concentration. TEAC values were expressed as µmoles/L and represent Trolox equivalents.

2.5.1.4. Total Radical Trapping Antioxidant Potential (TRAP)

Total antioxidant activity of saliva was determined by using the total radical trapping antioxidant potential (TRAP) method (Langley *et al.*, 1993). This method utilises oxygen electrode cells (Rank Bros Digital Model 10, Bottisham, UK) to measure the consumption of oxygen during oxidation of a substrate. 3ml phosphate buffered saline (pH 7.4) was added to the electrode chamber, the stirrer was activated and the chart recorder was calibrated to read 100% saturation. A Hamilton syringe was then used to inject 30µl of 0.4M 2-azobis (2-amidinopropane) hydrochloride (ABAP). A solution of 100 µl sample and 10 µl linoleic acid was mixed in a glass vial and 50 µl of this was injected into the chamber with a further Hamilton syringe.

The principle of this method lies in the oxidation of the linoleic acid by the ABAP, thus consuming oxygen from the chamber. The chart recorder measures the rate of oxygen consumption. The antioxidant capacity of the sample will inhibit the oxidation and therefore the rate of oxygen consumption. Once a linear rate of oxygen consumption is achieved on the chart recorder and the antioxidant capacity of the sample has been exhausted, 25 µl of 0.4mM Trolox is added to the chamber. This acts as an internal standard by which the antioxidant capacity of the sample is determined. The measured value of TRAP (TRAPmeas) was determined by using the equation shown in figure 2.2. Units of TRAP were expressed as µmoles/L.

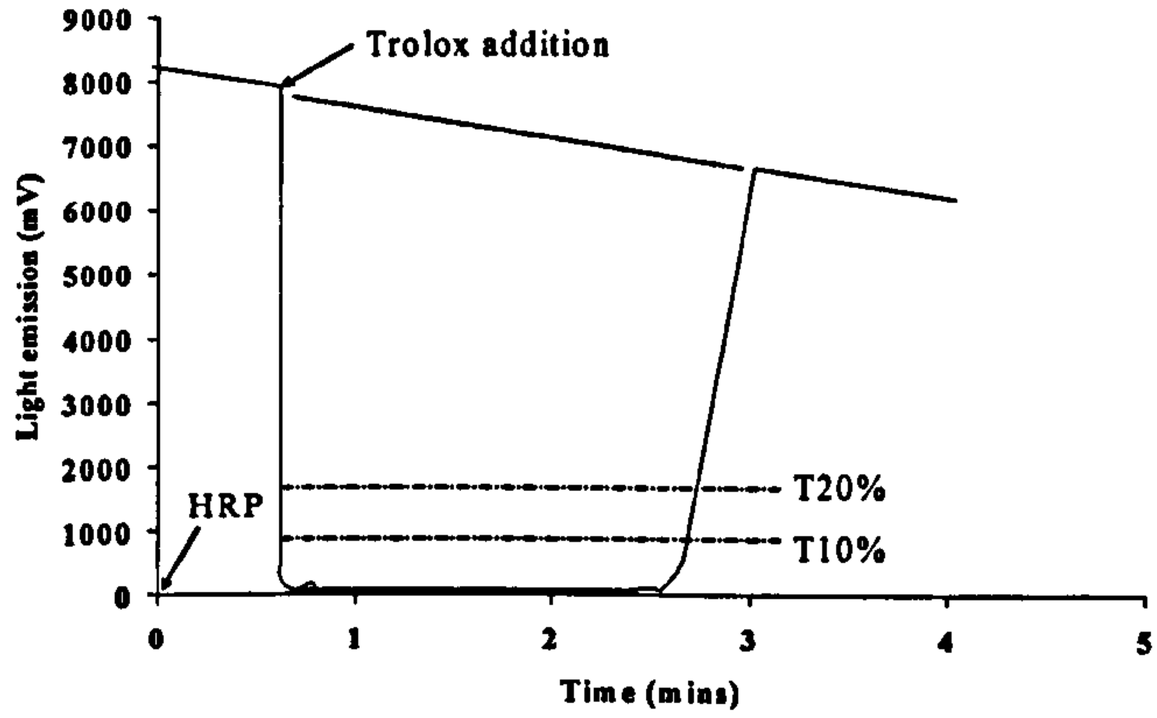
Figure 2.2. Schematic representation of TRAP assay (Langley *et al.*, 1993)



2.5.1.5. Enhanced Chemiluminescent Assay (ECL)

This method is based on the detection of chemiluminescent luminol after oxidation by hydrogen peroxide, catalysed by horseradish peroxidase (HRP) as described by Chapple *et al.* (1997). Two solutions were made (buffer 1 and buffer 2). Both buffers consisted of phosphate buffered saline (pH 7.6) with buffer 1 containing 50mg/L bovine serum albumin (BSA) whilst buffer 2 had 5g/l BSA. A solution of 50 units HRP was mixed with buffer 2. Before use, this solution was diluted further by adding 25µl in 5ml of buffer 1. ECL buffer was mixed according to the manufacturer's instructions (Amersham Biosciences, Buckingham, UK) and 100µl placed into a cuvette with 1ml of buffer 1. The reaction was initiated by adding 20µl of the HRP solution and vortexing. The cuvette was placed immediately into the luminometer. After the reading was stable at between 5500 and 7000 mV the cuvette was removed and 20µl antioxidant solution added (either saliva or Trolox standards) and vortexed before placing back into the luminometer within 10 seconds. The luminescence was followed on the chart recorder until 20% of the original value was achieved (see Figure 2.3). Activity was determined from a standard curve and expressed as µmoles/L Trolox equivalents.

Fig. 2.3. Schematic of light emission recording of ECL assay (Chapple *et al.*, 1997)



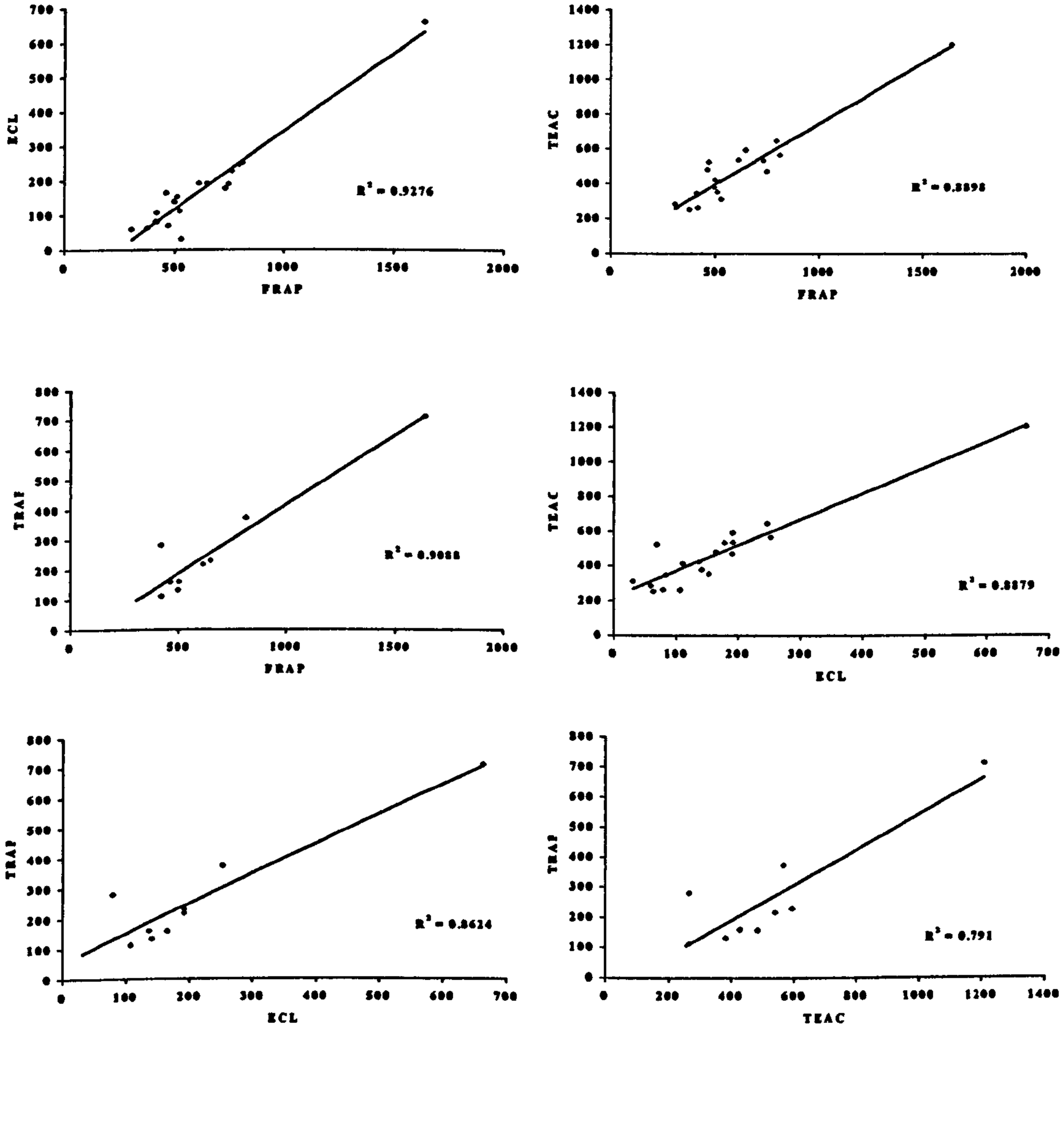
HRP – horse radish peroxidase.

2.5.1.6 Selection of FRAP and ECL for TAA determination

The four methods for determination of TAA were compared to determine their effectiveness, reproducibility and the extent to which they agree with each other (Fig. 2.4). FRAP was found to be effective at low antioxidant concentrations, cheap and quick to use, highly reproducible and to have a low coefficient of variance. TEAC was also effective at low antioxidant concentrations and quick to use. However, the cost of the kit was prohibitive for the scale of the planned studies. ECL was highly sensitive to low antioxidant concentrations and easy to use, although not as rapid as FRAP or TEAC. In addition, the materials required for ECL are expensive. TRAP was found to be poorly reproducible and very time-consuming. However, as it is not colourmetric, it is possible to use turbid solutions, which is of some advantage if testing products used in oral hygiene for antioxidant activity (see section 5.5)

After comparison testing, it was found that FRAP was the ideal method for determining TAA in such a large study cohort. ECL was also used for some in-vitro analysis, due to its high sensitivity.

Figure 2.4. Correlations for methods of TAA determination



Data shown are correlations of the 4 methods of TAA determination and were generated using a Pearsons two-tailed correlation test.

2.5.2 Scavenging antioxidants

2.5.2.1 Determination of ascorbate

Salivary and serum ascorbate was determined using an adaptation of the Ferrozine method (Butts and Mulville, 1975). For saliva, ascorbate concentration was determined from a standard curve of 0-20µM. 50µl of saliva was mixed with 150µl of water. 50µl of ferric reagent (1g Ferric ammonium sulphate in 250ml 20mM sulphuric acid) was added, followed by 450µl of ferrozine reagent (0.55g 3-(2-Pyridyl)-5,6-bis(4-Phenylsulfonic acid) 1,2,4 Triazine in 500ml sodium acetate buffer – pH 4.0) and the absorbance read within 15 seconds. 1mM ascorbate standard was prepared from 17.6 mg ascorbate and 25mg oxalic acid in 100ml of water. Salivary ascorbate delivery rates were calculated as for TAA in section 2.5.1.1.

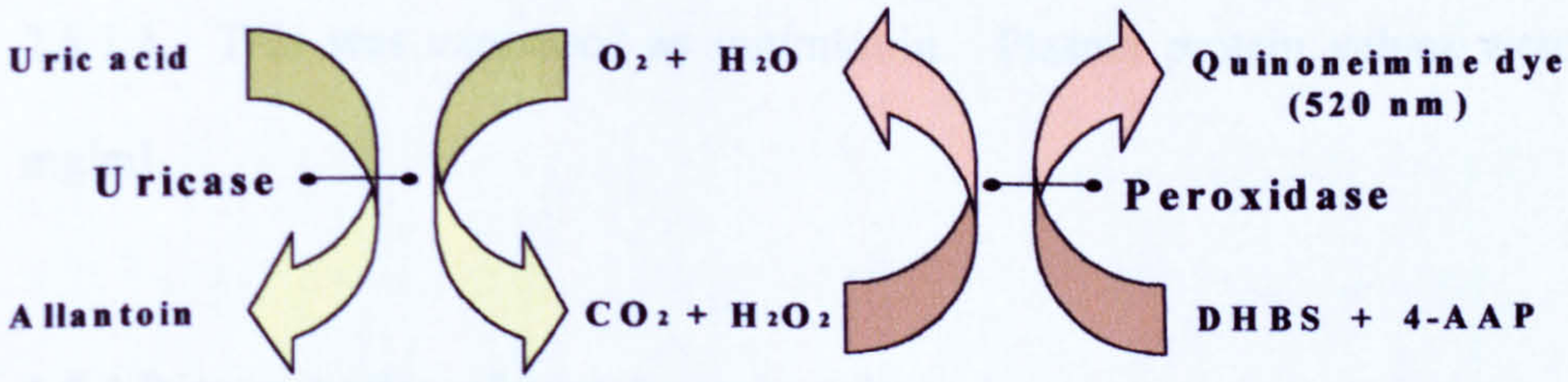
2.5.2.2 Determination of albumin

Salivary and plasma albumin concentrations were determined using Sigma kit 631, adapted for use with saliva in a plate reader. This is based on the method of Doumas *et al.* (1971) and uses bromcresol green (BCG) reagent. Albumin binds to BCG, with the colour being proportional to the albumin concentration. Standards and samples were tested in duplicate. 200µl of BCG reagent was added to 30µl of saliva and 10µl serum samples in microtitre plates and mixed for 20 seconds at 37°C. Absorbance was read on a plate reader (Bio-Rad, UK) at 620nm and the concentration determined from a standard curve (0-1mg/ml) prepared from bovine serum albumin. Salivary albumin delivery rate was calculated as for TAA in section 2.5.1.1.

2.5.2.3 Determination of uric acid

Salivary and serum uric acid was determined using Sigma Diagnostics procedure number 685, adapted for plate reader, based on the uricase – peroxidase – quinoneimine dye method of Fossati *et al.* (1980) (see Fig. 2.5).

Fig 2.5 Principles of the uricase-peroxidase-quinoneimine method for determination of uric acid



Legend. DHBS = 3,5-dichloro-2-hydroxybenzene-sulfonate.

4-AAP = 4-aminoantipyrine

All samples were assayed in duplicate. 200µl of reagent was added to 5µl of saliva and serum and incubated at 37°C for 5 minutes. Absorbance was read on a plate reader (Bio-Rad, UK) at 540nm and compared to a standard curve (0-1.5µM) prepared from uric acid

standard (Sigma Diagnostics number 685-1). Salivary urate delivery rates were calculated as for TAA in section 2.5.1.1. Plasma urate values were expressed as μM/ml.

2.5.3 Determination of protein

Salivary and serum protein were determined using the Bradford assay (Bradford, 1976), adapted for a microplate reader. Samples were assayed in duplicate. Bradford reagent was made by dissolving 40 mg of Coomassie Brilliant Blue in 20 ml of ethanol. 40 ml of phosphoric acid was added and mixed well. This was then made up to 500 ml with water and allowed to mature for two weeks prior to use. 100μl of Bradford reagent was added to 5μl of saliva and serum and the absorbance measured on a plate reader (Bio-Rad, UK) at 540nm. Protein concentration was determined from a standard curve (0-1mg/ml) prepared from bovine serum albumin. Salivary protein delivery rates were calculated as in section 2.5.1.1. This was expressed as mg/ml/min. Plasma protein values were expressed as mg/ml.

2.5.4 Determination of protein carbonyls

Saliva protein carbonyls were determined, as a biomarker of oxidative damage, using the method of Park *et al.* (1991). All samples were assayed in duplicate. 500μl of 20% trichloracetic acid (TCA) was added to 100μl of saliva and allowed to stand for 15 minutes. This was then centrifuged at 13000 rpm for 5 minutes at 4°C. The protein pellet was resuspended in 500μl 2M HCL, 0.1% dinitrophenylhydrazine (DNP). For each sample with DNP added, a blank sample without DNP was also processed. Samples were incubated at room temperature for 1 hour, and then 500μl of TCA was added and allowed to stand for 15 minutes before centrifuging as above. The pellet was then washed three times with 1:1 (vol) ethanol:ethyl acetate to remove excess DNP, allowing standing time of

10 minutes before centrifuging as above. The washed pellet was then dissolved in 6M guanidine-HCL for 15 minutes at 37°C and centrifuged. The supernatant was extracted and absorbance read at 370nm on a spectrophotometer (Shimazdu UV-160, Japan) after blanking with the matched sample without DNP. The results were calculated using the following equation:

$E370=21000M^{-1}\ cm^{-1}$ and expressed as nmoles carbonyls/mg.

2.5.5 Determination of enzyme activity

2.5.5.1 Catalase

Determination of catalase was based on the method of Aebi (1984) and measures the reduction of hydrogen peroxide by catalase over one minute. A buffer containing 50mM potassium phosphate (pH 7.0) was made. A substrate solution was mixed by adding 340µl 30% hydrogen peroxide to 100ml of buffer on the day of the procedure. 100µl of saliva was placed into a LP4 5ml polycarbonate tube. 900µl of buffer was added and vortexed. 1ml of substrate solution was added and mixed by rapid inversion. The sample in a glass cuvette was placed in a spectrophotomer and the absorbance recorded over one minute at 240nm. This was calculated against a standard curve constructed from catalase standards between the range of 0 and 300 units/ml. The activity was expressed as units catalase/min/mg protein.

2.5.5.2 Other antioxidants

Saliva was also analysed for further antioxidant enzyme activities and for the tripeptide glutathione. No detectable glutathione (Griffith, 1985), or activity of glutathione peroxidase (Beutler, 1979), glutathione reductase (Langley-Evans *et al.*, 1997) or superoxide dismutase (Marklund, 1985) were present in saliva. Rat tissue samples were assayed as positive controls for these assays.

2.5.6 Determination of prostaglandin E2

Salivary Prostaglandin E2 was determined using the Biotrak enzymeimmunoassay kit (BIA) from Amersham Biosciences (Buckinghamshire, UK). This method utilises a goat anti-mouse Ig coating on the microtitre plate well surface. Mouse anti-PGE2 in the assay reagent is added, together with either standard or sample and a PGE2-peroxidase conjugate. Competition for binding sites occurs between the unlabelled PGE2 and Peroxidase-labelled PGE2. The PGE2-peroxidase reacts with a substrate containing tetramethylbenzidine (TMB) to produce colour at 450nm. The PGE2 in the sample will inhibit PGE2-peroxidase from binding and therefore reduce the colour change.

2.5.7 Determination of progesterone

Salivary progesterone was determined using a DRG enzymeimmunoassay kit EIA-1561 from DRG Instruments GmbH, Germany. This method uses a polyclonal progesterone antiserum coat in the wells of a microtitre plate. The sample progesterone competes for binding sites with a progesterone conjugated with horse-radish peroxidase. A substrate solution is added after washing the cells and the sample progesterone concentration is inversely proportional to the colour produced.

2.5.8 Determination of estradiol

Salivary estradiol was determined using a DRG enzymeimmunoassay kit EIA-2693 from DRG Instruments GmbH, Germany. This method uses a polyclonal estradiol antiserum coat in the wells of a microtitre plate. The sample estradiol competes for binding sites with an estradiol conjugated with horse-radish peroxidase. A substrate solution is added after washing the cells and the sample estradiol concentration is inversely proportional to the colour produced.

2.6 Statistical analysis

All data were analysed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Individual tests used are shown in the appropriate chapters.

CHAPTER 3

Antioxidant properties of saliva

3.1 Introduction

The antioxidant composition of saliva has received little attention, despite the fact that some of its constituents may play an important role in the prevention of oral diseases (Moore *et al.*, 1994; Guarnieri *et al.*, 1991; Piyathilake *et al.*, 1995). The collection of saliva is simple, non-invasive and has the potential to provide data that may indicate an individual's susceptibility to many oral diseases (Chapple *et al.*, 1997; Battino *et al.*, 1999; Swain *et al.*, 2002) and possibly other systemic conditions such as cardiovascular disease (Kinane and Lowe, 2000) and oral cancer (Piyathilake *et al.*, 1995).

Previous research has made tentative steps towards characterising the antioxidant profile in supposedly healthy subjects and in individuals with periodontal disease. However, these studies were conflicting in their results and failed to fully evaluate the disease state and diagnostic methods used. Moore *et al.* (1994) reported no significant difference in the total antioxidant activity (TAA) of diseased and healthy dental patients. This conclusion was reached despite the fact that the sample groups were small (7 subjects) and the disease state was poorly defined. Moreover, the storage temperature of the saliva samples could have led to the degradation of antioxidant capacity (Chapple *et al.*, 1997). In a similar study, Chapple *et al.* (1997) found salivary antioxidant status was significantly lower in diseased patients compared to healthy controls.

Nevertheless, many aspects regarding saliva collection, antioxidant profile, disease characterisation and diagnostic methods still require further research. The antioxidant composition of saliva requires a definitive evaluation, whilst a universal standard for the measurement of disease is necessary in order for the data to be meaningful. As yet, published ranges for the normal concentrations of scavenging antioxidants in saliva have not been defined from large population bases. This study aimed to characterise the antioxidant profile of saliva, both stimulated and unstimulated, determine the extent of diurnal variation in saliva flow rate and antioxidant capacity and to compare different methods in the calculation of TAA.

3.2 Objectives

The antioxidant profile of saliva has not been extensively researched. The antioxidant composition may provide information helpful in the diagnosis and treatment of periodontal disease and may present biomarkers useful in the study of other inflammatory conditions. The specific objectives to be addressed in this chapter are:

- To determine the antioxidant profile of saliva.
- To ascertain an effective method for the determination of TAA in saliva.
- To compare saliva volumes and composition of unstimulated and stimulated saliva.
- To investigate the diurnal variation of saliva flow to ensure a standardised collection time.

3.3 Materials and Methods

3.3.1 Saliva collection protocol

All saliva collections followed the same procedure based on Navazesh (1993) as described in section 2.4. Briefly, unstimulated saliva was collected over a five-minute period and stored at -80°C until analysed.

3.4 The use of Salivette tubes on saliva samples

Unprocessed saliva contains an array of debris and is highly viscous. This leads to difficulties in accurate pipetting of the small volumes necessary when using microtitre plates (coefficient of variance = 16.12%). To increase the accuracy during pipetting, the saliva was processed in Salivette (Sarstedt, Leicester, UK) tubes. This produced a reduction in viscosity and the removal of debris (coefficient of variance = 4.40%).

3.4.1 Protocol for use of Salivette tubes

The antioxidant profile of fresh saliva was compared with saliva processed in the two types of Salivette tubes available (natural cotton insert and citric acid impregnated cotton insert) to assess the most suitable tube. Saliva was collected and centrifuged in either a standard centrifuge tube or one of the three Salivette tubes at 4000 *g* for 10 minutes at 4°C. The supernatant was then analysed for TAA, ascorbate, urate and albumin concentrations (see section 2.5).

3.4.2 Correlation of methods for the determination of TAA

Four methods were evaluated for the determination of TAA (see section 2.5.1). FRAP was chosen due to its ease of use, cost effectiveness and speed. Correlations were made in order to assess the effectiveness of the FRAP method when compared to other methods of TAA determination. All correlations between FRAP and ECL, TEAC and TRAP were significant ($p<0.01$). The results can be seen in Table 3.1.

Table 3.1. Correlations of methods of TAA determination

	ECL	TEAC	TRAP
Correlation (2-tailed)	0.963	0.978	0.953

Data are R values calculated using Pearson Two-Tailed Correlation test. $P<0.001$ in all cases.

3.4.3 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Standard deviation of values was compared between groups using Levene's test of homogeneity of variance. Differences in mean values between males and females were calculated using the Students T-test for independent samples. Correlation coefficients were calculated using Pearson's two-tailed correlation test.

3.4.4 Results

The salivette tube containing the plain cotton insert provided the closest antioxidant profile to that of un-filtered saliva. This suggests that the analytes in question were not absorbed into the insert. The results are shown in Table 3.2 below. Despite the fact that all three processing methods were significantly different from each other, the difference was relatively small between the unprocessed sample and the cotton filter salivette. This was due to a decrease in urate concentration after processing. However, the advantages in decreased viscosity and vastly reduced coefficient of variance after using the cotton filtered salivette were deemed to be of greater importance. The salivette containing the citric acid impregnated insert vastly altered the TAA in the sample. All three individual antioxidants were reduced after using this insert and therefore it was discarded for use in this study.

Table 3.2. Saliva antioxidant profile using plain and salivette tubes

Filtration state	TAA (FRAP) (μM)	Urate (μM)	Ascorbate (μM)	Albumin (μM)
Unfiltered saliva	954.62 ± 2.55	501.67 ± 27.56	6.79 ± 0.43	3.03 ± 0.31
Salivette (cotton insert)	839.62 ± 5.26*	401.67 ± 23.89	6.58 ± 0.29	2.86 ± 0.18
Salivette (citric acid insert)	181.77 ± 4.34*	308.33 ± 26.35	No reading	2.43 ± 0.21

Data are shown as mean ± standard error of the mean (S.E.M). * denotes significant difference to TAA of unprocessed sample ($p<0.05$). Data generated using a paired samples T-test.

3.5 Antioxidants in saliva

Previous research reporting the antioxidant composition of saliva has been limited in both size and population range. In this study, the antioxidant profile of saliva was evaluated in 147 individuals. Mean saliva volume over the five-minute collection period was 2.376±0.099ml (see Table 3.3). Male saliva production was significantly higher than female ($p<0.01$). Similarly, saliva flow rate was significantly higher in males compared to females ($p<0.01$).

Table 3.3. Saliva volumes collected over five minutes and flow rates.

	Overall (± SEM)	Male (± SEM)	Female (± SEM)
Saliva volume (ml)	2.376 ± 0.099 range 0.60 – 7.70	2.727 ± 0.153 * range 0.90 – 7.70	2.029 ± 0.113 range 0.60 – 4.90
Saliva flow (ml/min)	0.475 ± 0.02 range 0.12 – 1.54	0.545 ± 0.031 † range 0.180 – 1.540	0.4058 ± 0.023 range 0.120 – 0.980

Data shown are mean ± S.E.M. * denotes significant difference between male and female saliva volumes as collected over a 5 minute period ($p<0.01$). † denotes significant difference between male and female saliva flow rates ($p<0.01$). Ranges are for reference. Data generated using a paired samples T-test.

Total antioxidant activity (TAA) using the FRAP method was determined in 147 subjects (73 male, 74 female; mean age 54.80±1.02). Mean salivary TAA was 624.98±19.64μM overall, with TAA in males being significantly higher than in females ($p<0.01$) (see Table 3.4). Mean urate concentration was 184.30±10.48μM. Urate in males was significantly

higher when compared with the urate concentration in females (p=0.003). Mean ascorbate and albumin concentrations were 8.10±0.42 and 10.67±0.97 respectively.

Table 3.4. Mean salivary antioxidant concentrations

Antioxidant	Overall (μM ± SEM)	Male (μM ± SEM)	Female (μM ± SEM)
TAA (n=147)	624.98 ± 19.64 range 218.00 – 1671.81	698.67 ± 31.34 * range 314.00 – 1671.81	552.28 ± 20.76 range 218.00 – 1211.00
Urate (n=130)	184.30 ± 10.48 range 2.81 – 708.88	215.08 ± 16.75 * range 3.070 – 708.88	154.45 ± 11.73 range 2.81 – 451.44
Ascorbate (n=113)	8.10 ± 0.42 range 1.10 – 24.74	8.50 ± 0.65 range 1.66 – 24.74	7.70 ± 0.53 range 1.10 – 20.96
Albumin (n=129)	10.67 ± 0.97 range 0.93 – 56.47	11.16 ± 1.37 range 0.93 – 54.71	10.21 ± 1.39 range 1.64 – 56.47

Data are shown as mean ± S.E.M. * denotes significant difference between male and female values (p<0.01). Ranges included for reference. Data generated using a paired samples T-test.

Mean TAA delivery rate was 0.3059 ± 0.019 μmoles/ml/min, with male TAA delivery rate being significantly higher when compared to that of females (p<0.01). Urate delivery rate was 0.0942±0.008 with male values significantly higher than females (p<0.01). Delivery rates for ascorbate and albumin were 0.0042±0.0003 and 0.005±0.0005 respectively, with male ascorbate delivery rate being significantly higher when compared to female (p=0.017) (see Table 3.5).

Table 3.5. Mean antioxidant delivery rates

Antioxidant Delivery rate	Overall (µmoles/ml/min ± SEM)	Male (µmoles/ml/min ± SEM)	Female (µmoles/ml/min ± SEM)
TAA (n=147)	0.3059 ± 0.019 range 0.0523 –1.5949	0.3933 ± .0.333 * range 0.1008 –1.5949	0.2196 ± 0.0132 range 0.0523 0.5108
Urate (n=130)	0.0942 ± .0084 range 0.0004 –0.5500	0.1267 ± 0.0148 * range 0.0135 –0.5500	0.0626 ± 0.0061 range 0.0004 0.2022
Ascorbate (n=113)	0.0042 ± 0.00032 range 0.00033 0.0171	0.0049 ± 0.0005 * range 0.0006 –0.0165	0.0034 ± 0.0004 range 0.0003 0.0171
Albumin (n=129)	0.00502 ± 0.000526 range 0.0005 –0.0324	0.0089 ± 0.0008 range 0.0005 –0.0324	0.0042 ± 0.0007 range 0.0006 0.0305

Data shown as mean ±S.E.M. * denotes significant difference between male and female antioxidant delivery rates ($p<0.05$). Ranges are for reference. Data generated using a paired samples T-test.

3.5.1 Stoichiometry of FRAP

FRAP has been used throughout this study to determine the TAA of saliva. It was necessary to define the contribution of urate, ascorbate and albumin to TAA as measured by FRAP. By determining the Stoichiometry of the reaction between antioxidants and the FRAP reagent (i.e. the number of free radicals that may be quenched per molecule of antioxidant under these conditions), it is possible to determine if the measured TAA in a sample is equivalent to what might be expected given the known concentration of antioxidants present. These factors are expressed relative to Iron (II). The stoichiometric factor for each antioxidant was determined by plotting known concentrations against an

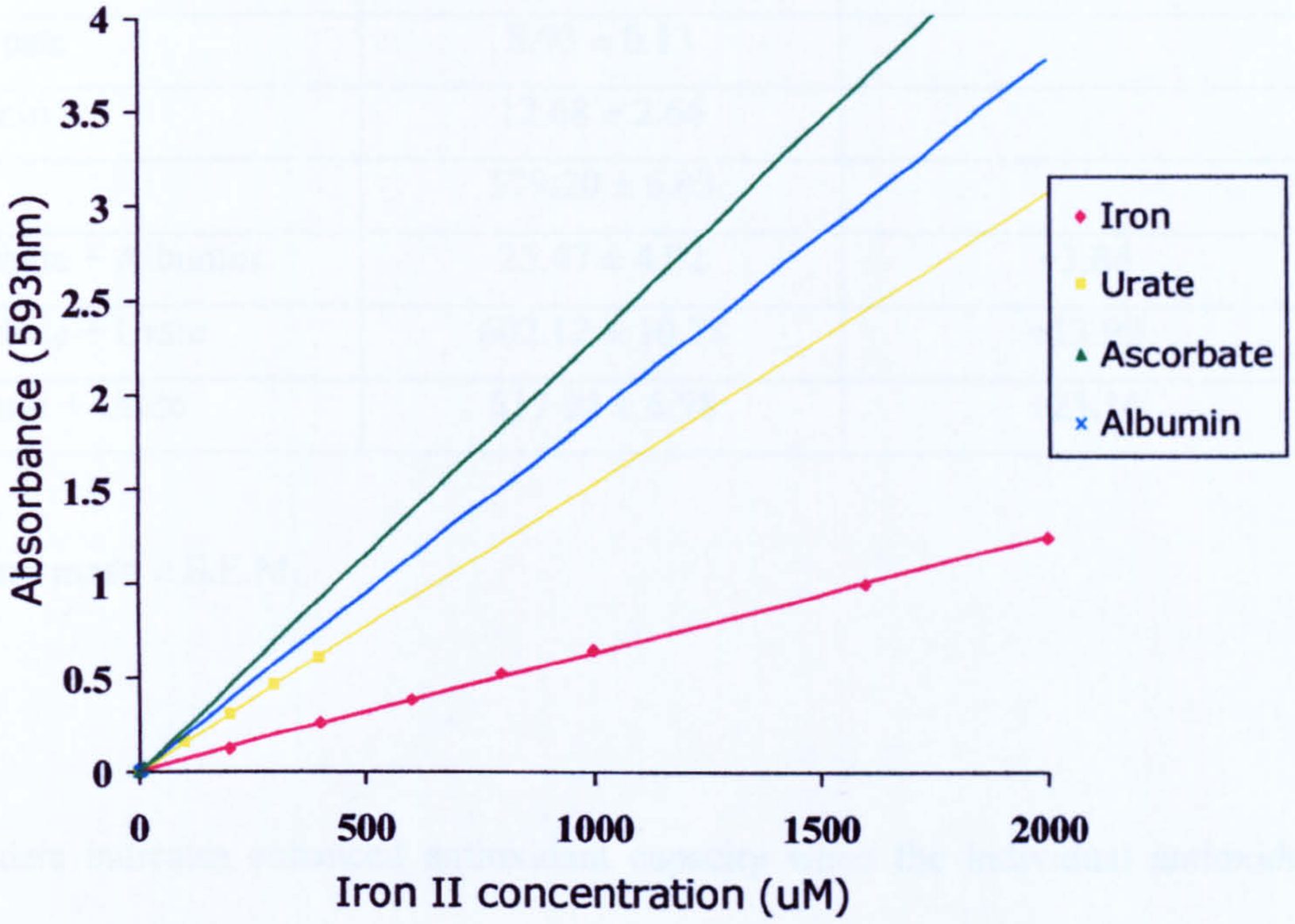
Iron II standard curve used for the FRAP assay (see Figure 3.1). The results can be seen in Table 3.6.

Table 3.6. Stoichiometry of individual antioxidants relative to Iron II sulphate as measured by FRAP

	Iron II sulphate	Urate	Ascorbate	Albumin
Stoichiometric factor	1.00	2.50	3.83	3.17

Data shown are factors by which the antioxidant concentration contributes to the TAA as measured by FRAP.

Fig. 3.1. Stoichiometric factors for individual antioxidant components in saliva



Trendlines represent the theoretical contribution of antioxidants compared to the Iron II standard.

These stoichiometric factors were applied to the individual antioxidant data in Table 3.4 and used to calculate the theoretical FRAP value for the samples. This was used to determine the contribution towards the TAA made by the individual antioxidants. The combined antioxidant activity of urate, ascorbate and albumin accounted for 85.47±3.57 % of TAA. Further analyses to investigate any possible interaction between the individual antioxidants were undertaken. Solutions of urate with ascorbate, urate with albumin and ascorbate with albumin were mixed at typical saliva concentrations. The results can be seen in Table 3.7.

Table 3.7. Interactions between antioxidants at saliva concentrations

Sample	Antioxidant activity (μM)	Difference from sum of individual AO (μM)
Ascorbate	8.93 ± 0.13	
Albumin	12.68 ± 2.66	
Urate	579.20 ± 6.03	
Ascorbate + Albumin	25.47 ± 4.02	+3.86
Ascorbate + Urate	602.12 ± 10.38	+13.99
Albumin + Urate	617.04 ± 6.98	+25.16

Data are mean ± S.E.M.

This data indicates enhanced antioxidant capacity when the individual antioxidants are combined, and may account some of the difference in TAA from the theoretical contributions seen in Figure 3.1. Synergistic effects of mixed antioxidants appear greatest when urate is combined with other agents.

3.6 Diurnal variation

Previous research has suggested that saliva flow follows a diurnal rhythm (Moore *et al.*, 1994). To investigate the daily variation in saliva flow and antioxidant capacity, saliva was collected as described in section 2.4 from six individuals (3 male/3 female) at 0300, 0700, 1200, 1700 and 2200 hours for three consecutive days. A cyclical diurnal rhythm was observed in which saliva volume peaked at 1700 hours and was at its lowest at 0700 hours. Mean saliva volumes can be seen in Table 3.8 and mean saliva flow rate can be seen in Figure 3.2. Saliva flow rate was significantly higher at 1200, 1700 and 2200 hours compared to 0300 and 0700 hours ($p<0.05$) (see Figure 3.2). TAA and ascorbate delivery

rates were significantly higher at 1200, 1700 and 2200 hours compared to at 0300 and 0700 hours ($p<0.05$). Urate delivery rate was significantly higher at 1200 and 1700 hours compared to 0300 and 0700 hours ($p<0.05$). Albumin delivery rates were significantly higher at 0300 hours compared to 0700, 1200, 1700 and 2200 hours ($p<0.05$). TAA and urate flow rates can be seen in Figure 3.3. Ascorbate and albumin flow rates can be seen in Figure 3.4.

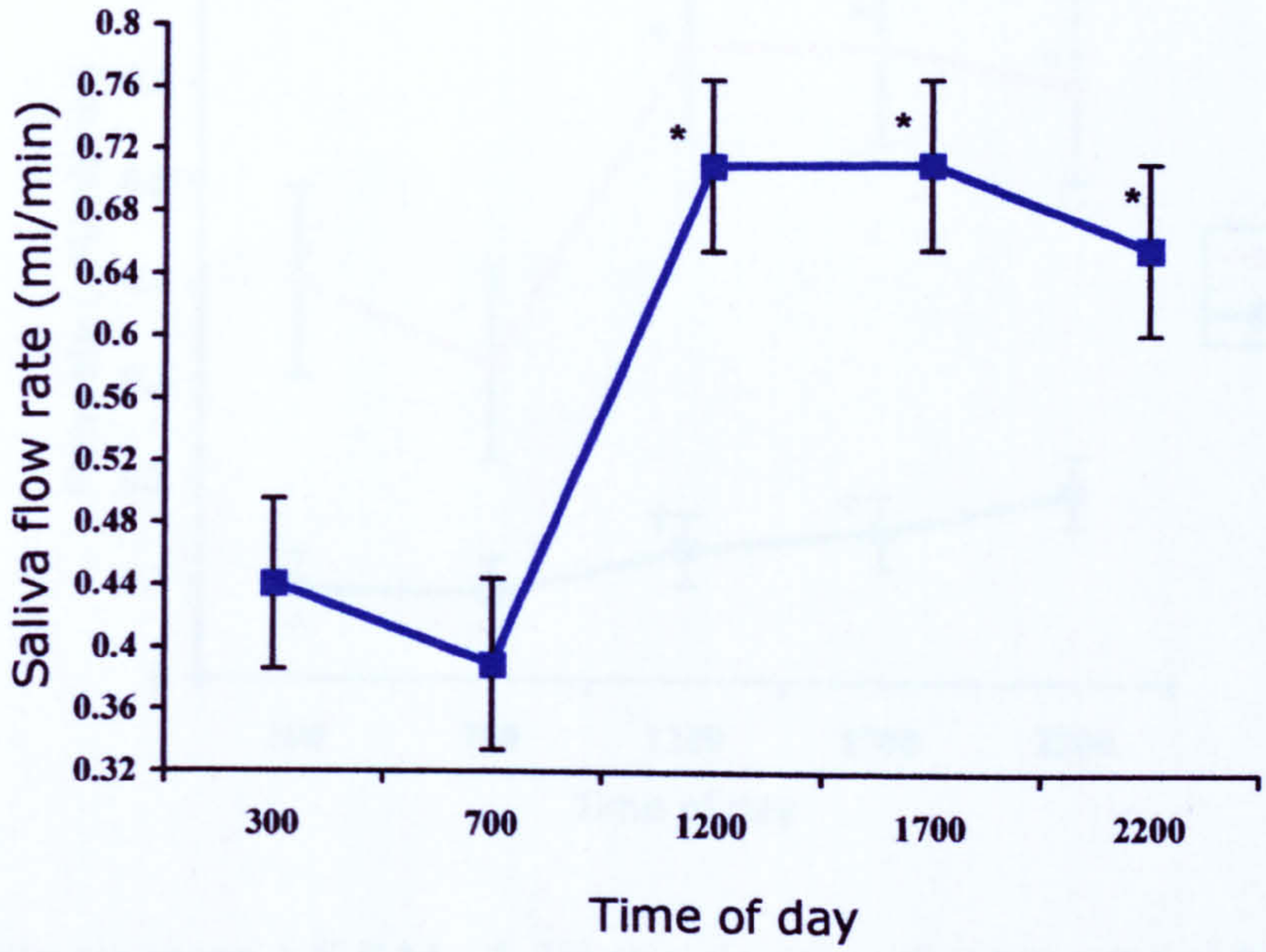
Table 3.8. Diurnal saliva volumes

	0300	0700	1200	1700	2200
Mean volume (ml)	2.202±0.294 range 0.410 – 4.492	1.943±0.183 range 0.545 – 3.610	3.549±0.238* range 1.763 – 5.413	3.555±0.339* range 1.739 – 6.299	3.282±0.318* range 0.794 – 5.856

Data shown are mean ± S.E.M. * denotes significantly higher volume compared to 0300 and 0700 hours ($p<0.05$). Ranges are for reference. Data generated using a paired samples T-test.

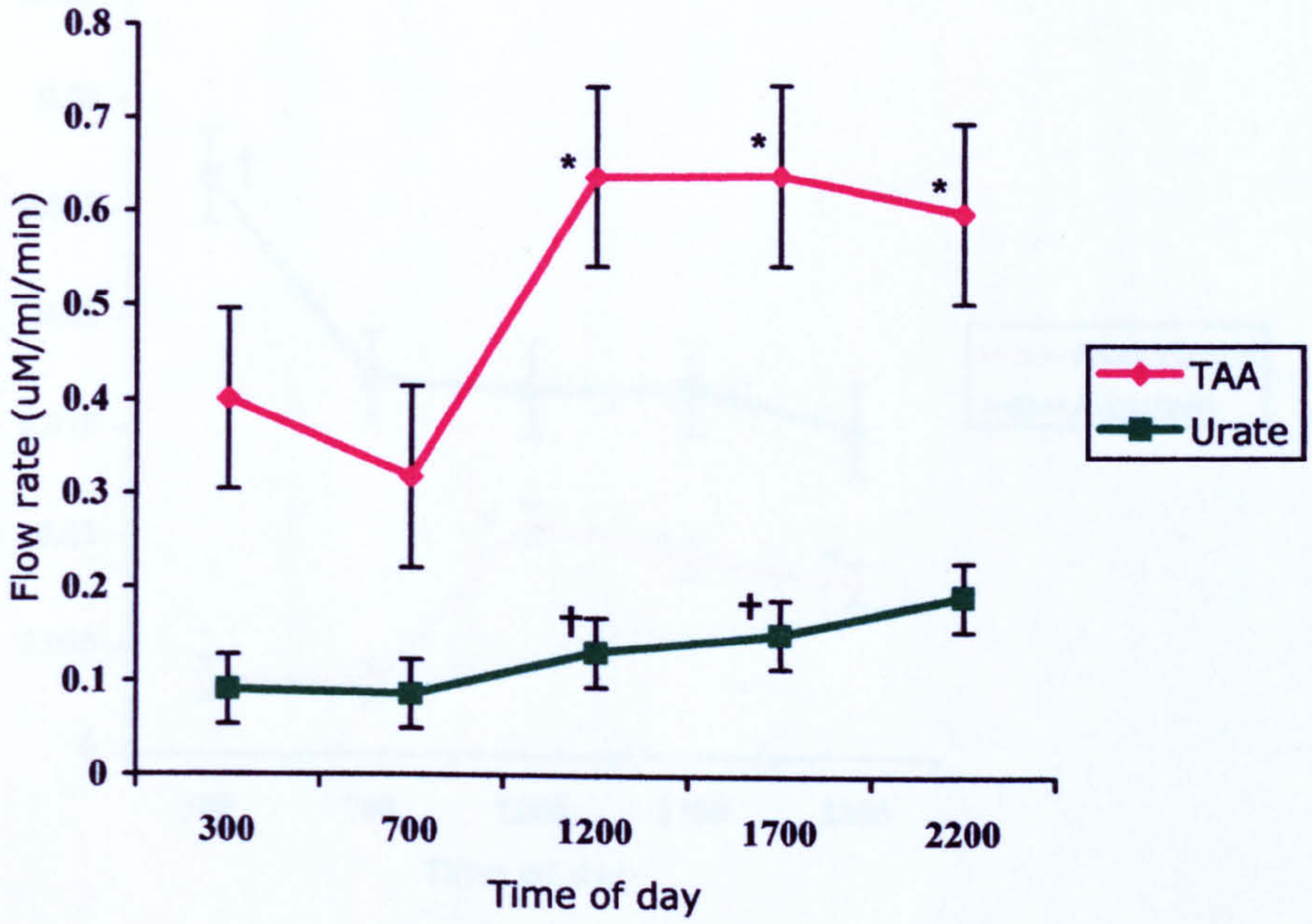
Mean saliva flow in males was higher than in females, with significant differences at 0300, 1700 and 2200 hours ($p<0.05$). Significant differences between males and females were also observed in TAA at 1200 hours, in TAA delivery rate at 0300, 1200 and 1700 hours, ascorbate delivery rate at 1200, 1700 and 2200 hours, in urate delivery rate at 1700 hours and in albumin delivery rate at 1200 hours.

Figure 3.2. Diurnal variation of saliva flow rate



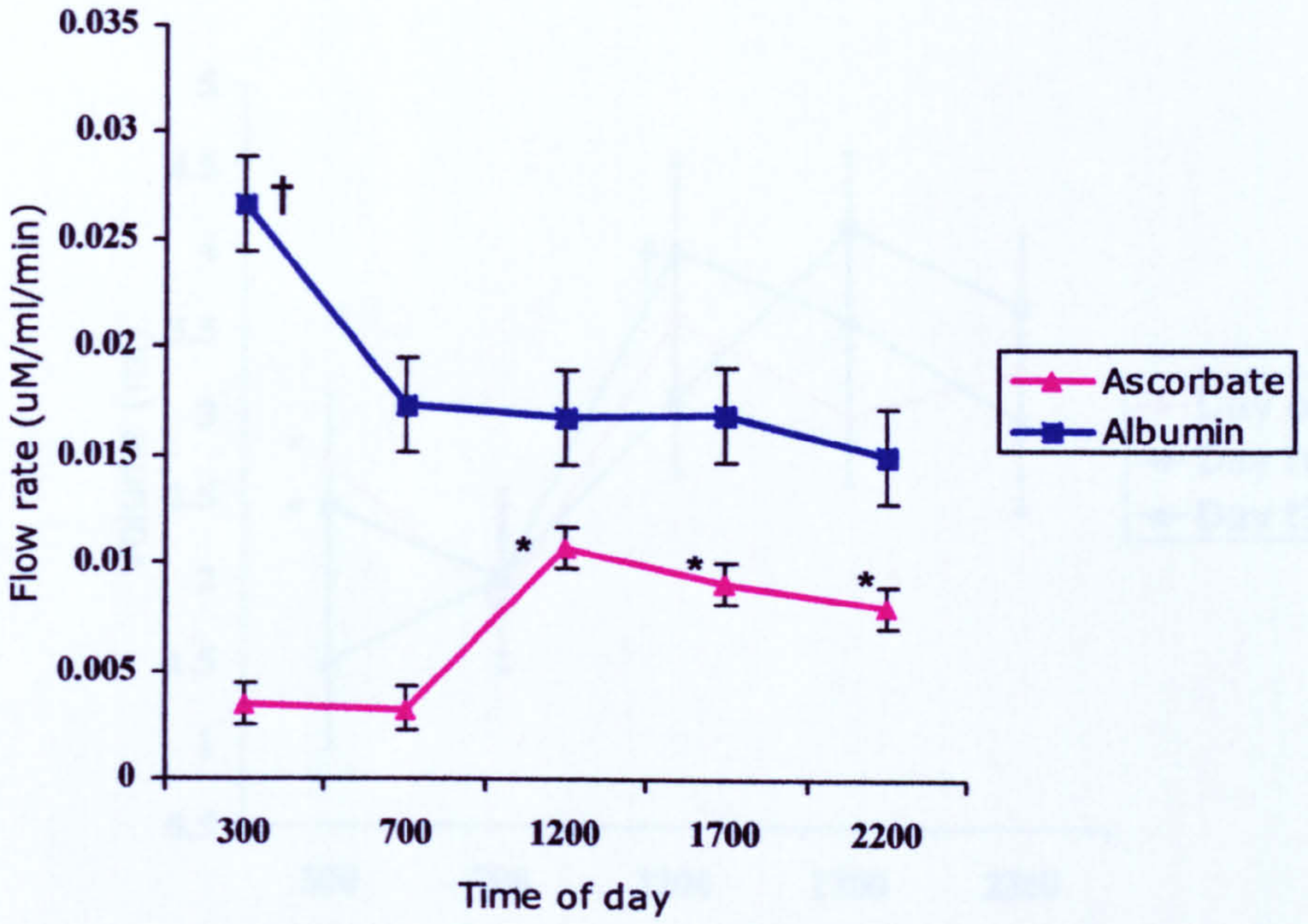
Data are mean ± S.E.M. * denotes significantly higher saliva flow rate compared to 0300 and 0700 hours ($p<0.05$). Data generated using a paired samples T-test.

Figure 3.3. Diurnal variation of TAA and urate flow rates in saliva



Data are mean ± S.E.M. * denotes significantly higher TAA flow rate compared to 0300 and 0700 hours ($p<0.05$). † denotes significantly higher urate flow rate compared to 0300 and 0700 hours ($p<0.05$). Data generated using a paired samples T-test.

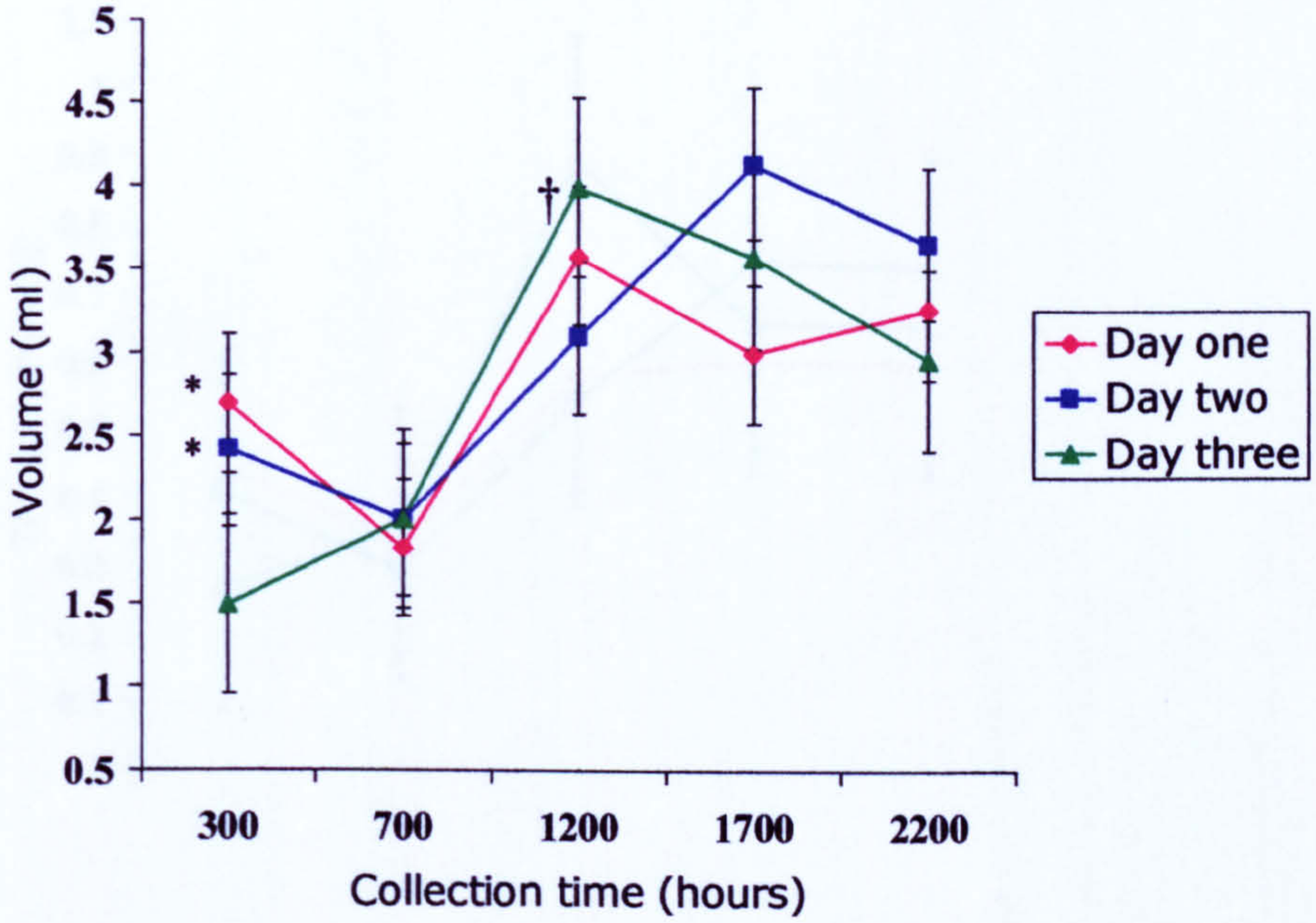
Figure 3.4. Diurnal variation of ascorbate and albumin flow rates in saliva



Data are mean ± S.E.M. * denotes significantly higher ascorbate flow rate compared to 0300 and 0700 hours ($p<0.05$). † denotes significantly higher albumin flow rate at 0300 compared to 0700, 1200, 1700 and 2200 hours ($p<0.05$). Data generated using a paired samples T-test.

Day to day variability in saliva volume was investigated to determine the extent of any possible daily variation at the time of collection. Variation in daily saliva flow can be seen in Figure 3.5. Saliva was significantly higher at 0300 hours on days one and two compared to day three, and at 1200 hours on day three compared to day two ($p<0.05$).

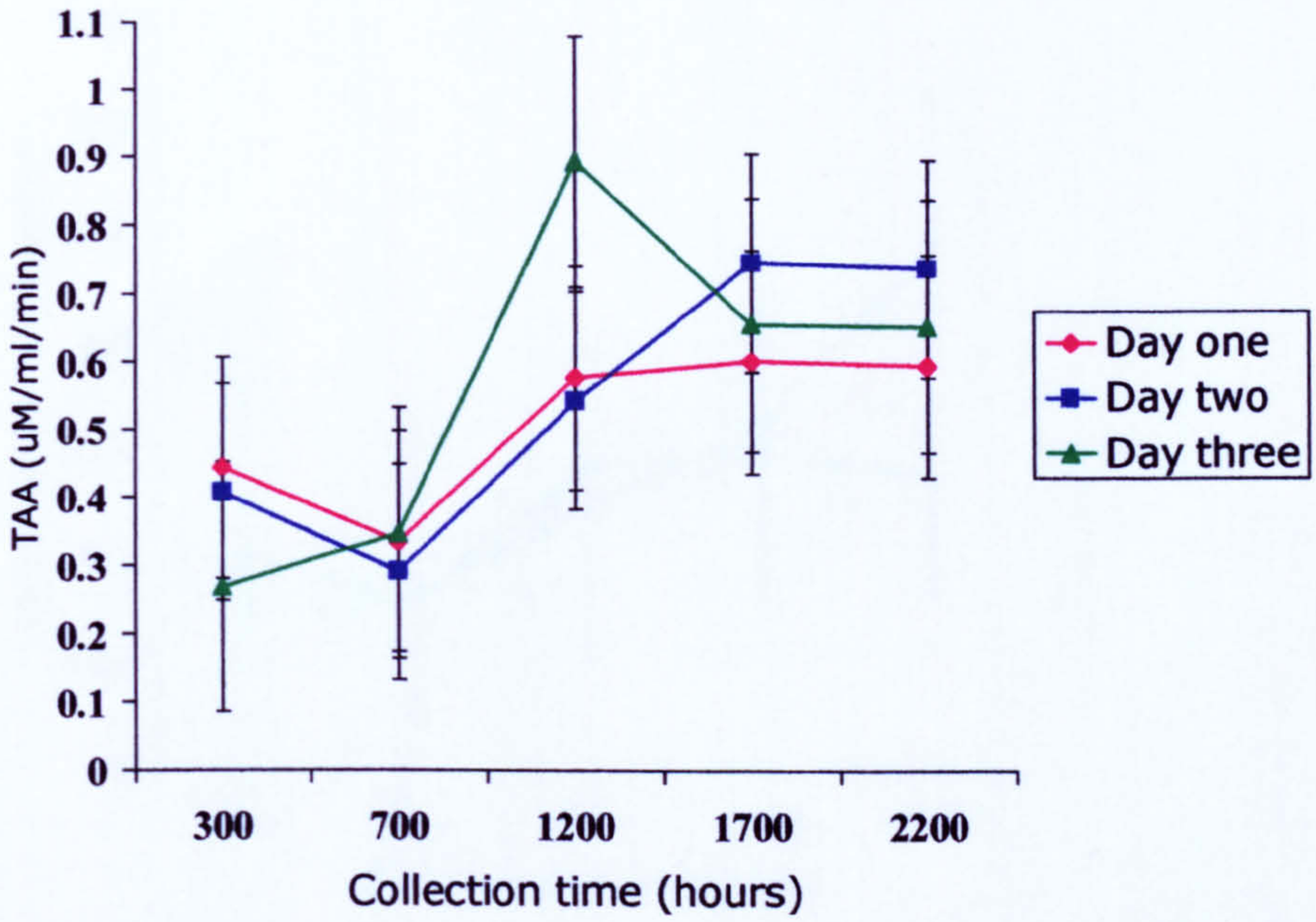
Figure 3.5. Variation in saliva volume over three days



Data are mean ± S.E.M. * denotes significantly higher volume compared to day three ($p<0.05$). † denotes significantly higher volume compared to day two ($p<0.05$). Data generated using a paired samples T-test.

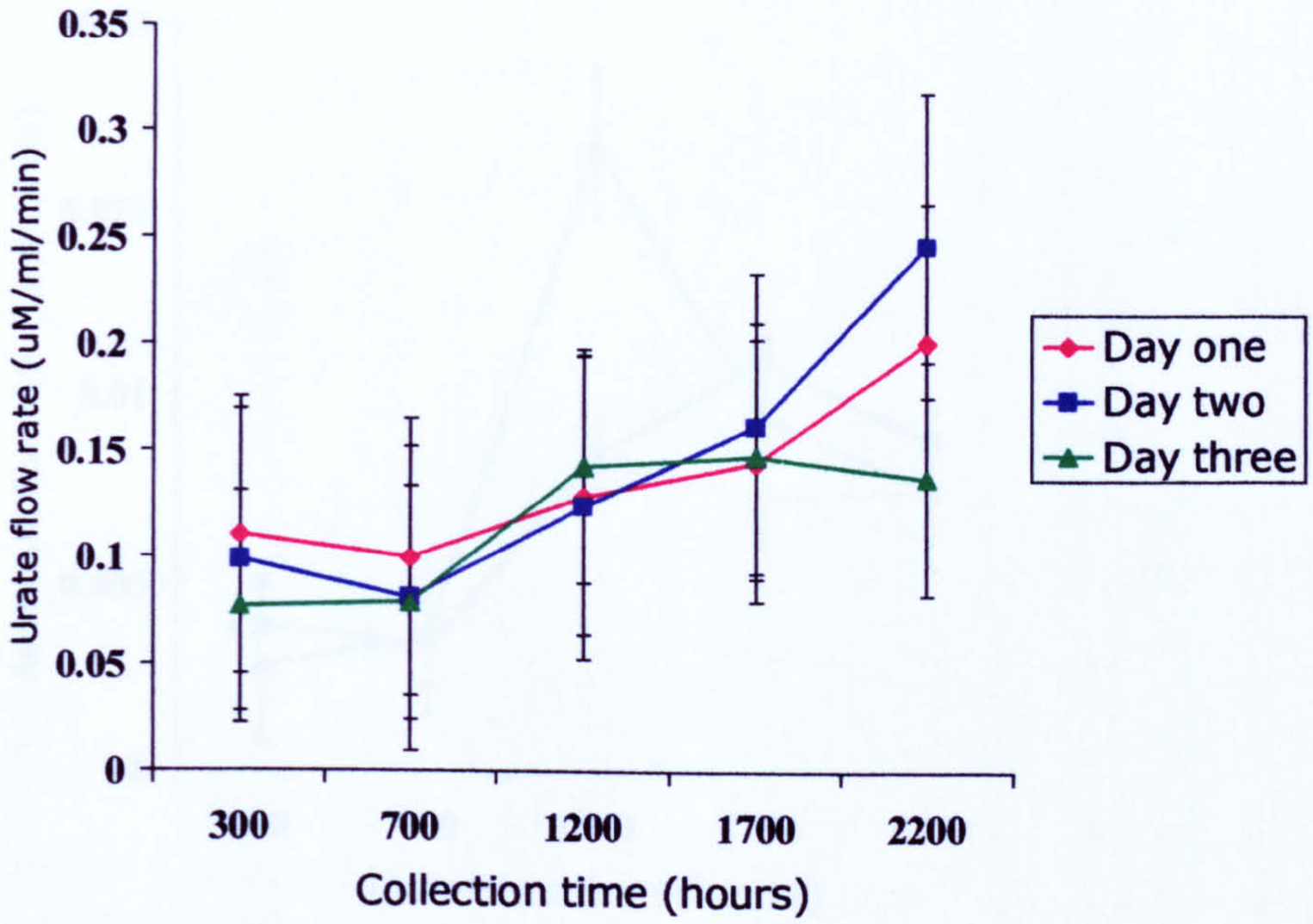
In addition, TAA, urate, ascorbate and albumin flow rate daily variability was determined. TAA variability can be seen in Figure 3.6. No significant differences were apparent in TAA flow rate between the three days. Similarly, no differences in urate flow rate were evident between the three days (see Figure 3.7). Ascorbate flow rate was significantly higher on day two compared to day three at 0300 hours ($p<0.05$) (see Figure 3.8). There were no differences in albumin flow rate between the three days (see Figure 3.9).

Figure 3.6 Variation in TAA flow rate over three days



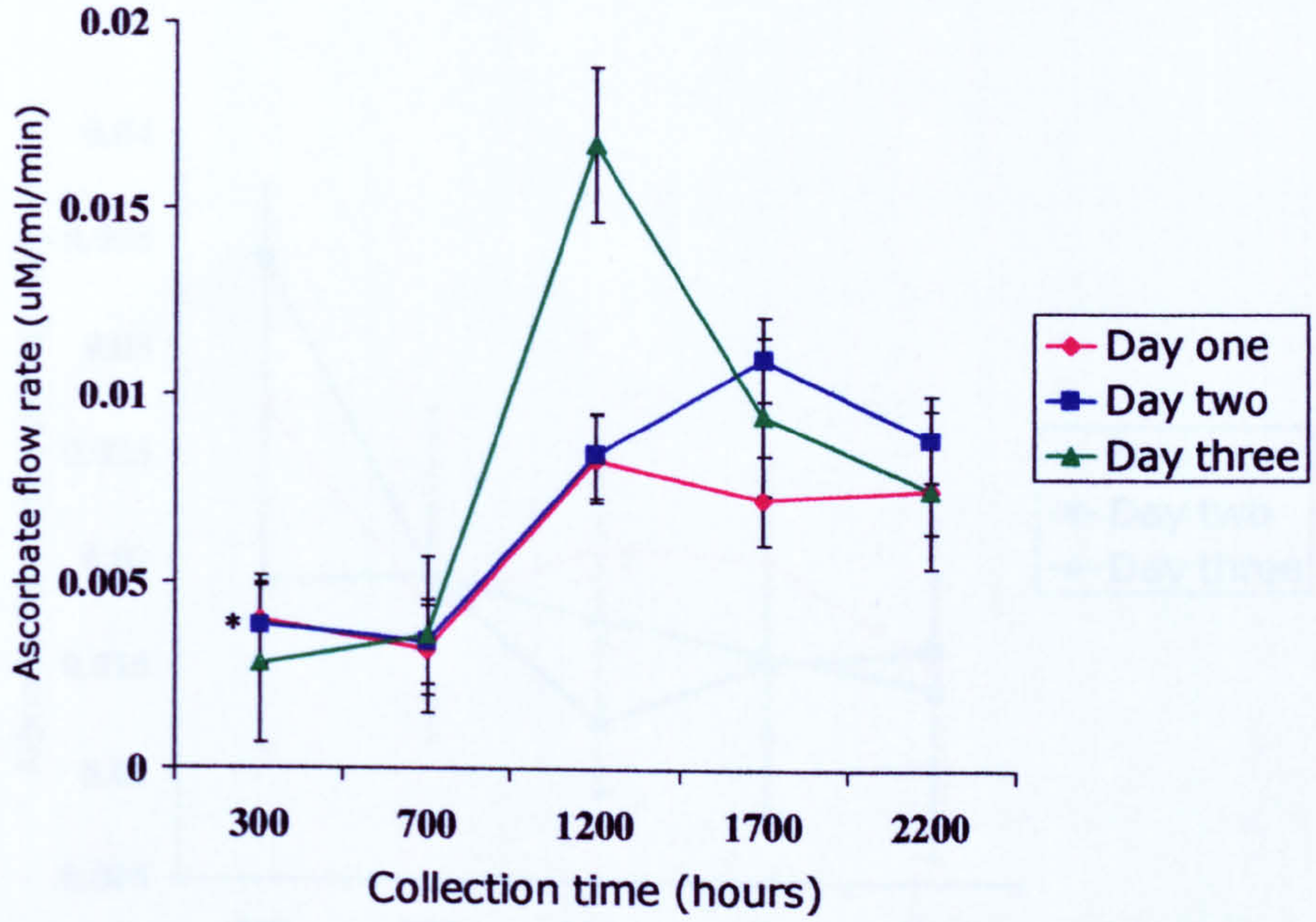
Data are mean ± S.E.M.

Figure 3.7 Variation in urate flow rate over three days



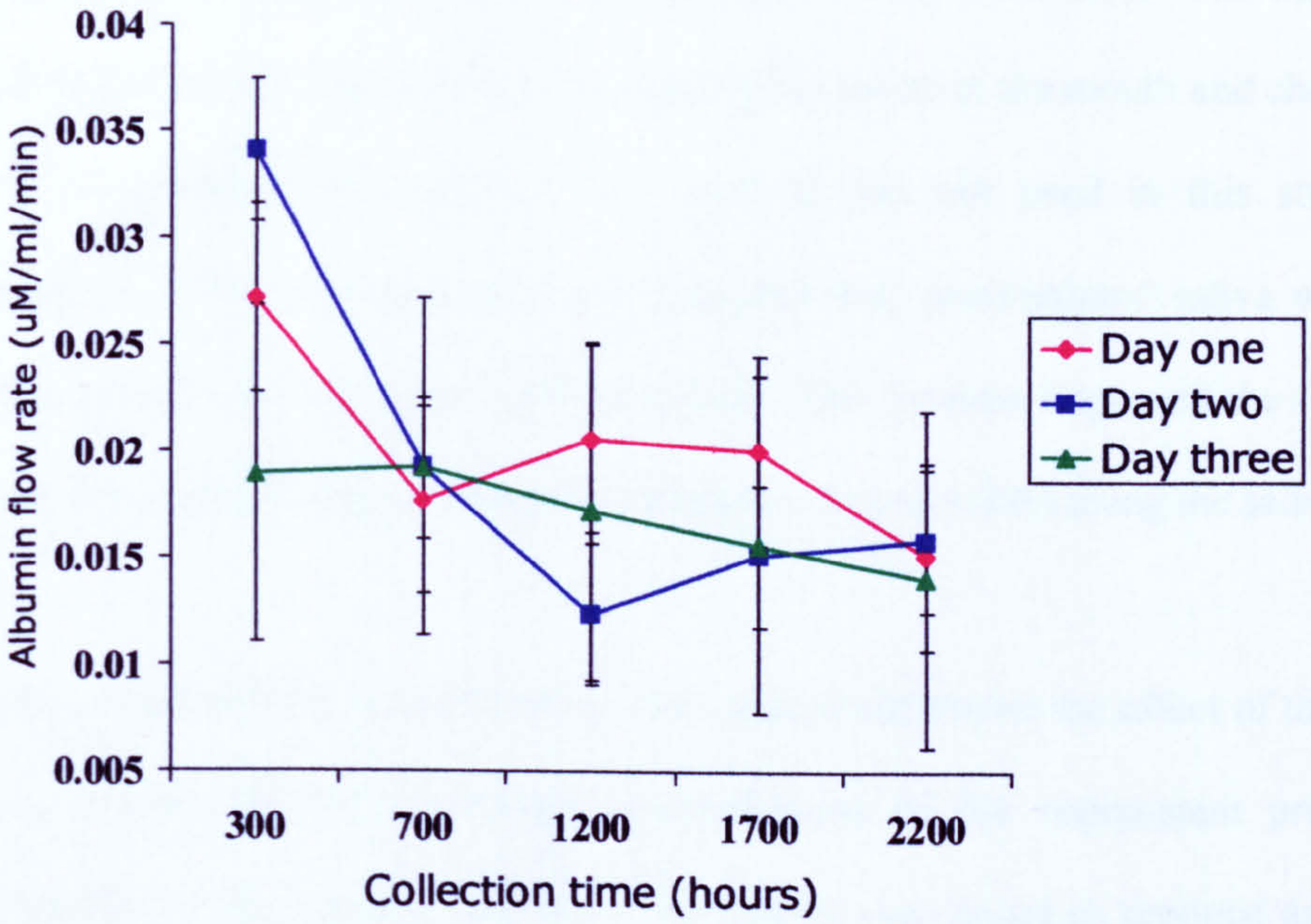
Data are mean ± S.E.M.

Figure 3.8 Variation in ascorbate flow rate over three days



Data are mean ± S.E.M. * denotes significantly higher ascorbate flow rate on day 2 compared to day three ($p<0.05$).

Figure 3.9 Variation in albumin flow rate over three days



Data are mean ± S.E.M.

3.7 Discussion

This study has investigated the antioxidant composition and delivery rates for antioxidants present in saliva. Salivary antioxidant status may provide an indication of potential susceptibility to disease conditions such as periodontal disease and oral cancers (Chapple *et al.*, 1997; Piyathilake *et al.*, 1995). The scavenging antioxidants present in saliva may provide protection from free radical-mediated oxidative damage within the oral cavity (Battino *et al.*, 1999). In addition, saliva flow plays an important role as it is the vector in which the antioxidants are delivered to oral tissues.

Unprocessed saliva has a high viscosity and may contain food particles and other debris. These components are detrimental to analytical processes. Saliva used in this study has been processed using 'Salivette' tubes (Sarstedt, Leicester, UK). The tubes are available with plain cotton inserts and cotton inserts treated with citric acid. The manufacturers' procedure for saliva collection involves placing the insert in the mouth and chewing gently until it is saturated with saliva. This method was not used in this study as only unstimulated saliva was analysed. To overcome this, unstimulated saliva was collected and then placed onto the insert until saturated. This process improved the coefficient of variance from 16.12% for pipetting unprocessed saliva to 4.40% using the salivette.

Analyses of saliva TAA concentrations were used to determine the effect of the inserts and filtering process on the antioxidant concentrations of the supernatant produced after centrifugation. The salivette with the cotton insert was found to produce an antioxidant profile similar to that of unfiltered saliva. The TAA of unfiltered saliva was 954 ± 2.55 μM whilst the cotton insert produced a TAA of 839.62 ± 5.26 μM. The citric acid insert impeded the FRAP and ascorbate assays, greatly reducing the antioxidant concentrations of these methods (TAA of 181.77 ± 4.34 μM) and no determination of ascorbate.

The Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996) was chosen for the determination of total antioxidant activity. FRAP was found to be inexpensive, quick to use, highly reproducible and have a low coefficient of variance. In addition, FRAP was effective at low antioxidant concentrations that are encountered in saliva. Moreover, TAA measured by FRAP was found to be significantly correlated with TAA determined using TEAC, ECL and TRAP. This suggests that although FRAP is

known not to detect some antioxidant species, such as glutathione (Janaszewska and Bartosz, 2002), it is an appropriate method to use in studies of salivary antioxidant status.

Saliva flow rates were calculated to enable antioxidant delivery rates to be determined. Mean saliva flow rate was 0.475ml/min. Male saliva flow was 0.545ml/min and was significantly higher compared to female saliva flow of 0.406ml/min ($p<0.01$). It is not known why females had a lower saliva production rate than males. Some factors that may be involved could include hormonal influences or simply a sense of embarrassment at having to engage in what is perceived to be an unsociable action. The consistency of the low volume recovery from women suggests that the former is the more likely explanation.

The major antioxidants found in saliva were urate, ascorbate and albumin. Urate was the major antioxidant component in saliva with a mean concentration of 184.30μM. Male urate concentrations were significantly higher (215.08μM) than those of females (154.45μM) ($p<0.01$). Ascorbate and albumin concentrations were lower at 8.10μM and 10.67μM respectively. There was a trend for higher ascorbate and albumin concentrations in males, but this was not significant. The higher TAA appeared to be due to the concentration of urate.

Antioxidant delivery rates were calculated as μmoles/ml/min. This figure was deemed more relevant than simple concentrations when referring to salivary antioxidant concentration, as it takes into account the saliva flow. This will indicate the rate at which the antioxidants can be delivered to the tissues in the oral cavity and so provide protection against oxidative damage. Mean TAA delivery rate was 0.3059μmoles/ml/min. Once again, in males, TAA delivery rate was significantly higher than in females ($p<0.01$). In

males urate delivery rate was 0.1267μmoles/ml/min and was significantly higher than the female rate of 0.0626μmoles/ml/min ($p<0.01$). Mean urate delivery rate was 0.0942μmoles/ml/min. Mean ascorbate was 0.0042μmoles/ml/min. In males, ascorbate delivery rate was 0.0049μmoles/ml/min and was significantly higher than in females (0.0034μmoles/ml/min). Mean albumin delivery rate was 0.0050μmoles/ml/min. There was no significant difference between the male delivery rate (0.0089μmoles/ml/min) and female delivery rate (0.0042μmoles/ml/min).

The disparity between male and female antioxidant flow rates was intriguing. Initially, this seemed to be merely a result of increased saliva flow rate. However, absolute concentrations of antioxidants were also higher in males than in females, so other mechanisms must be responsible. As yet, these are unknown. There may be involvement of sex hormones in the regulation of female saliva flow rate and antioxidant status.

Previous research has indicated that saliva production follows a diurnal rhythm (Moore *et al.*, 1994). It is clear that saliva flow rate may have an influence on the antioxidant delivery rate, as the two are statistically and functionally related. Therefore, it was necessary to determine the nature and magnitude of daily saliva flow variations in order to standardise the collection times to remove the possibility of flow variation influencing the antioxidant delivery rates. Mean saliva volume was significantly lower at 0300 and 0700 hours compared to 1200, 1700 and 2200 hours ($p<0.05$), as were TAA and ascorbate delivery rates. Urate delivery rate was significantly lower at 0300 and 0700 hours compared to 1200 and 1700 hours. Albumin delivery rate was significantly lower at 0300 hours compared to 0700, 1200, 1700 and 2200 hours. Saliva flow peaked at 1700 hours and was lowest at 0700 hours. Once again, there were marked differences in the saliva

flow rates of males and females. Mean saliva flow was higher at all time points in males, with significant differences at 0300, 1700 and 2200 hours ($p<0.05$).

These data clearly indicate that the flow rate of saliva and the delivery of antioxidants to the oral cavity are related to the time of day. Flow rates are lower in the hours of sleep than by day. This is most likely to reflect the consumption of food and the intake of fluids that would alter hydration status, which is a determination of saliva production (Walsh *et al.*, 2004), but could also be attributable to hormones such as cortisol. Cortisol is secreted according to a diurnal pattern that mirrors that noted for saliva flow rate (Selmaoui and Touitou, 2003). In addition, saliva flow rates have been shown to decrease with an increase in cortisol concentration (Ishii and Nagagawa, 2001).

Diurnal saliva samples were collected over three days to investigate the extent of day-to-day variability. Significant variation occurred at 0300 and 1200 hours. However, as saliva volumes are taken into account when calculating antioxidant flow rates, this would be unlikely to have a detrimental effect on their delivery rates. In addition, the saliva collection times used throughout the majority of this thesis did not occur at these times. The cause of this variation may have been simply a result of hydration status. This is known to have a significant effect on saliva production rates (Walsh *et al.*, 2004).

This data has confirmed the results of previous research as to the antioxidant profile of saliva and the difference between male and female saliva flow rate. The most important outcome in this chapter has been the development and characterisation of a saliva collection protocol for use in further studies. It has introduced the use of salivette tubes as a method of making saliva less viscous and creating less error, whilst still making use of

unstimulated samples, hence refining sample processing technique. The cyclic diurnal rhythm of saliva flow has also been highlighted, emphasising the need for a standard approach to sampling in studies of salivary antioxidant status in relation to health. In the light of these findings presented, subsequent saliva collections will be made at specific times in order to reduce the error incurred by flow rate changes throughout the day. Minimal day to day variation in TAA suggests that single determinations provide a useful indicator of individual antioxidant status. In particular, this study has confirmed that the FRAP assay can be sensitive enough for measurement of low concentrations of antioxidants found in saliva, is relatively cheap and easy to use. This makes it an ideal tool for the determination of salivary antioxidant status.

CHAPTER 4

The association of salivary antioxidant status with periodontal disease

4.1 Introduction

Periodontal diseases such as gingivitis and periodontitis are some of the most common chronic conditions affecting the worldwide adult population (Ridgeway, 2000; Brown and Loe, 1993). Gum inflammation is symptomatic of gingivitis, and is caused by deposits of plaque on the tooth surface. Gingivitis affects 50% of the adult population (Ridgeway, 2000). Periodontitis is a progression of gingivitis and is characterised by gum recession, degradation of gum tissue and alveolar bone loss (Brown and Loe, 1993).

Periodontal disease is initiated by three major bacterial pathogens, *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Bacteroides forsythus*, which invade the oral cavity and colonise the gum area (Lamont and Jenkinson, 1998). Of these, *P. gingivalis* is the most significant as it has the capacity to induce immune responses that compromise the integrity of host tissue, whilst inactivating the repair process (Jenkinson and Dymock, 1999; Fravalo *et al.*, 1996). The bacterial stimulus results in the recruitment of activated neutrophils into the infected area, where they release reactive oxygen species into the surrounding extracellular space (Chapple *et al.*, 1997). *P. gingivalis* also immobilizes the neutrophils, therefore concentrating the ROS-mediated tissue damage (Fredriksson *et al.*, 1998;Guarnieri *et al.*, 1991). A further compounding factor is *P. gingivalis'* ability to produce antioxidant enzymes to combat the cytotoxic action of the neutrophil-derived free radicals (Lamont and Jenkinson, 1998).

The tissue damage caused by free radicals has been proposed to be higher in those with periodontal disease as a result of a reduced antioxidant capacity (Chapple *et al.*, 1997; 2002). An amplified neutrophil response may be a causative factor involved in the extensive tissue damage encountered in some cases of periodontal disease (Fredriksson *et al.*, 1998). Nevertheless, a diminished antioxidant capacity could result in elevated oxidative damage to the gingiva. This reduction in antioxidants may be caused by factors such as smoking or a poor diet, which are known to impact upon circulating antioxidants (Tappia *et al.*, 1995).

Previous studies have found a reduction in the total antioxidant capacity of whole saliva in patients with periodontal disease (Chapple *et al.*, 1997), and lower concentrations of glutathione in gingival crevicular fluid (Chapple *et al.*, 2002). However, there is some disparity between these results and those of Moore *et al.* (1994) who found similar antioxidant profiles in patients with periodontal disease and those classed as healthy. Furthermore, although there are suggestions that antioxidant capacity is lower in individuals with periodontal disease, there has been no assessment as to whether increased free radical-mediated tissue injury is a feature of the condition.

The aim of this study was to determine whether a relationship exists between total antioxidant status in whole saliva and periodontal disease status, and establish the relationship between periodontal disease and biomarkers of oxidative injury.

4.2 Objectives

The determination of periodontal disease severity in previous studies has been both ill-defined and compromised by small study cohorts. There has also been some disparity in

the methods used to determine the TAA in saliva. In addition, no previous research has investigated the biomarkers of oxidative tissue injury in relation to periodontal disease. The principle objectives to be addressed in this chapter are:

- To categorise periodontal disease severity using the CPITN assessment criteria in a large study population and determine the relationship between disease severity and the TAA of saliva.
- To determine the contribution to TAA made by individual antioxidants in saliva.
- To develop and utilise the measurement of protein carbonyls as a biomarker of oxidative tissue damage.

4.3 Materials and Methods

4.3.1 Recruitment of cohort

One hundred and twenty nine dental patients attending for routine examination at the Wilson House Dental Practice in Newport Pagnell, UK were recruited for the study. The age range of the subjects was 39-76 years (mean 58.5 years), and the cohort consisted of 64 males and 65 females. All subjects were white Caucasians. Those patients taking nutritional supplements were excluded from the study, as were those who had just received dental treatments. This was to remove the possibility of artificially high intakes of nutrients and contamination from debris or anaesthetic respectively. All participants gave written informed consent before they took part in the study. Recruitment took place over an eleven-month period between September 2001 and August 2002. Saliva samples were always collected between 1pm and 5pm, in order to minimise the impact of diurnal variation in antioxidant status (see section 3.6). Ethical approval was obtained from the Milton Keynes Medical Ethics Committee.

4.3.2 Saliva collection protocol

All saliva collections followed the same procedure based on Navazesh (1993) as described in section 2.4. Unstimulated saliva was collected over a five-minute period and stored in dry ice (-79°C) whilst at the dental practice. Upon completion of the collections, the samples were taken and stored at -80°C until analysed.

4.3.3 Saliva processing

Before analysis, saliva was processed using salivettes as described in section 2.4, to remove debris and reduce viscosity. The supernatant was then analysed for TAA, ascorbate, urate and albumin concentrations and protein carbonyl content (see section 2.5). Salivary prostaglandin E_2 concentrations were determined (see section 2.5.6), as an index of local inflammation.

4.3.4 Classification of periodontal health status

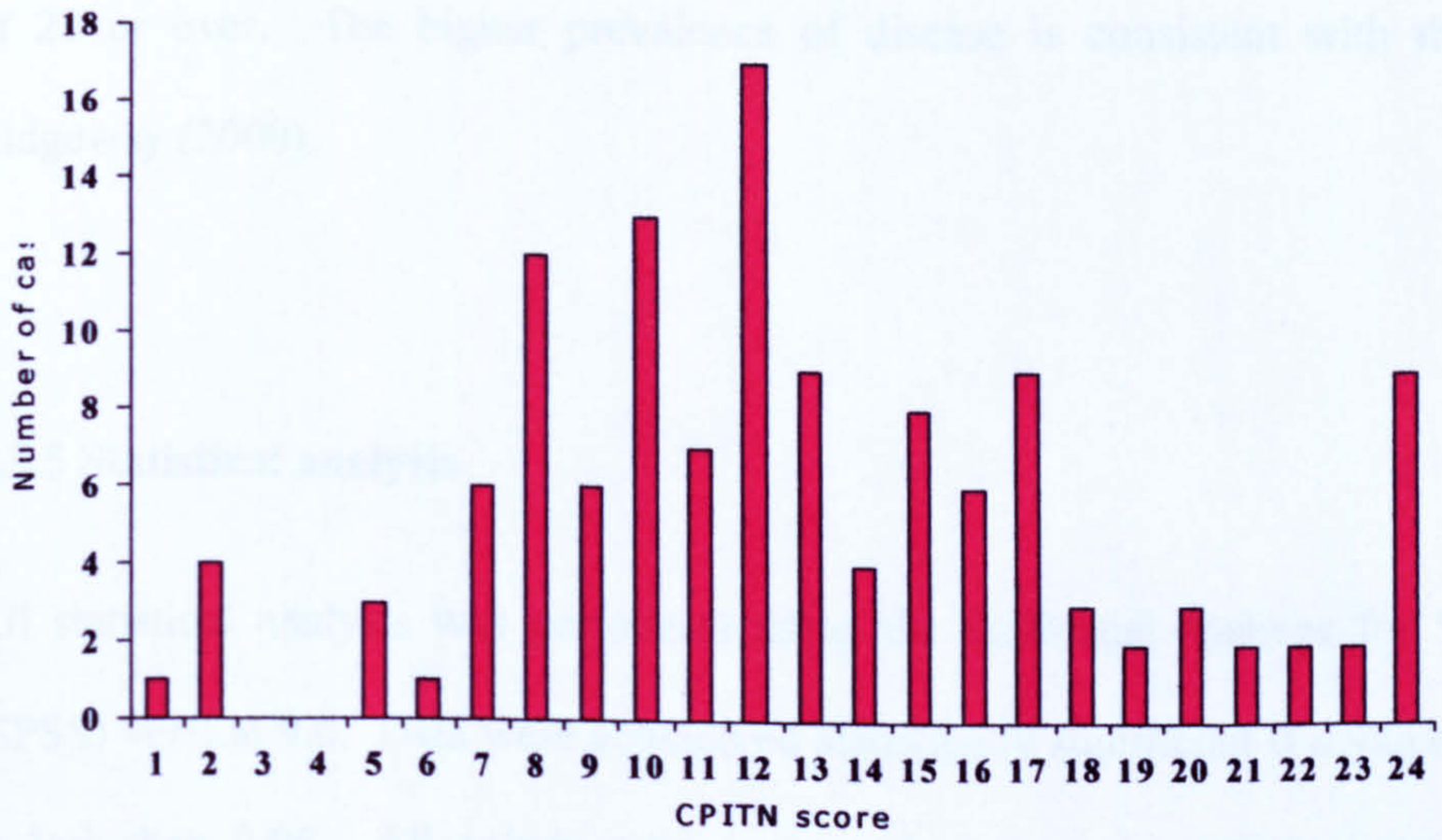
To accurately assess the extent of periodontal disease in dental patients, an adaptation of the Community Periodontal Index of Treatment needs (CPITN) was used (Ainamo *et al.*, 1982). This method involves the insertion of a graduated probe between the tooth and gum at a standard of no more than 0.25N to measure the depth of any periodontal pocket (Almas *et al*, 1991). In addition, a visual inspection of the sub-gingival tooth surface was made to assess the accumulation of plaque and calculus. The determination of periodontal pocket depth was made for sextants of the dentition and excluded third molars unless the second molars were missing. The scoring criteria ranged from 0 (no bleeding, pocketing or plaque retention) to 4 (extensive disease, pockets >5.5mm). The sum of the sextant scores was calculated and subtracted from a theoretical maximum of 24 to give the final score.

Thus, a CPITN score of 24 would indicate no disease, with a low score signifying disease.

The distribution of CPITN scores in the study population is shown in Figure 4.1.

Figure 4.1. Distribution of CPITN scores in the study population

Severe disease (n=46)	Moderate disease (n=37)	Healthy or mild disease (n=46)



Total number of cases = 129. CPITN scores – 0 = severe disease to 24 = healthy.

The distribution shows only the sum of the individual sextant scores as this was the only information made available by the dentist involved. This provides an overview of the periodontal health of the patient. Nevertheless, it is possible that scores towards the middle of the distribution may be arrived at in different ways. For instance, a CPITN score of 12

may indicate that all sextants had an individual score of 2. This would be classified as mild disease, with some plaque retention but no evidence of periodontal pockets. However, a score of 12 may also indicate a score of 4 in three sextants, which equates to severe disease, with the others being completely healthy. Whilst this scenario is unlikely, differences may occur due to eating habits that could lead to asymmetrical disease distribution within the oral cavity. The distribution shown in Figure 4.1 also emphasises the fact that the arbitrary distribution of subjects into diseased and healthy groups by Moore *et al.* (1994) is inappropriate. In this study population, representing a random selection of those attending for routine checks, only 15 patients (12%) had CPITN scores of 21 or over. The higher prevalence of disease is consistent with that reported by Ridgeway (2000).

4.3.5 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were considered statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Where covariates (salivary flow, sex) had a significant impact upon the outcome of the analyses, data are presented as marginal means adjusted for these variables, calculated using a univariate analysis. In all other cases, means were compared using one-way ANOVA with an LSD posthoc test, or an independent samples t test, as appropriate. The relationship between periodontal disease status and antioxidant-oxidative balance was also assessed using stepwise linear regression models, with a significance level for inclusion of $P<0.05$ and a removal probability of 0.1.

Binary logistic regression was used to determine the contribution of total antioxidant capacity to the risk of developing clinically significant periodontal disease (CPITN<18). The CPITN figure of 18 was used as a threshold for disease as this would indicate an average score for each sextant of 3 (Plaque retention and pockets >3.5mm but <5.5mm). Even if the disease distribution within the mouth is not uniform, a score of below 18 would indicate that at least 3 of the sextants had severe disease and pocketing >5.5mm, whilst the other 3 sextants displayed plaque retention. Using this threshold, 82% (n=106) subjects were classified as diseased. Tertiles of total antioxidant flow rate, sex and smoking habit were treated as categorical data in this analysis. The logistic regression model was generated with all three variables entered (Miles and Shevlin, 2001).

4.4 Results

4.4.1 Patient disease classification and antioxidant status

After dental examination and disease classification of the study cohort, the CPITN scores were used to group the patients into tertiles. These were categorised as healthy or mild disease (CPITN >14, $n = 46$), moderate disease (CPITN 11-14, $n = 37$) and severe disease (CPITN < 11, $n = 46$). The mean age was similar in each group but more smokers were present in the severely diseased tertile compared to the moderate disease group. There were no smokers in the healthy or mild diseased tertile (see Table 4.1).

TAA flow rate was significantly higher in the healthy or mild disease tertile compared to the severely diseased group ($p<0.05$) after a correction for sex was applied (see Table 4.1). Severe disease was associated with a 22% reduction in TAA flow rate. TAA, ascorbate

flow rate and albumin flow rate did not differ significantly between the three groups. Urate flow rate was significantly higher in the healthy or mild disease tertile when compared to the severely diseased group ($p<0.05$). Salivary protein carbonyl concentrations, once adjusted for sex, were significantly higher in the severely diseased tertile compared to the other two groups ($p<0.05$) (see Figure 4.2). Salivary flow rates were similar in all groups (see Table 4.1).

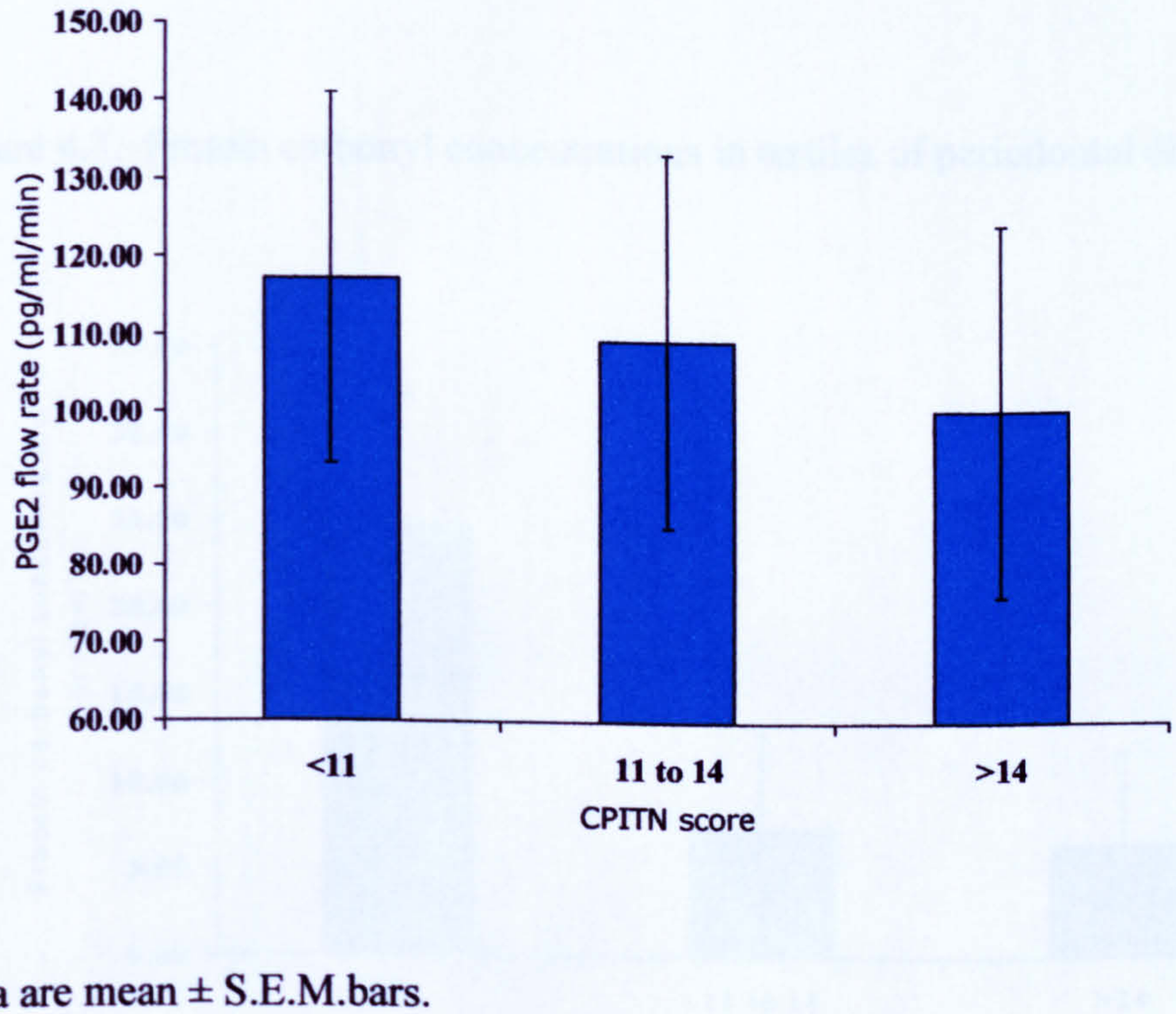
Table 4.1. Salivary antioxidant status in tertiles based on CPITN score

	Tertile of CPITN score		
	<11 (n=46)	11-14 (n=37)	>14 (n=46)
Age (years)	59.6 ± 1.3	57.4 ± 1.5	60.3 ± 1.3
TAA (µmol/litre)	593 ± 29	601 ± 32	605 ± 29
TAA flow rate ($\mu mol \cdot ml^{-1} \cdot min^{-1}$)	0.21 ± 0.02	0.26 ± 0.02	0.27 ± 0.02*
Ascorbate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	3.59 ± 0.44	3.59 ± 0.50	3.52 ± 0.48
Albumin flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	4.99 ± 0.84	4.96 ± 0.99	4.11 ± 0.95
Urate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	66.9 ± 10.8	86.6 ± 12.5	101.8 ± 12.2*
Salivary volume (5 mins)	2.23 ± 0.21	2.21 ± 0.17	2.22 ± 0.15
Smokers (n (%))	7 (15.2)	3 (8.1)	0

Subjects were grouped into tertiles according to their CPITN score. Data shown for age and salivary flow are unadjusted means ± (S.E.M). Antioxidant data are adjusted for sex; protein carbonyls are adjusted for sex and salivary flow rate. * denotes significant difference ($p < 0.05$) when compared to lowest tertile.

Prostaglandin E_2 is a known mediator of bone resorbtion (Raisz *et al.*, 1974), and has been linked with the degradation of alveolar bone tissue typical of severe periodontal disease (Leibur *et al.*, 1999). A one-way ANOVA was used to determine whether prostaglandin E_2 flow rate in whole saliva was associated with periodontal disease. A clear trend of increasing prostaglandin E_2 flow rate with severity of periodontal disease was observed in the tertile groups (see Figure 4.2). However, this trend failed to reach statistical significance. This is not altogether surprising as only a small number of samples (29 from a cohort of 129) were used in the determination of prostaglandin E_2, due to the cost of the assay.

Figure 4.2. Prostaglandin E_2 flow rate in tertiles of periodontal disease

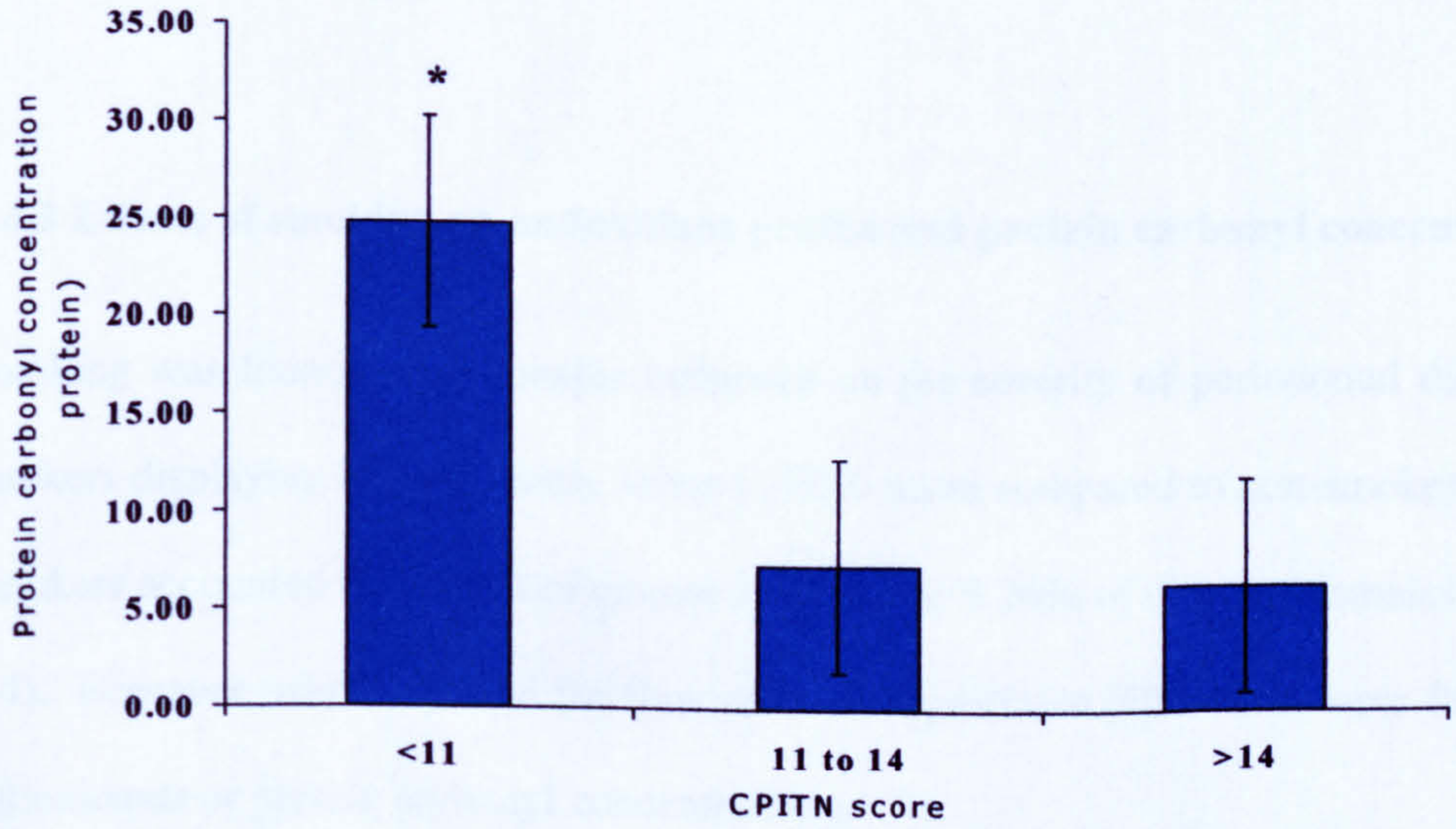


Data are mean ± S.E.M.bars.

4.4.2 Salivary antioxidant status and periodontal disease in male and female participants

Analysis of saliva samples exposed a significant difference in antioxidant profile between male and female patients. TAA was significantly higher in males when compared to females ($p=0.002$). Male patients also had significantly higher TAA flow rates and urate flow rates tended to be higher in males compared to females, but this failed to achieve statistical significance. Salivary flow rate was higher in males compared to that of females ($p=0.001$). Mean protein carbonyl concentration was higher in females but this was not significant. CPITN scores were similar in both male and female groups, as was the prevalence of disease (see Table 4.3).

Figure 4.3. Protein carbonyl concentrations in tertiles of periodontal disease



* denotes significant difference in carbonyl concentration relative to all other groups ($p<0.05$).

Table 4.2 Salivary antioxidant status and periodontal disease in male and female participants

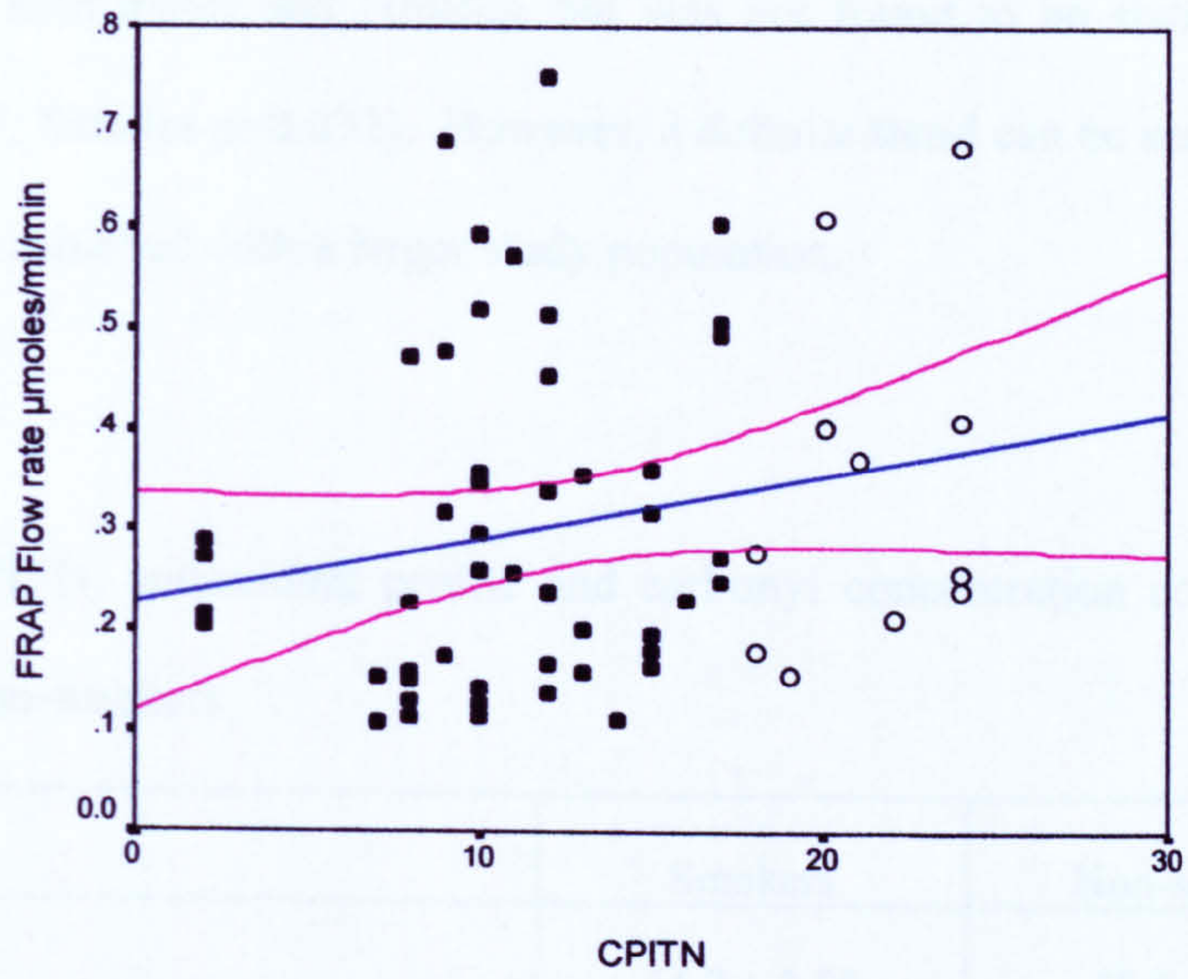
	Men (n = 64)	Women (n = 65)	P
Age (years)	59.8 ± 1.0	57.2 ± 0.9	0.058
CPITN score	12.3 ± 0.7	13.6 ± 0.6	0.173
TAA (µmol/litre)	654 ± 25	545 ± 23	0.002
Protein carbonyls (fmol/g of protein)	8.46 ± 1.71	19.26 ± 7.09	0.104
TAA flow rate ($\mu mol \cdot ml^{-1} \cdot min^{-1}$)	0.31 ± 0.02	0.19 ± 0.01	<0.001
Ascorbate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	4.27 ± 0.44	2.88 ± 0.30	0.011
Albumin flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	5.44 ± 0.74	4.03 ± 0.74	0.180
Urate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	108.8 ± 12.0	58.7 ± 6.6	<0.001
Salivary flow rate (ml/min)	2.58 ± 0.16	1.87 ± 0.11	0.001
Smokers (n (%))	5 (7.8)	5 (7.7)	
Diseased (n (%))	52 (81.3)	54 (83.1)	

Data shown are means ± SEM. Subjects were classified as diseased if CPITN score was below 18.

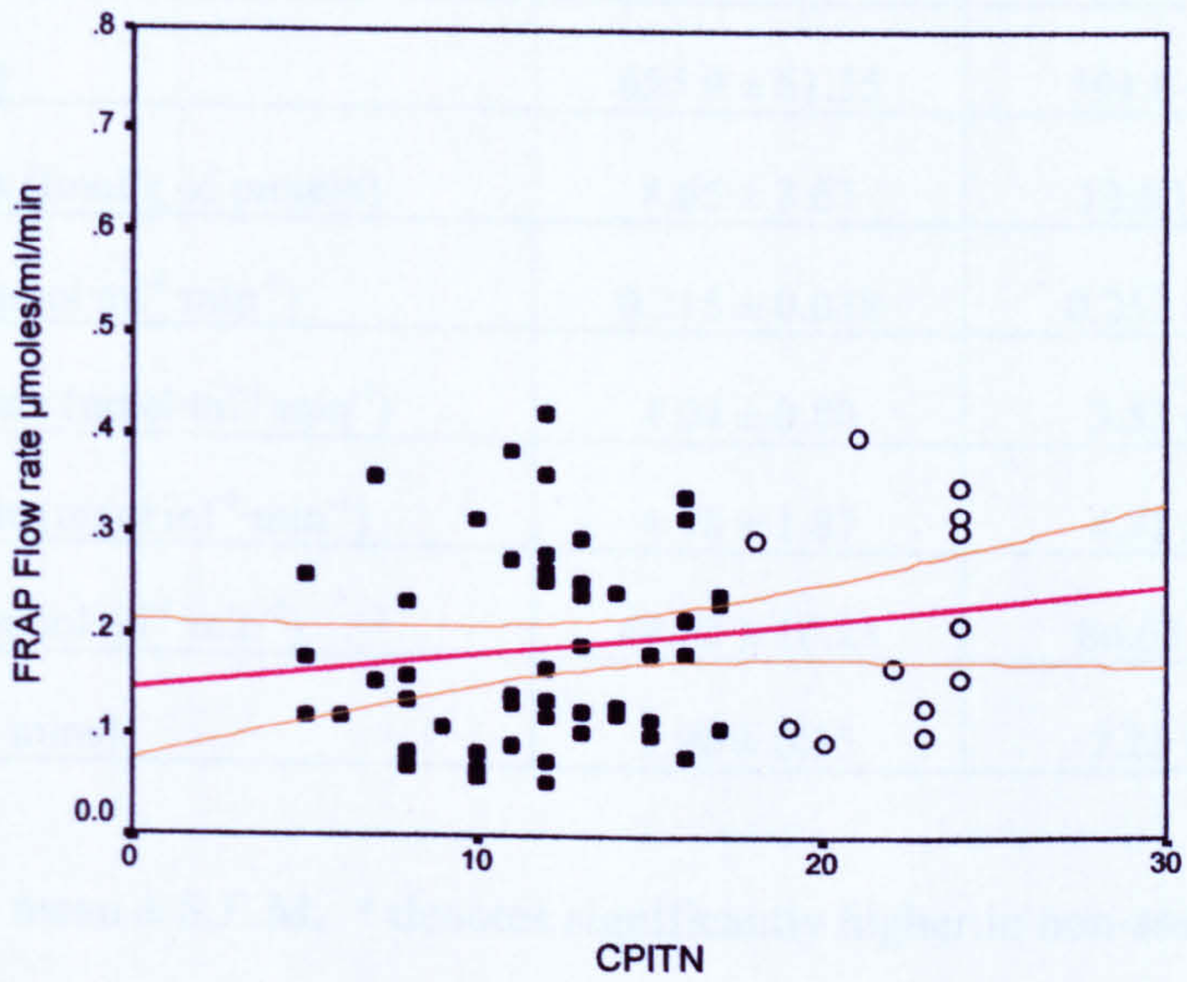
4.4.3 Effects of smoking on antioxidant profile and protein carbonyl concentration

Smoking was found to be a major influence on the severity of periodontal disease, with smokers displaying a significantly lower CPITN score compared to non-smokers ($p<0.05$). Smokers accounted for 9.62% of diseased males and 9.26% of diseased females (see Table 4.4). However, when adjusted for flow rates, no significant differences were found in any antioxidants or protein carbonyl concentrations.

Figure 4.4. Male and female CPITN and TAA flow rate correlations



Male
P=0.057
R=0.207



Female
P=0.072
R=0.185

Figure shows trendline and 95% confidence intervals. CPITN scores are 0=severely diseased to 24=healthy. N=64 males, 65 females. Open circles indicate healthy, closed circles indicate diseased.

4.4.4 CPITN and TAA flow rate correlations

As can be seen in Figure 4.4, TAA flow rate is associated with a higher CPITN score. This is apparent in both males and females, but was not found to be statistically significant (males p=0.057; females p=0.072). However, a definite trend can be seen and significance may have been achieved with a larger study population.

Table 4.3. CPITN, antioxidant profile and carbonyl concentration comparison between smokers and non-smokers

	Smokers	Non-smokers
Age (years)	54.7 ± 2.60	58.8 ± 0.68
CPITN score	7.30 ± 1.34	13.46 ± 0.48*
TAA (μmol/litre)	655.9 ± 61.55	594.8 ± 18.44
Protein carbonyls (fmol/g of protein)	8.65 ± 3.67	13.60 ± 3.57
TAA flow rate ($\mu mol \cdot ml^{-1} \cdot min^{-1}$)	0.215 ± 0.038	0.252 ± 0.014
Ascorbate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	4.04 ± 0.80	3.53 ± 0.29
Albumin flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	4.78 ± 1.87	4.71 ± 0.55
Urate flow rate ($nmol \cdot ml^{-1} \cdot min^{-1}$)	49.32 ± 10.23	86.63 ± 7.75
Saliva volume (5 mins)	1.90 ± 0.35	2.25 ± 0.11

Data shown are mean ± S.E.M. * denotes significantly higher in non-smokers compared to smokers ($p<0.05$).

4.4.5 Regression modelling

To determine the risk factors that had the strongest impact on periodontal disease state, a stepwise linear regression model was employed. Briefly, this allows the influence of many independent variables to be evaluated simultaneously and will exclude those that do not have a significant influence on the dependant variable. This statistical approach produced two statistically significant models as shown in Table 4.4. Smoking was found to have the greatest single influence on patients' CPITN score (p=0.003), with protein carbonyl concentration also emerging as a significant factor in the model (p=0.013). With smoking and protein carbonyl concentrations together in the model, the relationship was reinforced (p=0.001).

Table 4.4. Stepwise linear regression modelling of predictors of periodontal disease status

Model	Significant factors	Standardised coefficients	*P*	F	P
1	Smoking	-0.410	0.003	10.1	0.003
2	Smoking	-0.426	0.001		
	Protein carbonyls	-0.315	0.013	8.9	<0.001

Dependant variable = CPITN score. Variables excluded were TAA, TAA flow rate, urate flow rate, ascorbate flow rate and saliva flow rate.

Using the same linear regression approach to investigate the elements involved in protein carbonyl concentration, it was shown that the principal influences were low CPITN score (p=0.018) and female sex (p=0.020). When combined, these factors strengthened the association (p=0.007) (see Table 4.5).

Table 4.5. Stepwise linear regression modelling of salivary protein carbonyl concentrations

Model	Significant factors	Standardised coefficients	*P*	F	P
1	CPITN	-0.294	0.035	4.716	0.035
2	CPITN	-0.318	0.018		
	Sex	-0.311	0.020	5.45	0.007

Dependant variable = protein carbonyl concentration. Variables excluded were age, smoking, TAA, TAA flow rate, urate flow rate, ascorbate flow rate, albumin flow rate and saliva flow rate.

Binary logistic regression modelling was employed after patients had been placed into two categories, little or no disease (CPITN 18 or over) or diseased (CPITN 17 or lower). In order to calculate the risk of disease associated with low antioxidant status, patients were grouped into tertiles of TAA flow rate (see Table 4.6). Risk of developing periodontal disease was significantly associated with the lowest TAA flow rate (p=0.043) compared to the baseline highest TAA flow rate group (Odds Ratio = 4.46, 95% confidence interval 1.04-19.15). There was no significant risk of periodontal disease associated with TAA flow rate in the mid range (0.15 – 0.278 μmol/ml/min). The association of low TAA flow rate with disease was persistent after adjustment for smoking and sex.

Table 4.6. Logistic regression modelling of the contribution of TAA flow rate to risk of periodontal disease.

	Odds Ratio	P	95% Confidence intervals
TAA flow >0.278 (µmol/ml/min)	1.0		
TAA flow 0.159-0.278 (µmol/ml/min)	1.60	0.414	0.52-4.94
TAA flow <0.159 (µmol/ml/min)	4.46	0.043	1.04-19.15

Categories were diseased (CPITN 17 or lower) and little or no disease (CPITN 18 and above). Significant association of low TAA flow (<0.159 µmol/ml/min) and periodontal disease ($p<0.05$). Model is adjusted for smoking and sex.

4.5 Discussion

This research has investigated the possible relationship between the antioxidant capacity of saliva and periodontal disease status. An enhanced salivary antioxidant capacity may provide protection against the free radical-mediated tissue damage that is proposed to occur in periodontal disease (Chapple *et al.*, 1997; Battino *et al.*, 1999). The work has a number of novel aspects that were intended to resolve the discrepancies between existing studies in this area. Primarily, this study used a much larger cohort than has previously been used (Moore *et al.*, 1994; Chapple *et al.*, 1997).

In addition, the detection of salivary protein carbonyl concentrations was used as a biomarker of oxidative damage. The measurement of protein carbonyls in saliva was a novel technique for assessing oxidative protein damage in the oral cavity. This was a particularly appropriate technique as it detects metabolites that dissolve readily in saliva.

Despite this, the protein carbonyl concentration in saliva may be an underestimate of true oxidative damage. Most protein oxidation in periodontal disease is likely to occur within the periodontal pockets. These may not be well bathed in saliva on a regular basis and so oxidised products are not readily available for detection. A higher protein carbonyl concentration may be found by more direct sampling around the periodontal pocket area, for example using GCF collection (Chapple *et al.*, 1997). However, this would be time-consuming, more invasive and would require specialist equipment. These findings have been achieved using easily collected whole saliva. The observed increase in protein carbonyls of 3-4 fold may be an indicator of quite severe free radical injury in diseased patients.

The limited research previously conducted in this area has not employed a standardised method for assessment of periodontal disease (Moore *et al.*, 1994; Chapple *et al.*, 1997). The inaccurate classification of disease state used in these studies may account for the conflicting results attained. The method of disease classification used in this study was the Community Periodontal Index of Treatment Needs (CPITN) and is utilised by the majority of dental practices in the UK. This method was developed in conjunction with the Oral Health Unit of the World Health Organisation (WHO) (Ainamo *et al.*, 1982), and provides a detailed assessment of the dental and gingival condition.

For much of the analysis the patients were placed into one of three groups, depending on their CPITN scores. These were categorised as healthy or mild disease, moderate disease and severe disease. The CPITN distribution seen in the study population (see Figure 4.1) highlights the fact that very few individuals area completely disease free (Ridgeway, 2000). Only 9 from a study cohort of 129 scored a maximum 24 points. This would imply

that previous studies using 'apparently healthy' controls may have been flawed and that the control group may have included diseased individuals (Moore *et al.*, 1994).

The CPITN system for the categorisation of periodontal disease is not a foolproof system. In the healthy or mild disease range, as indicated in this study as a CPITN score of between 14 and 24, it is quite possible to have severe localised disease. However, this is unlikely as it would involve the isolation of the pathogenic factor to just one or two sextants. The low variation in TAA flow observed in the study population seems to reinforce this fact, as one would expect this factor to decrease in the presence of increased oxidative activity found in severely diseased gingiva. Despite the inherent shortcomings, the CPITN system remains the most effective method of periodontal examination of large populations (Ainamo *et al.*, 1982).

TAA, ascorbate, albumin and urate delivery rates were calculated along with protein carbonyl concentrations. TAA delivery rate was significantly higher in the healthy or mild disease group (0.27 ± 0.02 μmol/ml/min) compared to the severely diseased (0.21 ± 0.02 μmol/ml/min) ($p<0.05$), after adjustment for sex. Urate delivery rate was also higher in the healthy or mild disease group (101.8 ± 12.2 nmol/ml/min) compared to the severely diseased (66.9 ± 10.8 nmol/ml/min). This, along with the regression analysis, indicates that much of the difference in TAA is explained by the increased urate delivery in healthier patients. No difference was observed in ascorbate and albumin delivery rates between the three groups. Mean age was similar in all three groups. More smokers were found in the severely diseased groups (15.2%) compared to those who had moderate disease (8.1%). No smokers were found in the healthy or mild disease group. However, as indicated by the

comparison of smokers and non-smokers, smoking habit did not explain variation in antioxidant status between the 3 groups.

Protein carbonyl concentration was significantly lower in the healthy or mild disease tertile (7.77 ± 2.38 fmoles/g protein) when compared to the severely diseased group (25.43 ± 11.09 fmoles/g protein) ($p<0.05$). When the carbonyl concentrations were adjusted for sex, both the healthy or mild disease and the moderate disease tertiles were significantly lower when compared to the severely diseased group ($p<0.05$). This indicates that severe periodontal disease, as characterised by a low CPITN score, is the principle factor involved in the presence of increased protein carbonyl concentrations. This association is reinforced when the differences between males and females have been taken into account (see Table 4.2).

Smoking is known to increase oxidative stress due to the free radicals present in the smoke (Reznick *et al.*, 1992). No smokers were present in the healthy or mild disease tertile. Smokers accounted for 8.1% of the moderate disease group and 15.2% of the severely diseased tertile. This may lead to the assumption that smoking was the primary factor associated with periodontal disease severity, and that smoking may have been responsible for the decreased antioxidant status and increased oxidation. However, despite the fact that smoking was a significant determinant of CPITN score ($p=0.003$), no significant relationship was found between smoking and protein carbonyl concentrations in the regression analysis and comparison of smokers and non-smokers. This would indicate that in the study population, smoking did not contribute to the oxidative damage as measured by protein carbonyl concentrations. The only antioxidant to be significantly lower in smokers compared to non-smokers was ascorbate ($p=0.041$), and this was not shown to be

associated with CPITN score. Furthermore, once adjusted for delivery rate, the significance in ascorbate concentration between smokers and non-smokers was lost. Plasma ascorbate concentrations are well known to be lower in smokers than in non-smokers. This is chiefly due to low habitual intakes rather than free radical-mediated depletion (Tappia *et al.*, 1995; Harats *et al.*, 1990).

Male and female salivary antioxidant profiles displayed differences similar to those observed in Chapter 3. No significant difference was found in age or CPITN scores between males and females. However, TAA was significantly higher in males (654 ± 25 μmol/litre) compared to females (545 ± 23 μmol/litre) ($p<0.002$). TAA delivery rate was also significantly higher in males (0.31 ± 0.02 μmol/ml/min) compared to females (0.19 ± 0.01 μmol/ml/min) ($p<0.001$). Both ascorbate and urate delivery rates were significantly higher in males (4.27 ± 0.44 nmol/ml/min; 108.8 ± 12.0 nmol/ml/min), compared to females values of (2.88 ± 0.30 nmol/ml/min; 58.7 ± 6.6 nmol/ml/min) ($p=0.011$ and <0.001) respectively. Furthermore, saliva flow rate was significantly higher in males (2.58 ± 0.16 ml) compared to females (1.87 ± 0.11 ml) ($p=0.001$). Smokers constituted 7.8% of the total number of male patients, whilst smokers made up 7.7% of female patients.

These differences between males and females are, as yet, unexplained. Previous studies regarding saliva flow rate have reported increased saliva volumes in males but these have not been significant (Mazengo *et al.*, 1994; Bretz *et al.*, 2001). Differences may be explained by salivary gland size, which is likely to be in proportion to body size, although there is no clear evidence of this. Hormonal influences could be a possible factor, and would warrant further investigation. However, previous research has found no significant

difference in saliva volume between male controls, females not using contraception and females using oral contraceptive pills (Laine *et al.*, 1991).

The differences in TAA and TAA, urate and ascorbate delivery rates between males and females also remain unexplained. Despite variation in antioxidant status, no differences were found in CPITN scores. It seems that females have a reduced antioxidant delivery rate but maintain a similar disease profile to that of male patients. Similar observations have been made in previous research. Chapple *et al.* (1997) also found higher TAA capacity in males compared to females, whilst Moore *et al.* (1994) reported higher saliva flow, and both TAA and urate concentrations and delivery rates. Ascorbate delivery rate was also higher in males compared to females. However, none of these factors achieved statistical significance; a fact that may be due to the low numbers involved in these studies.

These data indicate that an increase in the severity of periodontal disease is associated with a decrease in salivary antioxidant delivery rate. This has previously been reported, but in a much smaller cohort (Chapple *et al.*, 1997). Moore *et al.* (1994) found no difference between apparently healthy and diseased patients. However, this study failed to accurately assess the disease state and incorporated storage methods that could lead to a depletion of antioxidant capacity (Chapple *et al.*, 1997). This is the first time that such a large cohort has been used in the study of salivary antioxidants and periodontal disease. In addition, the use of an internationally recognised method for the determination of periodontal health has added to the validity of the study. Previous research has relied upon visual inspections and ostensibly superficial evaluation of the severity of periodontal disease (Moore *et al.*, 1994). This research confirms the previous work of Chapple *et al.* (1997), but has used a much larger study population and a recognised periodontal disease assessment procedure.

A novel approach in this research has been the use of protein carbonyl concentrations as a biomarker of oxidative tissue damage (Parihar and Pandit, 2003; Mutlu-Turkoglu *et al.*, 2003). Patients with severe periodontal disease exhibited greater oxidative injury, as measured by an increase in protein carbonyl concentration. This process has not been previously reported, but is in agreement with the proposed hypothesis that free radicals mediate the initiation and progression of periodontal disease (Sculley and Langley-Evans, 2002). This is further reinforced by a recent study of protein carbonyl concentrations in smokers and non-smokers. Cigarette smoke is known to contain oxidative compounds that could increase the level of free radical-mediated tissue damage (Reznick *et al.*, 1992). Protein carbonyl concentration in whole saliva was higher in smokers compared to non-smokers (Reznick *et al.*, 2003).

However, the present findings did not confirm this, with CPITN score and sex being the only significant factors influencing protein carbonyl concentration (see Table 4.5). Perhaps a more effective method of assessing protein carbonyl concentrations would be to take samples of gingival crevicular fluid. This can be achieved using absorbent paper strips and centrifuging in an appropriate media to reclaim the required metabolites (Chapple *et al.*, 1997). This would provide oxidised metabolites in greater concentrations and from their source, rather than waiting for them to be expelled into the saliva. However, in the course of simple sampling techniques, this would involve a more specialist approach and be more time consuming.

It is not known whether the lower TAA delivery rates found in patients with more severe periodontal disease and higher protein carbonyl concentrations are a causative factor in disease, or a result of antioxidant depletion due to high oxidative stress. Certainly, in cases

of periodontal disease in individuals who display an amplified inflammatory response, antioxidant capacity may well be exhausted due to the unusually high concentration of free radicals being released by activated neutrophils (Fredriksson *et al.*, 1998). However, in most examples this is unlikely to be the case. A more likely interpretation is that lower antioxidant capacity within the oral cavity can result in the more rapid progression of periodontal disease due to a reduced capacity to remove ROS. The existence of free radicals in the gingival area promotes the inflammatory response in the host. This leads to an increase in pro-inflammatory cytokines such as IL-1, IL-6 and TNF α, and expression of transcription factors such as NFκB and NFIL-6 (Grimble, 1998). The recruitment of ever-increasing numbers of PMN in response to this and the associated elevation in ROS production could quickly overwhelm the antioxidant capacity in the oral cavity. The lower TAA may therefore be a product of both enhanced depletion, and factors that determine the steady state of antioxidant production.

The majority of free radical-mediated oxidative damage occurs at the epithelial surface where the gingiva meets the tooth. This area is suffused in GCF, which emanates through the relatively permeable epithelial surface (Chapple *et al.*, 2002). High performance liquid chromatography (HPLC) has shown that glutathione is present in GCF at concentrations of between 0.5-2.5mM, and is lower in patients with periodontitis (Chapple *et al.*, 2002). These findings were consistent with studies of the alveolar epithelial lining of the lung, in which glutathione is found in the epithelial lining fluid (ELF) (Bernard, 1991). Therefore, glutathione seems to play an important antioxidant role in exposed epithelial surfaces (Chapple *et al.*, 2002). Indeed, glutathione may be a factor involved in reducing the expression of the transcription factor NFκB, and therefore decreasing the proliferation of

proinflammatory cytokines (Schreck, 1991; Hunter and Grimble, 1994). Glutathione, however, is not detectable in whole saliva and so was not considered in the present study.

In order to investigate the importance of salivary antioxidant status in determining periodontal disease progression, intervention studies are needed. For example, if it were possible to artificially raise the TAA of saliva in a diseased individual, it may be useful to observe the disease state and inflammation of the gingiva over time. This would indicate any protective effects of an increase in salivary antioxidants. It may also be possible to study individuals on a long-term basis, before any disease was apparent. Using prospective cohorts, this would indicate if an individual's salivary TAA capacity had an impact on the disease initiation and progression.

The measurement of TAA and protein carbonyl concentrations in saliva may provide an indication of existing periodontal disease, and a possible marker of a patients' susceptibility to the disease in the future, above and beyond existing disease diagnostic techniques. If artificially increasing the TAA within the oral cavity is possible, either through dietary supplements or topically administered agents, this would provide an additional line of defence against periodontal disease, in addition to established oral hygiene routines. TAA and protein carbonyl determination in saliva offers a non-invasive, cheap and effective assessment of periodontal disease and may be useful in predicting future disease progression. Its use may alert medical practitioners to the extent or potential risk of periodontal disease. This could allow an intervention system to be developed that could increase TAA in saliva and help to reduce the extent of free radical-mediated oxidative tissue damage.

At present, the factors that determine the antioxidant status in saliva are unclear. It is apparent that salivary ascorbate concentration is not a simple correlation of plasma (Sculley and Langley-Evans, 2002). The major salivary antioxidant is urate, which again may not simply diffuse from plasma. The final studies of this thesis will explore the potential contribution of diet and other antioxidant sources to salivary TAA.

CHAPTER 5

Nutritional and hormonal influences on salivary antioxidant status

5.1 Introduction

Antioxidants appear to play an important role in oral health. This has already been discussed in the preceding chapters. Increased salivary antioxidant capacity may provide protection against free radical-mediated oxidative attack (Chapple *et al.*, 1997, 2002), and hence prevent diseases of the mouth. Therefore, a strategy for increasing salivary antioxidant capacity could be of benefit in reducing the extent of oxidative damage by scavenging the free radicals before they attack the tissue within the oral cavity (Battino *et al.*, 1999).

One method of elevating salivary antioxidant capacity may be to increase dietary antioxidant intake. This has been found to induce an increase in plasma antioxidant concentrations (Higdon and Frei, 2003; Terao, 1999). However, previous research and work presented earlier in this thesis suggests that the antioxidant profile of saliva does not mirror that of plasma (Chapple *et al.*, 1997). Nevertheless, a diet rich in antioxidants may provide an increase in salivary antioxidant status sufficient to be effective in the scavenging of free radicals.

Another approach could be to bathe the gingiva in an antioxidant solution. The antioxidant capacity of mouthrinses has been recently studied *in vitro* (Battino *et al.*, 2002), and such

agents would seem to be an ideal vector for antioxidant delivery, in addition to their antibacterial action. A further method of antioxidant delivery to the gingiva could be its inclusion of active agents in toothpaste. This would also serve to introduce antioxidants to the source of oxidative damage. This is of significance when considering the consequences of brushing teeth. The brushing action not only cleans the teeth, but also the gingiva. This could potentially induce an inflammatory response due to the localised trauma to the gingival surface. Antioxidants delivered to this area may quench ensuing oxidative damage.

One interesting factor that has been highlighted during this research is the repeatedly lower antioxidant capacity of females compared to males, irrespective of the level of periodontal disease. As yet this has no explanation. It is possible that saliva and antioxidant production may be influenced by hormonal factors, although there is no previous research in this area.

The aims of this study are to investigate the effects of increasing dietary antioxidant intake on salivary antioxidant capacity, determine the antioxidant capacity of a variety of toothpastes and mouthrinses and to study the salivary antioxidant profile throughout a one-month menstrual cycle.

5.2 Objectives

The effects of dietary-derived antioxidants on salivary TAA remain unknown. Previous research suggests that mouthrinses contain antioxidant compounds that may be of benefit in reducing oxidative tissue damage to the gingiva. No studies have investigated the

antioxidant properties of toothpastes. Saliva flow has been shown to be sensitive to hormonal variations. The principle objectives of this chapter are:

- To investigate the effects of dietary antioxidants on the antioxidant profile of saliva
- To determine the antioxidant capacity of mouthrinses and toothpaste suspensions.
- To record saliva and antioxidant flow rates throughout the menstrual cycle and examine their relationship to β-estradiol and progesterone concentrations.

5.3 Materials and Methods

5.3.1 Dietary antioxidant supplementation and salivary antioxidant status

5.3.1.1 High and low antioxidant meals and salivary antioxidant status

Previous research has demonstrated that an increase in dietary antioxidants can produce an enhanced plasma antioxidant capacity (Higdon and Frei, 2003; Terao, 1999). However, this rise in plasma antioxidant capacity does not appear to be matched by salivary antioxidants (Chapple *et al.*, 1997). In this study, the salivary antioxidant profile was evaluated after the consumption of a meal low in antioxidants, followed by another that was high in antioxidants, after fasting. Six individuals (3 male/3 female) took part in this study. Mean age was 27.83 ± 2.51 years. Meals were provided as breakfast following a fast of 12 hours, on two separate occasions.

5.3.1.2 Saliva collection protocol

All saliva collections followed the same procedure based on Navazesh (1993) as described in section 2.4. Unstimulated saliva was collected over a five-minute period and stored at -

80°C until analysed. A baseline saliva sample was taken prior to consumption of a meal after an overnight fast. Saliva was then collected at 30, 60, 120 and 240 minutes after consumption of the meal. During this period, no food or drink other than plain water was permitted.

5.3.1.3 Saliva processing

Before analysis, saliva was processed using salivettes as described in section 2.4, to remove debris and reduce viscosity. The supernatant was then analysed for TAA, ascorbate, urate and albumin concentrations (see section 2.5).

5.3.1.4 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were considered statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Data were analysed for difference between the two meals and 5 time points by using a paired samples T test.

5.3.2 Low and high antioxidant meal

The low antioxidant meal consisted of 30g 'Special K' cereal, 140ml semi-skimmed milk, one slice of thick toast with low fat spread and water. The high antioxidant meal was the same, but with the addition of 200ml orange juice, 200ml tea with milk and fruit jam with the toast. The nutritional content of the two test meals can be seen in Table 5.1. The high antioxidant meal therefore provided more ascorbate and between 25-41mg/200ml of flavonoids, of which approximately 10-25mg/200ml is derived from quercitin (Hertog *et al.*, 1993).

Table 5.1. Nutrient content of low and high antioxidant meals

	Low antioxidant meal	High antioxidant meal
Energy	322.66 kcal	450.31kcal
Protein	13.68g	16.68g
Fat	7.52g	7.77g
Carbohydrate	53.55g	84.0g
Vitamin A	141.27µg	147.43µg
Vitamin C	1.46mg	81.46mg
Vitamin E equivalents	0.84mg	1.18mg
Selenium	13.78µg	16.28µg
Flavonoids	0	25-41mg

All nutrient data was calculated using Comp-Eat version 5 (Carson Bengston Consultants Limited). Flavonoid intakes estimated from published values for commercial brands of black tea (Hertog *et al.*, 1993).

5.4 Results

5.4.1 Saliva volume

Mean saliva volumes can be seen in Table 5.2. With both low and high antioxidant meals, saliva volume was significantly higher at 30 minutes compared to baseline volumes ($p=0.005$ low AO/$p<0.01$ high AO). With the low antioxidant meal, saliva volume was significantly higher at 120 and 240 minutes compared to baseline volumes ($p=0.029$ / $p=0.012$ respectively), and significantly lower at 240 minutes compared to 30 minutes ($p=0.028$). With the high antioxidant meal, saliva volume decreased significantly at 60

minutes compared to 30 minutes (p=0.009). There were no significant differences in saliva volumes between the low and high antioxidant meals.

Table 5.2. Mean saliva volumes over 5 minutes

	Baseline (ml)	30 mins (ml)	60 mins (ml)	120 mins (ml)	240 mins (ml)
Low AO	2.54 ± 0.21	4.24 ± 0.53*	3.11 ± 0.44	3.54 ± 0.36*	3.43 ± 0.31*†
High AO	3.02 ± 0.35	4.51 ± 0.36*	3.71 ± 0.47†	3.79 ± 0.31	3.36 ± 0.24

Data are means ± S.E.M. AO = antioxidant. * denotes significant difference to baseline volumes ($p<0.05$). † denotes significant difference to 30 minutes ($p<0.05$).

5.4.2 Saliva antioxidant profile after low and high antioxidant meals

There were no significant differences in salivary TAA between the low and high antioxidant meal samples. However, after consumption of the low antioxidant meal, salivary TAA flow was significantly higher after 30 and 120 minutes (p=0.017 and p=0.02 respectively) than at baseline, and significantly lower after 240 minutes compared to 30 minutes (p=0.022). The high antioxidant meal induced an elevation in salivary TAA from baseline, peaking at 60 minutes and then returning to baseline concentrations. However, this trend was not significant (see Figure 5.1).

Figure 5.1. TAA flow rate in low and high antioxidant meals

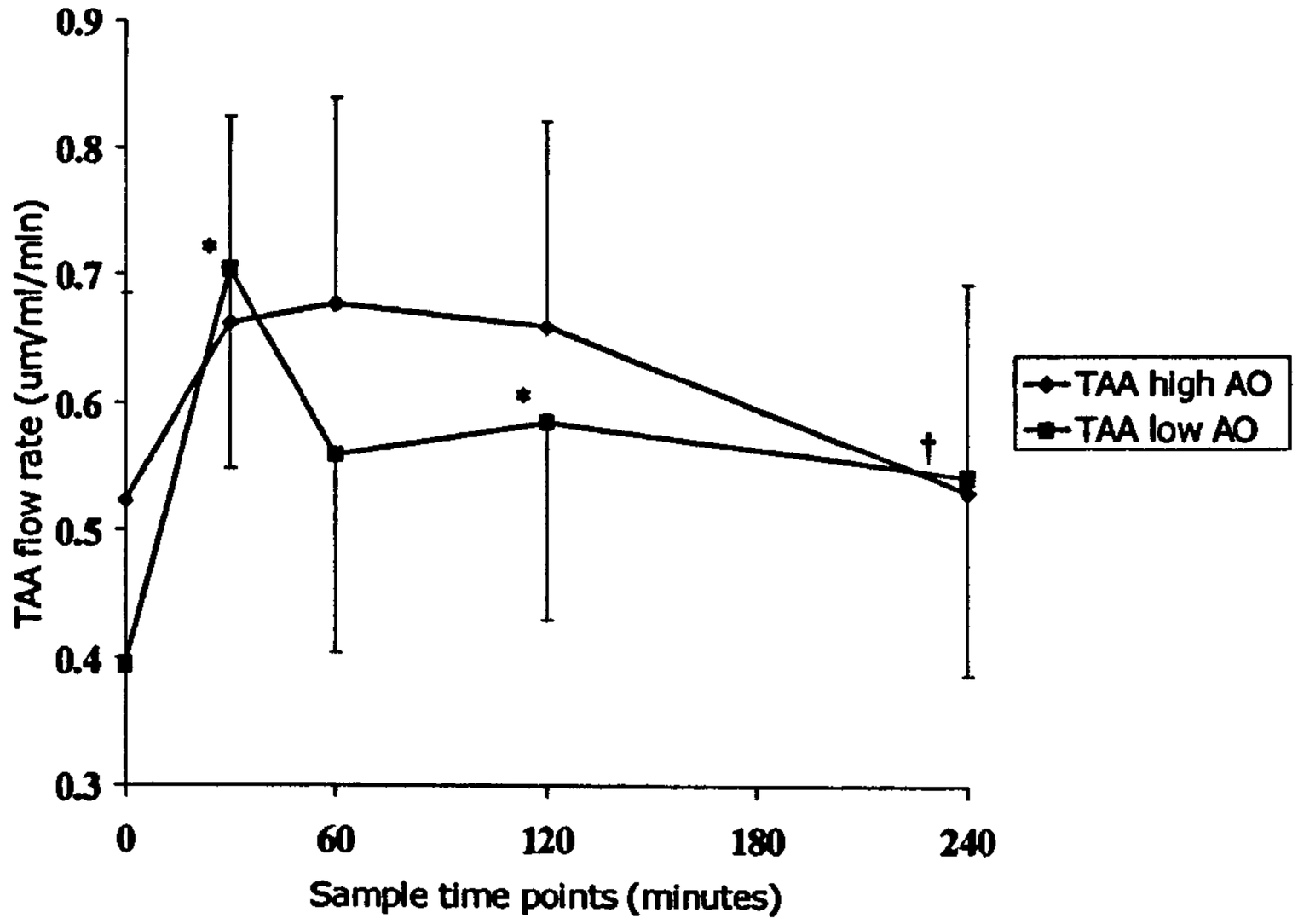


Figure shows TAA flow rates and upper and lower S.E.M bars. * denotes TAA flow rate significantly higher at 30 and 120 minutes compared to baseline and † denotes TAA flow rate significantly lower at 240 minutes compared to 30 minutes ($p<0.05$) for low antioxidant meal.

Urate flow rate was significantly higher at 120 minutes compared to baseline concentrations after consumption of the low antioxidant meal ($p=0.013$). No differences were observed after consumption of the high antioxidant meal, although flow rate increased after 60 minutes and then returned to baseline rate at 240 minutes. Urate flow rate was significantly higher after the high antioxidant meal at 60 and 240 minutes compared to the low antioxidant meal ($p=0.047$ and $p=0.004$ respectively) (see Figure 5.2).

Figure 5.2. Urate flow rate in low and high antioxidant meals

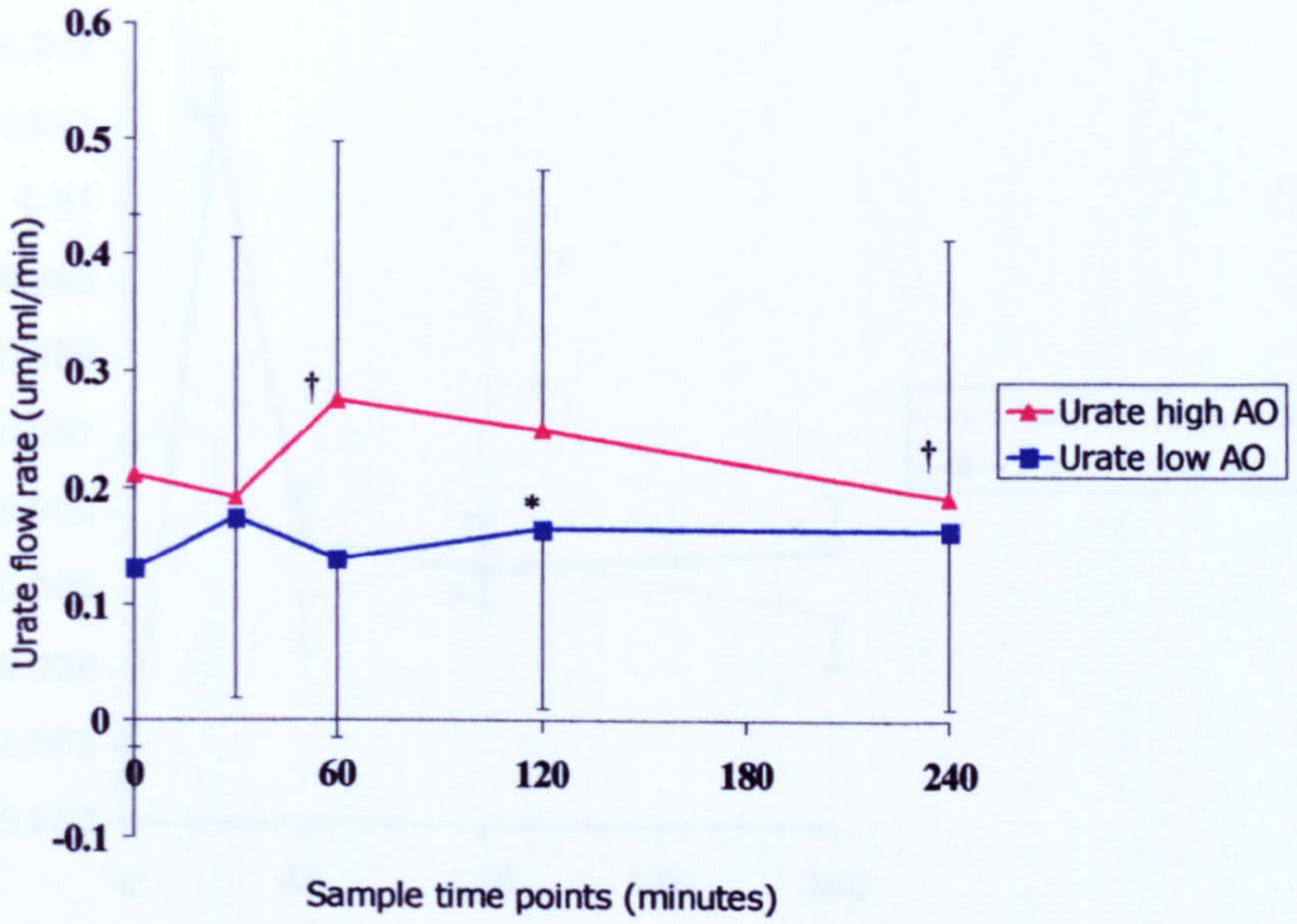


Figure shows Urate flow rates and upper and lower S.E.M bars. * denotes significantly higher urate flow rate at 120 minutes compared to baseline ($p<0.05$). † denotes significantly higher urate flow rate after the high antioxidant meal compared to the low antioxidant meals ($p<0.05$).

After consumption of the low antioxidant meal, ascorbate flow rate was significantly higher at 30 and 120 minutes compared to baseline ($p=0.049$ and $p=0.001$ respectively). Ascorbate flow rate was also significantly higher at 30 minutes compared to baseline after ingestion of the high antioxidant meal ($p<0.001$) (see Figure 5.3). There were no significant differences in ascorbate flow rate between the high and low antioxidant meals.

Figure 5.3. Ascorbate flow rate in low and high antioxidant meals.

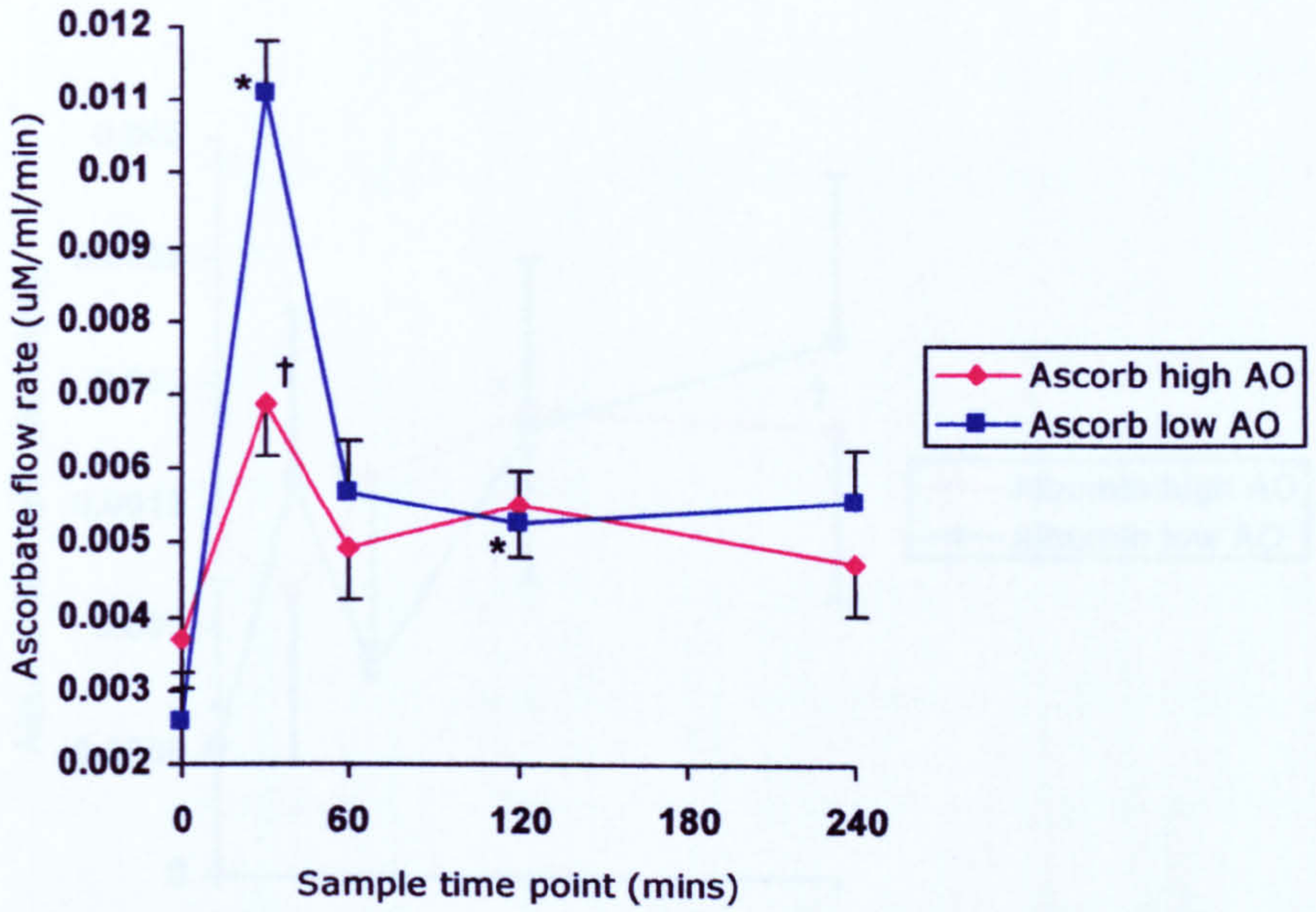


Figure shows ascorbate flow rates and upper and lower S.E.M bars. * denotes significantly higher ascorbate flow rate at 30 and 120 minutes compared to baseline ($p<0.05$) after low antioxidant meal. † denotes significantly higher ascorbate flow rate at 30 minutes compared to baseline, 60 and 240 minutes ($p<0.05$) after high antioxidant meal.

There were no significant changes in albumin flow rate after the low antioxidant meal. Albumin flow rate was significantly higher at 120 minutes compared to both baseline and 30 minutes ($p=0.02$ and $p=0.017$ respectively), and higher at 240 minutes compared to 30 minutes ($p=0.019$) after the high antioxidant meal. The albumin flow rate was significantly higher after consumption of the high antioxidant meal compared to the meal low in antioxidants at baseline and after 60 minutes ($p=0.015$ and $p=0.029$ respectively) (see Figure 5.4).

Figure 5.4 Albumin flow rate in low and high antioxidant meals

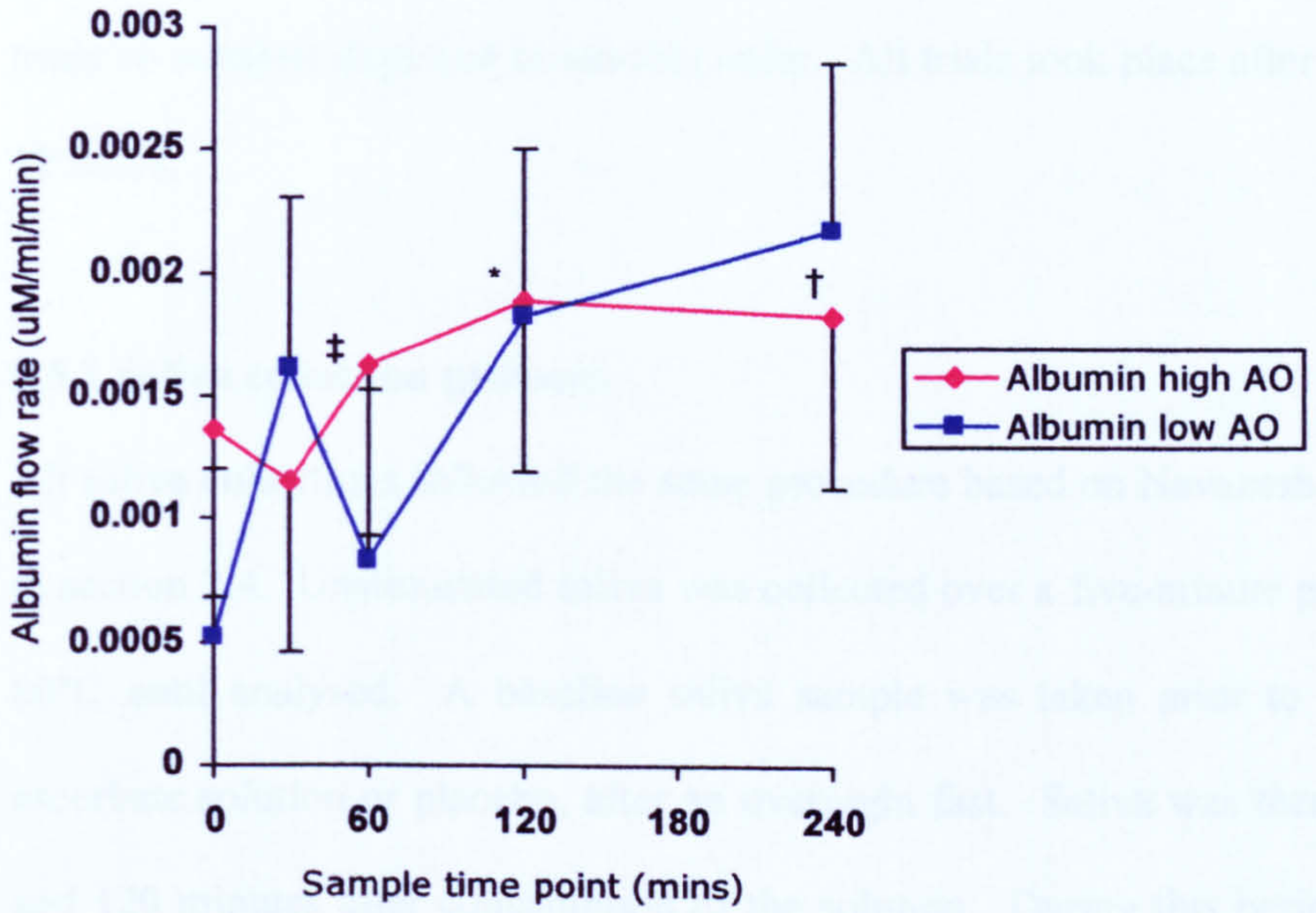


Figure shows albumin flow rates and upper and lower S.E.M bars. * denotes significantly higher albumin flow rate at 120 minutes compared to baseline and 30 minutes ($p<0.05$). † denotes significantly higher albumin flow rate at 240 minutes compared to 30 minutes ($p<0.05$). ‡ denotes significantly higher albumin flow rate at 60 minutes after consuming the high antioxidant meal compared to the low antioxidant meal ($p<0.05$).

5.5 Ascorbate supplementation and salivary antioxidant capacity

In section 5.3.2, ascorbate delivery rates were shown to have increased significantly after consumption of either high or low antioxidant meals. To what extent these increases were dependant on the dose was unclear. This study was designed to investigate the effects on salivary TAA of four different doses of ascorbate, to determine if an increase in ascorbate consumption resulted in proportional elevation of salivary TAA. Six individuals took part

in this study (4 male/2 female, mean age 28.83 ± 2.63). Each person consumed a drink made up of 100ml water and 5g sugar with either 80, 500 1000mg of ascorbate. A placebo of just water and sugar was also administered. All subjects therefore participated in four trials on separate days and in random order. All trials took place after an overnight fast of 12 hours.

5.5.1 Saliva collection protocol

All saliva collections followed the same procedure based on Navazesh (1993) as described in section 2.4. Unstimulated saliva was collected over a five-minute period and stored at -80°C until analysed. A baseline saliva sample was taken prior to consumption of the ascorbate solution or placebo, after an overnight fast. Saliva was then collected at 30, 60 and 120 minutes after consumption of the solution. During this period, no food or drink other than plain water was permitted.

5.5.2 Saliva processing

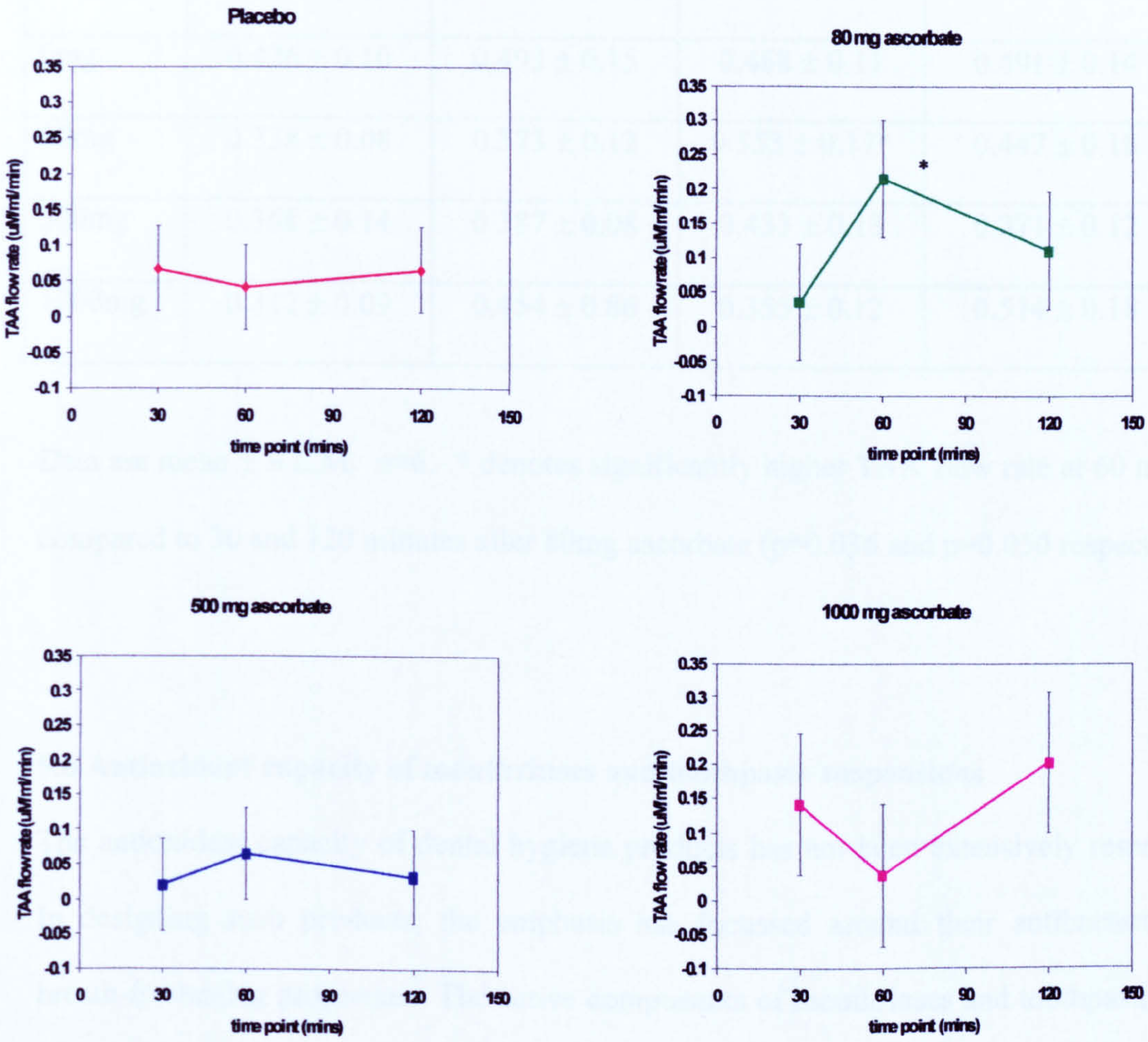
Before analysis, saliva was processed using salivettes as described in section 2.4, to remove debris and reduce viscosity. The supernatant was then analysed for TAA, and ascorbate concentrations (see section 2.5).

5.5.3 Results

Salivary TAA flow rate changes following ascorbate administration can be seen in Figure 5.5. Consumption of the placebo dose had no significant effect upon salivary TAA flow. TAA flow increased from a baseline concentration of 0.426 ± 0.10 μM/ml/min to 0.493 ± 0.15 μM/ml/min after, 30 minutes before decreasing. The 80 mg dose of ascorbate produced a significant increase in TAA flow rate from baseline (0.338 ± 0.08 μM/ml/min)

to 0.553 ± 0.17 μM/ml/min after 60 minutes. This then reduced to 0.447 ± 0.18 μM/ml/min after 120 minutes. The 500 mg ascorbate load produced an increase in salivary TAA flow rate from 0.368 ± 0.14 μM/ml/min at baseline to a peak of 0.433 ± 0.13 μM/ml/min after 60 mins. However, this trend failed to achieve statistical significance. After consumption of the 1000 mg ascorbate load, salivary TAA flow increased from 0.312 ± 0.09 μM/ml/min at baseline to a peak of 0.514 ± 0.18 μM/ml/min after 120 minutes. Again, this increase failed to achieve statistical significance (see Table 5.3).

Figure 5.5. TAA flow rate change relative to baseline after ascorbate supplementation



Data shown represent change in TAA flow rate relative to baseline value. Data are mean ± S.E.M bars. * denotes significantly higher TAA flow rate at 60 minutes compared to 30 and 120 minutes ($p=0.036$ and $p=0.050$ respectively).

Table 5.3. Ascorbate supplementation and TAA flow rate (μM/ml/min)

	Baseline	30 mins	60 mins	120 mins
0mg	0.426 ± 0.10	0.493 ± 0.15	0.468 ± 0.11	0.491 ± 0.14
80mg	0.338 ± 0.08	0.373 ± 0.12	0.553 ± 0.17*	0.447 ± 0.18
500mg	0.368 ± 0.14	0.387 ± 0.08	0.433 ± 0.13	0.371 ± 0.12
1000mg	0.312 ± 0.09	0.454 ± 0.86	0.355 ± 0.12	0.514 ± 0.18

Data are mean ± S.E.M. n=6. * denotes significantly higher TAA flow rate at 60 minutes compared to 30 and 120 minutes after 80mg ascorbate (p=0.036 and p=0.050 respectively).

5.6 Antioxidant capacity of mouthrinses and toothpaste suspensions

The antioxidant capacity of dental hygiene products has not been extensively researched. In designing such products, the emphasis has focussed around their antibacterial and breath-freshening properties. The active components of mouthrinses and toothpastes have therefore concentrated on fluoride content and antibacterial compounds. Battino *et al.* (2002) has recently studied the antioxidant capacity of several 'off the shelf' mouthrinses. The results were expressed as the volume of mouthrinse required to produce an inhibition of half of the free radical cation production (I_{50}). One mouthrinse failed to provide any antioxidant activity, whilst those remaining produced I_{50} at volumes ranging from 297μL to 8.34μL. Individual components of the mouthrinses were also assessed for antioxidant capacity. Amongst these, methyl salicylate was found to have the highest antioxidant activity with an I_{50} value of 0.27μM. There were no data available regarding the

antioxidant capacity of toothpaste. The aim of this study was to determine the TAA of mouthrinses and toothpastes and identify the active antioxidant components.

5.6.1 Method for determination of mouthrinse and toothpaste antioxidant capacity

The TAA of four commercially available mouthrinses (Corsodyl, Listerine, Oraldene and UniChem) and eight toothpastes (Aquafresh, Boots Smile, Clinomyn, Colgate Fresh Stripe, Colgate Milk Teeth, Euthamol, Macleans and Pearl Drops) were determined in this study. The mouthrinses were analysed undiluted. The toothpastes were initially diluted to 100mg/ml in distilled water. After mixing, the solution was centrifuged at 13000rpm for 5 mins at 4°C. The supernatant was aliquotted and stored at -80°C until analysed.

5.6.2 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were considered statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Data were analysed for differences between the mouthrinse and toothpaste solutions by using a one-way ANOVA.

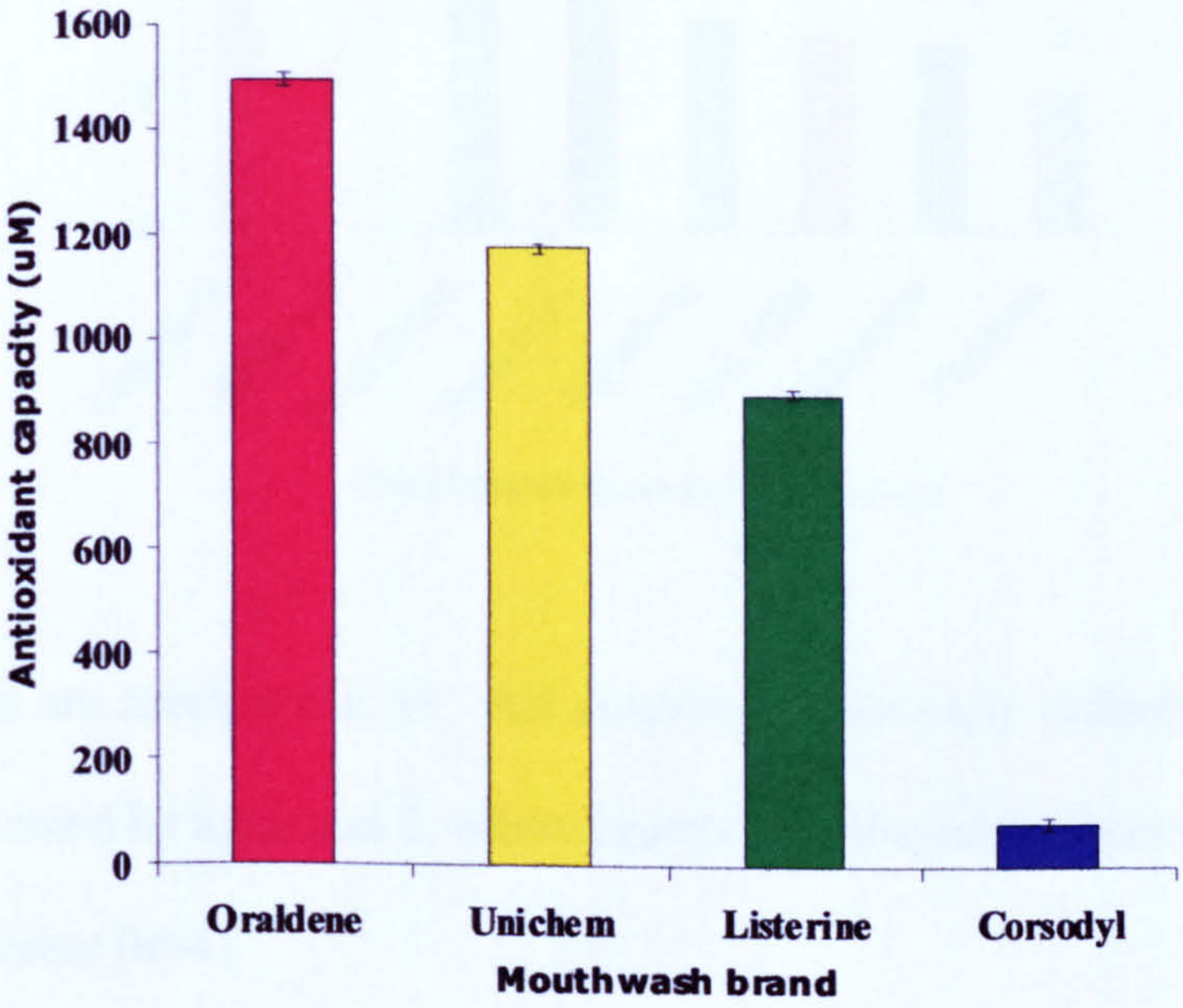
5.6.3 Results

5.6.3.1 Mouthrinses

Oraldene had the highest TAA of 1490.68 ± 30.11μM. This was followed by Unichem, with a TAA of 1169.65 ± 7.7μM. Listerine had a TAA of 895.48 ± 3.17μM with Corsodyl having the lowest TAA at 83.00 ± 4.27μM (see Figure 5.6). TAA values for all mouthrinses were significantly different from each other ($p<0.05$). Of the active antioxidant components of the mouthrinses, Oraldene, Unichem and Listerine all contained

methyl salicylate, with Oraldene and Listerine also containing ethanol. Corsodyl's only active antioxidant component was ethanol.

Figure 5.6. Total antioxidant activity of mouthrinses

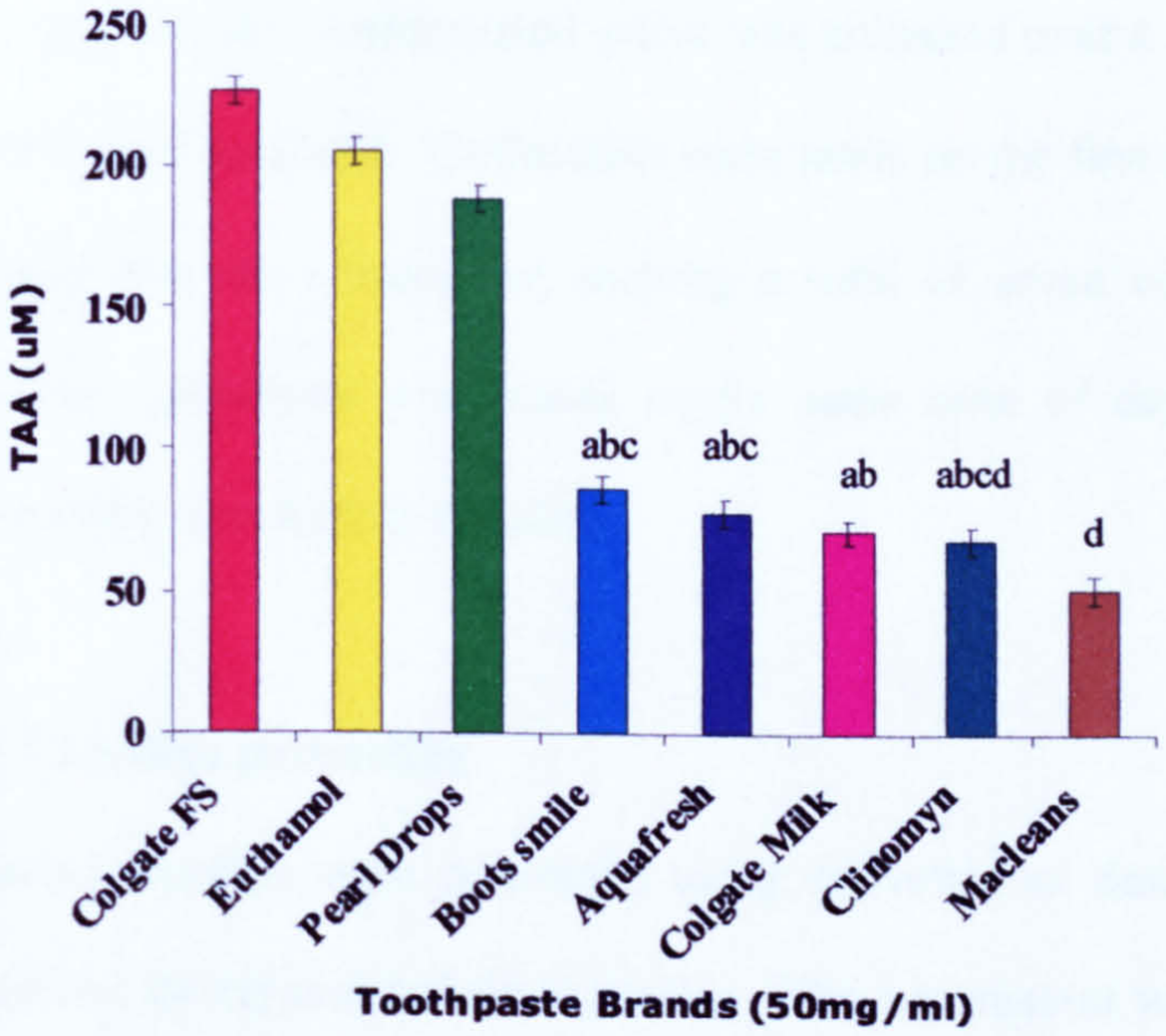


Data are mean ± S.E.M. All mouthrinse TAA were significantly different to each other ($p<0.05$) ($n=4$).

5.6.3.2 Toothpastes

The TAA of the eight toothpaste samples can be seen in Figure 5.7. Colgate Fresh Stripe (FS) possessed the highest antioxidant activity of 226.04 ± 4.10μM. The toothpaste with the lowest activity was Macleans with a TAA of 51.00 ± 0.001μM. The primary antioxidant ingredient found in toothpaste was sodium fluoride. This was present in all but Euthymol and Colgate Milk Teeth toothpastes.

Figure 5.7. TAA of toothpaste solutions



Data are mean ± S.E.M. All samples significantly different to others ($p<0.05$) except as indicated by a,b,c and d, where brands with the same superscript letter are not significantly different (n=4).

5.7 Salivary antioxidant profile throughout the menstrual cycle

A consistent factor that has been evident during this research has been the lower TAA in females compared to their male counterparts. This has been independent of periodontal disease status. In this study, the antioxidant profile was investigated in 5 women throughout a one-month menstrual cycle. Five female postgraduate students from the University of Nottingham were recruited for this study. The age range of the subjects was 21 to 32 years (mean 26.6 ± 2.11 years).

5.7.1 Saliva collection protocol

All saliva collections followed the same procedure based on Navazesh (1993) as described in section 2.4. Unstimulated saliva was collected over a five-minute period and stored at -80°C until analysed. Collections were made on the first day of a new menstrual cycle and every four days thereafter, making a total of seven collections for the monthly cycle. Saliva collections were made at the same time of day (8.00am) on each occasion to eliminate any diurnal variation.

5.7.2 Saliva processing

Saliva samples were processed using salivettes as described in section 2.4 in order to remove debris and reduce viscosity. The supernatant was then analysed for TAA, urate, ascorbate and albumin concentrations (see section 2.5).

5.7.3 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 9.0. Data were considered statistically significant if p values were equal to or less than 0.05. All values were expressed as means ± standard error of the mean (S.E.M). Data were analysed for differences between the seven samples by using a paired samples T test.

5.7.4 Results

5.7.4.1 Saliva volumes during the menstrual cycle

Mean saliva volume increased from 2.47 ± 0.68 ml on the first day of the cycle, peaking at 3.31 ± 1.13 ml on day 12, before reducing to 3.00 ± 0.61 ml by day 20. Saliva volume then increased to 3.17 ± 0.73 ml on the final sample time point on day 24. Saliva volumes

were significantly lower on the first day compared to samples on days 12, 16 and 20 ($p<0.05$). Mean saliva volumes and the range can be seen in Table 5.4.

Table 5.4. Mean 5 -minute saliva volumes during one-month menstrual cycle

Sample time (days into cycle)	Range (ml)	Volume over 5 minutes (ml)
0	1.11 - 4.92	2.47 ± 0.68
4	1.57 - 5.67	3.21 ± 0.73
8	1.68 - 7.79	3.31 ± 1.13
12	2.17 - 5.67	3.28 ± 0.64*
16	1.28 - 6.14	3.08 ± 0.87*
20	1.64 - 5.24	3.00 ± 0.61*
24	2.02 - 6.00	3.17 ± 0.73

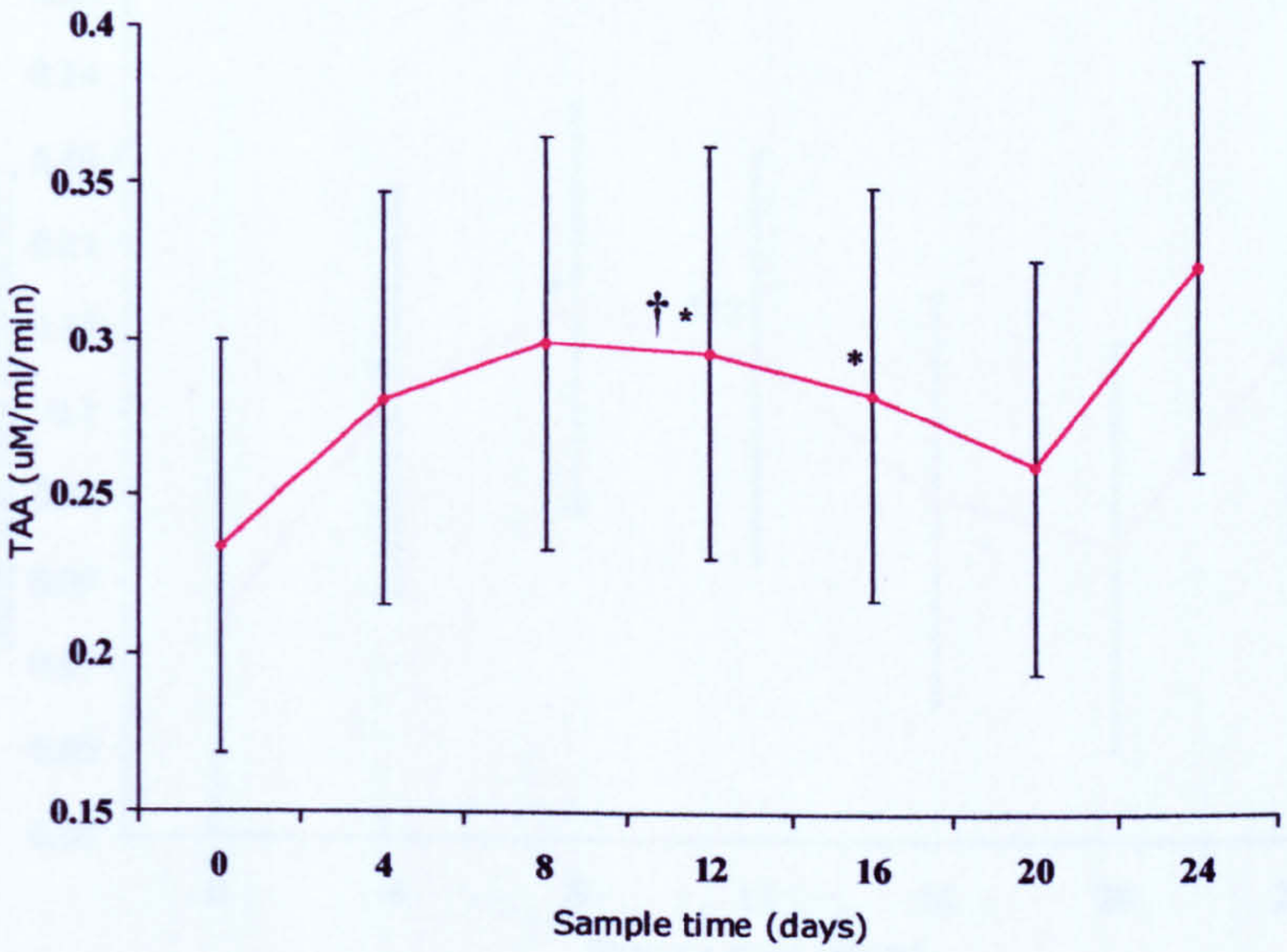
Data are mean ± S.E.M. n=5. * denotes significantly higher volume compared to sample time 0 ($p<0.05$).

5.7.4.2 Salivary TAA flow rate during the menstrual cycle

The salivary TAA flow rate was assessed throughout the menstrual cycle and can be seen in Figure 5.8. Mean salivary TAA flow increased from an initial concentration of 0.234 ± 0.071μM/ml/min on the first day to peak at 0.299 ± 0.081μM/ml/min on day 8. TAA flow then decreased steadily to 0.260 ± 0.049 μM/ml/min on day 20 before increasing again to 0.324 ± 0.081μM/ml/min on day 24. Salivary TAA flow rate was significantly higher on

days 12 and 16 compared to the first day (p=0.028 and p=0.005 respectively), and significantly higher on day 12 compared to day 20 (p=0.02).

Figure 5.8. TAA flow during the menstrual cycle



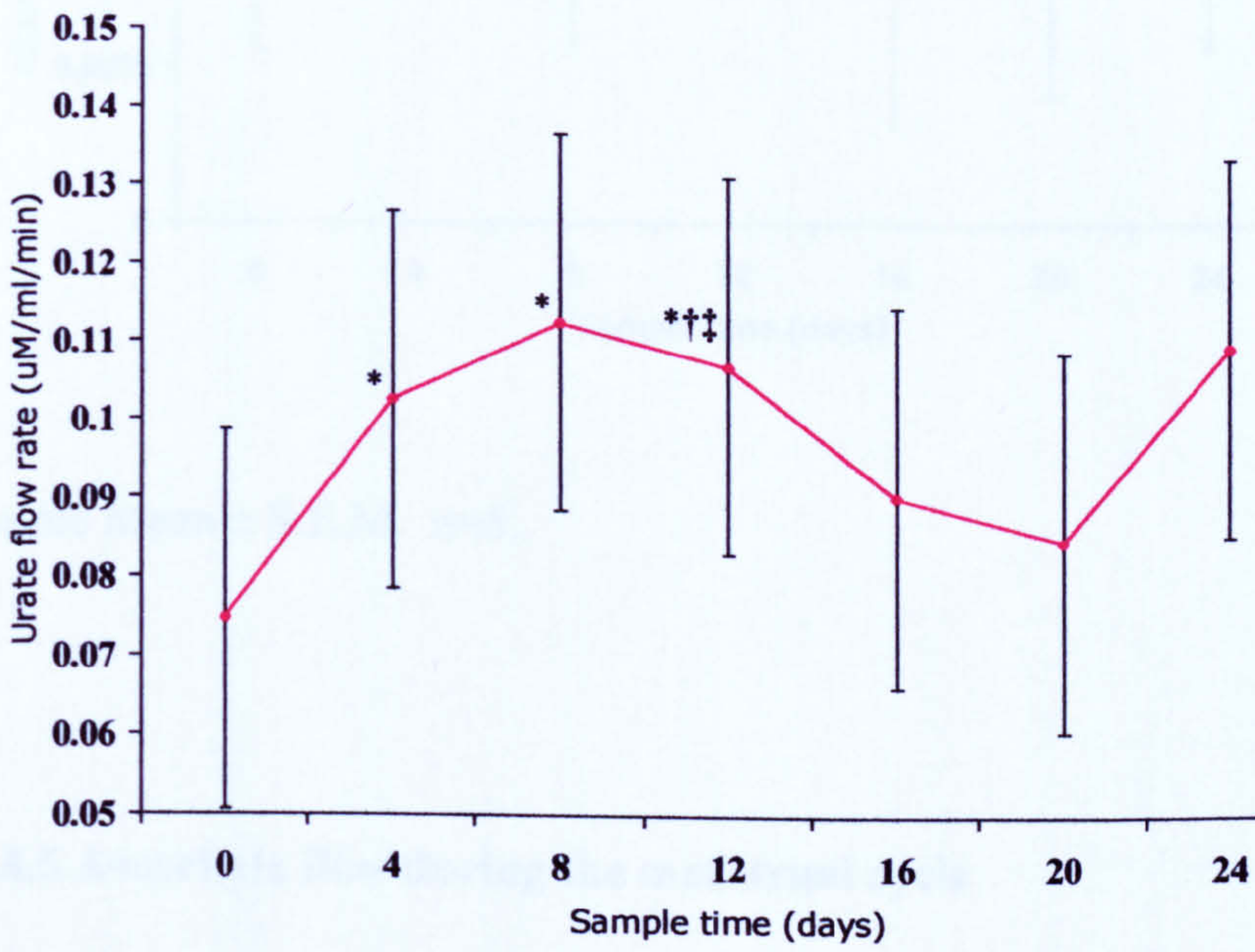
Data are mean ± S.E.M. n=5. * denotes significantly higher TAA flow rate compared to day 0 and day 20 (p<0.05). † denotes significantly higher TAA flow rate compared to day 20 (p<0.05).

5.7.4.3 Salivary urate flow during the menstrual cycle

Salivary urate flow rate followed a cyclical pattern that matched that of TAA flow rate. Mean salivary urate flow increased from 0.075 ± 0.026μM/ml/min on the first day of the cycle to peak at 0.112 ± 0.032μM/ml/min on day 8. This then decreased to 0.085 ± 0.018μM/ml/min on day 20 before increasing again to 0.109 ± 0.026μM/ml/min by day 24

(see Figure 5.9 below). Urate flow rate was significantly higher on days 4, 8 and 12 compared to the first day ($p=0.14$, $p=0.44$ and $p=0.50$ respectively). Urate flow rate was also significantly higher on day 12 compared to days 16 and 20 ($p=0.026$ and $p=0.043$ respectively).

Figure 5.9. Urate flow rate during the menstrual cycle

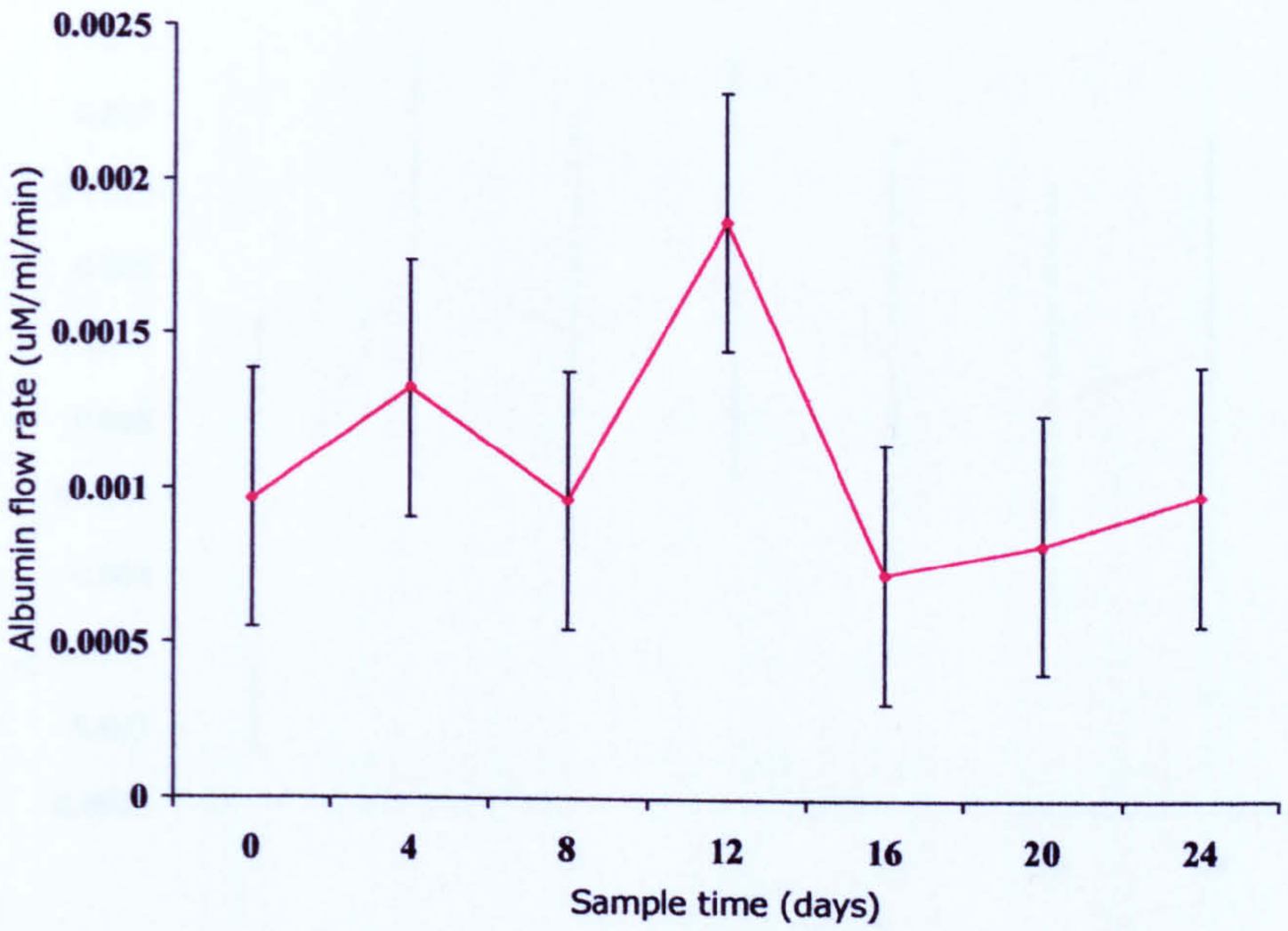


Data are mean ± S.E.M. n=5. * denotes significantly higher urate flow compared to day 0 ($p<0.05$). † denotes significantly higher urate flow compared to sample day 16 ($p<0.05$). ‡ denotes significantly higher urate flow compared to sample day 20 ($p<0.05$).

5.7.4.4 Albumin flow rate during the menstrual cycle

Mean albumin flow rate can be seen in Figure 5.10. Albumin flow peaked at 0.0019 ± 0.0007 μM/ml/min on day 12. No significant differences in albumin flow were observed between any days during the menstrual cycle.

Figure 5.10. Albumin flow rate during the menstrual cycle

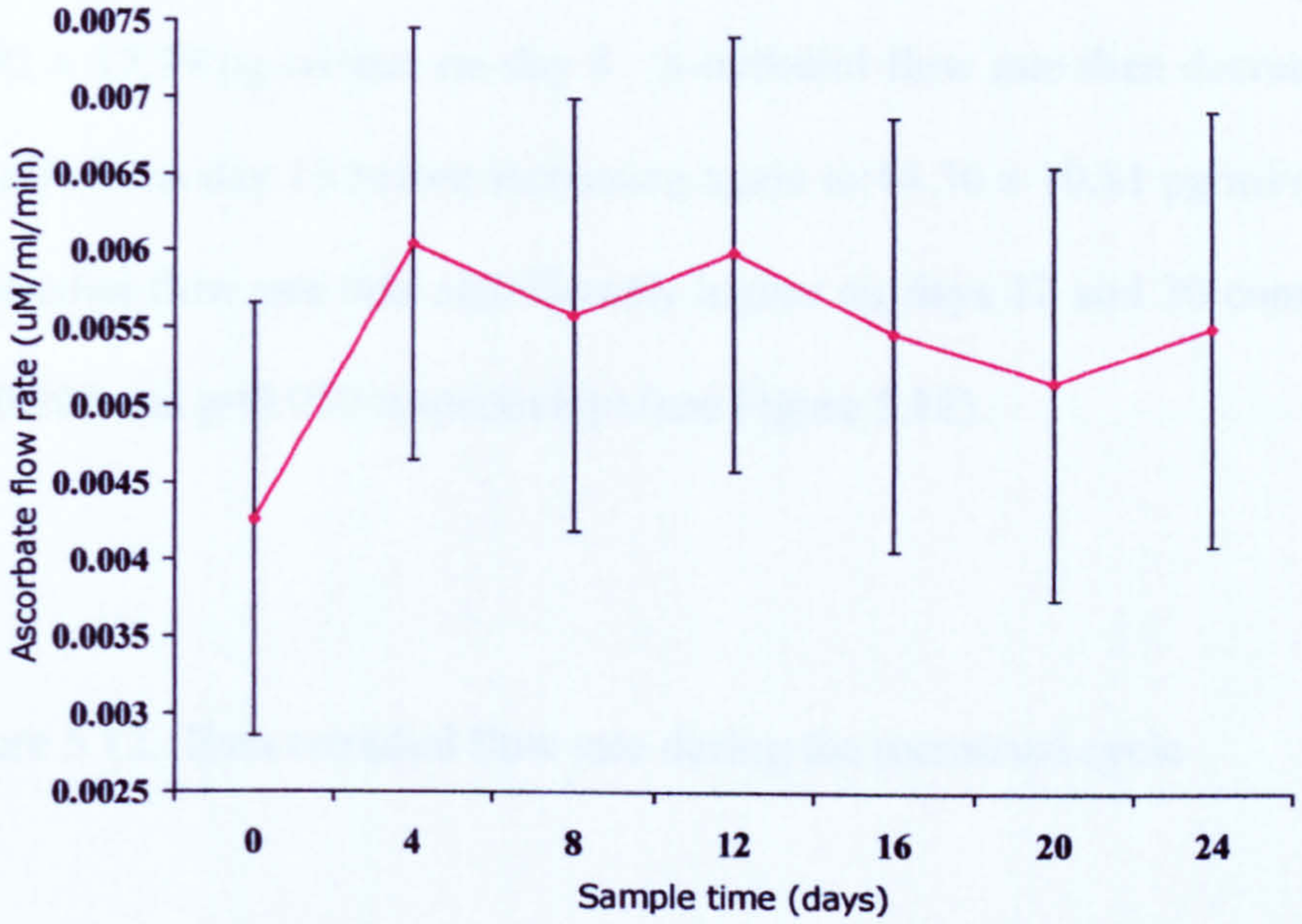


Data are Mean ± S.E.M. n=5.

5.7.4.5 Ascorbate flow during the menstrual cycle

Mean ascorbate flow rate can be seen in Figure 5.11. Ascorbate flow increased sharply from 0.0043 ± 0.0.17 μM/ml/min on the first day to peak at 0.0060 ± 0.0007 μM/ml/min on day 4. This flow rate remained stable until day 12 (0.006 ± 0.0013 μM/ml/min), after which flow rate decreased to 0.0051 ± 0.0009 μM/ml/min on day 20. No significant differences in ascorbate flow were observed between any days during the menstrual cycle.

Figure 5.11. Ascorbate flow rate during the menstrual cycle



Data are Mean ± S.E.M. n=5.

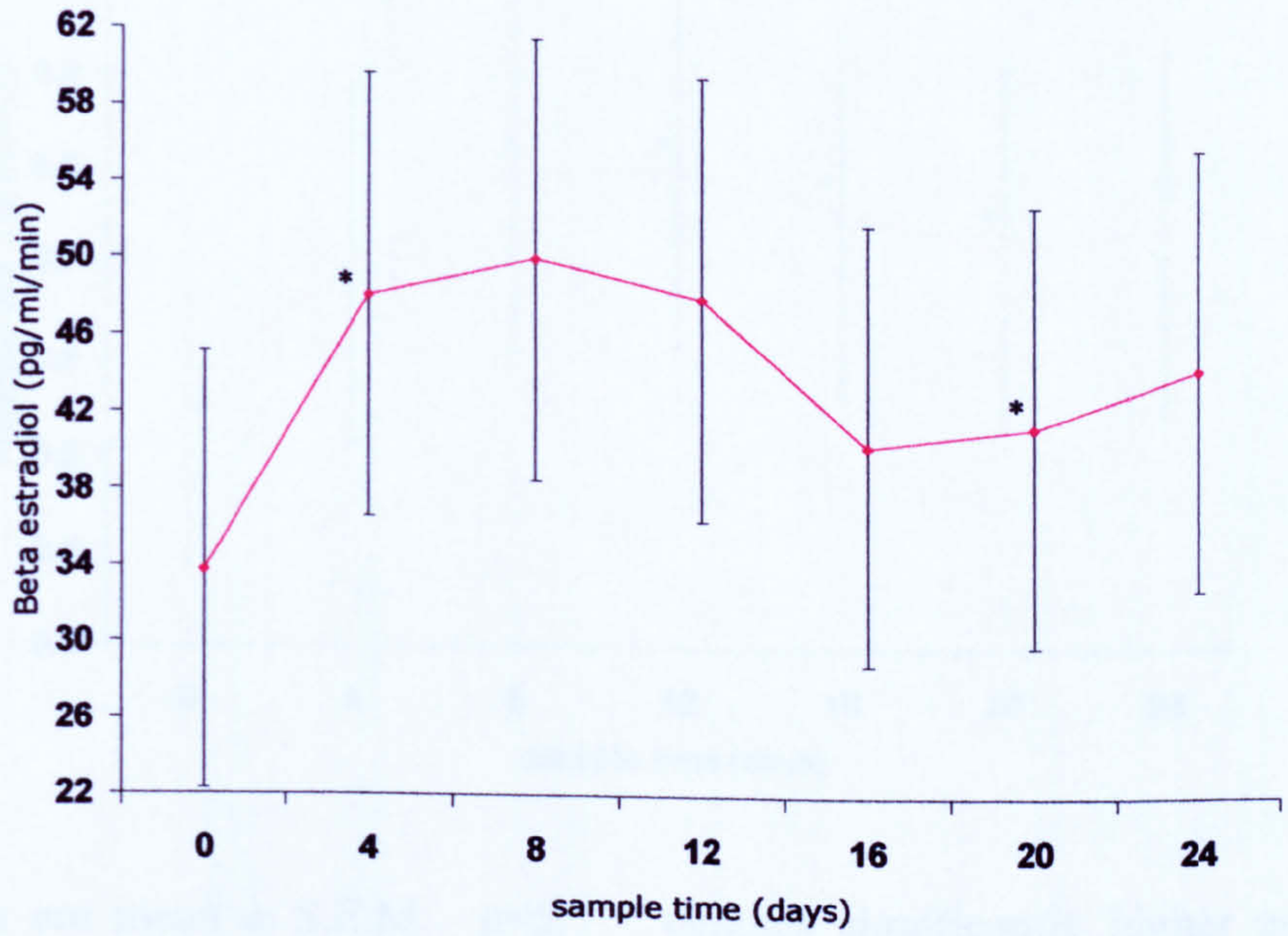
5.8 Beta Estradiol and progesterone flow rates during the menstrual cycle

Following analysis of salivary antioxidant flow rates during the menstrual cycle, it became evident that saliva flow, TAA flow and urate flow rates all followed a similar cyclical pattern. Previous research has found that hormone replacement therapy (HRT) increases saliva production in postmenopausal women (Laine and Leimola-Virtanen, 1996). This would be consistent with the possibility that female sex steroids were related to the pattern observed in this study. To investigate the influence of female sex hormones on saliva production and antioxidant profile, saliva samples collected during the same cycle as had been used in sections 5.6.4.1 to 5.6.4.5 were analysed for β-estradiol and progesterone concentrations.

5.8.1 β-estradiol flow rate during the menstrual cycle

Mean β-estradiol flow rate increased after the first day (33.75 ± 7.80 pg/ml/min) to peak at 49.92 ± 17.79 pg/ml/min on day 8. β-estradiol flow rate then decreased to 40.33 ± 13.18 pg/ml/min on day 16 before increasing again to 44.30 ± 10.81 pg/ml/min on day 24. Mean β-estradiol flow rate was significantly higher on days 12 and 20 compared to the first day ($p=0.001$ and $p=0.050$ respectively) (see Figure 5.12).

Figure 5.12. Beta estradiol flow rate during the menstrual cycle

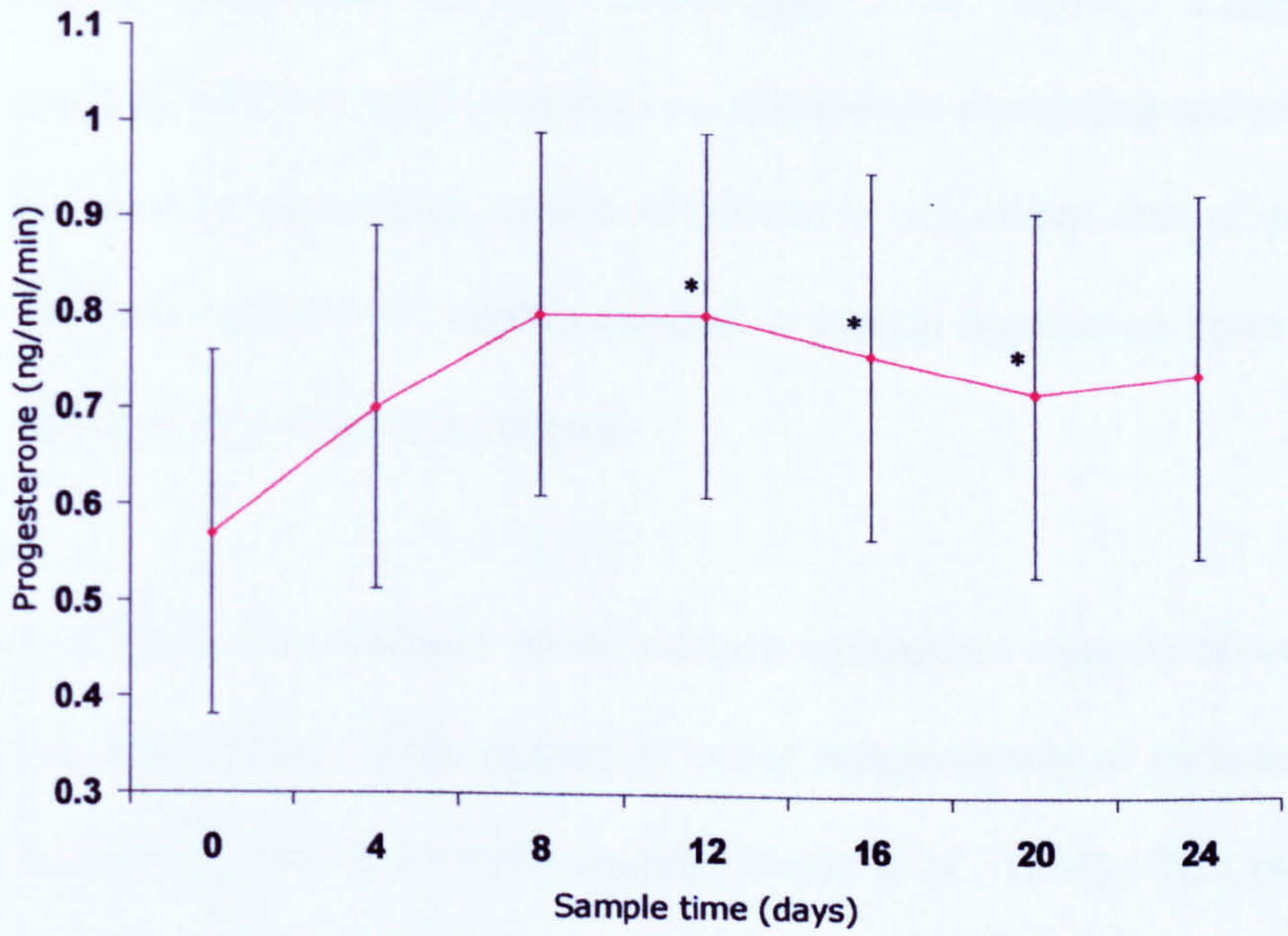


Data are mean ± S.E.M. n=5. * denotes significantly higher β-estradiol flow rate on days 4 and 20 compared to day 0 ($p<0.05$).

5.8.2 Progesterone flow rate during the menstrual cycle

Mean progesterone flow rate increased from 0.570 ± 0.17 ng/ml/min on the first day to peak at 0.799 ± 0.31 ng/ml/min on day 8. This gradually reduced to 0.718 ± 0.15 ng/ml/min on day 20 before increasing again to 0.737 ± 0.17 ng/ml/min on day 24. Salivary progesterone flow rate was significantly higher on days 12, 16 and 20 compared to the first day ($p<0.001$, $p=0.037$ and $p=0.010$ respectively) (see Figure 5.13).

Figure 5.13. Progesterone flow rate during the menstrual cycle



Data are mean ± S.E.M. n=5. * denotes significantly higher progesterone flow rate compared to day 0 ($p<0.05$).

5.9Discussion

These studies have taken preliminary steps towards investigating the efficacy of increasing salivary antioxidant status by nutritional and topical delivery of antioxidant-containing foods and dental products. This enhanced antioxidant capacity may offer protection against oxidative tissue damage that is suggested to take place in periodontal disease (Chapple *et al.*, 1997; Battino *et al.*, 1999). Previous research has demonstrated that an elevation in plasma antioxidants such as carotenoids and ascorbate was achieved by increasing the dietary intake of fruits and vegetables (Broekmans *et al.*, 2000; Higdon and Frei, 2003). In addition, plant phenols from olive oil were demonstrated to increase plasma antioxidant capacity (Bonanome *et al.*, 2000). Langley-Evans (2000) has demonstrated that tea flavonoids can also elevate circulating antioxidant levels. Although the salivary antioxidant profile is known to not mirror that of plasma (Chapple *et al.*, 1997), the effects of supplementation or topical application upon TAA in saliva do not appear to have been investigated.

In addition, the constantly lower salivary antioxidant capacity of women compared to men was investigated. This appears to occur independently of periodontal disease status and has been evident in previous studies (Moore *et al.*, 1994). This may in part be explained by differences in urate metabolism. In a study of urate concentrations in nasal lavage fluid recovered over five minutes, male subjects had significantly higher ($p<0.05$) urate concentrations (8.4 ± 1.3μM/L) compared to females (4.5 ± 0.6μM/L) (Housley *et al.*, 1996). In addition, a weak negative correlation was found between urate concentration and age in females ($r^2 = -0.685$) but not in males. This would indicate a possible gender difference in purine metabolism that may be under hormonal control. This is of significance as urate is the major antioxidant found in saliva (Moore *et al.*, 1994; Chapple

et al., 1997). Findings from earlier in this thesis confirm this viewpoint as both urate concentration and flow rate were considerably lower in women than in men. To investigate this further, saliva volume and antioxidant capacity were recorded from women throughout the menstrual cycle in an attempt to determine any hormonal control of saliva flow or composition. Concentrations of progesterone and β-estradiol were determined to investigate any possible influence they may have had on salivary TAA.

As previously mentioned, the antioxidant capacity of mouthrinses and toothpastes were also investigated. Surprisingly little research has been conducted in this area, which is interesting as mouthrinses and toothpastes present a direct vector for topical antioxidant application to the gingival tissue. Only mouthrinses have been studied and found to vary greatly in their antioxidant capacity (Battino *et al.*, 2002), although this has only been demonstrated *in vitro*.

The meals containing high and low concentrations of antioxidants were used to investigate their possible effects on salivary antioxidant status. The meals consisted of a typical breakfast of cereals and toast (see Section 5.2.2), the major difference between them being the inclusion of orange juice, tea and fruit jam. These additions raised vitamin A intake from 141.27μg to 147μg, vitamin E equivalents from 0.84mg to 1.18mg and vitamin C from 1.46mg to 81.46mg. Selenium was increased from 13.78μg to 16.28μg, although the use of FRAP to measure TAA excludes glutathione peroxidase determination. Flavonoids, provided at approximately 25-41mg in the high antioxidant meal, are potent antioxidants known to be detected by the FRAP assay (Langley-Evans, 2000).

Saliva volumes after consumption of both high and low antioxidant meals increased significantly over baseline volumes ($p<0.05$). As the meals were provided at breakfast time, this increase may be in part explained by the normal diurnal variation in saliva production (See section 3.6). The TAA of saliva after consumption of the high or low antioxidant meal was assessed to determine any effect of dietary antioxidant intake on salivary antioxidant status. The high antioxidant meal induced a steady rise in salivary TAA from baseline (0.523 ± 0.19μM/ml/min), peaking at 60 minutes (0.678 ± 0.19μM/ml/min) and then reducing gradually to a concentration similar to that of baseline after 240 minutes (0.529 ± 0.11μM/ml/min). Despite the increase from baseline and the gradual reduction after consumption of the high antioxidant meal, this trend failed to achieve statistical significance. The low antioxidant meal resulted in a significant increase in salivary TAA from baseline. There were no significant differences in TAA between the high and low antioxidant meal groups.

These results present something of a paradox, as it appears that consumption of a meal low in antioxidants induced a larger increase in salivary TAA than the high antioxidant meal. However, the low antioxidant meal baseline TAA was less than that of the high antioxidant group (0.394 ± 0.12μM/ml/min compared to 0.523 ± 0.19μM/ml/min), although this difference was not significant.

Consumption of the high antioxidant meal produced an increase in salivary urate flow from a baseline of 0.210 ± 0.10μM/ml/min to 0.274 ± 0.10μM/ml/min after 60 minutes. This change failed to achieve statistical significance. The low antioxidant meal resulted in a significant increase in salivary urate flow at 120 minutes (0.165 ± 0.06μM/ml/min) compared to baseline flow (0.131 ± 0.06μM/ml/min) ($p<0.05$). However, urate flow rate

was significantly higher after consumption of the high antioxidant meal at 60 and 120 minutes compared to the low antioxidant meal ($p<0.05$). This, however, reflected differences in the baseline TAA values on each day. These significant increases in salivary urate flow agree with previous research on plasma urate concentration after consumption of a meal after fasting. Urate was the only antioxidant to increase significantly in the postprandial condition (Cao and Prior, 2000), although the meal was low in other antioxidants.

Ascorbate flow rate after consumption of both the high and low antioxidant meal increased significantly over baseline concentrations ($p<0.05$). There were no significant differences in ascorbate flow rate between the high and low antioxidant meal groups. These transient increases in ascorbate flow rate have also been detected in plasma after ingestion of ascorbic acid. Ascorbate was demonstrated to be absorbed rapidly but with plasma concentrations quickly returning to baseline levels (De Lorenzo *et al.*, 2001). In the present study, this seems to have occurred with salivary ascorbate flow, with flow rate increasing from baseline but rapidly falling after 30 minutes.

Albumin flow rate did not differ significantly after consumption of the low antioxidant meal. The high antioxidant meal produced significant increases in albumin flow rate at 120 minutes compared to baseline and 30 minutes and at 240 minutes compared to 30 minutes ($p<0.05$). Albumin flow rate was significantly higher after consumption of the high antioxidant meal at baseline and 60 minutes compared to the low antioxidant meal ($p<0.05$).

The main outcome of this study is therefore that the antioxidant content of a meal has no significant influence on salivary antioxidant status. Eating appears to stimulate saliva flow and hence increases delivery of antioxidants, possibly derived from plasma and gingival crevicular fluid, to the mouth but this effect does not depend on immediate antioxidant content of the diet. There is a possibility that salivary antioxidant status reflects a longer-term, habitual diet. However, this seems unlikely as circulatory antioxidants have been shown to respond rapidly to dietary intake (Higdon and Frei, 2003; Terao, 1999). The increase in TAA flow rate observed may be due to the accompanying increase in saliva flow rate. Nevertheless, the increases in TAA flow rate tend to be dependant on urate, with often no accompanying increase in ascorbate and albumin flow rates, as seen in section 5.6, indicating that antioxidant composition of saliva is determined in a complex manner.

The study investigating the effects of ascorbate loading on salivary antioxidant status was chosen not only because all ascorbate is derived from the diet but also because plasma ascorbate concentrations have been demonstrated to increase after consumption of vitamin C (De Lorenzo *et al.*, 2001; Choy *et al.*, 2003). However, the salivary ascorbate flow rate did not appear to be related to the dose of ascorbate administered, nor did ascorbate loading significantly change TAA status. One reason for this may be the rapid clearing and excretion ascorbate as found in plasma by previous researchers (De Lorenzo *et al.*, 2001). Ascorbate is found in salivary acinar cells (Enwonwu, 1992) and, as humans cannot synthesise ascorbate, must be delivered in plasma. It is probable that the short clearance time seen in plasma is reflected in salivary ascorbate concentration. It may be necessary to take more frequent saliva samples after consumption in order to measure the acute response to ascorbate supplementation. Salivary ascorbate concentrations are about

10-fold lower than in plasma and their contribution to salivary TAA is small. Perhaps the clearance of large doses means that ascorbate could never produce an appreciable increase in TAA.

Mouthrinses are marketed almost exclusively on their antibacterial action and ability to freshen the breath of users. However, mouthrinses present an ideal opportunity to bathe the gingival tissues in an antioxidant solution. As yet, little research has been conducted into the antioxidant capacity of mouthrinses. Battino *et al.* (2002) investigated the antioxidant capacity of mouthrinses *in vitro*. Using a method of measurement that determined the volume required to inhibit half of the free radical cation production (I_{50}) in a model system, the mouthrinse with the highest antioxidant capacity had an I_{50} of only 8.34μL. The mouthrinse with the lowest antioxidant capacity required 297μL to achieve the same inhibition. This would suggest that there is a vast difference in the antioxidant capacity of different brands of mouthrinse. When the active antioxidant ingredients or the mouthrinses were evaluated, it was clear that those containing methyl salicylate provided the greatest antioxidant capacity. This is not surprising as methyl salicylate was found to be a potent antioxidant in these preparations, followed to a lesser extent by zinc chloride, allantoin, ethanol and sodium fluoride (Battino *et al.*, 2002). When used as directed, the use of the brand with the higher antioxidant capacity may provide an increased level of protection against oxidative damage in the mouth.

This study determined the antioxidant activity of 4 readily available mouthrinses. Antioxidant capacity was measured using the FRAP assay (Benzie and Strain, 1996) and expressed as Iron II equivalents. The antioxidant capacity ranged from 83.00 ± 4.27 μM to 1490 ± 30.11 μM. This represents almost an 18-fold increase in antioxidant activity of the

highest antioxidant mouthrinse over the lowest. Of the four mouthrinses tested, two were also studied in previous research (Battino *et al.*, 2002). The results from this study were similar to that of Battino and co-workers in that Listerine, whose main antioxidant component is methyl salicylate, possessed a high antioxidant capacity whilst Corsodyl, whose only active antioxidant ingredient was ethanol, was found to have the lowest activity. However, these studies have only demonstrated antioxidant effects *in vitro* and further research is needed to investigate the effects of mouthrinses *in vivo*, both in terms of initial antioxidant concentration around the gingiva and the time taken to reduce to baseline concentration. Nevertheless, if mouthrinse antioxidants can provide a localised increase in antioxidant capacity in the oral cavity, they may provide a tool for the reduction of the free radical-mediated tissue damage seen in conditions such as periodontal disease.

Similarly, there appears to be little research on the antioxidant capacity of toothpastes. There are, however, studies that have used vitamin A incorporated into a toothpaste and measured uptake of the vitamin in buccal mucosa cells (Sobeck *et al.*, 2002, 2003). In both studies, a significant uptake of vitamin A was observed in the mucosa cells after 3 days ($p<0.05$). In addition, the 2003 study observed a significant increase in plasma vitamin A concentration after 17 days ($p<0.05$). Wolinsky *et al.* (2000) used a dentifrice containing green tea bioflavonoids to determine the effects of antioxidants on plaque formation *in vitro*. After 4 days, the antioxidant-containing dentifrice had significantly reduced the plaque biomass compared to the non-antioxidant control. Together, these studies show that antioxidants provided via toothpastes retain biological activity and have the capacity to deliver active agents to the local tissues of the mouth.

Like mouthrinses, toothpastes may provide an ideal vector for antioxidant delivery to the gingival tissues. Of additional importance is the fact that the action of brushing may cause localised trauma to the gingiva. This may initiate an inflammatory response with the resultant PMN recruitment and the possibility of free radical release into the inflammation area. A toothpaste containing antioxidants may help to quench these free radicals before they could perpetuate oxidative damage to the gingival tissues. This study determined the antioxidant capacity of 8 commonly available toothpastes. The antioxidant activity ranged from 51.00 ± 0.001 μM to 226.04 ± 4.10 μM.

The main antioxidant found in the toothpastes studied was sodium fluoride, although the concentration was not stated by all brands and so comparison of this to the TAA was not possible. However, euthymol was one of only two toothpastes not to contain sodium fluoride, but possessed the second-highest TAA. It appears not to contain any other antioxidant component, although some ingredients are not fully listed. These data, like those obtained from the mouthrinses, were obtained *in vitro* and further studies are necessary to investigate the effects *in vivo*. However, this research has demonstrated that different brands of toothpaste and mouthrinse contain vastly differing concentrations of often unidentified antioxidants, which may be of benefit in conditions such as periodontal disease.

The difference in saliva and TAA flow rates between males and females has been consistently observed during this research. Females have displayed a lower saliva flow rate, lower TAA flow rate but no significant reduction in periodontal health. Previous research has demonstrated the effects of estrogen treatment on postmenopausal women, where saliva flow rate increases after estrogen supplementation (Eliasson *et al.*, 2003).

Leimola-Virtanen *et al.* (2000) reported the expression of estrogen receptors in oral mucosa and salivary glands. The expression of estrogen receptor mRNA in these tissues suggested a specific role for estrogen in the regulation of saliva production.

To determine the effect of estrogen and progesterone on saliva flow rate and antioxidant flow rates, saliva samples were collected during the menstrual cycle and analysed for estrogen, progesterone, saliva, TAA, urate, albumin and ascorbate flow rates. Saliva flow rate, TAA flow rate and urate flow rate all followed the same cyclical pattern across the 28-day study. Although the variation across the cycle was small, significant peaks in saliva and antioxidant flow rate were noticed at the transition from follicular to luteal phases of the cycle.

During the menstrual cycle, TAA and urate flow rates displayed a positive correlation (R^2=0.80; p=<0.01), which is consistent with earlier findings in this thesis. The contributions of β-estradiol and progesterone flow rate on TAA were assessed using stepwise linear regression analysis. Progesterone was found to have a greater influence on TAA flow rate (r=0.660; p=<0.001) than that of β-estradiol (r=0.305; p=0.028). When combined, the total contribution of progesterone and of β-estradiol to the variability in TAA flow rate was highly significant (p=<0.01; R^2=0.888).

These data suggest that the TAA flow rate in the study cohort was strongly influenced by the concentrations of progesterone and β-estradiol in saliva. This is in agreement with previous research (Laine and Leimola-Virtanen, 1996; Leimola-Virtanen *et al.*, 2000; Eliasson *et al.*, 2003). However, this does not explain the difference observed between males and females in terms of TAA flow rate and periodontal disease status. As yet, this

still remains unexplained. With urate contributing around 70-80% of the TAA of saliva, it would seem that this is the major factor involved in the gender discrepancy. However, women seem to suffer no increase in periodontal disease progression despite the reduction in salivary TAA flow rate.

In order to assess whether the influence of female sex hormones was the result of a direct antioxidant action and to determine whether testosterone has any antioxidant capacity that might explain the difference between men and women, progesterone, β-estradiol and testosterone were analysed, at concentrations found in saliva, *in vitro* for TAA capacity. None were found to have any antioxidant activity at these concentrations. Therefore, the repeatedly lower TAA flow rate in women, regardless of periodontal disease score, remains unexplained. However, the use of hormone replacement therapy in postmenopausal women may help to increase saliva and TAA flow rate, thereby providing an elevated level of protection against oxidative tissue damage as characterised by periodontal disease.

These studies have shown that some the mechanisms responsible for salivary antioxidant flow remain ambiguous. This work has demonstrated that consumption of high concentrations of antioxidants in a meal do not appear to increase salivary antioxidant flow rate over that of a low antioxidant meal. This is in contradiction to expected changes in plasma antioxidant concentration. The antioxidant capacity of mouthrinses and toothpastes were found to vary significantly *in vitro*. If this antioxidant activity can be transferred to the tissues within the mouth, these dental hygiene products have the capacity to reduce oxidative damage. The study of the antioxidant profile during the menstrual cycle produced two main findings. Firstly, the TAA flow rate was strongly influenced by

progesterone and, to a lesser extent, by estrogen. In addition, these female sex hormones were found to have no significant effect upon antioxidant capacity at the concentration ranges found in saliva.

The question still remains as to what causes the salivary TAA flow rate difference between males and females. This may be due to differences in purine metabolism and the resultant lower concentrations of urate. However, female periodontal disease status does not appear to be impaired despite the lower TAA flow rate. Further research may involve a study of ascorbate consumption but using a shorter sampling time to determine any changes in the short term, before the ascorbate is cleared and excreted. Additional work may focus on the male and female saliva TAA flow rate differences, particularly on purine metabolism.

CHAPTER 6

General Discussion

6.1 Introduction

The main aims of this thesis were to determine the antioxidant profile of saliva and investigate the association between salivary antioxidant status and periodontal disease in a large study cohort. Previous research has proposed a possible link between salivary antioxidant status and the severity of periodontal disease (Moore *et al.*, 1994; Chapple *et al.*, 1997). Oxidative injury is a primary cause of periodontal disease and the mechanisms of host interactions with the bacterial pathogens were evaluated, with an emphasis on the inflammatory response. In addition, dietary and hormonal influences were examined to establish possible mechanisms for modulation or enhancement of salivary antioxidant capacity. This research also studied the antioxidant capacity of mouthrinses and toothpastes to ascertain their possible effectiveness in elevating the antioxidant capacity within the oral cavity by topical application.

This general discussion will review the main findings of the research and discuss the importance and potential benefits of salivary antioxidant status to periodontal disease.

6.2 Antioxidant components of saliva

The primary antioxidants found in saliva were urate, ascorbate and albumin. Of these, urate was found to be the chief antioxidant (Chapter 3). This agrees with previous research

by Moore *et al.* (1994) and Chapple *et al.* (1997), who found urate to contribute around 70% of the TAA in saliva. Male members of the study cohort were found to have significantly higher TAA compared to females. Previous research had reported a trend of higher TAA in males, but had not found it to be significant (Moore *et al.*, 1994). The higher TAA in males was the result of increased urate concentrations, with no difference between males and females for ascorbate and albumin levels.

The higher salivary urate concentrations observed in males over females remains unexplained. Certainly, females who display a high plasma uric acid concentration are more susceptible than males to insulin resistance (Chou *et al.*, 2001). This may lead to the assumption that urate is lower in females as a protective mechanism, reducing the risk of diabetes and its associated disorders. Higher uric acid concentrations have also been observed in male urine over that of females (Parks *et al.*, 2003). This data also translated into higher TAA and urate flow rates. Saliva volumes over 5 minutes were significantly higher in males than in females.

The differences in saliva flow rate and TAA between males and females also remain largely unexplained. Saliva flow is known to be influenced by estrogen levels (Eliasson *et al.*, 2003), leading to the proposition that the reduction in flow was under hormonal control. However, the differences in saliva flow may be explained by saliva gland size. Unfortunately, this was not examined in this study but could explain the lower saliva flow of females over males.

The use of salivary antioxidant flow rate was a novel inclusion in this study, and deemed a more accurate indicator of antioxidant delivery in the mouth compared to a simple

measurement of concentration. Flow rate takes into account the volume of saliva produced, and so can give a true indication of antioxidant delivery to the tissues within the oral cavity. A simple concentration may give an inaccurate prediction of antioxidant status if saliva flow rate is unusually high or low.

6.3 Salivary antioxidant status and periodontal disease severity

Previous research has implicated bacterial colonisation as the initial step in the development of periodontal disease (Lamont and Jenkinson, 1998). The host response to this invasion involves the recruitment of neutrophils and their release of ROS (Chapple *et al.*, 1997). The damaging effects of neutrophil-derived ROS are a major factor involved in periodontal disease (Fredriksson *et al.*, 1998). A reduction in salivary antioxidant status has been proposed to result in an increase in oxidative tissue damage, and therefore in periodontal disease severity. Indeed, previous research has reported that a reduction in antioxidant activity in saliva was associated with an increase in periodontal disease (Chapple *et al.*, 1997).

However, a major shortfall in previous studies (Moore *et al.*, 1994; Chapple *et al.*, 1997) has been the use of a small study group and poorly defined disease status. This study used a large cohort of 129 dental patients. In addition, a standardised assessment technique was utilised (CPITN) and performed by one dentist in order to reduce error. The major findings of this study were that salivary antioxidant flow rate was significantly higher in those with healthy or mild disease compared to those with severe periodontal disease. Severe periodontal disease was associated with a 22% reduction in antioxidant flow rate. Interestingly, the simple calculation of TAA did not differ between the disease groups. This may account for the lack of significant differences observed previously (Moore *et al.*,

1994), who used only TAA concentration and not flow rate. To reinforce the theory that oxidative damage is associated with periodontal disease severity, this research observed significantly higher concentrations of protein carbonyls in those with severe periodontal disease compared to those with healthy to moderate disease.

From this data, it is clear that an elevated salivary antioxidant flow rate can provide protection against the oxidative processes involved in periodontal disease. It also highlights the increased oxidative tissue damage seen in severely diseased individuals, as measured by protein carbonyls. Recently, protein carbonyls have been utilised to measure oxidative damage in the saliva of smokers (Reznick *et al.*, 2003). It is still unclear as to whether the lower salivary antioxidant capacity observed in those with severe periodontal disease is a cause or effect of increased oxidative tissue damage. Indeed, some individuals do exhibit an amplified neutrophil response to bacterial pathogens that could lead to the host antioxidant defences being quickly overwhelmed (Fredriksson *et al.*, 1998). However, the more likely scenario is that a reduced antioxidant capacity within the oral cavity results in the rapid progression of free radical-mediated tissue damage and an increase in periodontal disease severity.

6.4 Periodontal disease and coronary heart disease

Periodontal disease shares many risk factors with coronary heart disease (CHD) (Beck, 1992). Some of these are associated with social and economic situations such as tobacco and alcohol consumption, financial position and education. However, periodontal disease and CHD share similar pathogenic mechanisms, chiefly those coupled to the inflammatory process (Saikku *et al.*, 1998). The same pro-inflammatory cytokines produced as a result of periodontal disease may also be involved in the development of atherosclerosis. This,

together with an increase in plasma clotting factors such as fibrin and fibrinogen, could increase the risk of thrombosis formation. An increase in salivary antioxidant status which reduces periodontal disease severity, and therefore inflammatory response mechanisms, may prevent these same mechanisms from impacting on the formation of arterial lesions and thrombosis development.

Plasma antioxidants have been found to be of benefit to CHD and its causative factors by reducing LDL cholesterol oxidation and auto-oxidation of glucose in a postprandial state (Kay and Holub, 2003). Indeed, plasma antioxidant concentrations were found to be lower in those suffering from coronary artery disease compared to healthy controls (Nojiri *et al.*, 2001). Therefore, the consumption of dietary antioxidants may help to attenuate the pathogenic mechanisms involved in both coronary artery and heart disease and periodontal disease.

6.5 Salivary antioxidants and oral cancer

In addition to periodontal disease, an increased salivary antioxidant status may offer protection against oral cancers. Smoking is the major cause of oral cancer. Salivary peroxidase, the primary antioxidant enzyme present in saliva, was found to be significantly reduced after smoking one cigarette (Reznick *et al.*, 2002), whilst ROS have been directly implicated in the development of oral cancers (Jeng *et al.*, 2001). This leads to the assumption that an elevation in salivary antioxidant status would increase the protection against cellular damage from cigarette smoke and ROS, and reduce the risk of oral cancer development.

6.6 Dental products and antioxidant capacity

Dental hygiene products possess the capability to increase the concentration of antioxidants within the mouth. Of the mouthrinses studied, Oraldene, Unichem and Listerine all have a TAA in excess of that of saliva samples from the healthiest periodontal patient group (1490 ± 68, 1169 ± 77 and 895 ± 48 respectively compared to 605 ± 29 of healthy periodontal tertile) (Chapter 4). Therefore, these mouthrinses may provide a localised increase in the antioxidant capacity of the mouth and gums that may reduce the extent of oxidative tissue damage associated with periodontal disease. However, what remains to be determined is the effectiveness of these mouthrinses *in vivo*. It is not known whether the TAA of these mouthrinses can be transferred to an elevation of the TAA within the mouth, or how long any increase may last. Certainly the possibility of increased antioxidant capacity, particularly after the trauma of brushing teeth, is attractive. This would be enhanced if a residual film of mouthrinse remains, resulting in an elevation in TAA over a longer period.

Toothpaste may also help to increase the TAA after brushing the teeth. A 50 mg sample of Colgate Fresh Stripe toothpaste, roughly the recommended amount used for brushing, possessed an antioxidant capacity of 226.04 ± 4.10 μM. Whilst much lower than the TAA of the highest three mouthrinses, this would still provide a useful increase in antioxidant activity. This is of additional importance as it would be delivered to the gingiva and gingival crevice – the site of inflammation associated with oxidative damage. Once again, the TAA of toothpaste suspensions was determined *in vitro*. Further study investigating the TAA *in vivo* would give further insight into the efficacy of toothpastes as a source of antioxidants into the mouth.

6.7 Hormonal influences

Saliva flow rate and TAA observed in this study have been consistently higher in males compared to females. This has also been found in previous research (Moore *et al.*, 1994; Chapple *et al.*, 1997), but failed to achieve statistical significance in these studies. This study observed a significantly lower saliva flow rate and TAA flow rate in females, without a corresponding reduction in periodontal health. Estrogen may play a role in saliva production, with receptors found on the oral mucosa and salivary glands (Valimaa *et al.*, 2004). However, this does not address the paradox of lower TAA flow rate without the associated decrease in periodontal health. Saliva flow, TAA and urate flow rates followed the same cyclical pattern through the menstrual cycle, with peaks observed around the changeover from the follicular to the luteal phase.

A positive correlation was observed between TAA and urate flow rates throughout the cycle. Both progesterone and estrogen were found to affect saliva and TAA flow rates during the menstrual cycle, with progesterone exerting the greater influence. Indeed, the combined effect of progesterone and estrogen accounted for almost 89% of the variability in TAA flow rate. This data suggests that female sex hormones may play a role in the flow rate of saliva and TAA status. However, the question still remains as to why females display a lower TAA flow rate but no loss in periodontal health over their male counterparts. Certainly, at saliva concentrations, neither progesterone, estrogen nor testosterone were found to contain any antioxidant activity. The mechanisms involved remain to be confirmed.

6.8 Conclusion

In conclusion, this thesis has reported the following information:

- The main antioxidants in saliva are urate, ascorbate and albumin, with urate contributing over 70% of the total antioxidant activity.
- Flow rates provide a more accurate indication of antioxidant delivery than a simple concentration.
- Males possess a higher saliva and antioxidant flow rate compared to females, but there is no evidence of detriment to periodontal health in women as a consequence of this.
- Periodontal disease severity was associated with a reduction in salivary antioxidant flow rate.
- Increased oxidative damage, as measured by protein carbonyls, was observed in those with severe periodontal disease compared to healthier individuals.
- Diet did not seem to influence antioxidant status in saliva as it does in plasma.
- Some dental hygiene products contain useful concentrations of antioxidants that may be of benefit in reducing oxidative tissue damage.
- Female saliva and TAA flow rates are influenced by estrogen and progesterone.

CHAPTER 7

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Appendix 1.

Publications arising from the work presented in this thesis.

1. D.V. Sculley, S.C. Langley-Evans (2002). Salivary antioxidants and periodontal disease status. *Proceedings of the Nutrition Society,* **61:** 137-143.

2. D.V. Sculley, S.C. Langley-Evans (2002). Association between salivary antioxidant status and periodontal disease. *Proceedings of the Nutrition Society,* **61:** 137A.

3. D.V. Sculley, S.C. Langley-Evans (2003). Periodontal disease is associated with lower antioxidant capacity in saliva and evidence of increased protein oxidation. *Clinical Science*, **105:** 167-172.

4. DV Sculley, SC Langley-Evans (2003). Salivary antioxidant status is independent of dietary intake. *Free Radical Research Communications,* **37:** 74.

5. DV Sculley, SC Langley-Evans (2003). Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Free Radical Research Communications,* **37:** 86.

Appendix 2

Milton Keynes NHS
Local Research Ethics Committee

Please ask for Ann Frew, Acting
Committee Secretary
Direct line: 01908-243992
[redacted]

Milton Keynes General Hospital
Standing Way
Eaglestone
Milton Keynes
MK6 5LD
Tel: 01908 660033
Fax: 01908 669349

Our ref: ARF/MKLREC/19/01
Wednesday, 09 May 2001

Mr. Dean Sculley,
Nene Centre for Research,
Graduate School,
University College Northampton,
Park Campus,
Broughton Green Road,
NORTHAMPTON NN2 7AL

Dear Mr. Sculley,

RE: SALIVARY ANTOXIDANTS AND PERIODONTAL DISEASE

Thank you for your recent submission to the Ethics Committee. I would like to formally apologise that the first submission sent to this office in February was lost, I have explained the circumstances to you regarding this and again please accept my apology for any inconvenience caused.

I can confirm that your protocol has been approved under Chairman's Action. The Chairman has asked for one alteration which is that the consent form is changed as follows; the term Medical Notes is replaced by the term Dental Notes. The Chairman has spoken to Dr. Langley Evans who has agreed to this.

The Ethics Committee are meeting on 30th May and the members will be asked to ratify the Chairman's Action accordingly.

The Committee complies with the ICH GCP guidelines on the composition, functions and operations of Independent Ethics Committees. The Committee is accountable to Buckinghamshire Health Authority. A copy of the constitution of the Committee is available on request.

If you have any query, or require further assistance, please do not hesitate to contact me.

Yours sincerely,

Ann Frew,
Acting Secretary,
Milton Keynes Local Research Ethics Committee.
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