THE ROLE OF LOCAL AND PERIPHERAL ANTIOXIDANTS IN THE PATHOGENESIS OF CHRONIC PERIODONTITIS IN SMOKERS

by

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ABSTRACT

Chronic periodontitis is one of the most common chronic inflammatory diseases of man and accounts for 60% of tooth loss. It is initiated by the subgingival biofilm and in susceptible individuals an abnormal inflammatory-immune response fails to resolve the inflammation and leads to destruction of the supporting tissues and the teeth. Risk factors for periodontitis may be systemic or local and of the systemic risk factors the most significant is smoking. Periodontitis patients appear to express a hyper-inflammatory phenotype involving excess or prolonged production of enzymes and reactive oxygen species (ROS) from cells of the innate immune response (primarily neutrophils). Neutrophil hyper-reactivity and hyperactivity, with respect to ROS production, has been demonstrated by several authors. Consistent with the exaggerated ROS production is the depletion of antioxidant defences against ROS within the periodontal pockets. Cigarette smoke is also reported to increase the oxidative burden and deplete antioxidant defences, but no data are available on gingival crevicular (GCF) antioxidant levels in smokers compared to non-smokers. This thesis explores the total antioxidant capacity (TAOC) of GCF and plasma in smokers and non-smokers with periodontitis and analyses the impact of smoking on the outcomes of periodontal therapy and upon local and peripheral antioxidant status in both groups. The working hypothesis is that an important mechanism underpinning the increased prevalence of periodontitis in smokers involves reduction of antioxidant defences due to smoking and thus increased oxidative stress and tissue damage. The cross-sectional data presented here suggest that smokers with periodontitis have a further compromise in GCF TAOC compared to age-, gender- and disease-matched non-smokers with periodontitis. The longitudinal data presented within this thesis suggest that the compromised GCF TAOC concentration seen in periodontitis irrespective of smoking status is likely to result from the inflammatory lesion, rather than predisposing to it. Moreover, the impact of the periodontal inflammation upon TAOC compromise appears more dominant than the effects of smoking.

DEDICATION

This thesis is dedicated to my husband, Rich, for all his unfailing support and love during this period of study and to my newly arrived daughter, Isobel, for giving me the sense of urgency to complete this work.

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ABBREVIATIONS:

A.a	-	Aggregatibacter actinomycetemcomitans
AAPH	-	2,2'-azobis (2,4-amidinopropane) dihydrochloride
ABST	-	2.2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)
AMVN	-	2,2'-azinobis (2,4-dimethylvaleronitrile)
ANOVA	-	Analysis of variance
ANUG	-	Acute necrotising ulcerative gingivitis
AO	-	Antioxidant
AP-	-	Activating protein
APC	-	Antigen presenting cells
ATP	-	Adenosine tri-phosphate
BOP	-	Bleeding on probing
BSA	-	Bovine serum albumin
$-^{\bullet}C-$	-	Carbon centred radical
Ca^{2+}	-	Calcium
CAL	-	Clinical attachment levels
COPD	-	Chronic obstructive pulmonary disease
CoQ_{10}	-	Co-enzyme Q10 (ubiquinone)
DCFH	-	2'.7'-dichlorodihydrofuorescein
DNA	-	Deoxyribosenucleic acid
ECL	-	Enhanced chemiluminescence
ELAM	-	Endothelium leucocyte adhesion molecule
fMLP	-	Formyl-methionyl-leucyl-phenylalanine
FRAP	-	Ferric reducing ability of plasma
GCF	-	Gingival crevicular fluid
GPx	-	Glutathione peroxidase
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathione

$H^{\bullet}$	-	Hydrogen radical
HOCl	-	Hypochlorous acid
H_2O_2	-	Hydrogen peroxide
$HO_2^{\bullet-}$	-	Perhydroxyl radical
$HOO^{\bullet}$	-	Alkoloxyl
HRP	-	Horseradish peroxidase
H_2S	-	Hydrogen sulphide
ICAM	-	Intercellular adhension molecule
Ig-	-	Immunoglobulin
IL-	-	Interleukin
IPS	-	Initial peak signal
K^+	-	Potassium
$L^{\bullet-}$	-	Luminol radical
LDL	-	Low-density lipoprotein
LSP	-	Lipopolysaccharides
LTB	-	Leukotrienes
MHC	-	Major histocompatibility complex
MMPs	-	Matrix metalloproteinases
NADPH	-	Nicotinamide adenine di-nucleotide phosphate
NETs	-	Neutrophil extracellular traps
$NO^{\bullet}$	-	Nitric oxide
$^1O_2$	-	Singlet oxygen
O_2	-	Molecule of di-oxygen
$O_2^{\bullet-}$	-	Superoxide anion
O_3	-	Ozone
$^{\bullet}OH$	-	Hydroxyl radical
OH^-	-	Hydroxide ion
$ONOO^-$	-	Peroxynitrite
ORAC	-	Oxygen radical absorbance capacity
PBS	-	Phosphate buffered saline
PGE	-	Prostaglandin

PMNL	-	Polymorphonulear leucoctye
PPD	-	Periodontal probing depth
PUFA	-	Polyunsaturated fatty acids
$RCOO^{\bullet}$	-	Acryloxyl
$RCOOO^{\bullet}$	-	Acrylperoxyl
REC	-	Recession
Redox	-	Reduction oxidation
RNS	-	Reactive nitrogen species
$RO^{\bullet}$	-	Aryloxyl
$ROO^{\bullet}$	-	Peroxyl
ROOH	-	Lipid hyroperoxide
ROS	-	Reactive oxygen species
R-Pe	-	Dichlorofluorescein-diacetate phycoerythrin
RSS	-	Reactive sulphur species
SOD	-	Superoxide dismutase
TAOC	-	Total antioxidant capacity
TEAC	-	Trolox equivalent antioxidant capacity
TIMP	-	Tissue inhibitors of matrix metalloproteinases
TNF	-	Tumour necrosis factor
TRAP	-	Trapping antioxidant parameter
Trolox	-	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylix
UV	-	Ultraviolet

INTRODUCTION

1. Introduction

1.1. Inflammatory periodontal diseases

Inflammatory periodontal diseases represent a range of inflammatory disorders that affect the supporting tissues of the teeth, namely the gingivae, periodontal ligament, cementum and alveolar bone and which ultimately lead to tooth loss. They arise due to complex interactions between the pathogenic bacteria of the subgingival biofilm and the host's inflammatory-immune response. Chronic periodontitis is still a major cause of tooth loss in the developed world (Papapanou 1999). In the UK 54% of the population examined in the Adult Dental Health Survey of 1998 had evidence of periodontitis, with 8% of adults suffering from advanced disease (Morris *et al* 1998). Current evidence indicates that the disease occurs in predisposed individuals that have an aberrant inflammatory/immune response to the microbial plaque adjacent to the gingival margin (Frederiksson *et al* 1998). The excessive or prolonged release of neutrophil enzymes and reactive oxygen species (ROS) is responsible for the majority of host-tissue destruction in periodontitis (Gustafsson *et al* 1997). To combat excessive ROS production the body possesses a variety of antioxidant (AO) defence mechanisms, which act in concert. Their role is to protect vital cell and tissue structures and bio-molecules from host-derived ROS as well as those of parasitic origin (Chapple *et al* 1996), by removing them as they form and repairing the damage they cause. A delicate balance exists between antioxidant defence and repair systems and pro-oxidant mechanisms of tissue destruction, and if the balance is tipped in favour of ROS activity, significant tissue damage ensues (Chapple *et al* 2002). Recent research into antioxidant defence in patients with periodontal disease has demonstrated both a reduced peripheral (plasma) and local (gingival crevicular fluid, GCF) total antioxidant capacity (TAOC) (Brock *et al* 2004). This thesis explores whether the compromised antioxidant defence systems predispose to chronic periodontitis or result from the inflammatory process and also what the impact of smoking is upon the TAOC of periodontitis patients.

1.2. Classification of inflammatory periodontal disease

Table 1 illustrates the nomenclature of periodontal diseases proposed by the International Workshop for Classification of Periodontal Diseases and Conditions in 1999.

Table 1.1: Classification of Periodontal Diseases

Classification of Inflammatory Periodontal Diseases
(Armitage *et al* 1999)

1. **Gingival Disease**
   a. Dental plaque induced gingival disease
   b. Non-plaque induced gingival lesion
2. **Chronic Periodontitis**†
   a. Localised
   b. Generalised
3. **Aggressive Periodontitis**†
   a. Localised
   b. Generalised
4. **Periodontitis as a manifestation of systemic disease**
   a. Associated with haematological disorders
   b. Associated with genetic disorders
5. **Necrotising Periodontal Disease**
   a. Necrotising ulcerative gingivitis
   b. Necrotising ulcerative periodontitis
6. **Abscesses of the Periodontium**
   a. Gingival abscess
   b. Periodontal abscess
   c. Pericoronal abscess
7. **Periodontitis associated with Endodontic Lesions**
   a. Combined periodontic-endodontic lesions
8. **Developmental or Acquired Deformities and Conditions**
   a. Localised tooth-related factors that modify or predispose to plaque-induced gingival disease/periodontitis
   b. Mucogingival deformities and conditions around teeth
   c. Mucogingival deformities and conditions on edentulous ridges
   d. Occusal trauma

† Can be further classified on the basis of extent and severity. As a general guide, extent can be characterized as Localised = ≤30% of sites involved and Generalised = ≥30% of sites involved. Severity can be characterized on the basis of the amount of clinical attachment loss (CAL) as follows: Slight = 1 or 2 mm CAL, Moderate = 3 or 4 mm CAL and Severe = ≥5 mm CAL

Plaque induced gingivitis and chronic periodontitis are the most prevalent of the inflammatory periodontal conditions encountered in clinical practice and will be the only conditions discussed in this thesis.

1.3. The aetiology and pathogenesis of gingivitis & periodontitis

Plaque has long been established as the causative agent for gingivitis by numerous studies dating back to the 1960s (Löe *et al* 1965). Plaque accumulation at the gingival margin for a period of between fifteen and twenty-one days, in the absence of oral hygiene measures, will lead to the development of the clinical signs of gingivitis in most individuals. These clinical signs; increased gingival erythema, odema and tendency of gingival soft tissues to bleeding upon gentle probing, are all reversible with the re-institution of appropriate oral hygiene measures (Löe *et al* 1965). Clinical changes induced by plaque accumulation may be subtle, but histologically the changes are quite marked as described by Page & Schroeder 1976 in their classical description of the initial, early and established lesions. Histological changes are seen as early as 24hrs within the gingival microvasculature; capillary beds open up, resulting in the accumulation of fluid exduate and swelling of the tissues. Inflammatory cell infiltration occurs in the connective tissues adjacent to the junctional epithelium, mainly comprising of lymphocytes, macrophages and neutrophilic polymorphonuclear leukocytes (PMNLs). As the cellular infiltrate continues to form, changes are seen in both the structural and cellular composition of the tissues. As the lesion develops an increase in gingival crevicular fluid flow rate is also seen (Lindhe 1997).

Although a clear correlation between the presence of plaque and gingivitis has been established it doesn't follow that all individuals with gingivitis will progress to develop periodontitis even in the presence of putative pathogens; this despite the consensus view expressed at the 2005 Ittigen Workshop that gingivitis and periodontitis are a continuum and that gingivitis is a 'pre-requisite' for periodontitis (Kinane *et al* 2005).

1.4. Dental plaque and the microbiology of periodontitis

Periodontitis is a unique infection in that, the site for bacterial colonisation, the teeth, have their structure partially within the connective tissues of the jaws and partially exposed to the external environment of the oral cavity. It is estimated that 700 species are capable of colonising the oral cavity and that an individual may harbour as many as 100 - 200 different species (Paster *et al* 2006). The teeth not only provide a non-shedding surface, which enhances the retention and accumulation of bacteria but also provide areas such as dentinal tubules, pits and cracks where pathogens may sequestrate from host defences. Within the gingival crevice the host's defence strategies may further be hindered by changes to hydrogen ion concentration (pH) (Zilm *et al* 2007), by proteolytic enzymes or by the reduction oxidation (Redox) potential (Eh)(Chapple & Matthews 2007).

Bacterial counts from subgingival plaque range from 10^3 in a healthy shallow sulcus to 10^8 in deep periodontal pockets, and counts within supragingival plaque can exceed 10^9 from a single tooth surface (Lindhe 1997). Despite the gross estimates of bacterial numbers colonising around or below the gingival margin, significant periodontitis does not develop in the majority of individuals and no clear relationship between numbers of colonising bacteria and the presence or severity of periodontal disease can be established. Moreover it is estimated that bacteria account for about 20% of the variants of disease, with the remainder being due to host factors (Grossi *et al* 1994).

A number of hypotheses have been proposed to explain the causal relationship between bacterial plaque and periodontitis. The most recent and widely accepted is the *ecological plaque hypothesis* (Marsh 1991 & 1994), which postulates that the physical characteristic and composition of the entire subgingival environment is the key to disease developing. Specific pathogens may be responsible for the initiation and progression of disease but they themselves are reliant upon non-pathogenic bacteria within their local community for survival. It is now widely recognised that dental plaque within the gingival crevice is organised into a biofilm and that the component

organisms are not merely passive neighbours, but that they are involved in a wide range of physical, metabolic and molecular interactions, which may be essential for attachment, growth, survival and virulence expression of the species at a particular site (Marsh 2005).

1.4.1. Putative pathogens

As plaque matures during the development of gingivitis and periodontitis, the subgingival biofilm exhibits a shift from a Gram positive and predominantly non-motile flora to a gram negative motile and anaerobic flora. These Gram negative bacteria within the biofilm possess a plethora of structural and secreted components (virulence factors) that are able to cause either direct destruction of host periodontal tissue or indirect damage via stimulation of an inappropriate host response. Extensive work on the periodontal microflora in chronic periodontitis has been carried out over several decades. Table 1.2 provides a list of the putative periodontal pathogens most frequently associated with active periodontitis.

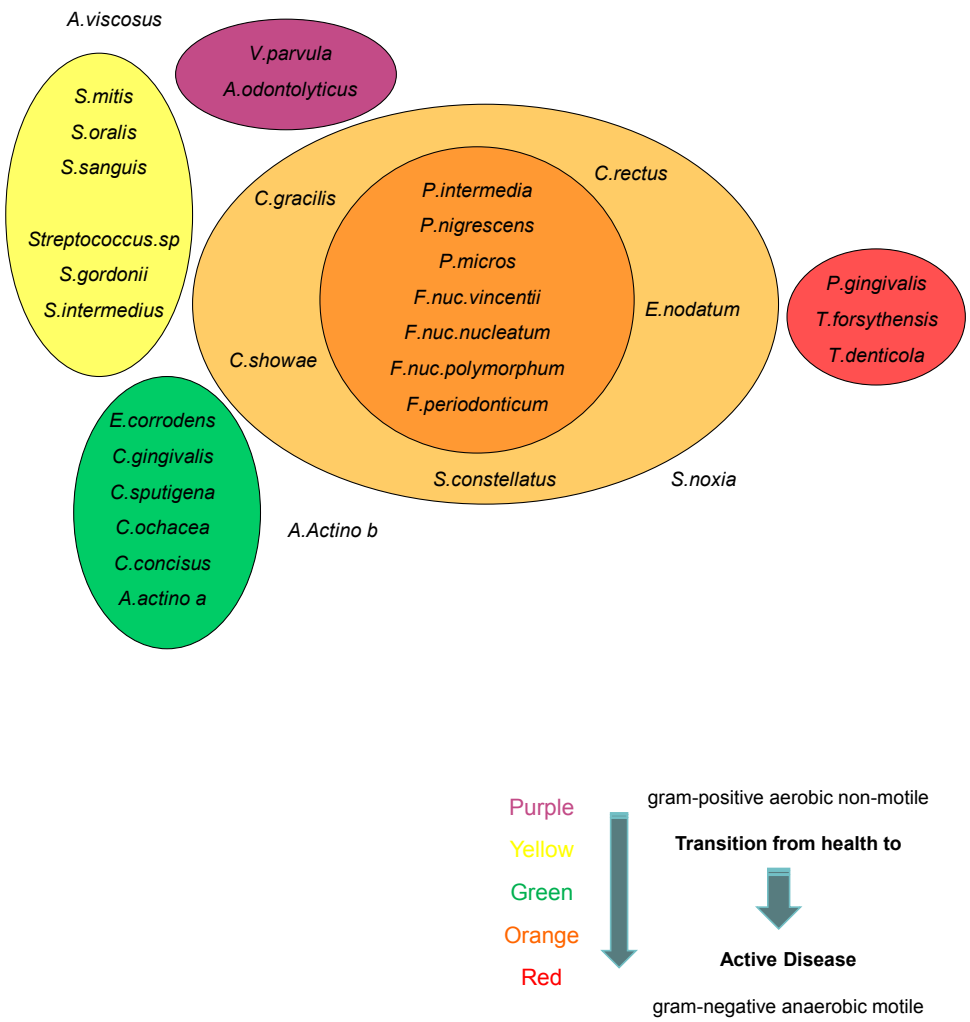
Table 1.2: Putative Periodontal Pathogens

Pathogenic Periodontal Species ***(Haffajee & Socransky 1994)***
Aggregatibacter actinomycetemcomitans (A.a)
Porphromonas gingivalis
Tanerella forsythus
Campylobacter rectus
Eubacterium nodatum
Fusobacterium nucleatum
Peptostreptococcus micros
Prevotella intermedia
Prevotella nigrescens
Streptococcus intermedius
Treponema spp (e.g. T. denticola)

More recent work by Sockransky and colleagues using cluster analysis and community ordination techniques examined the relationships amongst bacterial species within subgingival plaque and related these “complexes”

(denoted by different colours) to the clinical stages of periodontal disease. Their work demonstrated that certain organisms cluster together in discrete micro-environments along the length of the periodontal pocket. The red complex consisted of Gram negative motile anaerobes and displayed a strong relationship with deep active pockets. While the orange complex consisted of Gram-positive and Gram negative rods and cocci with some motility and were significantly associated with increasing pocket depths. The organisms within the purple, yellow and green complexes had no motility and consisted mainly of Gram positive organisms and were not associated active pocketing in periodontitis (Socransky *et al* 1998) (Figure 1.1.).

Fig. 1.1: Microbial complexes in subgingival plaque as described by Socransky *et al* 1998



In order for a pathogen to be causally linked with periodontitis, certain criteria need to be satisfied:

- The pathogen must be of a virulent clonal type
- The pathogen must possess the chromosomal and extra-chromosomal genetic factors to initiate disease
- The host must be susceptible to the pathogen
- The pathogen must be present in numbers sufficient to exceed the threshold of the host
- The pathogen must be located in the right place
- Other bacterial species must foster, or at least not inhibit, the process
- The local environment must be one which is conducive to the expression of the species' virulence properties

(Socransky & Haffafajee 1992)

1.4.2. Microbial virulence factors

Bacterial virulence factors fall into three broad categories:

- Enzymes, such as collagenase and hyaluronidase, are capable of breaking down the epithelial inter-cellular cement, thus allowing invasion into the connective tissues where greater damage can be effected than from within the external environment of the gingival crevice.

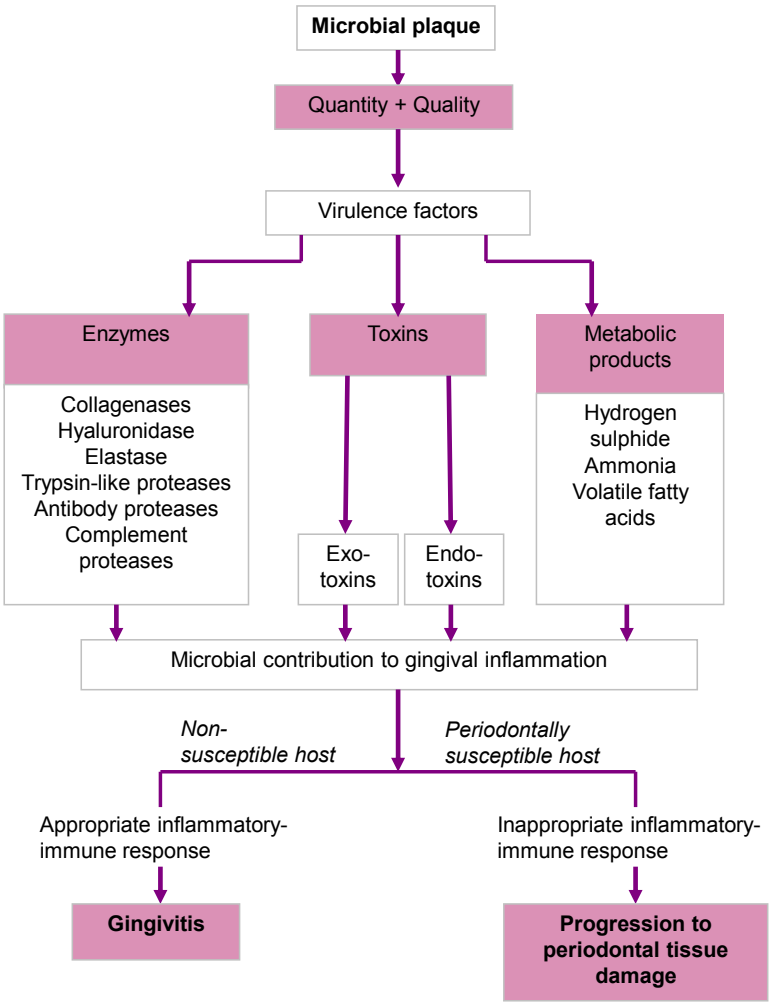
- Toxins may modulate inflammatory responses by direct killing of host cells or by potentiating the release of excess pro-inflammatory cytokines and chemoattractants. They also have the ability to prevent phagocytosis and are cytotoxic to leucocytes (Madianos *et al* 2005). They are elaborated as either endotoxins or exotoxins.

  Endotoxins are released from the cell wall of gram negative bacteria upon death, but a degree of slow release also occurs within vesicular or soluble forms. Endotoxins are also termed lipopolysaccharides (LPS) and are amongst the most potent stimulants of the inflammatory and immune response of the host.

Exotoxins are released during the bacterial life cycle and include "leucotoxin", capable of destroying polymorphonulcear leucocytes (PMNLs or neutrophils) and epitheliotoxin.

- Metabolic waste products such as ammonia, volatile fatty acids, indole, hydrogen sulphide (H_2S) and butyric acid may also damage host cells and tissues. Some putative pathogens are capable of metabolising protective anti-inflammatory and antioxidant peptides to form toxic compounds such as H_2S. (Chapple & Gilbert 2002). In addition ammonia provides a rise at the low pH of periodontal pockets due to alterations in microbial metabolic pathways (Zilm 2007).

Fig. 1.2: Schematic representation of how periodontal micro-organisms may cause tissue damage (Adapted from Chapple & Gilbert 2002)



These virulence factors are capable of causing direct damage to the tissues of the periodontium and stimulate host cells to activate a wide range of inflammatory and immune responses; the latter, although designed to eliminate the bacterial infection, may in fact, also cause tissue damage when dysregulated.

1.5. Host response and its contribution to the aetiology of periodontitis

The presence of the bacterial biofilm within the gingival crevice or periodontal pocket will in the first instance initiate the innate (non-specific) immune response. One of the innate immune system's primary lines of defence is the physical barrier created by the rapid turnover of junctional epithelium, plus it's permeability to gingival crevicular fluid (GCF) and neutrophilic polymorphonuclear leucocytes (PMNL). As well as forming a physical barrier, the junctional epithelium releases cell signalling molecules, to establish the initial inflammatory response. Inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), intereukin-8 (IL-8) and tumour necrosis factor α (TNFα) initiate the inflammatory response in the underlying connective tissues and induce neutrophil and macrophage chemotaxis as a second line of defence to antigens localised within the gingival crevice. These pro-inflammatory cytokines from the epithelial cells, together with bacterial virulence factors that diffuse into the connective tissues, stimulate resident host cells such as dendritic cells, tissue macrophages, fibroblasts and mast cells to produce and release more of the same pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, prostaglandin (PGE_2)), leukotrienes (LTB_4) and histamine, which in turn activate the endothelial cells of the micro-vascular beds to express surface adhesion molecules that are important in leukocyte extravasation (Madianos *et al* 2005).

Intercellular adhesion molecule 1 (ICAM-1) and endothelium leucocyte adhesion molecule 1 (ELAM-1) are expressed on the vascular endothelial cells and are important adhesion molecules, which bind to complimentary PMNL receptors (selectins and integrins) aiding the migration of PMNLs through the epithelium and into the gingival crevice (Moughal *et al* 1992).

Histamine and PGE_2 cause vasodilatation allowing more blood cells and plasma proteins (e.g. complement) to be brought to the area of infection. The presence of bacterial lipopolysaccharides also initiates the alternative pathway of the complement cascade, C3a and C5a enhance the inflammatory response by causing further histamine release from mast cells and C3b facilitates PMNL adhesion to bacteria, further aiding phagocytosis (opsonisation).

PMNLs are the predominant leukocytes within the periodontal lesion (Van Dyke *et al* 1985) and once at the area of infection can either phagocytose opsonised bacteria or degranulate extracellulary thereby releasing their enzymes, antimicrobial peptides and ROS prior to undergoing programmed cell death (apoptosis) in order to the eliminate the pathogen. The monocytes, which enter the tissues as Langerhans cells, act as scavengers phagocytosing the dead bacteria and PMNLs. They also play an important role in activating the acquired immune response by acting as antigen presenting cells (APC) (Chapple & Gilbert 2002).

When macrophages encounter pathogens they phagocytose them and release onto their surface antigenic material that binds to the host cell's major histocompatible complex (MHC) class II receptors, allowing recognition by effector T-cells. These T-cells proceed to mount a specific immune response, including memory cell production, lymphokine production, direct lysis of bacteria (T-cell cytotoxicity) and assisting in B-cell function (CD4 T-helper cells). B-cells differentiate into plasma cells which produce immunoglobulins, that are released into the blood stream and tissues where they 'home in' on and bind to target bacteria, and with the help of the complement system, phagocytose and destroy them. IgG is the most important immunoglobulin in periodontal disease and the Fcγ-RII receptors of PMNLs show a strong avidity to IgG-opsonised bacteria (Kobayashi *et al* 2000), thus binding the antigen/antibody complex and facilitating phagocytosis and downstream killing.

The early inflammatory lesion is dominated by PMNLs, but with time the mature lesion changes and T & B lymphocytes are activated providing specifically targeted and controlled killing of bacteria through the acquired immune response. The innate immune response continues at the same time providing a less specific and less effective defence mechanism. If left untreated the size of the inflammatory lesion exceeds a certain threshold within each host and tissue damage ensues particularly if the host's enzyme-inhibitor (e.g. tissue inhibitors of matrix metalloproteinases TIMPs) and antioxidant defences are compromised.

1.5.1. Mechanisms of tissue damage

There are many mechanisms of tissue damage that result from the presence of bacterial plaque within the gingival crevice/pocket and their antigenic products that diffuse through the junctional epithelium. They can be summarised as:

- Direct damage by the bacteria
- Indirect damage via bacterial-induced inflammatory process
- Indirect damage by the acquired immune response (largely though the B-cell/plasma cell systems where PMNLs are the effector cells)

Periodontal pathogens posses a wide array of virulence factors as discussed previously, such as proteolytic enzymes, which are capable of degrading the extra-cellular components of the periodontium, such as collagen, elastin, fibrin and fibronectin. *A*.a produces a leucotoxin, which can lyse neutrophils and macrophages, while *P.gingivalis* produces an array of proteases including gingipains and those capable of destroying complement and immunoglobulins. The LPS cell walls of gram-negative bacteria are capable of invoking both innate and acquired host immune responses.

In generating an inflammatory exduate the resident host cells exude not only cytokines but also matrix metalloproteinases (MMPs), which degrade collagen within the connective tissues thereby creating room for the inflammatory

exduate and facilitating the movement of immune cells within the connective tissues. The balance between the production of the family of matrix metalloproteinases and their inhibitors (TIMPs) may be tipped by pro-inflammatory cytokines such as Il-1β in favour of excessive damage of all the components of the extracellular matrix (Page *et al* 1997). Additionally, pathological levels of PGE_2, IL-1β, TNFα and IL-6 produced by resident fibroblasts and leukocytes mediate alveolar bone loss (Schwartz *et al* 1997).

During the PMNLs respiratory burst, molecular oxygen is reduced via the NADPH-oxidase to form superoxide and other reactive oxygen species (ROS). Lyosomal antimicrobial compounds are then discharged into the cell's vacuole and activated by the ROS to kill the ingested organism within (Ahluwalia *et al* 2004, Segal 1993). Before apoptosis is completed, these biologically active products may be released into the external environment allowing extracellular killing of micro-organisms but also causing damage to surrounding host cell and tissue structures (Lindhe 1997).

1.6. The threat of oxygen

Oxygen is essential for life but breathing pure oxygen at atmospheric conditions for more than 48 hours will lead to respiratory distress and even death (Acworth & Bailey 1995). An adult exposed to pure oxygen at 1atm pressure for as little as 6 hours will show signs of oxygen stress; chest soreness, cough and a sore throat, whilst longer exposure leads to alveolar damage (Halliwell 1994a). The potentially toxic effects of oxygen have long been recognised but not fully understood. Research by Binger in the late 1920s on oxygen toxicity in mammals identified experimental work in the previous century on respiratory problems induced by altered levels of oxygen consumption. The most conclusive of these studies were those of Bert in 1878, who documented, "oxygen at high tension is a powerful poison" (Binger *et al* 1927). However, it was not until the late 1940s - 1950s that oxygen toxicity was fully appreciated by the medical community, when retrolenral fibroplasias in premature babies were attributed to the high O_2 concentration

of the incubator in 1954 (Halliwell 1994a). In the late 1960s and early 1970s studies of newborn bronchopulmonary dysplasia and adult respiratory distress syndrome confirmed the association (Knight 1998).

It is not the molecular oxygen *per se* that is toxic more the highly reactive reduced adducts of oxygen. The presence of free radicals in biological systems was not generally considered likely until 1954 when R. Gershman and D. Gilbert proposed that many of the damaging effects of oxygen could be attributed to oxygen free radicals (Halliwell 1984). The discovery of superoxide dismutase in 1969 by McCord and Fridovich lead to affirmation and further development of this hypothesis (McCord & Fridovich 1969).

Oxygen radicals and other oxygen-derived species are constantly generated either by "accidents of chemistry" or for specific metabolic purposes *in vivo* (Halliwell 1994a). It has been calculated that for every 100 tons of oxygen metabolised approximately two tons form reactive oxygen species, and for every oxygen molecule crossing into a cell each day 1 in 100 will damage proteins and 1 in 200 damage DNA. These ROS are also capable of damaging lipid and it is the damage to these biological molecules that renders excess ROS release dangerous, especially when the body's natural antioxidant defences are compromised (Acworth & Bailey 1995).

1.6.1. Basic free radical reactions

Free radicals have been defined as "any species capable of independent existence that contain one or more unpaired electrons, an unpaired electron being one that is alone in an orbital" (Halliwell 1991). Free radicals may be formed in three ways: i) by the addition of a single electron to a molecule, ii) by the loss of a single electron, iii) by homolytic cleavage of a covalent bond.

$A + e^- \rightarrow A^{\bullet -}$ electron transfer

$X{:}Y \rightarrow X^\bullet + Y^\bullet$ homolytic fission

Electron transference is the most common mechanism in biological systems as it requires less energy input than homolytic cleavage. In heterolytic fission the electrons of the covalent bond are retained by only one of the fragments of the parent molecules and this results in ion formation, the ions being charged, rather than free radicals (Cheeseman *et al* 1993).

Free radicals can be positively charged, negatively charged or neutral and the unpaired electron is symbolised by a superscripted dot in the chemical symbol. Electrons are more stable when paired together in orbitals and free radicals are more reactive than non-radical species. When two radicals meet (resulting in their disappearance) a termination reaction occurs whereby their unpaired electrons combine via a covalent bond.

$O_2^{\bullet -}$ (superoxide) + $NO^{\bullet}$ (nitric oxide) → $ONOO^-$ (peroxynitrite) *Reaction 1*

However most molecules in the body are not radicals, and when a radical meets a non-radical molecule it will reduce, oxidise or simply add to the non-radical resulting in the other molecule involved becoming a radical itself. Therefore, the important feature in radical reactions is that they proceed in a chain reaction, one radical begets another and so on (Halliwell 1989 & 1994b).

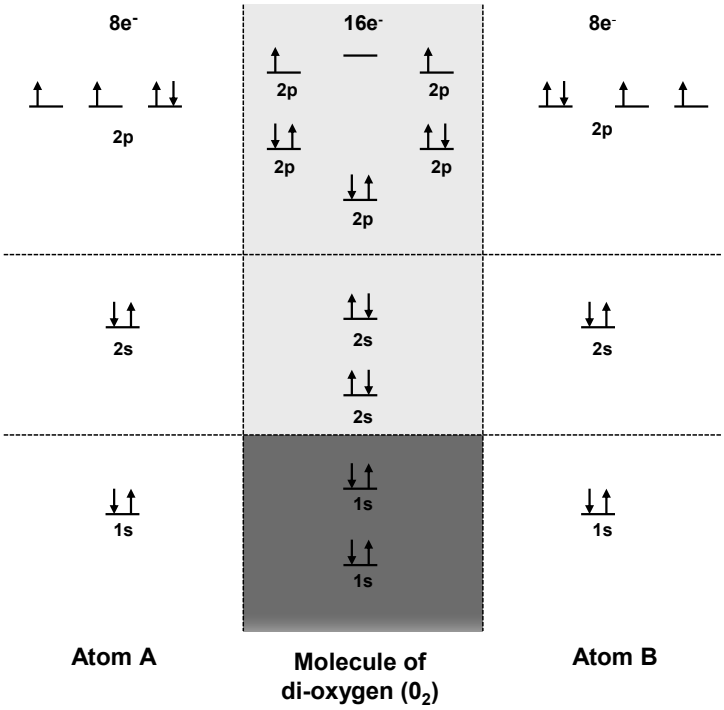
By their nature, free radicals are highly reactive and diverse species including not only oxygen species but also nitrogen and sulphur species, while the hydrogen radical ($H^{\bullet}$ the same as the hydrogen atom) is the simplest free radical containing only one proton and one electron (Halliwell 1994b). This thesis will focus on the oxygen derived reactive species (ROS) but some mention of the other species is necessary due to interactions between different classes (see reaction 1).

1.6.2. Atomic and molecular oxygen

Atoms have shells containing negatively charged electrons, which require energy to prevent them being pulled into the nucleus. Each shell can have up

to four orbital patterns around the nucleus, spinning in either direction. Orbitals are filled in order of increasing energy and may hold only two electrons with opposite spins (the Pauli Exclusion Principle). Atomic oxygen has eight electrons distributed 1_s^2, 2_s^2, 2_p^4. In the first shell the electrons are paired in an s-orbital, in the second shell one pair of electrons are also in an s-orbital while the remaining four electron pairs are in p-orbitals. In the p-orbitals there is only one paired electron group, while the other two p-orbitals contain individual electrons (Webster & Nunn 1987). Molecular di-oxygen is formed from the joining of two oxygen atoms and is regarded as a stable bi-radical, as it has sixteen electrons occupying two atomic shells, but the outer $2e^-$ are unpaired with a parallel spin (see Fig.1.3).

Fig 1.3: The organisation of electrons within the shells and orbitals of di-oxygen. Two oxygen atoms (Atom A & Atom B) are shown on either side of the molecule of di-oxygen (O_2) which results from their combination. (Adapted from Webster & Nunn 1987)



The result of this arrangement is a molecule with the desire to pair up its outer unpaired electrons, making it a powerful oxidising agent, but because of the spin restriction caused by the parallel spin of the outer unpaired $2e^-$ it can not

accept electron pairs, as $2e^-$ do not exist in isolation with parallel spins, this spin restriction forces molecular oxygen to only accept one electron at a time (Webster & Nunn 1987, Chapple & Matthews 2007).

The removal of an electron constitutes oxidation, while the substance receiving the electron becomes reduced. Thermodynamically oxygen wants to take on additional electrons (two per atom, four per molecule) to produce a water molecule which has much lower free energy, but due to its stepwise acceptance of a single electron at a time, oxygen free radical formation occurs. The addition of the first electron (e^-) to an oxygen molecule results in the formation of the superoxide anion:

$$O_2 + e^- \rightarrow O_2^{\bullet -} \qquad \textit{Reaction 2}$$

The addition of a second electron (e^-) results in the formation of the ROS hydrogen peroxide (H_2O_2), although not a radical, it will readily receive two more electrons (reaction 4 & 5) thereby making it a cytotoxic oxidant:

$$O_2^{\bullet -} + e^- \; 2H^+ \rightarrow H_2O_2 \qquad \textit{Reaction 3}$$

The addition of a third electron (e^-) results in the formation of the hydroxyl radical, one of the most potent free radicals known, which can indiscriminately oxidise virtually any organic molecule:

$$H_2O_2 + e^- \rightarrow {}^{\bullet}OH + OH^- \qquad \textit{Reaction 4}$$

The addition of a fourth electron (e^-) results in the formation of water:

$${}^{\bullet}OH + e^- + H^+ \rightarrow H_2O. \qquad \textit{Reaction 5}$$

(McCord 2000, Chapple & Matthews 2007)

1.6.3. Origins and formation of reactive oxygen species

ROS are generated by a wide variety of sources, and are formed in all living organisms either as a result of normal metabolism (endogenous sources) or

accidentally and as a consequence of exposure to external environmental stimuli (exogenous sources) such as ionizing radiation, UV light, therapeutic drugs and pollutants such as vehicle exhaust fumes. Behavioural activities such as tobacco smoking or beetle nut use will also add to oxidative damage by ROS. Endogenous sources are from two main processes:

- as a bi-product of metabolic pathways
- functional generation by host cells, primarily defence cells (phagocytes) plus cells of the connective tissues (osteoblasts and fibroblasts).

1.6.4. Reactive oxygen species

Reactive Oxygen Species (ROS) is a collective term which includes all oxygen derived free radicals, including those reactive intermediate oxygen species formed which are not true radicals but capable of radical formation in the intra- and extracellular environment (Chapple & Matthews 2007). Table 1.3 illustrates the family of true radicals and reactive species derived from oxygen now collectively referred to as ROS.

Table 1.3: Table of Reactive Oxygen Species modified from Battino *et al* 1999

Reactive Oxygen Species

Radical		Non-radicals	
Superoxide	$O_2^{\bullet -}$	Singlet Oxygen	$^1O_2$
Hydroxyl	$^{\bullet}OH$	Ozone	O_3
Alkoloxyl	$HOO^{\bullet}$	Hypochlorous acid	HOCl
Aryloxyl	$RO^{\bullet}$	Hydrogen peroxide	H_2O_2
Arylperoxyl	$ArO^{\bullet}$		
Peroxyl	$ROO^{\bullet}$		
Acryloxyl	$RCOO^{\bullet}$		
Acylperoxyl	$RCOOO^{\bullet}$		

Convention is to use • to signify an unpaired electron & - or + for the molecular charge

1.6.4.1. Singlet oxygen ($^1O_2$)

Two singlet oxygen states exist, by removal of one electron and alleviation of the spin restriction from molecular oxygen. Singlet oxygen $O_2{}^1\Delta_g$ is not a true radical as it has no unpaired electrons, but has great importance in biological systems, whereas singlet oxygen $O_2{}^1\Sigma_g{}^+$ has a short life span and decays to Δ_g rapidly (Halliwell *et al* 1984, Darr *et al* 1994). Singlet oxygen $O_2{}^1\Delta_g$ is highly reactive in lipid membranes but little is known of its role in tissue damage and any possible role in periodontal inflammation has yet to be identified (Chapple 1997).

1.6.4.2. Superoxide ($O_2^{\bullet-}$)

If a single electron is added to molecular oxygen the superoxide ($O_2^{\bullet-}$) anion is formed (see reaction 2). This reaction is brought about as an accidental bi-product of metabolism within the mitochondria, the main sites of oxygen metabolism during ATP production. During the mitochondrial electron transport system, electrons leak at a constant rate and reduce oxygen to $O_2^{\bullet-}$. At least two sites have been identified in the electron transport chain, Complex I and ubisemiquinone, where leakage may occur and superoxide formation results (McCord 2000). It is estimated the 1-2% of the oxygen consumed by the mitochondria is only partially reduced by leaked electrons and converted to $O_2^{\bullet-}$ (Cadenas *et al* 2000). Since we consume large quantities of oxygen even at rest, it is estimated that we may produce over 2kg of superoxide per year, this figure increases in those with chronic inflammation (Halliwell 1994a & 1994b). Hence the mitochondria contain a specific enzyme system, mitochondrial superoxide dismutase, to reduce $O_2^{\bullet-}$ back to the less reactive H_2O_2 and ultimately water (via a second enzyme called catalase).

However, the most important source of $O_2^{\bullet-}$ is the functional generation by phagocytic cells (neutrophils, monocytes, macrophages and esinophils) to inactivate invading bacteria or viruses (Halliwell 1994a). Its production occurs within the hexo-monophosphate (nicotinamide adrenine di-nucleotide phosphate, NADPH-oxidase) shunt, that shunts glucose-6-phosphate from

the glycolysis pathway and utilizes molecular oxygen and NADPH to form the superoxide radical anion $O_2^{\bullet-}$ (Chapple & Matthews 2007). NADPH-oxidase consists of a number of subunits at rest between the cytosol and the intercellular vesicle membrane, when the PMNL is stimulated by an antigen, cytokine or other mediator, the respiratory burst ensues and the cytobolic subunits migrate to the vacuole or cell membrane and assemble into the active oxidase, resulting in the delivery of $O_2^{\bullet-}$ into the phagocytic vacuole. Although superoxide may contribute to the microbial killing process other more potent ROS are rapidly generated from this precursor (Bergendi *et al* 1999).

Superoxide can act as both an oxidant and a reductant, undergoing a dismutation reaction in which one $O_2^{\bullet-}$ acts as a reductant while the other as an oxidant. This spontaneous dismutation occurs rapidly at a neutral pH (K2 = 1 x 105 M-1 second-1) (Darr *et al* 1994). $O_2^{\bullet-}$ is considered a weakly reactive radical by comparison with the hydroxyl radical but nevertheless can attack a number of biological targets and it's ability to spontaneously dismutate to hydrogen peroxide and singlet oxygen leads to damage by these radicals (Chapple 1997).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow {}^1O_2 + H_2O_2 \qquad \textit{Reaction 6}$$

Via a complex reaction with hydrogen peroxide catalysed by metal ions (Haber-Weis reaction) superoxide may be converted to the highly reactive hydroxyl radical (Cheeseman *et al* 1993, Chapple 1997).

$$O_2^{\bullet-} + H_2O_2. \xrightarrow{\text{Fe or Cu ions}} {}^{\bullet}OH + OH^- + O_2 \qquad \textit{Reaction 7}$$

Superoxide can also interact with nitric oxide to form the highly potent peroxynitrite anion (reaction 1) (Chapple 1997).

1.6.4.3. Hydrogen peroxide (H_2O_2)

Although not a true radical and being formed following the two electron reduction of ground oxygen (reaction 3), hydrogen peroxide has widely been

reported as cytotoxic in mammalian cells at levels of ≥50μM (Halliwell *et al* 2000). Hydrogen peroxide is generated by the dismutation of superoxide $O_2^{\bullet-}$ (mostly from active phagocytes) *in vivo*, both non-enzymatically and catalysed by superoxide dismutase (SOD). It resembles water in its molecular structure and is able to diffuse across cell and nuclear membranes unlike $O_2^{\bullet-}$ (Halliwell 1994a). H_2O_2 is however poorly reactive, it can only act as a mild oxidising or reducting agent and does not oxidise most biological molecules readily. The greater threat from H_2O_2 comes from its indiscriminate conversion to the hydroxyl radical ($^{\bullet}OH$) either by exposure to ultraviolet light or through interaction with transition metal ions, most importantly in the classical Fenton reaction (Halliwell *et al* 2000). In the presence of iron II or copper I it is reduced and the hydroxide ion (OH^-) and hydroxyl radical ($^{\bullet}OH$) are formed via Fenton chemistry (Blake *et al* 1987).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad \textit{Reaction 8}$$

More recently (and of high significance in biological systems) it has been recognised that H_2O_2 is a key cell signalling molecule and is involved in the up-regulation of the expression of certain genes through redox-regulated gene transcription factors for example IL triggers the displacement of an inhibitory subunit from the cytoplasmic transcription factor NF-κB (Halliwell 1994a), facilitating nuclear translocation of free NF-κB and down-stream transcription of pro-inflammatory cytokine genes e.g.IL-1 and IL-8. It also plays a role in the promotion of epithelial cell electrolyte transport (Conner *et al* 1996), and where inflammation is present it may increase adhesion molecule expression, cause proliferation of cells, induce apoptosis and modulate aggregation of platelets (Chapple & Matthew 2007).

Hydrogen peroxide is removed primarily by the antioxidant enzyme catalase, which acts predominately intracellularly, by glutathione peroxidase within the mitochondria and extracellularly, and the thioredoxin-linked peroxidases (Chapple & Matthew 2007). Although most cells are exposed to some levels of H_2O_2 from mitochondrial and phagocytic sources, some tissues especially in the oral cavity may be exposed to higher concentrations due to other

exogenous sources. Beverages, such as green or black tea and coffee, contain concentrations of H_2O_2 above 100µm, which may diffuse into the cells (Halliwell *et al* 2000). Oral bacteria are also a source of H_2O_2 and salivary peroxidise, by its conversion to hypothiocyanite, is able to stop this bacterial production and protect the epithelial cells in contact with the pathogens (Carlsson 1987).

1.6.4.4. Hydroxyl radical ($^{\bullet}OH$)

The hydroxyl radical ($^{\bullet}OH$) or related perhydroxyl radical ($HO_2^{\bullet-}$) are the most potent ROS and are known to cause damage and destruction to a variety of cellular and tissue components (Chapple & Matthews 2007). It may be formed from superoxide via a Haber-Weiss reaction with hydrogen peroxide (reaction 7), or from hydrogen peroxide via Fenton reactions (reaction 8). However formed, the hydroxyl radical is extremely reactive, attacking and damaging almost every molecule within living cells while persisting for less than a microsecond (Halliwell 1991) (Table 1.4).

Table 1.4: Period of Half-life of Reactive Oxygen Species modified from Bergendi *et al* 1999

Period of Half-life of Free Radical & Intermediates

ROS		Half-life (sec)
Singlet oxygen	$^1O_2$	1×10^{-5}
Superoxide	$O_2^{\bullet-}$	Enzyme decomposition
Hydrogen peroxide	H_2O_2	Enzyme decomposition
Hydroxyl	$^{\bullet}OH$	1×10^{-9}
Nitric oxide	$NO^{\bullet}$	1 to 10
Peroxynitrite	$ONOO^-$	0.05 to 1.0
Alcoxyl	$RO^{\bullet}$	1×10^{-6}
Peroxyl	$ROO^{\bullet}$	7

Whilst some radicals are stable enough to diffuse some distance from their site of generation, $^{\bullet}OH$ is so reactive that it is believed to react within 1-5 molecular diameters of its site of formation (Pryor 1986). Thus, it will react at the site of formation and has been shown to oxidise proteins and promote DNA strand scission (Conner *et al* 1996). Reactions of $^{\bullet}OH$ with biological molecules, most of which are non-radicals, set off chain reactions. The best characterised of these is the lipid peroxidation reaction, which occurs when $^{\bullet}OH$ is generated near to membranes and attacks the fatty acid side chain of the membrane phospholipid, creating a lipid peroxyl radical and lipid hydroperoxides. If lipid peroxidation progresses unchecked it can lead to cell necrosis.

1.6.5. Sources of Fenton-reactive metal iron *in vivo*

Since the formation of the majority of hydroxyl radicals is through a metal-dependant reduction of hydrogen peroxide via Fenton chemistry, the damage and significance of $^{\bullet}OH$ radical activity depends upon its locus of formation and the availability of metal ions. Cells and organisms handle iron salts very carefully to ensure “free iron” rarely occurs. Iron is always bound to proteins, membranes, nucleic acids or low-molecular weight chelating agents. However, ferritin-bound iron can be mobilised from proteins by superoxide and $^{\bullet}OH$ formation occurs. Whilst at pH of 5.6 iron maybe mobilised from transferrin, which is achievable in the microenvironments of adherent phagocytes (Halliwell *et al* 1986) and by certain bacteria (Roberts *et al* 2005).

The majority of iron is stored in haemoglobin and around 10% in myoglin (Halliwell et al 1984) but there is no clear evidence to suggest whether these may serve as Fenton catalysts.

1.6.6. Nitrogen-derived free radicals

The bulk of free radical knowledge and research to date has focused on reactive oxygen intermediates, brief mention of the nitrogen derived free

radicals is included due to the interactions between the two groups, and in particular peroxynitrite formation ($ONOO^-$).

1.6.6.1. Nitric oxide ($NO^•$)

Nitric oxide is synthesised from the amino acid L-arginine within vascular endothelial cells, by macrophages and certain brain cells (Halliwell 1994a), it is a small lipophilic molecule, which is able to cross cell membranes. $NO^•$ activity is regulated by cytokines and during inflammation it regulates several humoral and cellular responses, having both anti-inflammatory and pro-inflammatory properties, dependant on the phase and type of inflammation (Moilanen *et al* 1995). When present in low concentrations nitric oxide acts as a vasodilator helping regulate blood flow and pressure, as well as a neurotransmitter for the central nervous system. It also plays a part in the neuroendocrine system. At high concentrations the nitric oxide radical may act as a cytostatic and is cytotoxic for bacterial, fungal and protozoal organisms as well as tumour cells (Darr *et al* 1994, Bergendi *et al* 1999, Cuzzocrea et al 2001).

Simultaneous production of $NO^•$ and $O_2^{•-}$ can lead to the production of the highly reactive peroxynitrite anion ($ONOO^-$), while not a true radical it is believed to be responsible for many of the reactions originally attributed to the radicals which produced it (Chapple & Matthews 2007).

1.6.6.2. Peroxynitrite ($ONOO^-$)

Peroxynitrite is a selective oxidant reacting slowly with most biological molecules (Beckman & Koppenol 1996). Peroxynitrite is formed by a diffusion-limited reaction between $NO^•$ and $O_2^{•-}$, it has a half-life of 1.9 seconds at pH7.4, which permits its diffusion over several cell diameters (Beckman *et al* 1990). Once near or inside a cell $ONOO^-$ is able to damage or deplete a number of vital cell components, such as DNA strand scission, lipids by peroxidation and antioxidant availability (Cuzzocrea *et al* 2001).

1.6.7. Sulphur derived free radicals (reactive sulphur species; RSS)

ROS may frequently react with cellular thiols to form disulphides, which are considered only mildly oxidising under physiological conditions but, under conditions of oxidative stress, sulphur maybe oxidised beyond this disulphide state to form disulphide S-monoxides and disulphide S-dioxide. These reactive sulphur species inhibit the function of thiol-dependent proteins and increase the reactivity of some of their parent compounds, especially H_2O_2 and disulphides (Giles *et al* 2002).

1.7. The role of ROS in tissue damage

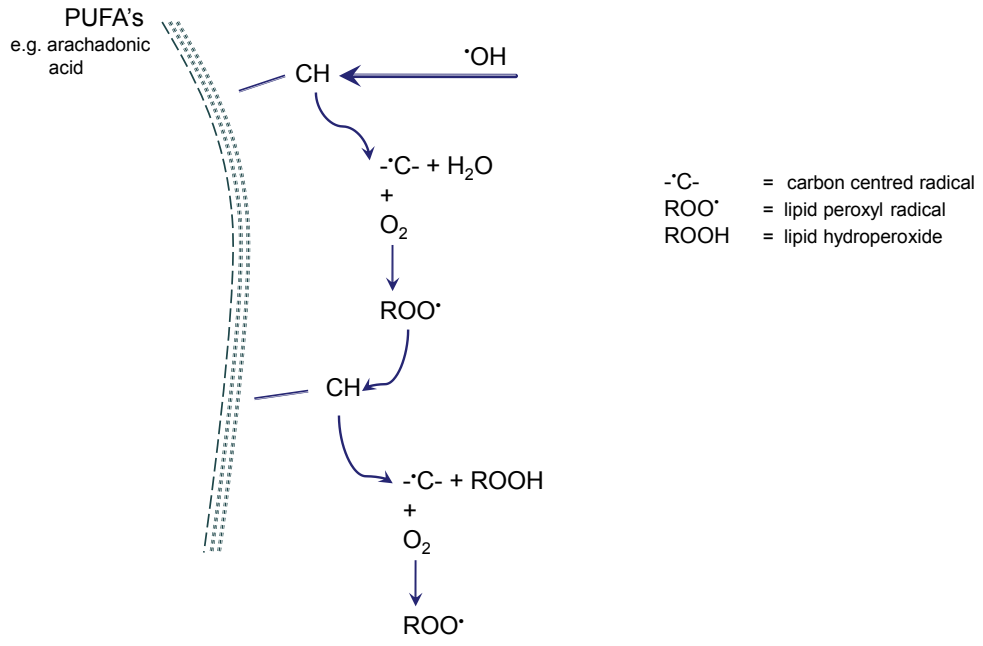
Whilst reactivity does not equate to toxicity, it is clear that many of the reactions involving ROS can produce damage *in vivo*, oxidant by-products of normal metabolism cause extensive damage to lipid, protein and DNA. Ames and co-workers estimate that each human cell receives as many as 10,000 radical 'hits' per day (Ames *et al* 1993).

1.7.1. Lipid perioxidation

The polyunsaturated fatty acids (PUFA) located in biological cell membranes, such as cytoplasmic cell membranes and mitochondrial membranes are prime targets for ROS (Çanakçi *et al* 2005). Most polyunsaturated fatty acids have their double bond conjugated and separated by a methylene group. The presence of this double bond next to the methylene group, makes the methylene C-H bond weaker and susceptible for hydrogen abstraction (Blake *et al* 1987). The hydroxyl radical and peroxynitrite anion are most effective at activating this process, which gives rise to the lipid peroxidation chain reaction, the events of which have been simplified into three major stages by Halliwell (1991): initiation, propagation and termination. The hydroxyl or perynitirite species attacks the PUFA side chains (e.g. arachidonic acid and decosachexaenoic acid) of the lipid membrane and abstracts the hydrogen atom (initiation) forming a carbon-centred radical (-$^{\bullet}$C-) in the membrane. These carbon centred radicals may either undergo molecular rearrangement

to give a conjugated diene structure or may combine with another such radical forming a covalent bond and creating cross-linking and disruption of the membrane structure. More commonly the carbon-centred side chain radical combines with oxygen creating yet another radical, the peroxyl radical ($ROO^{\bullet}$). The peroxyl radical is reactive enough to attack adjacent PUFA side chains (propagation), abstracting hydrogen and generating another carbon-centred radical and a lipid hydroperoxide (ROOH). The carbon-centred side chain radical goes on to form another peroxyl radical in the presence of oxygen, which in turn attacks another PUFA side chain and thus a self-perpetuating chain reaction occurs in which hundreds of lipid hydroperoxides are formed (see fig.1.4).

Fig. 1.4: The lipid peroxidation chain reaction initiated by hydroxyl radicals.
(Adapted from Chapple & Matthews 2007)



The accumulation of lipid hydroperoxides in the cell membrane can have a serious effect on the membranes' fluidity, affecting the activity of transmembrane enzymes, receptors, transporters and other membrane proteins. This results in changes in membrane selectivity and permeability and may even cause it to collapse (Halliwell *et al* 1991, Çanakçi *et al* 2005).

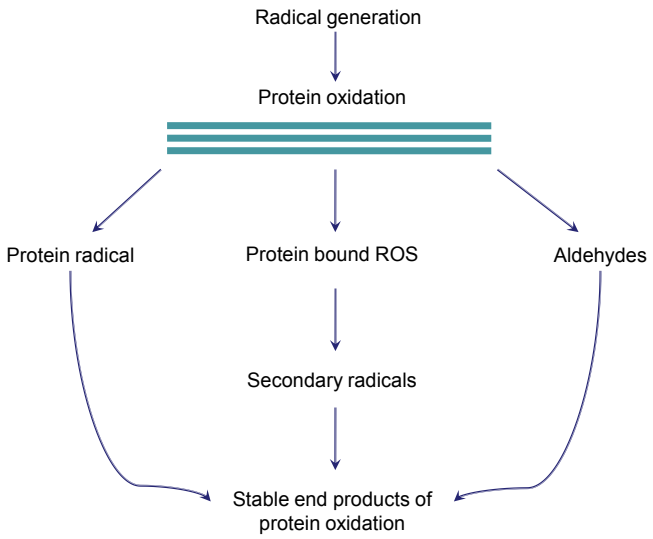
Lipid hydroperoxides can also decompose into highly cytotoxic secondary products such as aldehydes, which are also able to cause damage to membrane proteins, inactivating receptors and membrane-bound enzymes (Halliwell 1991).

The lipid-soluble radical scavenger vitamin E (α-tocopherol) is the most effective agent in the termination of the lipid peroxidation chain reaction and is vital to ensure membrane integrity (Chapple & Matthews 2007).

1.7.2. Protein oxidation

Proteins are the most abundant cell constituents, making them an important target for ROS, as even minor structural modification of a single protein can lead to changes in biological activity within the cell (Çanakçi *et al* 2005). Oxidized proteins are often functionally inactive and are more susceptible to proteinases due to their unfolding, however certain oxidized proteins are poorly handled by cells and together with an altered rate of production from ROS, their accumulation and damaging effect can be seen during aging and in certain chronic conditions such as diabetes (Dean *et al* 1997).

Fig 1.5: A schematic representation of the possible effects of ROS on proteins. (Adapted from Dean et al 1997)



Oxidation of amino acid residue side chains, formation of protein-protein cross-linkage and oxidation of the protein backbone leading to protein fragmentation are all seen as a result of ROS exposure (Berlett & Stadtman 1997). Hydroxyl radicals ($^{\bullet}OH$) are the predominant species to initiate protein oxidation but the course of the oxidation process is governed by the availability of O_2 and $O_2^{\bullet-}$ or its protonated form ($HO_2^{\bullet}$). Work has shown that transition metal ions can substitute for certain radicals in some reactions (Berlett & Stadtman 1997).

1.7.3. Nucleic damage (DNA)

Both nuclear and mitochondrial DNA are susceptible to damage from endogenous ROS. Damage to DNA is predominantly by $^{\bullet}OH$ and NOO^{-}, which are able to generate a multitude of products from all four bases, while $^{1}O_2$ attacks guanine preferentially and $O_2^{\bullet-}$ does not attack DNA (Halliwell 1994a, Cuzzocrea *et al* 2001). H_2O_2 is relatively unreactive with DNA in isolation but able to cause damage via the generation of oxidants from iron-mediated Fenton reactions (Imlay & Linn 1988, Henle & Linn 1997).

DNA damage by ROS/RNS can cause structural alterations such as nicking, deletion, rearrangement, insertions, sequence amplifications and base-pair mutations. One of the most common base lesions is the base-pair mutation of purine and pyrimidine, which can convert guanine to 8-hydroxyguanine, which is often measured as an index marker of DNA damage (Cuzzocrea *et al* 2001, Chapple & Matthews 2007). As well as causing damage to DNA, ROS have been recognised as key activators of gene transcription factors and therefore play a role in the regulation of genes encoding for pro-inflammatory or protective actions (Battino *et al* 1999).

1.7.4. Carbohydrate damage

Oxidative damage by radicals on carbohydrates is known to occur to a lesser extent. Glucose, whether free or bound to protein, can oxidize to produce reactive oxidants, $O_2^{\bullet-}$ and H_2O_2 (Hunt *et al* 1993). Sugars such as glucose,

deoxy sugar and nucleotides readily react with $^{\bullet}OH$, while some radicals are capable of causing fragmentation of carbohydrates, e.g. hyaluronic acid resulting in a drop in viscosity (Blake *et al* 1987).

1.8. Role of ROS in periodontal disease

The role of ROS in the pathogenesis of a variety of inflammatory diseases and tissue damage, both direct and indirect, has become a major area of research over the last decade. Halliwell devised postulates, similar to those of Koch in 1884, which stated the criteria to be fulfilled before ROS can be concluded as the primary mediator of tissue damage for a given disease: -

- "the ROS or the oxidative damage must always be demonstrated at the site of injury"
- "the time course of formation of ROS or of the oxidative damage it causes should be consistent with the time course of the tissue injury, preceding or accompanying it"
- "direct application of the ROS over a relevant time course to the tissue at concentrations within range found *in vivo* should reproduce most or all of the tissue injury and oxidative damage observed"
- "removing the ROS or inhibiting formation should diminish the tissue injury to an extent related to the degree of inhibition of the oxidative damage caused by the ROS"

  (Halliwell 2000, Halliwell & Whiteman 2004)

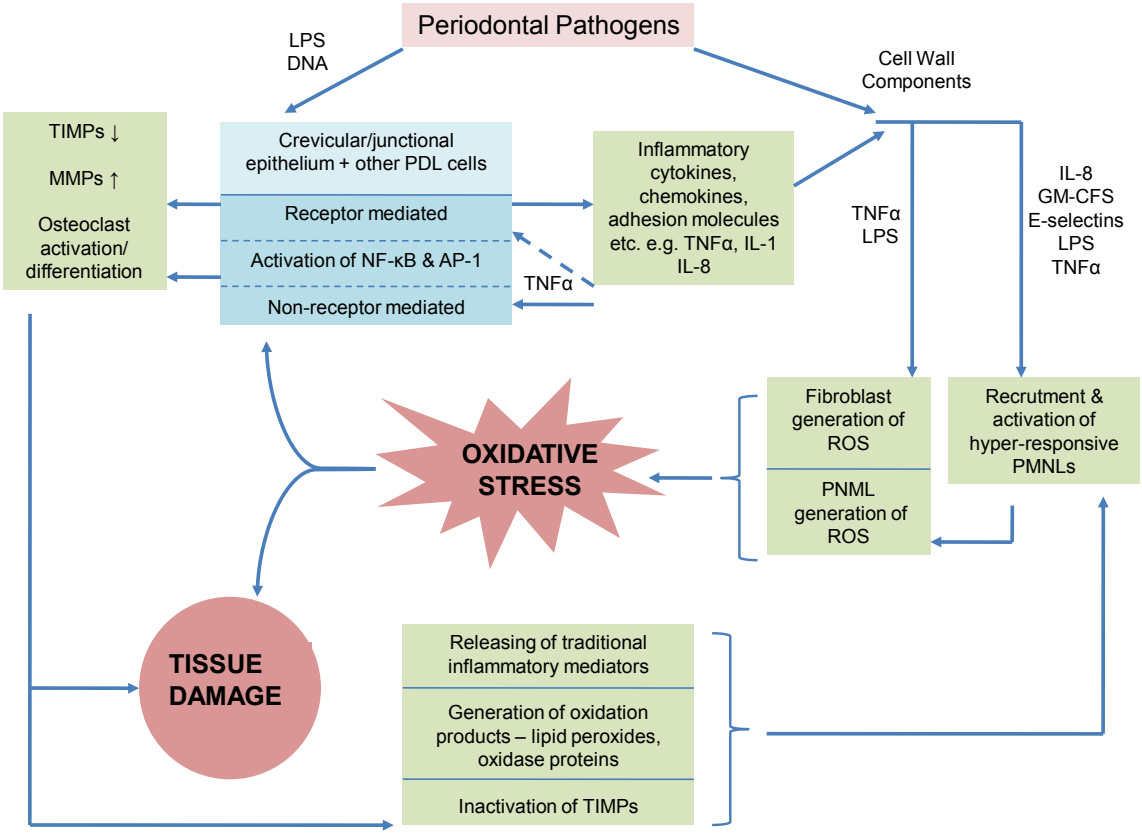
These postulates assume levels of ROS generated are large enough to cause direct tissue damage, but are limiting and do not relate to potential for ROS-mediated indirect damage through redox-sensitive signalling pathways (Chapple & Matthews 2007).

1.8.1. Direct actions of ROS in periodontal destruction

Excessive production of ROS principally by PMNLs in periodontal disease causes indiscriminate damage to cellular, DNA molecules, lipid membranes and proteins, as well as extracellular matrix components of the periodontal

tissues (Çanakçi *et al* 2005). Studies have shown ROS degradation of a number of extracellular components including proteoglycans and their constituent glycosaminoglycans including hyluronan. ROS have also been demonstrated to be capable of degrading bone proteoglycans and collagen degradation, resulting in a reduction of collagen gelation, increased aggregation, cross-linking and collagen insolubility (Waddington *et al* 2000, Çanakçi *et al* 2005).

Fig 1.6: Schematic diagram showing the role of ROS in generating chronic inflammation and tissue damage in response to periodontal pathogens (Chapple & Matthews 2007).



1.8.2. Indirect action of ROS in periodontal destruction

In addition to direct intracellular and extracellular damage, ROS are capable of causing cell injury indirectly by enhancing pro-inflammatory gene expression, including cytokines (e.g. TNFα. IL-1), chemokines (e.g. IL-8) and cellular adhesion molecules. The transcription factors NF-κB and activating protein-1 (AP-1) are redox sensitive and ROS are thought to modulate their

activity. ROS are also believed to increase apoptosis. Induction of apoptosis may be seen in response to DNA damage, which occurs through ROS, particularly $NO^{\bullet}$. An increase in $NO^{\bullet}$ has been reported in both experimental and human models of periodontitis (Çanakçi *et al* 2005, Chapple & Mathews 2007).

1.8.3. ROS produced by neutrophils

PMNLs are widely believed to be the initial and predominant host defence cell against the pathogenic bacteria of periodontal disease. When primed by an antigen, bacterial or otherwise, the PMNL undergoes a respiratory burst, whereby an uptake of oxygen arises through the activation of NADPH-oxidase within the part of the plasma membrane which forms the phagosome. The NADPH oxidase catalyses the oxidisation of NADPH to NADP, which releases two electrons resulting in the reduction of oxygen to superoxide, which serves as a precursor for further ROS formation (Halliwell 2006).

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -} \qquad \textit{Reaction 9}$$

The $O_2^{\bullet -}$ initially formed spontaneously dismutates or is converted to hydrogen peroxide H_2O_2 via one of the three superoxide dismutase enzymes.

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H+ \xrightarrow{\pm SOD} {}^1O_2 + H_2O_2 \qquad \textit{Reaction 10}$$

The hydrogen peroxide formed from this reaction acts as a substrate for neutrophil myeloperoxidase and is converted to hydrocholorous acid (HOCl) a highly biologically active ROS (Chapple & Mathews 2007). The H_2O_2 may also undergo Fenton reactions in the presence of Fe^{2+} or Cu^{2+} ions to form the hydroxyl radical ($^{\bullet}OH$) (reaction 8).

These ROS, along with lyosomal antimicrobial compounds (myeloperoxidase, lysozyme, lactoferrin, cationic proteins etc) are discharged into the cell's vacuole to kill the ingested organism within. Before apoptosis is completed, these biologically active products may be released into the external environment allowing extracellular killing of micro-organisms (Lindhe 1997).

However in more recent years research has indicated the production of ROS within neutrophils may not be directly responsible for the destruction of the engulfed microbe, but may act indirectly through the activation of the lysosomal proteases. The release of ROS into the phagosome induces a charge across the membrane which must be compensated for. The movement of compensating K^+ ions through a Ca^{2+} channels produced conditions within the phagosome conducive with microbial killing and digestion by enzymes release from the cytoplasmic granules (Ahluwalia *et al* 2004, Segal 1993).

PMNLs require an optimum oxygen tension (1% O_2 concentration) and suitable pH (approximately 7.0 – 7.5) for sufficient $O_2^{\bullet-}$ production. Studies on the environment within the periodontal pocket have demonstrated an average O_2 tension of 1.8% and average pH of 6.92, such levels are acceptable to accommodate the production $O_2^{\bullet-}$ by PMNLs (Waddington *et al* 2000). Other research has demonstrated in chronic periodontal disorders that peripheral neutrophils are both hyper-responsive to FCγ-receptor stimulation and hyper-reactive, releasing excessive ROS (Gustafsson *et al* 2006, Matthews *et al* 2006, Matthews *et al* 2007).

Another way in which ROS have been implicated in microbial killing is the release of neutrophil extracellular traps (NETs), which are released as the PMNL's membrane breaks. This cell death, known as 'netosis', is believed to be distinct from apoptosis and necrosis and is dependent on the production of ROS from the NADPH oxidase (Fuchs *et al* 2007). NETs allow the PNMLs to fulfil their antimicrobial function even beyond their lifespan and maybe another way in which collateral tissue damage ensues.

1.8.4. Other cellular sources of ROS in the periodontal tissues

All cells produce ROS as part of normal physiological functions. The prime source in periodontal disease is from the mononuclear and neutrophilic polymorphonuclear phagocytes, but there is evidence that other cells of the periodontal tissues may contribute to local oxidative stress (Chapple &

Matthews 2007). Fibroblasts have been shown to produce superoxide ($O_2^{\bullet-}$) in response to bacterial cell wall components and cytokines such as IL-1 and TNF (Meier *et al* 1989, Skaleric *et al* 2000). The production of ROS by fibroblasts has been shown to be further increased in the presence of calcium (Ca^{2+}) (Skaleric *et al* 2000). Calcium levels are high in the Howship's lacunae (Silver 1988) and the activity of the osteoclast at the alveolar crest may also increase Ca^{2+} levels perpetuating further fibroblast ROS release.

Gingival epithelial cells have been shown to express an NADPH oxidase (Nox) distinct from the phox isoforms of phagocytes, although its activity is 20 fold less than those reported for phagocytes. Sustained production within a periodontal pocket may represent a significance source of local ROS (Chamulitrat *et al* 2004).

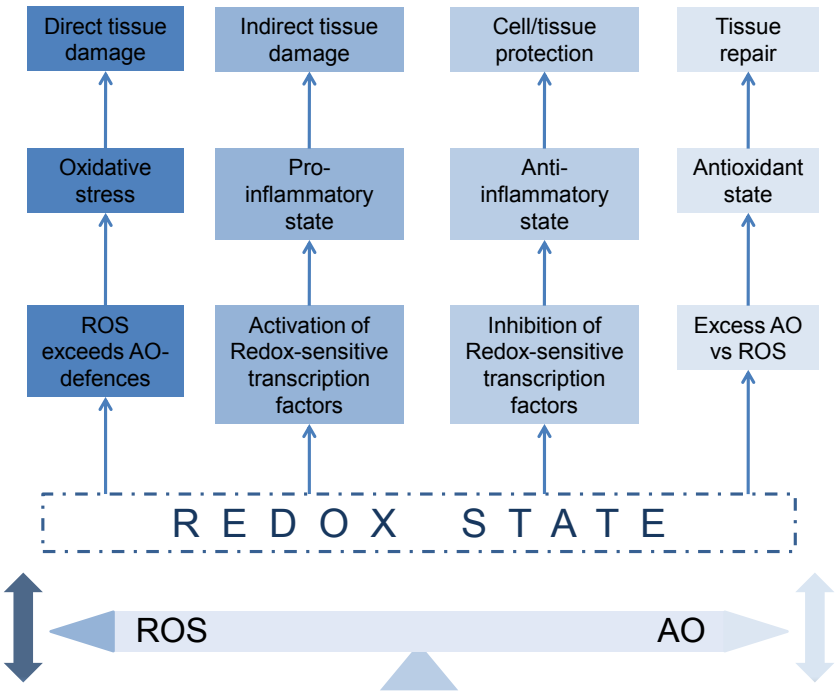
ROS have also been reportedly produced by osteoclasts at the ruffle border/bone interface suggesting a direct role in bone resorption (Chapple 1997). However, other research suggests, ROS, such as $O_2^{\bullet-}$ and H_2O_2 are also involved in the activation of the osteoclasts via activation of the transcription factor NF-κB, prior to bone resorption taking place rather than though direct degradation of the bone matrix (Hall *et al* 1995). Finally, type-1 interferon has been recently implicated in the hyperactive PMNLA phagocyte, implying either a priming role for viruses or an autoimmune compound to periodontitis pathogenesis (Wright *et al* 2008).

1.9. Host defence against free radicals - Antioxidants

Reactive oxygen species possess two main roles: the redox regulation of cell signalling/functions and the detrimental effect on certain substrates, the link between these two distinct functions is the body's antioxidant defence systems, which evolved to limit free radicals in biological systems. The human body possess a plethora of antioxidants to defend against free radical activities and in normal physiology there is a dynamic equilibrium between the two, the so-called 'redox balance'. It is only when ROS activity exceeds

antioxidant defence capabilities or antioxidant defences are reduced that the balance shifts in favour of the ROS, resulting in oxidative stress and possible tissue damage (Chapple & Matthews 2007) see fig 1.7.

Fig 1.7: The biological effect of shifts in the balance of activity between reactive oxygen species (ROS) and antioxidant (AO) species. (Adapted from Chapple & Matthews 2007)



Antioxidants may be regarded as "those substances which when present at low concentrations, compared to those of an oxidisable substrate, will significantly delay or inhibit oxidation of that substrate" (Halliwell & Gutteridge 1989). Antioxidants may be classified in several ways: -

- according to their mode of function into either preventative or scavenging antioxidants (table 1.5)
- according to their location of action, intracellular, extracellular or membrane associated (table 1.6)
- with regard to their solubility, lipid or water (table 1.7)
- by their structural dependents (table 1.8)
- by their source/origins, dietary or non-dietary (table 1.9)

Table 1.5. **Antioxidants classified by mode of action**

Mode of Action	Examples
Preventative Antioxidants	Enzymes: superoxide dismutase (SOD 1,2 and 3), catalase, glutathione peroxidise, DNA repair enzymes e.g. poly(ADP-ribose) polymerase
	Metal ion sequestrators: albumin, lactoferrin, transferrin, hapoglobin, ceruloplasmin, hexpexin, carotenoids, SOD, catalase, glutathione peroxidise, glutathione reductase, uric acid, polyphenolic flavenoids
Scavenging (chain breaking) antioxidants	Ascorbate, carotenoids, uric acid, α-tocopherol, polyphenols (flavenoids), bilirubin, albumin, ubiquinone (reduced form), reduced glutathione and other thiols

Table 1.6. **Example of key antioxidants classified by location**

Location	Examples
Intracellular	Superoxide dismutase 1 and 2, catalase, glutathione peroxidise, DNA repair enzymes e.g. poly(ADP-ribose) polymerase, others, reduced glutathione, ubiquinone (reduced form)
Extracellular	Superoxide dismutase 3, selenium-glutathione peroxidise, reduced glutathione, lactoferrin, transferring, hapoglobin, ceruloplasmin, albumin, ascorbate, carotenoids, uric acid
Membrane associated	α-tocopherol

Table 1.7. **Key antioxidants classified by solubility**

Solubility	Examples
Water soluble	Hatoglobin, ceruloplasmin, albumin, ascorbate, uric acid, polyphenolic flavenoids, reduced glutathione and other thiols, cysteine, transferrin
Lipid soluble	α-tocopherol, carotenoids, bilirubin, quinines (e.g. reduced ubiquinone)

Table 1.8. **Antioxidants classified by structure they protect**

Structure	Examples
DNA protective antioxidants	Superoxide dismutase 1 and 2, glutathione peroxidise, DNA repair enzymes e.g. poly(ADP-ribose) polymerase, reduced glutathione, cysteine
Protein protective antioxidants	Sequestration of transition metals by preventative antioxidants
	Scavenging by competing substrates
	Antioxidant enzymes
Lipid protective antioxidants	α-tocopherol, ascorbate, carotenoids (including retinol), reduced ubiquinone, reduced glutathione, glutathione peroxidase, bilirubin

Table 1.9. **Some key antioxidants classified by their origin**

Location	Examples
Exogenous antioxidants (diet only)	Carotenoids, ascorbic acid, tocopherol (α, γ, β, δ), polyphenols (e.g. flavenoids, catechins) folic acid, cysteine
Endogenous antioxidant (synthesised by the body)	Catalase, superoxide dismutase, glutathione peroxidise, glutathione-S-transferase, reduced glutathione, ceruloplasmin, transferrin, ferritin, glycosylase, peroxisomes, proteases
Synthetic	e.g. *N*-acetylcysteine, penicillamine, tetracyclines

All tables taken from Chapple & Matthews 2007.

The preventative antioxidants function by enzymatic elimination of superoxide and hydrogen peroxide or by sequestration of metal ions, preventing Fenton reactions and subsequent hydroxyl radical formation (Halliwell & Gutteridge 1990). The scavenging/chain breaking antioxidants are the most important in extracellular fluids, inhibiting chain initiating and chain propagating radicals as they form (Brock 2005). The lipid soluble antioxidants act at the cell membrane and protect against lipid peroxidation, while water-soluble antioxidants are more important within extracellular tissue fluids. Several

antioxidants have dual or triple actions such as ascorbate (vitamin C), which acts as a chain breaking/scavenging antioxidant as well as a preventative antioxidant by its ability recycle α-tocopherol (vitamin E) from its oxidised form (Niki 1987) and to bind metal ions, thus making classification to some extent limited due to the multilayered defence systems that exist (Chapple & Matthews 2007).

1.9.1. Superoxide Dismutase (SOD)

The general acceptance of free radicals in biological systems was brought about by the discovery of the enzyme superoxide dismutase (SOD) in 1969 by McCord and Fridovich. This enzyme catalyses the conversion of $O_2^{\bullet-}$ to H_2O_2 (reaction 10) via a dismutation reaction making use of the fact that superoxide ($O_2^{\bullet-}$) is both an oxidant and a reductant, keen to take on or release its extra electron. Superoxide dismutase uses one $O_2^{\bullet-}$ radical to oxidize another (McCord 2000).

Three different types of SOD exist, all of which are able to activate the dismutation reaction up to 10,000 times faster than the spontaneous dismutation of $O_2^{\bullet-}$ (Battino *et al* 1999). SOD1 is a Cu^{2+}/Zn^{2+} enzyme present within the cytosol, SOD2 is a Mn^{2+} dependant enzyme located in the mitochondria and SOD3 is an extracellular enzyme found at low levels at the extracellular surface (Cuzzocrea *et al* 2001).

Since all the SOD enzymes accelerate H_2O_2 production, which yields $^{\bullet}OH$ a more volatile radical than $O_2^{\bullet-}$, SOD must work in conjunction with other enzymes to remove H_2O_2 from human biological systems. This is achieved by two enzyme families – the catalases and the glutathione peroxidises (Battino *et al* 1999).

1.9.2. Catalase

Catalase is found within the peroxisomes and acts intracellularly, because H_2O_2 is a weak reductant as well as an oxidant, catalase dismutates it to form

water and oxygen (reaction 11) or uses it as an oxidant when working as a peroxidase (reaction 12) (McCord 2000, Battino et al 1999).

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \qquad \textit{Reaction 11}$$

$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \qquad \textit{Reaction 12}$$

Although catalase removes H_2O_2 with great efficacy, the most important H_2O_2 removing enzyme within mammalian cells is the selenoprotein glutathione peroxidise (Halliwell 1994a), and together they maintain intracellular levels of H_2O_2 at 10^{-9} – 10^{-7}m (Blake *et al* 1987).

1.9.3. Glutathione

Glutathione is a ubiquitous tri-peptide, which can be synthesised within the cell, although it's component amino acids are essential and obtained from the diet (Chapple & Matthews 2007). It represents the most prominent low molecular weight thiol (up to 5 – 10mM) present in the cell and exists in oxidized (GSSG) and reduced (GSH) forms (Cnubben *et al* 2001). GSH plays a essential role in the glutathione peroxidase (GPx) antioxidant enzyme system. GPx utilises the reducing power of GSH to remove hydrogen peroxide, converting two GSH molecules to one GSSG molecule and water (reaction 13) (Meister & Anderson 1983).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad \textit{Reaction 13}$$

Whilst GSH acts as the substrate for removal of H_2O_2 via GPx, it is also reconstituted from GSSG by glutathione reductase (GR) at the expense of NADPH, permitting the continuous action of glutathione peroxidase (Cnubben *et al* 2001).

$$GSSG + H^+ + NADPH \rightarrow 2GSH + NADP^+ \qquad \textit{Reaction 14}$$

GSH also plays an important role in maintaining intracellular redox balance, regulating pathways that are affected by oxidative stress, acting as a neurotransmitter, aiding in the preservation and restoration of other

antioxidant species, such as vitamin C & E and it can effect inflammatory cytokine production by regulating expression/activation of redox sensitive transcription factors e.g. nuclear factor-κB (Chapple & Matthews 2007).

The preventative enzyme based antioxidants illustrate the compartmentalisation that exists between intra- and extracellular antioxidants, since the mid 1980's it was concluded that the action of SOD, catalase or glutathione peroxidase in the removal of $O_2^{\bullet-}$ or H_2O_2 contributed little to any antioxidant activity in extracellular fluids (Halliwell & Gutteridge 1986). The extracellular levels of GSH are indeed low, approximately 1 – 4μM (Svardal *et al* 1990), however recent work has detected millimolar levels of GSH in gingival crevicular fluid (Chapple *et al* 2002) and high levels contributing to the total antioxidant status of cervical epithelium (Cope *et al* 1999), which may indicate a fundamental defence role for GSH at exposed epithelial surfaces (Chapple *et al* 2002). Indeed GCF GSH levels appear severely compromised in periodontitis relative to health (Chapple *et al* 2002).

1.9.4. Ascorbic acid (vitamin C)

Ascorbic acid (vitamin C) has a multitude of antioxidant properties. It is a powerful scavenger of $O_2^{\bullet-}$, $HO_2^{\bullet}$, $^{\bullet}OH$, HOCl, and able to scavenge water-soluble peroxyl radicals ($RO_2^{\bullet}$), as well as scavenge and quench $^1O_2$ in aqueous solutions (Halliwell & Gutteridge 1990). Ascorbic acid repairs and therefore prevents damage by radicals arising from the $^{\bullet}OH$ radicals' actions on uric acid. It is also able to prevent Fenton reactions by decreasing heme breakdown and the subsequent release of Fe^{2+} (Chapple & Matthews 2007). Carcinogenic nitrosamines can be reduced to inactive products and protection afforded against oxidants present in tobacco smoke by ascorbic acid (Halliwell & Gutteridge 1990). Ascorbate has also been shown to decrease pro-inflammatory gene expression via effects on NF-κB activation (Griffiths & Lunec 2001).

Ascorbic acid is able to regenerate α-tocopherol (vitamin E) by reducing the α-tocopherol radical that forms at membrane surfaces (Chapple 1996). It is

an excellent reducing agent, most of it's antioxidant features are credited to this, but unfortunately it is also able to reduce copper and iron ions, as well as accelerate $^{\bullet}OH$ formation in the presence of H_2O_2 (Battino *et al* 1999). Fortunately in healthy humans free metal ions are not readily available in extracelluar fluids (Halliwell & Gutteridge 1990).

Vitamin C is an essential nutrient and the recommended daily intake of ascorbate is 40mg – 60mg (Levine *et al* 1995), plasma levels have been recorded at 30 – 60μM (Rumley et al 1998), while levels in GCF are reportedly three times higher (Meyle & Kapitza 1990). Ascorbate is converted to ascorbyl radicals via radical attack and then breaks down to dehydroascorbate. Dehydroascorbate can be transformed back to ascorbate by enzyme systems, either directly by reduced GSH or by NAD-semi-dehydroascorbate reductase, which ulitises GSH. These regenerative enzyme systems are located intracellularly, therefore extracellular ascorbic acid may be readily exhausted in conditions of oxidative stress (Bergendi *et al* 1999).

1.9.5. α-Tocopherol (vitamin E)

Vitamin E is a ubiquitous, lipid-soluble, low molecular weight antioxidant present within the lipid constituents of cell membranes and plasma lipoproteins and thus has a role both intra- and extracellularly (Halliwell 1994b). Vitamin E was discovered in 1922 by Evan & Bishop and its active ingredient was later isolated and named tocopherol by Evans *et al* in 1936 (Wang & Quinn 1999) . Their research led to the discovery of seven further isomers, which were organised into familial groups as four tocopherols (prefixed with α, β, γ and δ) and four tocotrienols (with identical prefixes). The Commission on Biochemical Nomenclature recommended that the term 'Vitamin E' be used for all tocopherol and tocotrienol derivatives qualitatively exhibiting the biological activities of α-tocopherol (Wang & Quinn 1999).

Vitamin E is generally considered the most important and effective lipid soluble antioxidant, maintaining cell membrane integrity from lipid

peroxidation by scavenging the peroxyl radical ($ROO^{\bullet}$). Its antioxidant activity arises from a single phenolic OH group, which when oxidized gives rise to the tocopherol radical (Chapple & Matthews 2007). The resultant tocopherol radical, although not completely unreactive, is less reactive than the $ROO^{\bullet}$ and not as adept at attacking the fatty-acid side chains, thus the lipid perioxidation chain reaction is slowed (Halliwell 1994a). The tocopherol radical can be reconstituted by co-enzyme Q10 (ubiquinol) in the lipid environment and ascorbic acid in the aqueous phase (Chapple & Matthews 2007). Although α-tocopherol accounts for only a small percentage of total antioxidant activity in plasma, its importance cannot be negated as it is the only lipid-soluble chain breaking antioxidant in plasma (Halliwell & Gutteridge 1986).

The role of α-tocopherol in the pathogenesis of periodontal disease is likely to be a minor one, due to its limited mobility and the fact that many of the ROS are generated in aqueous solution, particularly those from phagocytes and vascular endothelium (Chapple 1997). Despite this limited antioxidant role, α-tocopherol possesses anti-inflammatory roles, which may be of baring, such as the inhibition of superoxide production from macrophages and neutrophils, inhibition of nitric oxide production from vascular endothelial cells and the inhibition of protein kinase C and subsequent platelet aggregation (Azzi et al 2002).

1.9.6. Carotenoids (vitamin A)

Carotenoids belong to the tetraterpene family and over 600 natural structural variants exist. They are synthesised by plants, fungi, bacteria and algae, but humans are unable to manufacture carotenoids and must therefore incorporate them as part of their diet (Tapiero *et al* 2004). Of these only about 20 are found in human plasma and tissues, including lycopene, α-carotene, β-carotene, lutein, crytoxanthine, retinol (vitamin A_1) and dehydroretinol (vitamin A_2), which are derived from green vegetables and fruit (Tapiero *et al* 2004, Chapple & Matthews 2007). Lycopene predominates in plasma and is derived almost exclusively from tomatoes and tomato products

in the Western diet. It has a high singlet oxygen ($^1O_2$) quenching capacity compared to other carotenoids and unlike other extracelluar antioxidants it is unaffected by smoking (Gerster 1997).

Carotenoids are highly lipophilic and at higher plasma concentrations have been shown to protect against various inflammatory and malignant diseases (Tapiero *et al* 2004), although studying the antioxidant capacity of carotenoids within the tissues has proven more difficult due to the abundance and efficiency of tocopherols against the peroxyl radical in vivo (Handleman 2001). Vitamin A is also controversial as an antioxidant because its action depends on the local oxygen tension. At the low partial pressures of oxygen found in mammalian tissues β-carotene acts as an antioxidant, but at high oxygen tension it loses much of it antioxidant capacity and may even behave as a prooxidant (Krinsky 2001), which is associated with substantial detrimental effects on the surrounding tissues (Omenn *et al* 1996).

1.9.7. Co-enzyme Q10

Co-enzyme Q10 plays an important role in the energy-transducing membrane of mitochondria as a mobile redox proton carrier. It exists in an oxidised form, ubiquinone or CoQ_{10} and a reduced form, ubiquniol or $CoQH_2$, both forms of which possess antioxidant properties (Niki 1997, Battino *et al* 1999). Co-enzyme Q10 deficiency has been demonstrated in the gingival tissues of patients with periodontal disease (Littarru *et al* 1971, Hansen *et al* 1976), but there has been a lack of studies to substantiate any periodontal benefit in supplementation (Chapple & Matthews 2007).

1.9.8. Uric acid

Uric acid is a powerful antioxidant, capable of both preventative antioxidant functions and it is also one of the major scavenging antioxidants found in plasma and saliva (Brock et al 2004). Uric acid protects erythrocytes from peroxidative damage, scavenges $^1O_2$, $^{\bullet}OH$ and HOCl (Ames *et al* 1981) and binds iron and copper ions in forms that prevent the occurrence of Fenton

reactions (Halliwell & Gutteridge 1990). However, the reaction of uric acid with certain oxidising species, such as $ROO^{\bullet}$ or $^{\bullet}OH$ can generate uric acid radicals which themselves are capable of causing biological damage (Halliwell & Gutteridge 1990).

Uric acid is oxidised to Allantoin enzymically or by hydroxyl radicals but the enzyme uricase is not present in humans therefore any Allantoin formed is due to 'abnormal' oxidation and measurement of the allantoin/urate concentration ratio has been posed as an important marker of free radical reactions taking place *in vivo* (Grootveld & Halliwell 1987).

1.9.9. Polyphenols

The polyphenol flavenoids affect a range of biological functions, such as capillary permeability and inhibition of enzymes, and have more recently been proposed as radical scavengers (Battino *et al* 1999). Polyphenol flavenoids are micronutrients absorbed from the diet, in particular, vegetables, red wine and tea (Weisburger 1999). The flavenoid family is vast, with over 400 known compounds (Prior & Cao 1999a), including flavones, derivatives of which include catechins, epigallocatchin gallate, and theaflavins in tea, other recognised phenolic compounds including quercetin from vegtables, and resveratrol in red wine (Weisburger 1999). Mechanisms contributing to dietary antioxidant functions may include direct scavenging of free radicals, intercepting radical chain propagation of lipid peroxides, scavenging of nitrogen species and transition metal ion chelation (Rice-Evans 1999). Flavenoids may also contribute to the redox regulation in cells via their reducing properties, independent of their antioxidant properties (Rice-Evans 2001).

1.10. Concept of "total antioxidant capacity" (TAOC)

The body's antioxidant systems are very integrated and highly complex displaying co-operative activities that may be overlooked if studies

concentrate on individual antioxidant systems leading to an inaccurate picture of the *in vivo* situation. Antioxidants also work in concert through redox cycling reactions, regenerating each other from their respective radical species, an example being the recycling of α-tocopherol by vitamin C (Chapple 1996). For these reasons research has now focused on measuring the global antioxidant defence or "total antioxidant capacity" of biological fluids. These assays provide information on the combined effect of the individual antioxidants and may account for the influence of antioxidant substances as yet undiscovered or those that are technically difficult to analyse (Chapple & Matthews 2007).

1.11. Smoking

Smoking has long been established as a major environmental risk factor for periodontal disease (Palmer *et al* 2005). Smokers are exposed to over 40'000 chemicals from their cigarette smoke and the combustion of tobacco also creates free radicals, 1×10^{16} radicals per cigarette or 1×10^{14} per puff (Pryor *et al* 1983). Several studies have demonstrated lower plasma concentrations of antioxidants in smokers *in vivo* (Alberg 2002) and a decrease in serum antioxidant concentration has been negatively associated with the prevalence of inflammatory periodontitis (Chapple *et al* 2006).

1.11.1. Smoking as a risk factor for periodontitis

The first association between tobacco smoking and periodontal disease came from the studies of Pindborg in 1947, which recognised that many of the individuals affected by acute necrotising ulcerative gingivitis (ANUG) were smokers (Palmer *et al* 2005). Since then a great deal of research into the detrimental effects of tobacco smoking has concluded it has widespread systemic effects, many of which may provide mechanisms that increases the individual susceptibility to periodontal disease and affect their response to treatment, by stimulating destructive/inflammatory responses and impairing protective/reparative responses (Ryder 2007).

Smokers of tobacco can be seen to be exposed to two levels of tobacco products, 'acute and chronic' exposure. Chronic low level tobacco products can be found in serum, saliva, gingival crevicular fluid, (GCF) and within the cell and extracellular matrix of the periodontal tissues. These low chronic concentrations will have an effect on the host response but during the act of smoking, the concentration of tobacco products is increased several hundred to a thousand times in these tissues and fluids, which may produce different effects compared to those of long-term chronic levels (Ryder 2007).

The effects of tobacco use on the microflora within the oral cavity have been studied extensively and data are somewhat contradictory, while many research groups have found no significant differences in the incidence and distribution in selected periodontal pathogens (Preber *et al* 1992, Stoltenberg *et al* 1993, Darby *et al* 2000, Bostrom *et al* 2001), others have shown changes in the prevelance of pathogenic bacteria within the biofilm (Haffajee & Socransky 2001) and discovered significant differences in recovery rates of these pathogens (Zambon *et al* 1996). More recent work has reported less of a reduction on periodontal pathogens in smokers compared to non-smokers following non-surgical management of periodontal disease, scaling and root surface debridment (Van der Velden *et al* 2003, Darby *et al* 2005). The later studies implicate smoking in changes to the local environment, promoting growth of certain pathogenic bacteria which may result in an altered host response.

Smoking has a long term chronic effect of impairing the vasculature of the periodontal tissues. Bleeding on probing is reduced in smokers (Bergstrom & Bostrom 2001) as is gingival redness and levels of GCF (Preber & Bergstrom 1985). The vascularity of the periodontal tissues changes in those individuals who smoke. Histological comparisons between smokers and non-smokers have revealed significantly larger numbers of vessels in inflamed tissues of non-smokers than smokers and that the total number of vessels expressing ICAM-1 was also reduced (Rezavandi *et al* 2002), which could affect the emigration of neutrophils into the tissues. Tobacco smoke exposure increased general circulating neutrophil numbers (Sørensen *et al* 2004), yet

numbers of neutrophils reaching the gingival sulcus appears reduced in smokers (Pauletto *et al* 2000), again demonstrating the impairment of transmigration from the periodontal microvasculature and the potential for PMNL accumulation in periodontal tissues.

Neutrophil receptors may also be affected by smoking. Neutrophils express functional receptors for several components and metabolites of tobacco, such as nicotine and cotinine (Benhammou *et al* 2000) and the numbers of these receptors is increased in smokers and declines post cessation (Lebargy *et al* 1996). Neutrophils also express receptors for endogenous factors such as IL-8, ICAM-1 and TNF-α. Smoking has been shown to deregulate systemic concentrations of soluble ICAM-1, a circulating adhesion molecule with immunomodulatory potential (Palmer *et al* 2002) and deregulate the release of IL-8 and TNF-α from peripheral neutrophils in periodontitis patients (Fredriksson *et al* 2002). Smokers with periodontitis also show impairment in granulocyte function, when challenged with bacterial products they release, such as serine proteases, elastase and MMPs which degrade connective tissues (Söder *et al* 2002).

Smoking has a detrimental effect on healing following both non-surgical and surgical modalities of periodontal treatment (Palmer *et al* 2005), which may be due to the increased levels and/or activity of proteolytic enzymes on the structural components of the periodontium, the elevation of destructive inflammatory cytokines, and/or the suppression of regenerative function (Ryder 2007). The cytokine IL-1β has been found to be at higher levels following non-surgical debridment in smokers (Goutoudi et al 2004), while higher levels of MMPs and relatively low levels of the enzyme inhibitors alpha-1-antitrypson and alpha-2-Macroglobulin were found in smokers following surgical management (Persson *et al* 2003). Studies on the effects of smoking on periodontal fibroblasts are difficult to assess as most use doses of nicotine or cotinine at much higher levels than would be expected in plasma, but overall the evidence does suggest smoking may inhibit fibroblast function, recruitment and adhesion to the root surface (Palmer *et al* 2005).

1.11.2. The effect of smoking on ROS production from neutrophils

The phagocytotic ability of neutrophils has been shown to be hindered by aqueous-phase smoke extracts, as well as suppression of the oxidative burst (Zappacosta *et al* 2001). Whilst a significantly lower level of ROS production has been shown, some research has also indicated higher chemotaxis (Sørensen *et al* 2004). The research on the effects of tobacco products on neutrophil expression of ROS is rather inconsistent and other researchers have suggested that tobacco constituents can exacerbate aspects of the respiratory burst, enhancing the production of ROS, particularly peroxynitirite $ONOO^-$ (Iho *et al* 2003). Components of tobacco smoke can have a profound effect on neutrophils increasing their formyl-methionyl-leucyl-phenylalanine (fMLP) receptors and leaving them 'primed', resulting in a two-fold increase in the release of elastase and superoxide in response to fMLP (Keothe *et al* 2000). An increased priming effect of TNF-α has also been demonstrated in smokers with periodontitis accompanied by an increased generation of radicals and up-regulated neutrophil function (Gustafsson *et al* 2000).

1.11.3. The effect of smoking on antioxidant status

There is compelling evidence to suggest that the antioxidant status of smokers is reduced, cigarette smoke may result in an increased metabolic turnover, due to the greater expenditure of antioxidant micronutrients from increased oxidative stress caused by the tobacco products, or alternatively smoking could decrease micronutrient absorption (Alberg 2002). Cigarette smokers have a significantly lower plasma antioxidant status compared to non-smokers independent of their dietary antioxidant intake (Dietrich *et al* 2003).

Smokers have been observed to have circulating concentrations of dehydroascorbate, a marker of vitamin C depletion at much higher levels than those of non-smokers (Lykkesfedlt *et al* 1997). Plasma levels of vitamin C and carotenoids are depressed in smokers (Chow *et al* 1986), despite a reduced consumption of vitamin C rich foods generally seen in individuals who smoke (Zondervan *et al* 1996). The lowered levels of dietary

antioxidants found in their plasma is seen independent of their dietary intake (Schectman *et al* 1989). Plasma levels of α-tocopherol are also significantly reduced in smokers particularly in the individual greater than thirty five years of age (Lui *et al* 1998).

Smoking even a single cigarette will significantly reduce salivary glutathione (GSH) concentrations (Zappacosta *et al* 1999 & 2002) and similar data exist for plasma concentrations (Rahman & MacNee 1999). Reduction in GSH levels in the local tissue in periodontitis patients who smoke has been reported, and a dose dependant reduction in GSH within the periodontal ligament has been described as a result of smoking (Chang *et al* 2003). GSH has been shown to protect against the cytotoxic activities of nicotine on periodontal ligament fibroblasts (Chang *et al* 2002).

1.12. Measuring ROS and antioxidant status in biological samples

There are currently no 'gold standard' methods for measuring ROS-mediated damage in human tissues or the antioxidant capacity of the individual (Chapple & Matthews 2007). Most free radicals and other reactive species persist for only a very short time *in vivo*, having an extremely short half life, 10^{-6}–10^{-9}s and therefore cannot be measured directly (Chapple & Matthews 2007). There are two approaches to detecting ROS (Halliwell & Whiteman 2004): -

- attempting to '*trap'* the species and measure the levels of trapped molecules
- measuring the levels of oxidative damage incurred by ROS

The spin traps/probes currently available cannot be used on humans because of unknown toxicity at the high levels required *in vivo.* Therefore they are used on body fluids or tissue samples. These *ex-vivo* spin traps include ascorbic acid, urate and aromatic traps, such as salicylates (Halliwell & Whiteman 2004). The majority of clinical research has examined the levels of oxidative damage caused by ROS by measuring the biomarkers of lipid

peroxidation, DNA or protein damage, rather than utilising spin traps. All have been extensively reviewed by Halliwell & Whiteman in 2004 and all have confounders leading to the need for careful interpretation of results.

The approach to measuring endogenous antioxidant defences in bodily fluids is either to assay a single compound in isolation, in groups or to measure the total antioxidant capacity (TAOC) (Woodford & Whitehead 1998). Antioxidant species predominate differentially in compartments of the body. In plasma a variety of antioxidant assays have been developed either focusing on the aqueous compartment (e.g. ascorbic acid, uric acid, proteins) or in isolated fractions of plasma, such as low-density lipoproteins (e.g. carotenoids, α-tocopherol) (Yeum *et al* 2004). However there are co-operative interactions between the two compartments and antioxidants do not work in isolation; therefore the sum of individual antioxidant activities will not represent the global capacity against ROS. Hence the development and use of assays measuring total antioxidant capacity are preferable to those measuring individual species, though careful interpretation is required as not all measure both the aqueous and lipophilic phase antioxidants and may differ in their sensitivity towards different species within the compartment (Chapple & Matthews 2007).

1.12.1. Total antioxidant capacity methodology

The published methods for estimating the total antioxidant capacity of bodily fluids all measure the inhibition of an artificially generated oxidative process. A free radical is generated in the solution containing a target for oxidation and the antioxidant within the sample quenches the target response by interacting with the radical species. The assays differ in their choice of free radical generator, target of oxidation and means of measuring the oxidized product (Woodford & Whitehead 1998).

In some methodologies it may be argued that reductants are being measured rather than antioxidants; clarification of these terms is therefore required. A reductant or reducing agent is a substance that donates electrons, causing a

reactant to be reduced. An oxidant or oxidizing agent accepts electrons, causing a reactant to be oxidized. Reductants and oxidants are chemical terms, while the terms antioxidant and pro-oxidant have meaning in the context of biological systems and not all reductants are antioxidants (Prior & Cao 1999b). An antioxidant is defined as "a substance which when present at low concentrations, compared to those of an oxidasable substrate, will significantly delay or inhibit oxidation of that substrate" (Halliwell & Gutteridge 1989). The term 'pro-oxidant' is a synonym for reactive species which are toxic substances able to cause oxidative damage to lipids, proteins and DNA, thus, chemically, a pro-oxidant is an oxidant of pathological importance. An antioxidant antagonises the pro-oxidant, resulting in products that have no or low toxicity (Prior & Cao 1999).

1.12.1.2. Assays for measuring water-soluble TAOC

There are two main approaches to measuring the total antioxidant capacity of hydrophilic compartment of plasma. The first involves using oxidants that act as pro-oxidants or radical inducing species, such as 2,2'-azobis (2,4-amidinopropane) dihydrochloride (AAPH). AAPH spontaneously decomposes at 37°C at a known rate constant, giving rise to a carbon-centred radical that reacts with oxygen to produce peroxyl radicals (Yeum *et al* 2004). It can be monitored by hydrophilic substrates such as: -

- DCFH (2',7'-dichlorodihydrofluorescein), which provides a fluorescent signal used in the total radical trapping antioxidant parameter (TRAP) assay or with the oxygen radical absorbance capacity (ORAC) assay (Prior & Cao 1999).

- R-Pe (dichlorofluorescein-diacetate phycoerythrin), which is a fluorescent protein also used with the TRAP assay (Prior & Cao 1999).

- Crocin, which interacts with the peroxyl radical leading to a bleaching reaction that is measured as an absorbance change (Tubaro et al 1998)

The oxidation of these hydrophilic substrates is inhibited by the antioxidant present in the plasma during an induction period and the antioxidant capacity measured by the delay or profile (e.g. area under the curve) of the reaction. The longer the delay in the substrates activity and signal generation, the greater the antioxidant capacity (Chapple & Matthews 2007).

The second approach to measuring TAOC in the aqueous phase of plasma is to use systems that generate a free radical chain reaction using an oxidant such as hydrogen peroxide rather than a pro-oxidant. An oxidisable substrate is used (e.g. luminol) and the ability of the antioxidants within the plasma to scavenge the radicals produced is measured by assessing the delay in signal or time for which the signal is absent. This measurement is then calibrated against a known/standard antioxidant species, most commonly the water soluble vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylix acid (Trolox) (Chapple & Matthews 2007). Assays which use this system include: -

- Ferric reducing ability of plasma (FRAP) assay, which measures the reduction of ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) at low pH, which causes a coloured complex, ferrous-tripyridyltriazine to be formed (Benzie & Strain 1996).

- Trolox equivalent antioxidant capacity (TEAC) assay, which measures the ability of plasma to quench the radical cation of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)) (Miller *et al* 1993).

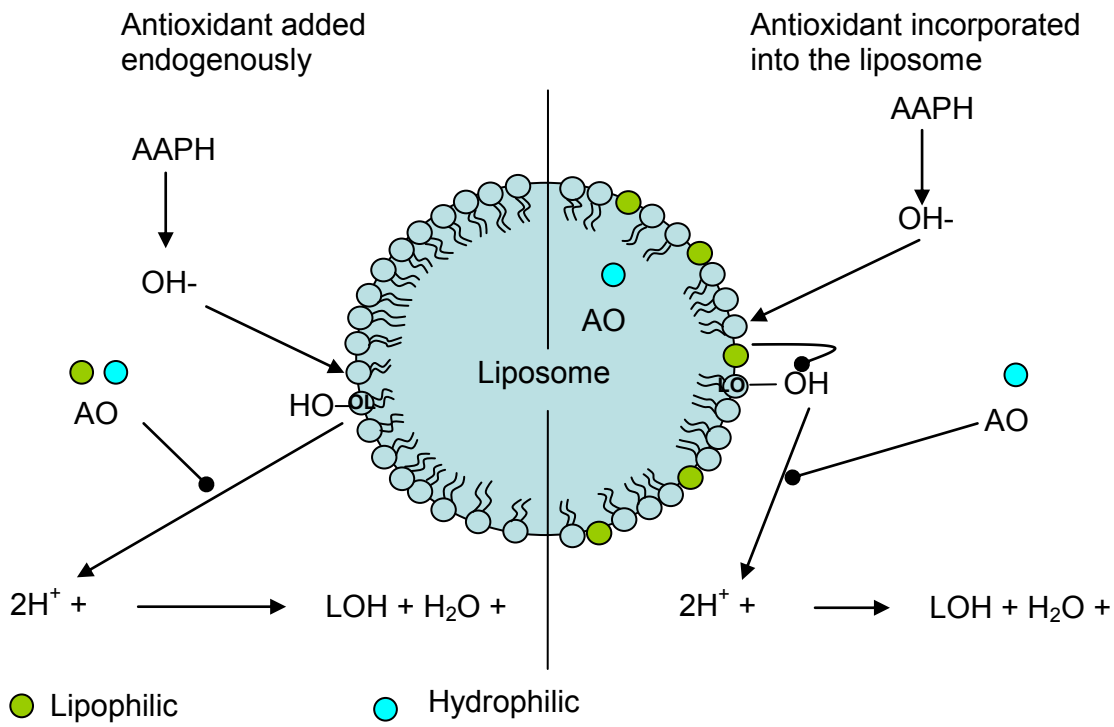
- Enhanced chemiluminescence (ECL) assay, which measures the ability of the antioxidants in the fluid sample to inhibit the enhanced light signal produced by the oxidation of the luminescent substrate luminal by hydrogen peroxide, using horseradish peroxidase as the catalyst. (Whitehead *et al* 1992, Chapple *et al* 1997a) A point in the light recovery curve is used to assess the total antioxidant capacity, of the sample added, as the return of light signals the exhaustion of the antioxidants within the sample added, an early point in the curve would signal the most

efficient antioxidant but the end point would include less efficient antioxidants (Chapple & Matthews 2007).

1.12.1.3. Assays for measuring lipid-soluble TAOC

The general approach to measuring the antioxidant capacity in the lipid compartment of plasma is to assess the oxidizability of isolated low-density lipoprotein (LDL) using a pro-oxidant radical inducer e.g. AMVN (2,2'-azobis (2,4-dimethylvaleronitrile)) to produce a lipid-soluble peroxyl radical. The lipid peroxidation is then measured by assessing the production of conjugated dienes (Nikki 1990).

Figure 1.8. Schematic diagram of the action of the antioxidant when incorporated into the liposomes or when added exogenously.



Our laboratory has produced a novel 'in house' method to measure the scavenging capacity of selected compounds in a lipid peroxidation prevention assay. The peroxyl radical generator AAPH is used to initiate a lipid peroxidation cascade and the inclusion of potential antioxidants in the assay

devopled by el-Saadani et al (1989), based on lipoprotein (LDL) oxidation. The modification alters the lipid source from LDL to proprietary radical formation. The antioxidant capacity is measured against lipid hydroperoxyl radical formation. By using liposome either with or without antioxidants incorporated into their structure, both the hydrophilic and lipophilic nature of the test compound to be assessed (figure 1.8.).

1.13. Gingival crevicular fluid (GCF)

Gingival crevicular fluid (GCF) is a plasma derived fluid which bathes the epithelial cells of the gingival crevice, continuously flowing at very slow rates (0.24-1.56µl/min) at non-inflamed sites (Chapple *et al* 2002a). GCF is a serum transudate in health and is formed by serum moving passively from the gingival capillaries, through the connective tissue of the gingiva and into the gingival crevice. During periodontal disease the flow rate and volume are seen to significantly increase, its composition changes to reflect the state of disease within the periodontal tissues becoming more like an inflammatory exudate and containing all the constituents of the local inflammatory response (Chapple *et al* 2002b). These constituents broadly fall into three overlapping categories; enzymes and ROS released by inflammatory or constitutive connective tissue cells, products of cellular or tissue degradation and inflammatory cytokines, mediators and other products secreted by activated leucocytes (Offenbacher *et al* 1993).

Few studies have examined the TAOC of GCF as a marker of local oxidative stress due to the difficulty in using such small volumes available for assay compared to saliva (Chapple 2007). Brock *et al* demonstrated the TAOC of GCF to be both qualitatively and quantitatively distinct from that of saliva, serum and plasma (Brock *et al* 2004) with GSH predominating in GCF (Chapple *et al* 2002a) and uric acid predominating in saliva (Moore *et al* 1994) and serum (Maxwell *et al* 2006).

1.14. Immediate background and aim of the study

Chronic periodontitis persists as major cause of tooth loss in the developed world (Papapanou 1999), and arises due to the complex interactions between the pathogenic bacteria within the subgingival biofilm and the host's inflammatory-immune response. One of the key elements in the host defence response is the presence of local neutrophil activity. Although the role of PMNLs is primarily protective, the majority of host tissue damage incurred during periodontitis results from an excessive or prolonged release of neutrophil enzymes and ROS, (Gustafsson *et al* 1997), rather than from the pathogenic bacterial products themselves.

The body protects itself from the potentially damaging effects of these ROS via its endogenous antioxidant defence mechanisms, which are able to scavenge or prevent the formation of these radical species. The oxidant-antioxidant balance may be tipped in favour of tissue destruction as a result of excessive ROS production and/or a diminished antioxidant defence capacity.

Our current knowledge on oxidative damage in periodontal disease outweighs that of the antioxidant defences. Investigation into the total antioxidant capacity would provide a clearer picture of the extent of pro-oxidant contribution to disease pathogenesis and about deficiencies in these systems which may place the individual at greater risk (Chapple 1997). Work by Brock *et al* into antioxidant defence in patients with periodontal disease has demonstrated both a reduced peripheral (plasma) and local (GCF) total antioxidant capacity (Brock *et al* 2004). Whether the compromised antioxidant defence systems predispose to chronic periodontitis or result from the inflammatory process has been investigated in non-smoking individuals only by the same group (Chapple *et al* 2007) but despite the pro-oxidant effects of smoke constituents, the impact of smoking upon the TAOC of periodontitis patients has not yet been explored. The present research was therefore undertaken to investigate: -

- The local (GCF) and peripheral (plasma) total antioxidant capacity in smokers with chronic periodontitis both before (baseline) and after (post-

treatment) conventional non-surgical therapy for the management of their periodontitis.

- The clinical and blood-borne markers of inflammation and plaque levels of smoking subjects at both baseline and post-therapy

Antioxidant data will be analysed both at the subject level and compared with previous reported non-smokers with and without periodontitis, and also at the site level within the periodontal patients who smoke (deep verses shallow).

1.14.1. Objectives:

The objective of this research is:

- To explore the impact of smoking upon TAOC in periodontitis patients
- To assess healing response clinically in smokers with periodontitis following conventional non-surgical therapy and to equate this with changes in the local and peripheral antioxidant defence status

The author acted as the therapist for all the patients, both the smokers reported within this thesis and the non-smokers, that formed the basis of previous publications (Brock *et al* 2004, Chapple *et al* 2007, Wright *et al* 2008).

MATERIALS & METHODS

Materials

2.1. Standard solutions and equipment for antioxidant assay

2.1.1. Phosphate buffered saline containing bovine serum albumin (PBS-BSA) - Running Buffer

Phosphate buffered saline was prepared by dissolving 7.5g NaCl, 100mg KH_2PO_4 and 750mg K_2HPO_4 in 900ml of water and the pH adjusted to 7.6 by drop wise addition of 0.5M sodium hydroxide. Once prepared 50mg of bovine serum albumin (BSA; Cohn fraction V, minimum 96% purity) was added in layers until it dissolved and the volume was made up to 1litre with deionised water. The PBS-BSA was stored at 4°C for a maximum of four weeks.

2.1.2. Synthesis and preparation of signal reagent

In research carried out using the Enhanced Chemiluminescence (ECL) antioxidant assay within our laboratory pre-2000, a commercial signal reagent called Amerlite™ was used (Johnson and Johnson Clinical Diagnostic Ltd; Amersham, UK). This consisted of a signal reagent buffer (pH 8.5, 30mls) and tablets A (containing luminol and para-iodophenol) and B (containing sodium perborate; which when dissolved in an aqueous solution yields hydrogen peroxide). The manufacture of the Amerlite reagent ceased in 1999, since which time we have synthesised our own “in-house” signal reagent according to the original patented formulation.

2.1.2.1. Signal reagent powder constituents

Signal reagents A and B (equivalent to tablets A and B in the Amerlite™ system) were prepared using the below formulations, the chemicals were mixed and ground to a fine powder and 1g aliquots were then transferred to foil-wrapped, sterile 1.5ml Eppendorf tubes and stored in a vacuum dessicator at 4°C.

Signal reagent A's formulation:	weight	(%)
Sodium chloride	5.56g	(92.65%)
Sodium benzoate	199.80mg	(3.33%)
Trisoduim citrate dehydrate (luminescence grade; Fluka)	90.00mg	(1.50%)
Sodium p-iodophenol	76.20mg	(1.27%)
Sodium luminol	75.00mg	(1.25%)

Signal reagent B's formulation:	weight	(%)
Sodium chloride	5.23g	(87.17%)
Sodium perborate tetrahydrate	480.00mg	(8.00%)
Sodium benzoate	199.80mg	(3.33%)
Trisodium citrate dehydrate (luminescence grade; Fluka)	90.00mg	(1.50%)

2.1.2.2. Signal reagent buffer preparation

Signal reagent buffer was formulated by weighing out the following constituents in order into a one litre flat bottomed round flask:

Potassium chloride	7.50g
Boric acid (molecular biology grade; Fluka)	6.20g
Trisodium citrate dehydrate (luminescence grade; Fluka)	1.00g
Sodium hydroxide (luminescence grade; Fluka)	0.80g

Approximately 900ml of ultrapure water was then added and components allowed to dissolve prior to pH adjustment 8.5 using 0.5M sodium hydroxide and increasing the volume to one litre with ultrapure water. 30ml aliquots of buffer were transferred to 50ml polypropylene centrifuge tubes (Sterlin; Appleton Woods), prior to storage in the dark at 4°C.

2.1.2.3. Preparation of working signal reagent

The final stage in the preparation of the signal reagent involved the addition of 60mg of reagent mix 'A' followed by 60mg of 'B' to 30mls of reagent buffer in

the 50ml centrifuge tubes. The top was replaced and sealed with Parafilm (Appleton Wood). The tube was then covered with aluminium foil and placed on a roller mixer for 20mins at 4°C, prior to storage in the dark at the same temperature. Once the final signal reagent was prepared it was stored for a maximum of two weeks.

2.1.3. Horseradish peroxidase stock solution 50IU/ml

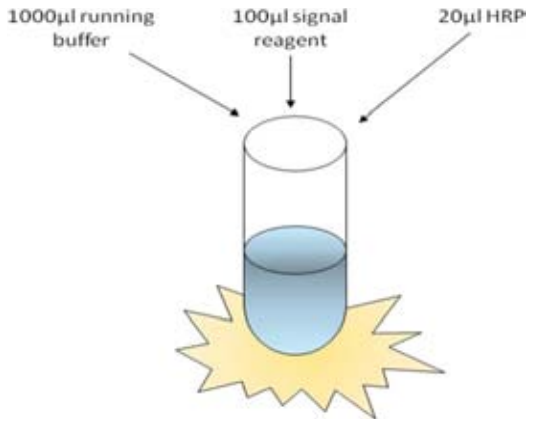
A 500IU bottle of type XII horseradish peroxidase (hydrogen peroxide oxidoreductantase, EC 1.11.1.7 - HRP) was dissolved in 10ml PBS-BSA (containing 5mg/ml BSA). 100µl aliquots were placed in foil-covered 500µl Eppendorf tubes (Appleton Woods) and stored at -20°C until required. Prior to assay the HRP solution was thawed at room temperature and a working HRP solution for that days testing made from this stock and kept in a foil wrapped Bijou container while the remainder was stored at 4°C for a maximum of two days.

Throughout the course of the study it became necessary to revert to a conjugated HRP (anti-RB IgGs with a binding site AP 311), similar to that used by Whitehead *et al* 1992, in order to improve HRP stability. The HRP-conjugate was therefore used in the later assays. However, given that calibrations curves were performed daily, this did not affect derived antioxidant measures.

2.1.4. Standard ECL assay mix

All chemiluminescence reactions were carried out in plastic cuvettes (12 x 75mm, Starstedt, Leicester, UK). In preparation for the assay 1ml of PBS-BSA running buffer (2.1.1.) was added to the cuvette and allowed to equilibrate in a tube cooler (Grant Boekel PCB2; Wolf Laboratories Ltd., Pocklington, UK) set at 19°C $\pm$ 0.5°C for a minimum of 30mins. Immediately prior to assay, 100µl signal reagent and 20µl working HRP solution were added to the PBS-BSA running buffer (figure 2.1.).

Fig. 2.1. Standard Enhanced Chemiluminescence assay mix



2.1.5. Trolox standards (80µM) for assay calibration

The external standard/calibrant for the assay was a water soluble tocopherol analogue 'Trolox' (6-hyroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). An 80µM Trolox stock solution was prepared by dissolving 20.02mg Trolox (Aldrich Chemical Co.) in one litre of deionised water (12-18h with stirring at 4°C). The solution was then aliquotted into 1.5ml Eppendorf tubes and stored for maximum of 18months at -80°C. As required a stock aliquot was thawed at room temperature and the required dilution made up by adding PBS-BSA; all standards were prepared from a single stock for use on that day and kept at 4°C between calibrations.

2.1.6. BioOrbit 1250 Luminometer

The enhanced Chemiluminescence (ECL) assays were performed using a luminometer (BioOrbit 1250 Luminonmeter; Labtech International, Sussex, UK). The luminometer is a single-sampling unit, comprising of a measuring head containing a photomultiplier tube and an electronic unit that amplifies the signal, which is expressed in mV. The luminometer is interfaced with an IBM-compatible computer and the output controlled using the 1250 *Luminometer Program*® software package. The software permits simultaneous recording and display of data; up to 5000 data points over the desired time period of up to one hour.

2.2. Classification and categorisation of patients

2.2.1. Indices of health & disease

2.2.1.1. Periodontal probing depth (PPD)

Full mouth periodontal pocket probing depths (PPD) were assessed by two examiners; N Ling-Mountford (NLM) and G Brock (GB). NLM examined the smokers with periodontitis (2.2.2.1.) and both control groups **B** (2.2.2.4.1. and 2.2.2.5.1.) using a Hu-Friedy UNC-PCP15 probe (Claudius Ash & Sons, Potters Bar Herts., UK) adapted to fit into a Brodontic handle to ensure constant probing force of no greater than 20g (0.2N). While GB examined both control groups **A** (2.2.2.4. and 2.2.2.5.) using a Hu-Friedy UNC-PCP15 probe without any adaption. Probing depths, defined as the measurement from the base of the pocket/crevice to the gingival margin, were recorded at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-palatal, mid-palatal and disto-palatal) in millimetres (mm). In order to reduce inter-examiner variability (as there was no formal examiner calibration) all PPD measurements were recorded in duplicate and where a difference of ≥1mm existed between readings a third measurement was taken. The mean of the closest two PPD measures per site were taken as the PPD of for that site. Each examiner assessed the same patients throughout the study, with no mixing of examiners within patient or control groups.

2.2.1.2. Recession (REC)

Full mouth recession charts (REC) were carried out by NLM using the adapted UNC-PCP15 probe in the smokers with periodontitis group (2.2.2.1.) and both control groups **B** (2.2.2.4.1. and 2.2.2.5.1.). Recession was measured at the same six points per tooth simultaneously with the PPD measurement. Recession was defined as the measurement/distance (in mm) from the gingival margin to the cemento-enamel junction (CEJ). All REC measurements were recorded in duplicate and where a difference of ≥1mm existed between readings a third measurement was taken. The mean of the

duplicated measures was used as the recession score for each site. Where the gingival margin lay coronally to the CEJ the recession distance was recorded as a negative value for attachment loss calculations.

2.2.1.3. Clinical attachment levels (CAL)

Full mouth clinical attachment levels were calculated by the addition of the PPD and the REC duplicate measurements in the smokers with periodontitis (2.2.2.1.) and both controls groups **B** (2.2.2.4.1. and 2.2.2.5.1.). The mean from these duplicate measures were recorded for each site (where a negative recession value indicated a gingival margin situated coronal to the CEJ).

2.2.1.4. Bleeding scores

Bleeding was assessed for the whole mouth by NLM in the smokers with periodontitis (2.2.2.1.) and both control groups **B** (2.2.2.4.1. and 2.2.2.5.1.), while GB assessed bleeding in both control groups **A** (2.2.2.4. and 2.2.2.5.). Each tooth was probed using a WHO 621 probe (Hu-Friedy – Claudius Ash) with no greater than 20g (0.2N) of pressure and scored dichotomously for the presence or absence of bleeding at four sites (mesial, distal, buccal and palatal). All teeth were probed and assessed in this manner and the total number of bleeding points was totalled and divided by four times the number of units probed, this figure was then multiplied by one hundred to give a whole mouth percentage (% BOP).

2.2.1.5. Plaque scores

Plaque levels were assessed by NLM in the smokers with periodontitis (2.2.2.1.) and both control groups **B** (2.2.2.4.1. and 2.2.2.5.1.). Plaque was recorded dichotomously full mouth at four sites per tooth (mesial, distal, buccal and palatal). All teeth were disclosed using a commercially available two tone disclosing liquid agent, Plaqsearch, (Oraldent - Kimbolton, Cambridgeshire UK) and assessed for the presence or absence of plaque. The total number of sites bearing plaque was totalled and divided by four

times the number of units viewed, this figure was then multiplied by one hundred to give a whole mouth percentage.

2.2.1.6. Radiographs

As part of the patient's initial consultation process, prior to entry into the study and clinical sampling, the radiographers at Birmingham Dental Hospital performed radiographs for each subject with chronic periodontitis. For the majority of subjects full mouth periapical radiographs were taken using a standard paralleling technique with film holders. In some cases however, orthopantanograms were recorded instead for logistical reasons. Radiographs were used to confirm diagnostic criteria and not as outcome measures.

2.2.2. Volunteer groups

Volunteers fell into three patient groups; a single test group of patients who smoked and had periodontitis, two positive control groups of non-smokers with periodontitis and two negative control groups of periodontally healthy patients. All three patient groups were used for the longitudinal clinical study to determine local (GCF) and peripheral (plasma) total antioxidant capacity (TAOC). Volunteers were allowed to withdraw from the study at any point without explanation or compromise to their care. Ethical approval was provided for the different study groups under separate applications (smokers with chronic periodontitis (2.2.2.1.) LREC5521, both control groups **A** (2.2.2.4. and 2.2.2.5.) LREC0405 and both control groups **B** (2.2.2.4.1. and 2.2.2.5.1.) LREC5643).

2.2.2.1. Smokers with chronic periodontitis

Volunteers (n= 21), who currently smoked ≥10 cigarettes per day and had been diagnosed with chronic periodontitis, were recruited from patients referred to the Unit of Periodontology, Birmingham Dental Hospital for diagnosis and treatment. The patients had to be current smokers at

recruitment and during the active treatment phase plus have a smoking history of a minimum of 10yrs.

2.2.2.2. Inclusion criteria

A diagnosis of chronic periodontitis had to have been established by the referring clinician within the Unit of Periodontology of the Birmingham Dental Hospital. The patients had to have:

- At least 20 standing teeth
- Probing depths of ≥5mm at >30% of sites
- Radiographic evidence of generalised bone loss of ≥30% at 30% of sites

2.2.2.3. Exclusion criteria

Recruitment and participation into the study were not permitted if the patients:

- Took vitamin or mineral supplements
- Were pregnant
- Had an incompatible medical history, such as a condition requiring antibiotic prophylaxis for invasive procedures
- Took medication which may affect outcomes of periodontal therapy
- Took medication affecting neutrophil function
- Took non-steroidal anti-inflammatory drugs

2.2.2.4. Non-smokers with chronic periodontitis – Group A

Volunteers (n=17) were used for comparisons employing the patient as a unit of analysis for TAOC. (in this group sampling sites were selected on the basis of index sites irrespective of PPD) Summary data from this group have been previously published (Brock *et al* 2004, Chapple *et al* 2007). These volunteers were patients within the Unit of Periodontology of the Birmingham Dental Hospital, and were age and gender matched to the smokers. Inclusion and exclusion criteria were the same as for smokers (2.2.2.2. and 2.2.2.3.) with the exception of their smoking status; they must not have been a current

smoker or a reformed smoker and must have been smoking habit free for a minimum period of 10 yrs.

2.2.2.4.1. Non-smokers with chronic periodontitis – Group B

A second non-smokers group of periodontitis volunteers (n=18) were also recruited from patients referred to the Unit of Periodontology, Birmingham Dental Hospital for diagnosis and treatment (in this group sampling sites were selected on the basis of deep sites). These patients were age and gender matched to the smokers. Their recruitment followed the same inclusion and exclusion criteria as group **A** (2.2.2.4.).

2.2.2.5. Periodontally healthy subjects – Group A

Volunteers (n=17) were recruited from staff of the School of Dentistry and South Birmingham Primary Care Trust who worked at the Birmingham Dental Hospital. They were employed for comparison with the periodontitis non-smokers (2.2.2.4., index sites). These subjects were age and sex matched to the smoking subjects and to the non-smoking periodontitis group **A**. In order to be included in the study as a healthy control subject, the volunteer was required to have a minimum of 20 teeth, with no probing depths of >3mm or evidence of attachment loss due to periodontal disease. Subjects were non-smokers or ex-smokers of ≥10years. Summary data from this group have been previously published (Brock *et al* 2004, Chapple *et al* 2007).

2.2.2.5.1. Periodontally healthy subjects – Group B

A second healthy control group of volunteers (n=18) were also recruited from the staff of the School of Dentistry and South Birmingham Primary Care Trust who worked at the Birmingham Dental Hospital and followed the same inclusion and exclusion criteria as the other healthy control group (2.2.2.5.). The purpose of this control group was to enable deep and shallow sites to be sampled for comparison with the deep and shallow sites in the non-smokers

with periodontitis (group **B**, 2.2.2.4.1.) and the smokers with periodontitis (2.2.2.1.).

Table 2.1. Comparison summary of GCF sampling of all volunteer groups

	Smokers with periodontitis	Non-smokers with periodontitis (group A)	Non-smokers with periodontitis (group B)	Non-smoker healthy controls (group A)	Non-smoker healthy controls (group B)
Index sites (patient as unit of analysis)	√	√	-	-	-
Deep sites (sites as unit of analysis)	√	-	√	-	-
Shallow sites (patient &/or sites as unit of analysis)	√	-	-	√	√

Subjects from any of the volunteer groups were allowed to withdraw from the study at any point.

Methods

2.3. Treatment regime

The indices and sampling for the periodontitis groups were recorded at the recruitment visits (baseline), to assess levels of disease, inflammation and oral hygiene levels. These were repeated three months after non-surgical periodontal management (review). The non-surgical periodontal therapy was performed over five treatment sessions; gross scaling and introduction of oral hygiene measures was followed by quadrant fine scaling and root surface debridment with both hand (Gracy curettes; LM Dental, J&S Davies, Potters Bar, Herts. UK) and ultrasonic (Dentsply UK Ltd, Addlestone, Weybridge, Surry, UK) instruments and under local analgesia with reinforcement of oral hygiene measure as required. The healthy control patients were sampled at baseline only and received a subsequent scaling and prophylaxis as required.

2.3.1. Smoking cessation

All smokers with periodontitis (2.2.2.1.) received a brief smoking intervention based on the "Four A's Approach" to smoking cessation. This included assessment of their smoking history, advice on the impact of smoking on their periodontitis, assessing their current interest in quitting smoking and review of any past attempts. Those smokers who showed interest in quitting their tobacco habit were offered support from the lead clinician (NLM), including motivational and behavioural advice plus information on the use of pharmacological aids (Nicotine replacement therapies, Zyban or Champix) in their quit attempt to reduce withdrawal symptoms. If the volunteer required a more intensive support system than could be offered during treatment, referral to the NHS Stop Smoking Services was offered. Smoking status and interest in cessation was assessed prior to treatment and following treatment at the three month review appointment if cessation has not occurred prior to this time.

2.4. Clinical sample collection protocols

2.4.1. Plasma

Volunteers were asked not to eat or drink anything with the exception of bottled water, plus to refrain from using any form of oral hygiene product from midnight the evening before the sampling visit. Venous blood was collected into 7ml Vacutainers™ tubes (two lithium heparin (119 I.U.) and one plain tube; Becton Dickinson, Cowley Oxon, UK) following venipuncture of one of the veins in the ante-cubital fossa. Plasma was then obtained from the heparinised samples, whilst the blood collected in the plain Vacutainers™ tube was sent to the laboratory of the Queen Elizabeth Hospital, Birmingham for analysis to establish constituent blood cell counts.

The heparinised venous blood samples, collected in the two lithium heparin Vacutainers™ tubes, were left to stand at room temperature for 30mins prior to centrifugation (Centra CL3R refrigerated bench Centrifuge, Thermo Quest Scientific Equipment Group Ltd, Basingstoke, Hampshire, UK) at 1000g for a further 30 minutes at 4°C. Subsequently plasma was retrieved using a Pasteur pipette (Appleton Woods), aliquotted into 1.5ml cryogenic vials and frozen at -80°C until required. The samples were defrosted by hand and used immediately when required.

2.4.2. GCF sampling

Gingival crevicular fluid (GCF) samples were collected using Periopaper strips (Oraflow Inc., Plainview, New York, USA) and measured using a pre-calibrated Periotron 8000® (Oraflow Inc.; methods 2.4.2.4.). The Periotron 8000® is an instrument designed to quantify submicrolitre volumes of fluid samples using “periopaper strips” and has been reported as a precise instrument when used with a standardised protocol. The same machine was calibrated and used throughout the study (Chapple *et al* 1999, Ciantar *et al* 1998). The Periotron consists of a pair of upper and lower counterparts or “jaws”, which close to hold a periopaper strip between them and a digital

display screen. Each jaw has an electrical charge, one positive, the other negative, and when a dry strip is inserted no electrical charge can flow. However, when a strip containing moisture is placed between the jaws, the charge is able to flow in direct proportion to the volume of fluid present, and is displayed as a digital reading. GCF volumes were determined from a look up table (Appendix 1).

Sampling was performed as previously described (Chapple *et al* 1993, 1997). Sterile Periopaper strips were mounted on the holders provided taking care not to touch the strips prior to use. Individual strips were placed into an "index site" (2.4.2.1.), a known deep pocket (2.4.2.2.) or shallow crevice (2.4.2.3.) of the test tooth by one of the three operators (NLM, GB, and MM). Isolation was achieved by placing cotton wool rolls in the buccal vestibule and drying the site with air using a 3-in-1 syringe; care was taken to ensure air was directed from the vestibule coronally to avoid disturbing the fluid within the gingival crevice. The periopaper strip was placed in the gingival crevice or pocket using college tweezers until gentle resistance was felt, taking care to ensure the entire leading edge of the strip was inserted rather than just one corner. Each strip was left *in situ* for 30 seconds then recovered and the GCF volume determined using a pre-calibrated Periotron 8000 (2.4.2.4.).

Volunteers were asked to follow the same overnight starving protocols as for blood sampling. Throughout the GCF sampling the patients were requested to refrain from talking or closing their mouths and the operator prevented contamination of the strip by the lips, tongue or cheeks with retraction of these tissues. Prior to storage, samples visibly displaying blood contamination were rejected and an alternative site chosen for sampling. The same GCF collection sites were used at baseline and post-operatively (Table 2.1).

2.4.2.1. Index sites

Six mesio-buccal sites, unless otherwise stated, were chosen in the maxillary arch, ideally the first molars, first pre-molars and canines. Neighbouring teeth were used as an alternative if necessary and mirrored in the opposing side of

the arch. Index sites were sampled in the smokers with periodontitis and non-smokers with periodontitis group **A** prior to a full periodontal assessment, therefore each site's periodontal status was at the time unknown. These samples were taken to enable statistical analysis using patient as the unit of analysis and reduce confounding by site characteristics, and were to be compared to control data from a previous clinical trial (periodontitis non-smokers 2.2.2.4. and periodontally healthy non-smokers 2.2.2.5.) in which the author had also been involved (Brock *et al* 2004, Chapple *et al* 2007).

2.4.2.2. Deep sites

Known deep pockets were sampled in both the smokers and non-smokers with periodontitis group **B** following a full periodontal assessment (2.3.1.1. and 2.2.1.4.), with greater than a 24 hours period for the gingiva to recover from probing. Four deep sites, defined as a site of "active" disease displaying bleeding on probing and with a probing depth of ≥5mm, were sampled per subject in the maxillary arch. This was to enable site-specific, rather than patient-based analysis, by comparing deep sites in the smokers and non-smoker periodontitis groups and also deep verses shallow sites (2.4.2.3.) within the smokers.

2.4.2.3. Shallow sites

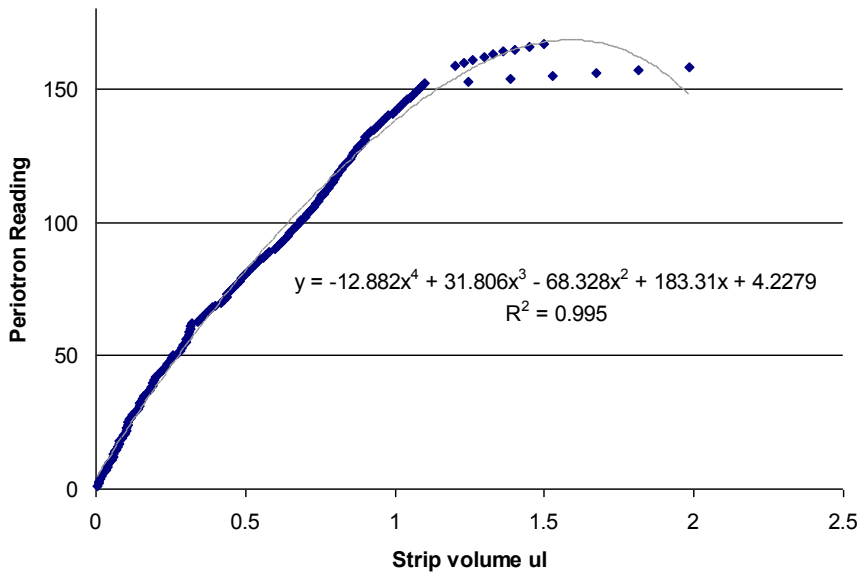
Four shallow sites were sampled in the periodontitis smokers and the healthy control groups; a shallow site being defined as having a probing depth of ≤3mm with no bleeding on probing. These sites were sampled following the full periodontal measures with a minimum of 24 hours for gingival recovery. Shallow sites were sampled to enable comparison with deep sites in the smokers in a site-based analysis.

2.4.2.4. GCF volume determination

GCF volumes were determined with a pre-calibrated Periotron 8000. Calibration was performed using known volumes of serum (from a single

donor) diluted 1:5 with physiological saline as described by Chapple *et al* (1999). Briefly, increasing incremental volumes of 0.01µl of 20% serum were delivered onto periopaper strips using a Hamilton microsyringe (Aldrich Chemical Co., Poole, UK) from 0.01 to 1.2µls and the Periotron reading recorded. Measurements were taken in triplicate for each volume and between each reading the Periotron was re-set to zero using the same dry strip throughout. A graph of volumes against Periotron readings was formulated and a calibration line produced that was described using a 4th order polynomial regression equation (figure 2.2). Based on this equation a "look-up" table (appendix one) was produced in order to determine volumes of samples obtained clinically from the Periotron display against calculated sample volumes.

Fig. 2.2. Periotron calibration curve



2.4.2.5. Periotron 8000 operating procedure

The same Periotron 8000 was used throughout the entire study. Before each sampling session the Periotron was switched on for a minimum period of 30mins prior to use, with a blank periopaper strip between the jaws and the digital reader set to zero. Immediately following a GCF sample collection the

periopaper strip was inserted between the jaws and left in place until a reading was produced. Once the Periotron volume was obtained the strip was immediately removed and placed in the relevant cryogenic vial, the Periotron jaws were dry-wiped, or cleaned using isopropyl alcohol wipes (Sterets™, Seton Healthcare Group, Oldham UK) and dried prior to reinsertion of a blank strip and re-zeroing. Care was taken to insert the sample and blank strip in the same orientation and to the same length between the jaws (Chapple *et al* 1999).

2.4.2.6. GCF sample elution and storage

Each periopaper strip was immediately transferred from the Periotron to the relevant "cryotube" containing 600µls or 400µls of PBS-BSA (50mg/L). Samples were stored in the cryotubes in multiples according to the nature of the site sampled (i.e. index, deep or shallow), with 100µl of PBS-BSA per strip. Elution was carried out over a 30min period at room temperature, as previously described as the optimal technique (Chapple *et al* 1997). The cryotubes were then "snap" frozen and stored in liquid nitrogen with the strips retained "*in situ*".

Before use the stored elutes were defrosted by hand, vortexed for 2 seconds and assayed immediately. For quantitative determinations, a known volume of eluate was used in conjunction with Trolox standards whose volumes matched those of the sampled eluate.

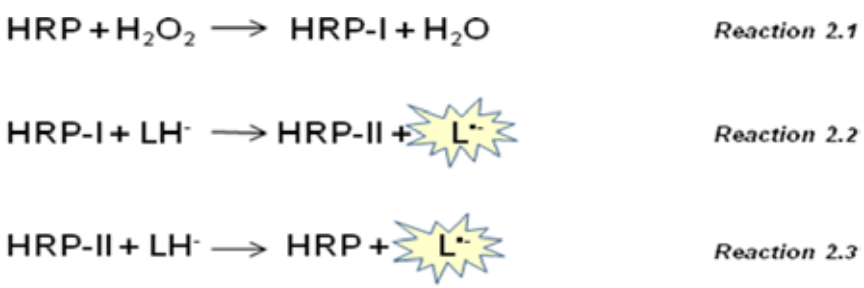
GCF samples were collected at baseline and 3 months following treatment for the periodontitis patients, so were all PPD, REC, CAL and indices measurements.

2.5. Total antioxidant capacity (TAOC) assay methodology

2.5.1. Introduction to chemiluminescence as a diagnostic tool

Luminescence is a generic term used for a range of processes that produce light when molecules in an electronically excited state decay to their ground state with the emission of photons. Chemiluminescence is often confused in the early literature with fluorescence; the two processes differ in the source of energy which produces the molecules in an excited-state. In chemiluminescence the energy is of a chemical reaction, whereas in fluorescence it is an incident radiation (Kricka & Thorpe 1983).

The use of synthetic chemiluminescent compounds in research is vast. One such compound, luminol (5amino-2,3-dihydro-1,4-phthalazine dione) was reported to possess luminescent properties by Albrecht in 1928 and is still widely used (Dodeigne *et al* 2000). Light emission occurs from luminol when it is oxidised by a range of oxidants, hydrogen peroxide and sodium perborate being the most commonly used (Whitehead *et al* 1979), when in an aqueous media a catalyst is required. Horseradish peroxidase (HRP) is relatively specific for the hydrogen acceptor H_2O_2 (Misra & Squatrito 1982). The horseradish peroxidase-catalysed chemiluminescent oxidation of luminol results in the formation of the luminol radical ($L^{\bullet-}$) and follows three main stages. Peroxidase reacts with the oxidant H_2O_2 to form compound I (reaction 2.1). Compound I reacts with the luminol anion ($LH^{\bullet}$) to form compound II (reaction 2.2), which in turn reacts with another molecule of $LH^{\bullet}$ to yield the original peroxidase enzyme (reaction 2.3). The luminol radical ($L^{\bullet-}$) then undergoes further reactions resulting in the formation of an endoperoxide, which then decomposes to yield an electronically excited 3-aminophthalate dianion, emitting light on return to its ground state (Thorpe & Kricka 1986, Mistra & Squatrito 1992). The light emitted is detected and measured using a luminometer (Thorpe & Kricka 1986).



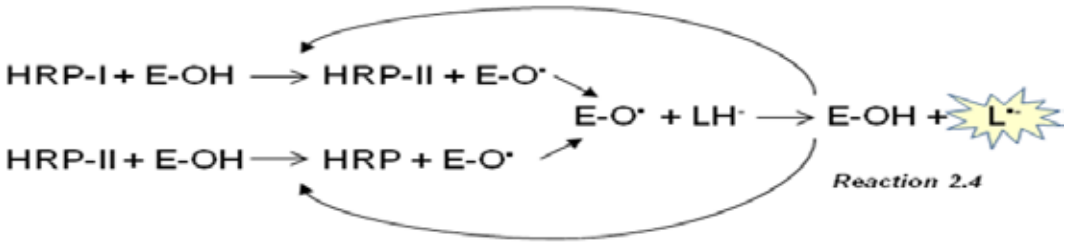
HRP-I = horseradish peroxidase compound I; HRP-II = horseradish peroxidase compound II; LH⁻ = luminol anion; L•⁻ = luminol radical

2.5.2. Enhanced chemiluminescence assays

Limitations in chemiluminescence, such as the relatively low intensity of light emission, which gives a poor signal to background ratio and the initial brief peak in light emission which decays rapidly, have been overcome by the use of chemical enhancers (Brock 2005).

2.5.2.1. Mechanism of enhanced chemiluminescence

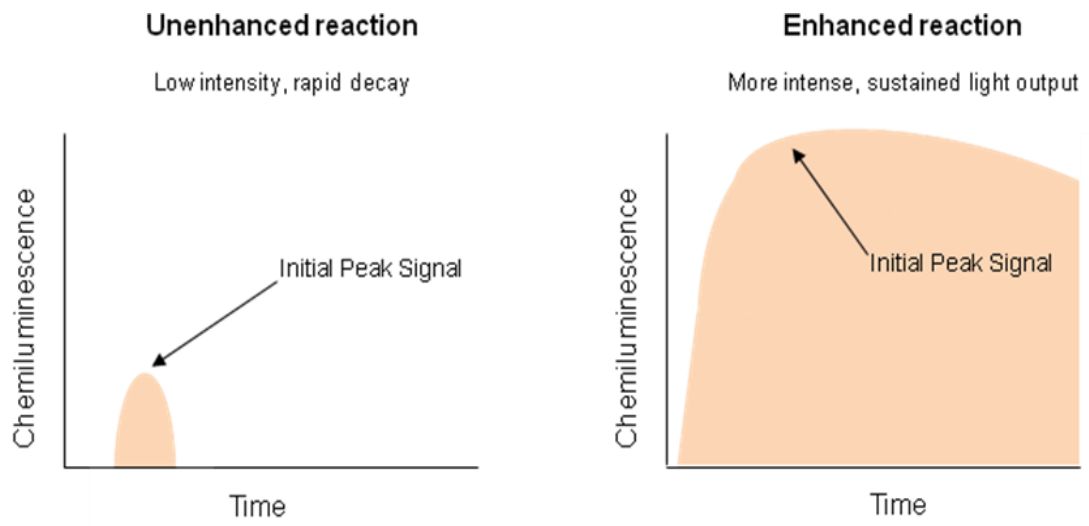
Light emission in an unenhanced HRP-catalysed oxidation of luminol is rate limited by the relatively slow reaction of compound II with luminol. Enhancers are believed to increase light emission by increasing the conversion of compound II back to active HRP because it acts as a more favourable substrate than the luminol for oxidation. The enhancer radical thus formed can oxidise the luminol itself producing a further luminol radical and increases light emission and in the process is reduced back to its ground state (reaction 2.4) (Thorpe & Kricka 1987).



E-OH = enhancer
E-O• = enhancer radical

The consequent light emission intensity may be increased by 500-1000 fold at pH 7 – 9.6 and is prolonged and stable (Thorpe et al 1985b, Thorpe & Kricka 1987; figure 2.3).

Fig. 2.3. A comparision of unenhanced and enhanced chemiluminescence



Several compounds may act as enhancers, although certain substituted phenol derivatives, including *para*-iodophenol (*p*-iodophenol) are particularly potent enhancers. Emission spectra of phenol-enhanced and unenhanced reactions are similar, confirming that the luminol is the emitting species rather than the enhancer (Thorpe *et al* 1985a).

2.5.2.2. Application of the ECL system as an antioxidant assay: inhibition by radical scavengers

The light emission in ECL, produced and driven by the continuous production of free radicals from the *p*-iodophenol enhancer, luminol and reactive oxygen species, may be almost totally suppressed by adding chain-breaking (radical scavenging) antioxidants to the assay (Whitehead *et al* 1992). Once all the added antioxidant has been quenched the luminol oxidation continues and the light output returns to its original level. As the radicals are constantly generated, the time period of suppression is linearly related to the quantity of

antioxidant present or added to the assay, allowing the quantification of total antioxidant capacity in biological fluids (Whitehead *et al* 1992). The light output and its suppression was measured against time using a BioOrbit 1250 luminometer (2.1.6.).

2.5.2.3. BioOrbit 1250 Luminometer protocol

The luminometer was allowed to warm up for 30mins prior to the day's assays. The software package was then run whilst the gain setting was adjusted to give an absolute reading of 10mV, using an internal 10mV standard. The background setting was then adjusted to 0mV using an empty assay tube.

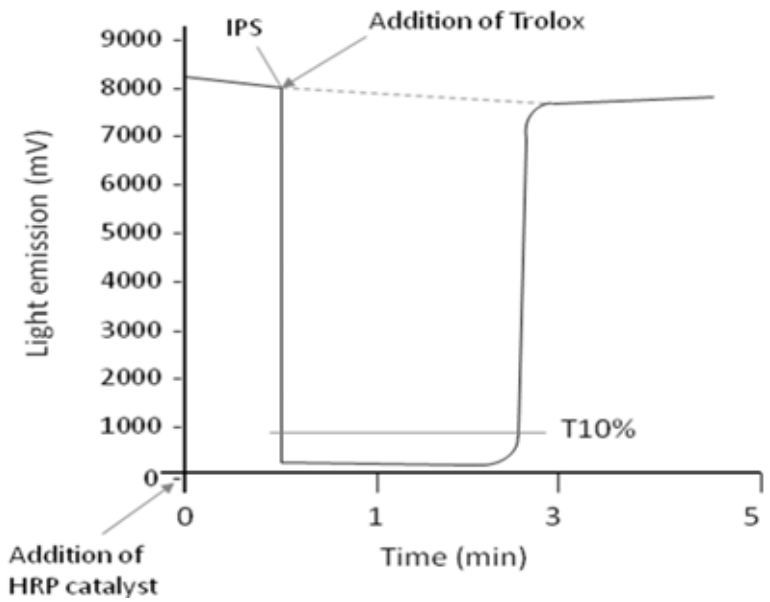
2.5.2.4. Preparation of working solution for the assay

Sufficient PBS-BSA (2.1.1.) for each days assay runs was transferred into universal containers and allowed to warm to room temperature prior to aliqoting into curvettes as part of the standard ECL assay mix (2.1.4.). The ECL signal reagent was prepared (2.1.2.), the HRP aliquot (2.1.3.) defrosted, the working dilution of HRP solution made by adding 15µl to 5ml PBS-BSA (50mg/L) buffer in a sterile bijou container and mixed by gentle inversion. The standard assay mix (2.1.4.) was then made up using the diluted HRP stock, pulsed-mixed for two seconds using a bench vortex at medium speed and immediately loaded into the chamber of the luminometer. The reaction was then allowed to proceed until the light emission reading stabilised at a peak value (initial peak signal, IPS; fig 2.2). An IPS of between 7,000 and 10,000mV was used for all assays (Chapple *et al* 1997). If the working dilution of HRP produced an IPS outside of these parameters it was rejected, a fresh dilution was made using higher or lower levels of stock HRP and the assay re-run. Once within the IPS limits, the working dilution of HRP was stable for use for a twelve hour period at room temperature.

2.5.2.5. Influence of the test solution (GCF & Plasma) upon ECL

The standard ECL (2.1.4.) mix was loaded into the luminometer chamber and luminol oxidation proceeded until the desired IPS was attained. The process was then stopped momentarily. The curvette was removed and a known volume of test solution (GCF or plasma) added and briefly vortexed for 2secs before returning to the luminometer chamber. The process was allowed to continue and data collection resumed immediately. The assay was terminated once the antioxidant capacity of the test sample was exhausted and the chemiluminescence intensity passed a fixed recovery value (10% of the IPS; figure 2.4.). The data collected were saved for subsequent analysis.

Fig. 2.4. Light emission kinetics prior to and following addition of 10µL of Trolox standard. (Adapted from Chapple *et al* 1997)



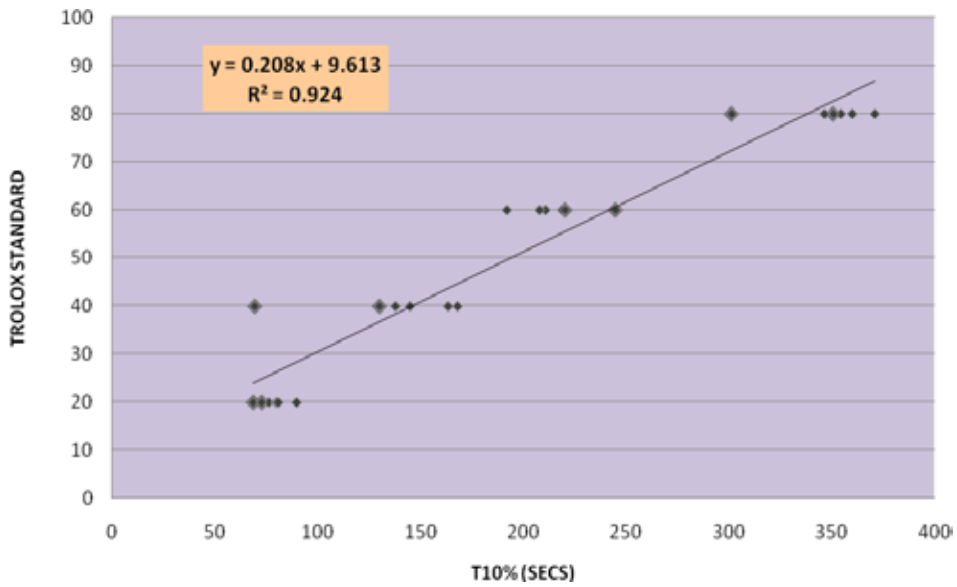
2.5.2.6. Definition of T10%

The 10% value was required to compare the total antioxidant capacity of the test samples (GCF and plasma) with the Trolox calibrant. It was taken as the time in seconds between the addition of the test sample to the ECL mix and the recovery to 10% of the IPS (Whitehead *et al* 1992, Chapple *et al* 1997).

2.5.2.7. Total antioxidant assay calibration and sample volumes

External Trolox calibrations were performed in triplicate, for each dilution, at the beginning and end of the working session, with an additional standard curve measure if a prolonged working day was required. 20µl volumes of a range of Trolox standards (20µM - 80µM) were used, except for the GCF samples where 100µl volumes were required using 0.625µM - 10µM Trolox solutions. A graph of T10% (time – secs) against Trolox concentrations (µM) was plotted and a calibration line derived, which was described by a linear regression equation (Fig. 2.5)

Fig. 2.5. A typical Trolox standard calibration plot, including begining, mid and end of day standards



Test sample volumes were always matched with those of the calibration. The GCF eluates were undiluted while plasma samples were diluted 1 in 10 with PBS-BSA to ensure a T10% value within the range of standards and also a workable assay time.

2.5.2.8. Calculation of total antioxidant capacity

Using the linear regression equation, calculated for the day, the T10% value of a test sample could be used to describe the total antioxidant concentration

in μM Trolox equivalents (Fig. 2.4). A multiplication factor was required to allow for dilution of plasma samples to determine the final Trolox equivalent values, as the plasma sample volumes were matched with those of the standard. For example, if the T10% value of the test plasma sample gave a total antioxidant concentration of 32.56μM Trolox equivalents, as this was a 1 in 10 dilution, then the final Trolox equivalent concentration was 325.6μM. The total antioxidant capacity per 30-seconds sample (nmoles Trolox) could also be calculated for the GCF test samples.

2.6. Statistical analysis of results

All data was analysed using a personal computer running Minitab™ version 15. P values of ≤0.05 were considered significant. Parametric data were analysed by analysis of variance (ANOVA) and where appropriate by paired T test (for paired data). As all the data were not normally distributed, statistical significance was verified using non-parametric test (Mann Whitney U-test).

RESULTS & DISCUSSION

3. Cross-sectional investigations:

Local and peripheral total antioxidant capacity (TAOC)

3.1. Context

Our current knowledge of the relationship between periodontal disease and antioxidant defence systems is limited (Chapple 1997) but work carried out by our research team has indicated a reduction in total antioxidant capacity both locally (GCF) and peripherally (plasma) in non-smokers with periodontitis compared with periodontally healthy controls (Brock *et al* 2004). Smoking is a risk factor for periodontitis (Ryder 2007) and increases the body's exposure to reactive species (Pryor *et al* 1983) as well as reducing the body's antioxidant defences against these species (Alberg 2002). To date, no research has been carried out to investigate the impact of smoking upon the TAOC of peripheral (plasma) or local (GCF) compartments in patients with periodontitis, with the exception of a study by Buduneli and co-workers 2006, which investigated the effects of smoking and gingival inflammation on salivary antioxidant capacity. They reported no change in salivary antioxidant status in gingivitis patients as a result of smoking or gingival inflammation. However, they could not preclude the possibility of an oxidant-antioxidant imbalance induced by tobacco within the periodontal tissues themselves and suggested analysis of GCF TAOC would better address this question (Buduneli *et al* 2006). This chapter therefore focuses on comparing the TAOC (both local and peripheral) of smokers with periodontitis and non-smokers with periodontitis and also with periodontally healthy non-smoker controls.

The data from the smokers with moderate periodontitis are initially compared to age- and gender-matched non-smokers with periodontitis and matched healthy controls (group **A**) from previously published research by our group (Brock *et al* 2004), in which the author was involved. Because the latter study group only exhibited slight periodontitis, a second and diseased-matched

group of non-smokers with moderate periodontitis were recruited along with matched healthy controls (group **B**).

3.2. Clinical and demographic data

Twenty one smokers with periodontitis (2.2.2.1, chapter 2) were enrolled into the study and age and gender-matched to seventeen non-smokers with periodontitis (group **A**; 2.2.2.4.) and seventeen non-smoker healthy controls (group **A**, 2.2.2.5.) from previously published work (Brock *et al* 2004, Chapple *et al* 2007). The smoker group contained fifteen females and six males, while the non-smokers and control group each contained ten females and seven males (Table 3.1).

The mean whole mouth periodontal probing depths in the non-smoker periodontitis group **A** were significantly lower than the smokers with periodontitis (p=0.002) indicating lower levels (mild) disease compared with the smokers with periodontitis. Due to the significant differences in periodontitis levels between these two groups the decision was made to recruit a second group of disease matched non-smokers with periodontitis and healthy controls. Therefore the twenty one smokers with chronic moderate periodontitis (2.2.2.1.) were also age and gender matched to twenty non-smokers with chronic moderate periodontitis, (group **B**; 2.2.2.4.1 chapter 2). Two of the non-smoker's samples were not assayed for technical reasons. Thus, twenty one smokers and eighteen non-smoker periodontitis patients plus eighteen aged and gender-matched periodontally healthy control subjects (group **B**, 2.2.2.5.1.) completed the cross-sectional study. These two groups each contained fourteen females and four males (Table 3.1). There was no significant age difference between any of the groups enrolled into the study (p=0.6).

The mean whole mouth periodontal probing depths plus range of subject means are illustrated in table 3.1. There was no significant difference between the mean probing depth values of the smokers with periodontitis and

those of the non-smoker periodontitis group **B** (p=0.4). However, as already stated, the mean whole mouth probing pocket depths in the non-smoker periodontitis group **A** were significantly lower than the smokers (p=0.002), but also significantly lower than the non-smoker periodontitis group **B** (p=0.004).

Table 3.1.: Clinical demographic data for the experimental groups

		Group A		**Group B**	
	Periodontitis (moderate) smokers	**Periodontitis (mild) non-smokers**	**Health**	**Periodontitis (moderate) non-smokers**	**Health**
Gender	15♀ & 6♂	10♀ & 7♂	10♀ & 7♂	14♀ & 4♂	14♀ & ♂4
Age yrs – mean ±SD (range)	44.8 ±8.5 (23 – 65)	43.0 ±9.3 (23 – 58)	44.3 ±10.6 (24 – 63)	47.1 ±6.4 (36 – 61)	46.6 ±6.1 (37 – 62)
Probing depths (mm) FM mean ±SD	3.79 ±0.72	3.05 ±0.54	≤3.0	3.63 ±0.50	≤3.0
Recession (mm) FM mean ±SD	0.72 ±0.49	Not recorded	-	0.35 ±0.31	-
CAL (mm) FM mean ±SD	4.06 ±0.83	Not recorded	-	3.99 ±0.70	-
% BOP FM ±SD	49.19 ±17.65	22.55 ±7.26	≤10.0	65.17 ±13.73	≤10.0
% Plaque FM ±SD	64.48 ±15.49	Not recorded	-	78.89 ±9.00	≤10.0

Whole mouth recession (REC) measures are also documented in table 3.1. Recession was significantly lower (p=0.008) in the non-smokers with periodontitis group **B** relative to the smokers with periodontitis. However, when clinical attachment loss (CAL) was calculated for these two groups no significant difference was found (p=0.7). Recession measurements were not taken in group **A** and therefore CAL measurements could not be calculated for this group.

The mean whole mouth percentage of sites bleeding on probing (% BOP) varied greatly between the different groups (Table 3.1). These differences in

BOP scores were significant, consistent with previous literature reports, between the smokers with periodontitis and the non-smokers with periodontitis groups **A** ($p<0.00001$) and **B** ($p=0.014$). However, a significant difference was also seen between the two non-smoker periodontitis groups **A** and **B** ($p<0.00001$), reflecting lower levels of disease in group **A** relative to group **B**.

The mean whole mouth percentage of sites noted with plaque present was recorded in both the smokers group (64.48%) and non-smokers with periodontitis group **B** (78.89%), these scores were significantly different ($p<0.002$, table 3.1). Plaque levels were not assessed in the non-smokers periodontitis group **A** and therefore no comparison could be made with the other groups.

3.3. GCF samples

Gingival crevicular fluid samples were taken from either index sites (2.4.2.1), employing the patient as the unit of analysis, or deep sites (2.4.2.2.), allowing site specific analysis of TAOC. Mean pocket probing depths for all sites sampled were also calculated (Table 3.2).

As expected, GCF volumes (per 30 second sample) were significantly higher in all the periodontitis groups compared to the both the healthy control groups **A** and **B** ($p<0.0001$) (Table 3.2).

3.3.1. Smokers with periodontitis versus group A (index sites – patient as unit of analysis)

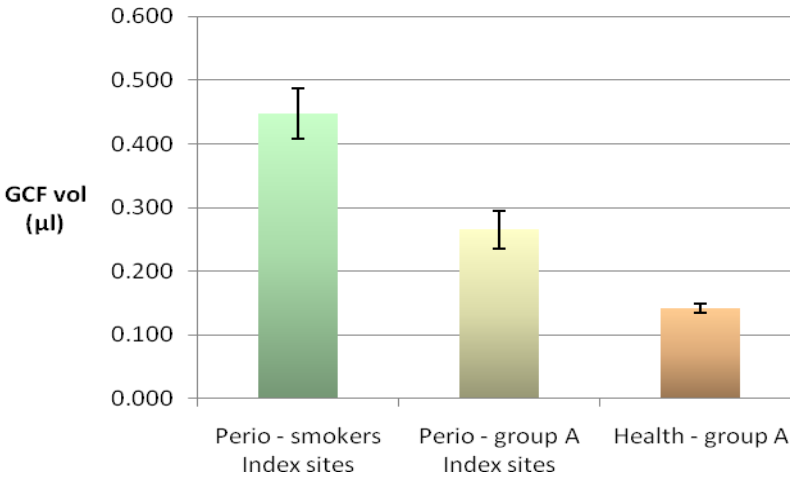
The GCF volumes for smokers were significantly greater 0.45µl (range 0.21µl-0.77µl) when compared to the non-smokers with periodontitis group **A** 0.27µl (range 0.12µl-0.59µl) ($p<0.0001$) (Table 3.2, Fig 3.1). The mean probing pocket depths of these index sites were also significantly higher ($p=0.017$) in the smokers with periodontitis 4.63mm (range; 2.67-6.83mm) than group **A**

3.79mm (range 1.83-6.17mm), which was expected as group **A** demonstrated only mild periodontitis.

Tablet 3.2.: Clinical demographic data for Deep and Index sites that were sampled for GCF TAOC analysis, plus the TAOC nM Trolox equivalent per 30 second sample

		Group A		Group B	
	Periodontitis smokers	**Periodontitis non-smokers**	**Health**	**Periodontitis non-smokers**	**Health**
PPD – Index sites mm (mean ±SD)	4.63 ±1.01	3.91 ±1.82	≤3.0	-	-
GCF vol µl – Index sites (mean +SD)	0.45 ±0.18	0.27 ±0.12	0.14 ±0.03	-	-
TAOC nM/30sec – Index site (mean ±SD)	0.29 ±0.27	0.14 ±0.06	0.17 ±0.08	-	-
PPD – Deep sites mm (mean ±SD)	6.04 ±0.66	-	-	6.42 ±0.97	≤3.0
GCF vol µl – Deep sites (mean +SD)	0.59 ±0.21	-	-	0.44 ±0.22	0.20 ±0.06
TAOC nM/30sec – Deep site (mean ±SD)	0.25 ±0.23	-	-	0.28 ±0.13	0.23 ±0.11

Fig. 3.1 Mean GCF volume (SEM) from smokers and non-smokers with periodontitis group A at index sites and age- and gender-matched healthy controls

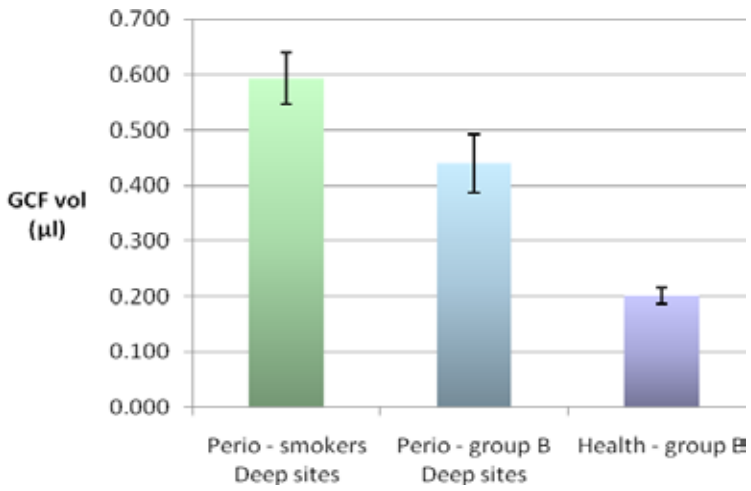


No significant correlation was found between the PPDs and GCF volumes in the smokers with periodontitis ($p>0.5$, $R^2=0.02$, $r=0.14$), while in the non-smokers with periodontitis group **A** showed a weak yet positive correlation ($p=0.007$, $R^2=0.21$, $r=0.46$).

3.3.2. Smokers with periodontitis versus group B (deep sites – diseased site as unit of analysis)

The GCF volumes of the deep sites in the smokers were significantly greater 0.59µl (range 0.20µl-1.14µl) than those in the non-smokers with periodontitis group **B** 0.44µl (range 0.23µl–1.11µl) ($p<0.012$) (Table 3.2, Fig 3.2). The mean probing pocket depth measurements for the deep sites in the smokers with periodontitis were 6.04mm (range 5.25-7.75mm) and in group **B** was 6.42mm (range 4.50-8.25mm). These two groups showed no significant difference ($p=0.16$) in the probing depth measurements of the selected deep pockets.

Fig. 3.2 Mean GCF volume (SEM) from smokers and non-smokers with periodontitis group B at deep sites and age- and gender-matched healthy controls



A strong positive correlation was seen in both the smokers probing depths and GCF volumes collected at the deep sites ($P= 0.004$, $R^2=0.37$, $r=0.61$) and

with the non-smokers with periodontal disease group **B** ($p<0.0001$, $R^2=0.58$, $r=0.76$).

3.4. Total antioxidant capacity (TAOC)

3.4.1. GCF TAOC per 30 second sample of smokers with periodontitis versus group A (patient as unit of analysis)

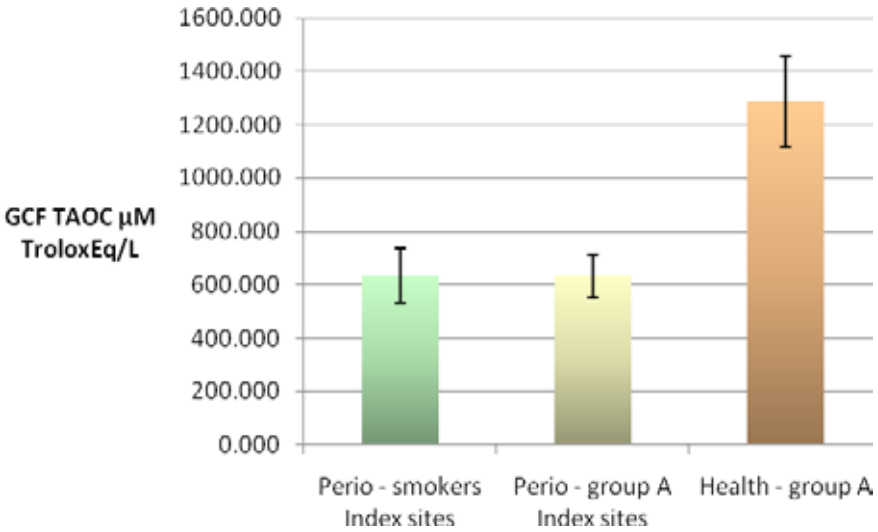
Technical problems with the assay resulted in a loss of one sample during the testing of the index sites and therefore results for the smokers with periodontitis were based on twenty of the volunteers. The GCF samples were taken over a 30 second time frame and assessed for TAOC within the sample. The mean TAOC per 30 second sample was significantly lower in the non-smokers with periodontitis group **A** (0.14 $\pm$0.06 nmoles/sample) compared to the smokers with periodontitis (0.29 $\pm$0.27 nmoles/sample, $p=0.05$). However the lower TAOC in the non-smokers with periodontitis group **A** did not reach significance when compared to the healthy control group **A** (0.17 $\pm$0.08 nmoles/sample, $p=0.17$), despite the larger volumes collected from the former periodontally involved group. The smokers with periodontal disease showed greater GCF TAOC per 30 second sample compared to the non-smoking healthy group **A** but this did not reach significance ($p=0.2$), despite the significantly larger volume of GCF samples in the smokers.

3.4.1.2. GCF TAOC concentration of smokers with periodontitis versus group A (patient as unit of analysis)

To allow for differences in volume collection of GCF, TOAC was also expressed as a GCF concentration (TAOC μM Trolox equivalents per litre) for all groups. There was no significant difference in the mean GCF TAOC of the index sites between the smokers with periodontitis 632.81μM ($\pm$459.59μM) and the non-smokers with periodontitis group **A** 632.39μM ($\pm$343.24μM) ($p>0.9$). However, the mean GCF TAOC of the index sites in the control group **A** 1287.43μM ($\pm$696.10μM) was significantly higher than both the

smokers (p=0.0002) and the non-smokers with periodontitis group **A** (p=0.0003) (Fig 3.3).

Fig. 3.3 Mean GCF TAOC µM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis group A at index sites and age- and gender-matched healthy controls



3.4.2. GCF TAOC per 30 second sample of smokers with periodontitis versus group B (diseased site as unit of analysis)

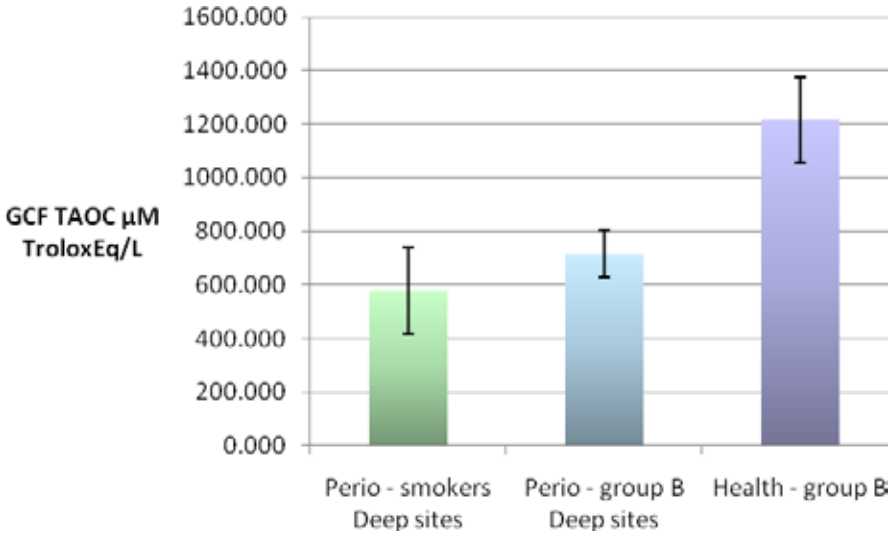
The greatest mean TAOC per 30 second sample of the deep sites was seen in the non-smoking periodontitis group **B** (0.27 ±0.13 nmoles/sample), which was not significantly higher than the smokers with periodontitis (0.25 ±0.23, p=0.15). While the lowest mean TAOC per 30 second sample of the sites sampled seen in the healthy control group **B** (0.22 ±0.11 nmoles/sample), which may reflect the lower level of GCF retrieved from the healthy site over the 30 second time period.

3.4.2.1. GCF TAOC concentration of smokers with periodontitis versus group B (diseased site as unit of analysis)

The mean TAOC of the GCF of the deep sites were then calculated as µM Trolox equivalent per litre. The deep site samples for the smokers with

periodontitis 577.89μM (±740.20μM) were significantly lower than both the non-smokers with periodontitis group **B** 716.13μM (±380.58μM) (p=0.02) and the control group **B** 1215.44μM (±687.07μM) (p=0.0007). A significant difference was also seen between the non-smokers with periodontitis group **B** and the control group **B** (p=0.027) (Fig.3.4).

Fig. 3.4 Mean GCF TAOC μM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis group B at deep sites and age- and gender-matched healthy controls



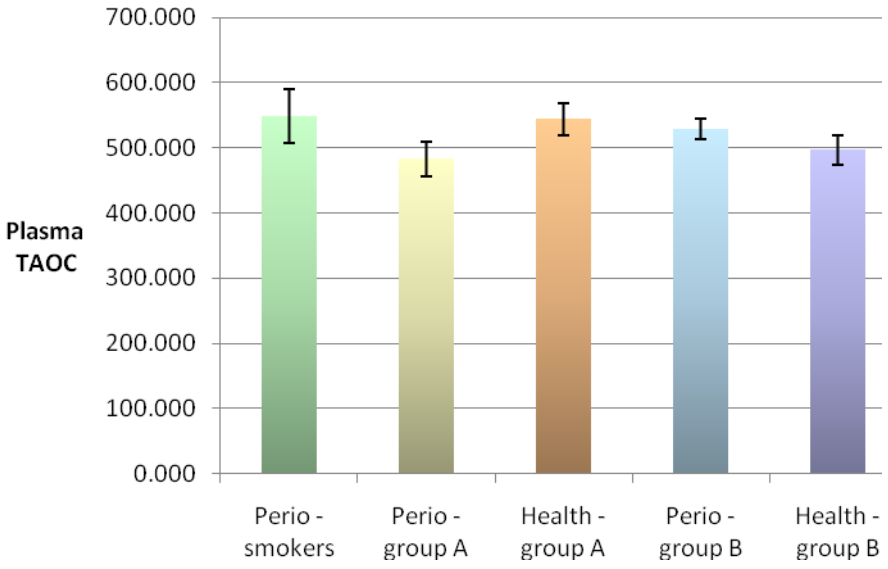
3.4.3. Plasma TAOC

Little difference was seen between all the groups in the TAOC of plasma samples (Fig. 3.5). The smokers with periodontitis displayed the greatest plasma total antioxidant concentration (548.68μM ±185.58μM), but this did not reach significance compared to all other groups (p>0.4).

The lowest plasma total antioxidant concentration was seen in the non-smokers with periodontitis group **A** (482.97μM ±110.80μM), which approached significance compared to their healthy control group **A** (544.44μM ±101.91μM, p=0.05) and which was significantly lower than both the non-smokers with periodontitis group **B** (528.95μM ±66.20μM, p=0.03) and healthy

group **B** (496.79μM ±95.50μM, p=0.03). However this lowered plasma TAOC did not reach significance compared to the smokers (p>0.3).

Fig. 3.5 Mean plasma TAOC μM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis and age- and gender-matched healthy controls



Peripheral (plasma) total antioxidant concentration was lower than local (GCF) total antioxidant concentrations and showed a gender bias towards being significantly higher in male subjects than females regardless of periodontal health or disease status (p<0.013, Fig 3.5 & 3.6). In the non-smokers with periodontitis group **B** the difference between the plasma total antioxidant concentration in males to females failed to reach significance (p=0.07).

By contrast the GCF samples showed no significant gender bias (p>0.1), despite the mean total antioxidant concentrations in female subjects (with or without periodontitis) appearing higher than those from male subjects (Fig 3.6). This gender bias is consistent with that found in our earlier work (Brock *et al* 2004). Within the smokers the same gender bias was found with the exception of the male smokers at index sites (Fig 3.6), which was higher than in the females at index sites, but again this difference between the genders failed to reach significance (p>0.4).

Fig.3.6. Total antioxidant concentrations in plasma and GCF (patient as unit of analysis): stratified for gender (mean SEM)

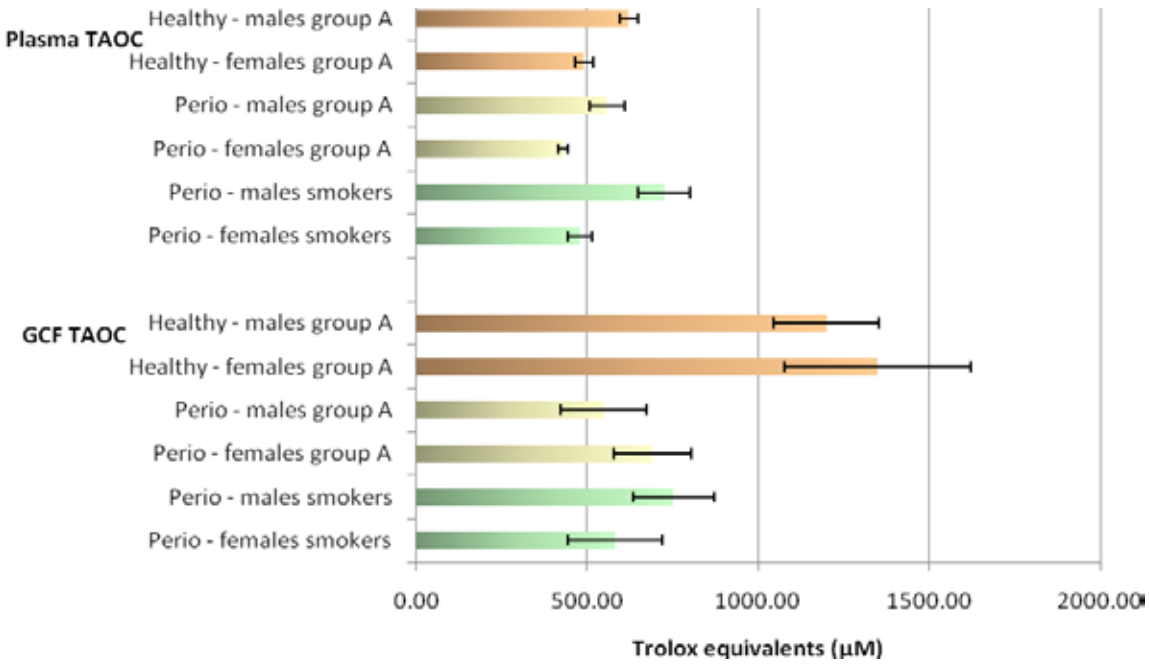
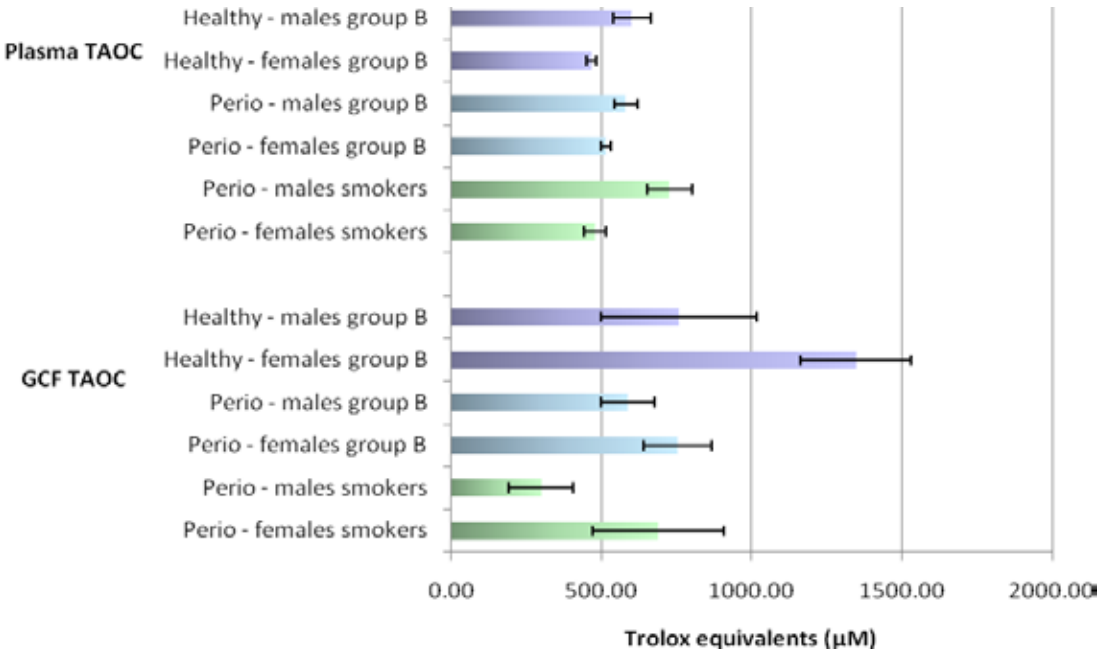


Fig.3.7. Total antioxidant concentrations in plasma and GCF (deep site as unit of analysis): stratified for gender (mean SEM)



3.5. Summary of findings

- GCF volumes were higher in smokers with periodontitis than non-smokers with periodontitis
- GCF TAOC concentrations were lower in both the smokers and the non-smokers with periodontitis groups compared to healthy control groups
- In disease matched subjects, the smokers showed a lower GCF TAOC concentration than non-smokers with periodontitis (group B)
- Little difference was seen in plasma TAOC in the periodontitis groups and the healthy controls

3.6. Discussion

This is the first study to quantify local (GCF) and peripheral (plasma) total antioxidant capacity in smokers with periodontal disease in order to compare them with non-smokers with equivalent levels of periodontal disease and periodontally healthy non-smokers.

The demographics of the patient groups were evenly matched and the decision to use only non-smokers as healthy controls rather than periodontally healthy smokers, was taken due to the difficulty in assessing periodontal health based on BOP, which is altered in smokers due to the long term chronic effect smoking has upon the vasculature of the periodontal tissues (Palmer *et al* 2005). However, the levels of periodontal disease were not comparable between the smokers and group **A**; group **A** displaying only mild periodontitis compared to moderate periodontitis in the smokers group. Therefore a second non-smokers control group, group **B**, were recruited in order to more closely match periodontal disease experience between smoker and non-smoker groups. In the second group, to further compare disease, both current disease (PPD) and historical disease (CAL levels) were assessed and analysis confirmed that there was no significant difference in disease levels between the smokers and non-smokers in group **B** ($p=0.7$).

The significantly lower levels of BOP found in the smokers with periodontal disease compared to both the non-smoking groups, is in agreement with previous research (Preber & Bergstrom 1985, Lie *et al* 1998, Bergstrom & Bostrom 2001). However, the difference in percentage of sites BOP between the two non-smoker groups may be explained by the lower disease experience in group **A**.

The percentage of sites with plaque recorded was lower in the smokers with periodontal disease compared to group **B** (plaque scores were not recorded in group **A**). Early research indicated higher levels of plaque accumulation in smokers (Kristoffersen 1970), but subsequent work dispelled this as an explanation for the increased disease prevalence in smokers and indicated similar levels of plaque accumulation, when controlling for other factors, in both smokers and non-smokers (Bergstrom 1981, Bergstrom & Preber 1986, Lie *et al* 1998). The lower plaque levels recorded in the smokers with periodontal disease within our study may be explained possibly by motivational factors, but its lack of impact upon level of periodontal disease experienced in this group may be due to the trend for smokers to harbour greater levels of periodontal pathogens compared to non-smokers without an increased level of plaque (Palmer *et al* 2005) i.e. the quality rather than the quantity of plaque.

GCF volumes were higher in the smokers, which was unexpected as reduced resting rates of GCF flow are reported in smokers (Persson *et al* 1999). However some research has reported an increase in GCF volumes during experimental gingivitis in smokers (Bergstrom & Preber 1986) and a transient increase is seen during an episode of smoking (McLaughlin *et al* 1993). These conflicting data may reflect differences arising due to the differential effects of acute and chronic smoking upon the gingival vasculature (Palmer *et al* 2005). In the long term, smoking impairs the vasculature of the periodontal tissues; however Baab & Öberg (1987) demonstrated an increase in gingival blood flow during smoking of approximately 25%. This increase was maintained for 5mins and then gradually declined to baseline flow levels. Meekin *et al* (2000) also found changes in the gingival blood flow rate of

light/occasional and heavier smokers after smoking a single cigarette, though these were not statistically significant. The smokers within the current research project were not restricted from smoking prior to GCF collection, which occurred at the beginning of the sampling appointment, this may therefore have had a confounding influence upon the gingival blood flow rates and in turn GCF volumes at the time of sampling.

The significantly reduced local (GCF) TAOC concentration in both the smokers with periodontitis and non-smokers with periodontitis compared to age- and gender-matched healthy controls is in broad agreement with preliminary observations by Chapple *et al* (2002). While the data demonstrate lowered local GCF TAOC in the periodontitis groups, the mechanism underlying the difference and whether the difference predisposed to, or results from periodontal inflammation remains unclear. Increased GCF volumes as a consequence of the inflammatory process itself may cause a dilution of antioxidant concentrations, but the data do not support this theory. The amount of TAOC sampled in 30 seconds appears unrelated to the volume collected (smokers r=0.4, non-smokers group **A** r=0.2). The data indicate that the index sites from periodontitis subjects yielded lower overall amounts of antioxidants despite the GCF volumes collected being double those of the non-smokers group **A** and treble those of the smokers with periodontitis compared to the healthy group **A**.

Analysis of the deeper sites in both the smokers and non-smokers with periodontitis group **B** also demonstrated a significantly reduced TAOC concentration in GCF samples compared to the healthy group **B**. Again the data demonstrate increases in GCF volumes, consistent with the higher levels of disease, but this did not appear to dilute the antioxidant concentration in the 30 second samples (smokers r=0.3, non-smokers group **B** r=0.04). The GCF volumes were again double in the non-smokers and treble in the smokers compared to the healthy control group, yet still reflected a reduced TAOC.

When the patient was utilised as the unit of analysis GCF TAOC concentration in the periodontitis groups were lower compared to healthy

groups but no difference was seen between the smokers and non-smokers with periodontitis group **A**, who were not disease matched. However, in the analysis of the diseased matched groups at deep sites, the smokers showed a significantly reduced total antioxidant concentration in GCF compared to the non-smokers with periodontitis group **B**. This further reduction in the amount of antioxidant present may be as a result of the additional load of ROS production from the use of tobacco, both chronically and acutely (Palmer *et al* 2005, Pryor *et al* 1983), as patients were not restrained from smoking prior to sampling.

The plasma TAOC showed little difference between all volunteer groups, with the exception of the smokers, who showed the highest TAOC but this was only significant compared to the non-smokers with periodontitis group **A**, who had the lowest TAOC of all the groups. These findings are in broad agreement with previous research that suggested higher levels in healthy subjects (Chapple *et al* 2002). Within the current study, the non-smokers with periodontitis group **A** displayed a lower plasma TAOC than both healthy control groups. Indeed in large scale epidemiological studies an increased serum antioxidant concentration was associated with a reduced relative risk of periodontitis even in never smokers (Chapple *et al* 2007). The increased plasma TAOC in smokers with periodontitis, relative to non-smokers with periodontitis was unexpected but may reflect up regulation of antioxidant defence systems in smokers as a compensatory physiological protective mechanism.

The plasma samples from male subjects possessed greater TAOC than those from females, whether periodontal status was considered or not, which is in agreement with previous findings (Brock *et al* 2004, Maxwell *et al* 2006). This gender bias is believed to be due to higher uric acid levels in males than in females (Woodford & Whitehead 1998), a major antioxidant in plasma. By contrast this gender bias was not evident in GCF TAOC, the female volunteers, with the exception of female smokers at index sites, displayed higher levels of TAOC than their male counterparts regardless of periodontal status. Since GCF is derived from plasma it is logical to assume the

composition would be similar; however the GCF profile to plasma seems to undergo significant modification, both quantitatively and qualitatively within the tissues, as the emerging GCF has a different antioxidant profile regardless of gender and uric acid is not the major component. Data by Chapple and co-workers (2002) support this concept and indicate that thiol-containing antioxidants, particularly reduced glutathione, are present in high concentrations in GCF. Research by Brock *et al* (2004) demonstrated that the levels of glutathione (reduced, oxidised and total) found within the GCF of periodontitis patients is reduced compared to healthy controls, which may have implications for the pathogenesis of periodontal disease and possibly it's management.

In conclusion, the cross sectional data reported here suggest a reduced protective effect of local (GCF) antioxidant capacity in volunteers with periodontal disease, particularly those who use tobacco, compared to matched healthy control subjects. While the potential consequences of a compromised local antioxidant concentration could be significant, the temporal relationship between reduced GCF TAOC and the development of periodontal inflammation cannot be elucidated due to the cross sectional nature of this analysis. The next chapter reports data from a longitudinal interventional study and aims to define more clearly the potential role of this reduced local antioxidant concentration in the pathobiology of periodontitis.

4. Longitudinal investigations:

Local and peripheral total antioxidant capacity

4.1. Context

Whilst the cross-sectional data described in chapter 3 demonstrate both a compromised local and peripheral total antioxidant capacity as a feature of inflammatory periodontitis and to a greater extent locally within the smoking group, the data could not differentiate between whether this predisposed to the periodontal inflammation or was a consequence of increased levels of reactive oxygen species (ROS) (i.e. oxidative stress) generated during the inflammatory process itself. Longitudinal investigation into both local and peripheral TAOC following root surface decontamination should help determine the temporal relationship between the reduced antioxidant capacity and the periodontal inflammation. This could help in the development of novel antioxidant therapies for adjunctive use in “at risk” patients. Furthermore, the longitudinal study should help to elucidate whether the additional reduction in TAOC in smokers remains significant once the inflammation has been reduced by non-surgical intervention. The aim of this longitudinal intervention study was therefore to determine the effect of a single course of conventional non-surgical periodontal therapy on local (GCF) and peripheral (plasma) total antioxidant capacity in previously untreated chronic periodontitis patients, who were current smokers.

4.2. Clinical and demographic data

Twenty of the smokers with periodontitis (2.2.2.1) and thirty five of the non-smokers with periodontitis (2.2.2.4 and 2.2.2.4.1) recruited for the cross-sectional study completed the longitudinal intervention study (table 4.1). One female volunteer from the smokers with periodontitis group withdrew from the longitudinal study just prior to the three month review appointment, due to work commitments. The reduction in number of volunteers in the smokers

with periodontitis had little impact upon the mean age of the group and no significant age differences where found between any of the groups (p>0.3).

Table. 4.1.: Clinical demographic data for experimental groups at exit from the study

	Periodontitis (moderate) smokers	**Group A Periodontitis (mild) non-smokers**	**Group B Periodontitis (moderate) non-smokers**
Gender	14♀ & 6♂	10♀ & 7♂	14♀ & 4♂
Age yrs – mean ±SD (range)	44.6 ±8.3 (23 – 65)	43.0 ±9.3 (23 – 58)	47.1 ±6.4 (36 – 61)

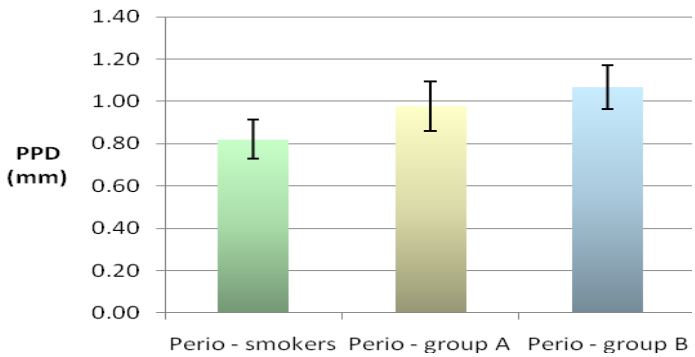
All smokers were offered smoking cessation intervention during the study and following the non-surgical management, during the healing period, four of the smokers decided to quit smoking permanently (20% success rate). These "ex-smokers" were included in the results as research is inconclusive as to what time frame post therapy is required for the periodontium to demonstrate healing comparable with that of never smokers (Krall *et al* 2006).

Table 4.2.: Mean whole mouth clinical measurements at Baseline & 3 months post therapy

	Periodontitis (moderate) smokers		**Group A Periodontitis (mild) non-smokers**		**Group B Periodontitis (moderate) non-smokers**	
	Baseline	**3mth review**	**Baseline**	**3mth review**	**Baseline**	**3mth review**
Probing depths (mm) FM mean ±SD	3.93 ±0.71	3.01 ±0.48	3.05 ±0.54	2.07 ±0.21	3.63 ±0.50	2.56 ±0.22
Recession (mm) FM mean ±SD	0.72 ±0.50	0.94 ±0.55	-	-	0.35 ±0.31	0.56 ±0.42
CAL (mm) FM mean ±SD	4.08 ±0.85	3.34 ±0.88	-	-	3.99 ±0.70	3.15 ±0.55
% BOP FM ±SD	49.90 ±17.80	25.15 ±17.48	22.55 ±7.26	4.19 ±3.79	65.17 ±13.73	14.61 ±8.41
% Plaque FM ±SD	65.45 ±15.22	37.25 ±13.18	-	-	78.89 ±9.00	39.61 ±16.06

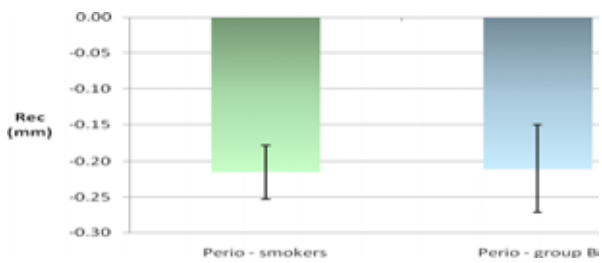
Following non-surgical management of the periodontal inflammation and a three month healing period, all three groups showed significant ($p<0.0001$) reductions in their mean full mouth probing pocket depths (Table 4.2, Fig 4.1); smokers with periodontitis 0.82mm (±0.40), non-smokers with periodontitis group **A** 0.98mm (±0.48) and non-smokers with periodontitis group **B** 1.07mm (±0.44). The greatest improvement was seen in the non-smokers with periodontitis Group **B**, though the improvements were not significantly different between any of the groups ($p>0.2$).

Fig 4.1.: Mean reduction in whole mouth probing pocket depths (PPD ± SEM) following non-surgical therapy & 3 months healing period



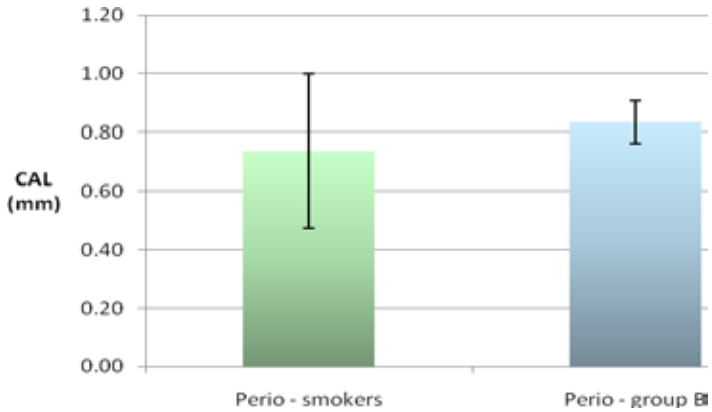
The full mouth recession measurements following periodontal management increased in both the smokers with periodontitis (0.22mm, ±0.17) and the non-smokers with periodontitis group **B** (0.21mm, ±0.26). Little difference was seen in the amount of recession between the two groups ($p>0.9$) (Table 4.2, Fig 4.2).

Fig 4.2.: Mean change in whole mouth recession (REC ±SEM) following non-surgical therapy & 3months healing period



The full mouth mean clinical attachment loss was seen to reduce significantly in both the smokers with periodontitis 0.74mm (±1.18, p=0.012) and the non-smokers with periodontitis group **B** 0.84mm (±0.31, p<0.0001). The greatest clinical attachment gain was seen in the non-smokers with periodontitis group **B**, but this was not significantly different from the smoker group (p>0.8) (Table 4.2, Fig 4.3). Recession and clinical attachment loss were not calculated for the non-smokers with periodontitis group **A**.

Fig 4.3: Mean whole mouth clinical attachment gain (±SEM) following non-surgical therapy & 3 months healing period

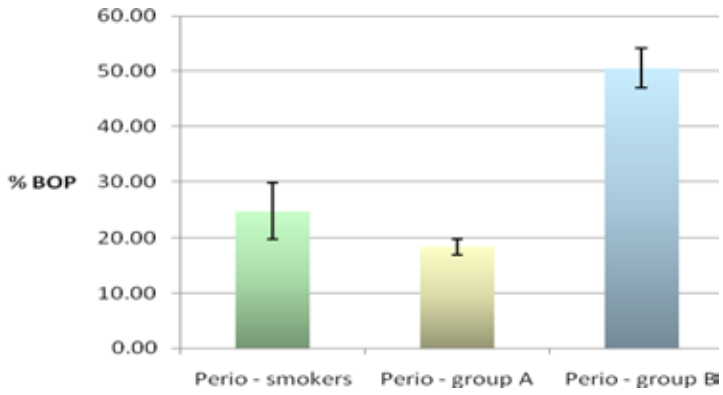


Given the large variance in CAL gain amongst the smokers, a correlation was performed between CAL gain and smoking habit, expressed as cigarettes per day and pack years respectively. The correlations were negative at -0.37 (R^2=0.14) and at -0.33 (R^2=0.11) and approached significance (p=0.08). Removal of the ex-smokers (the four newly quit subjects) from the statistical analysis had little impact on these data.

Full mouth percentage of sites bleeding on probing (% BOP) reduced significantly post-periodontal therapy in all three experimental groups (Table 4.2, Fig 4.4); smokers with periodontitis 24.75% (±22.59, p<0.0001), in the non-smokers with periodontitis group **A** 18.37% (±5.99, p<0.0001) and non-smokers with periodontitis group **B** 50.56% (±15.01, p<0.0001). The greatest reduction in bleeding on probing was seen in the non-smokers with periodontitis group **B**, which was significantly greater than in the other two

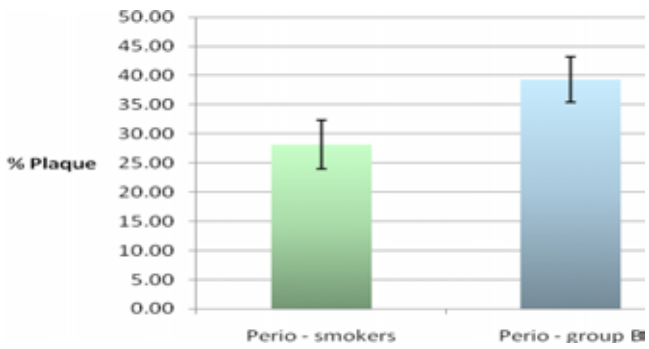
experimental groups (p<0.0003). The smallest improvement in bleeding on probing following periodontal therapy was seen in the non-smokers with periodontitis group **A.**

Fig 4.4.: Mean reduction in whole mouth bleeding on probing (SEM) following non-surgical therapy & 3 months healing period



Full mouth percentage sites with plaque reduced significantly (Table 4.2, Fig 4.5) following non-surgical management of the periodontal disease in both the smokers 28.20% (±18.71, p<0.0001) and in the non-smokers group **B** 39.28% (±16.38, p<0.0001). The greatest improvement in plaque score was seen in the non-smokers with periodontitis group **B**, but this was not a significantly greater improvement than that seen in the smokers with periodontitis (p=0.14).

Fig 4.5.: Mean reduction in whole mouth sites with plaque (SEM) following non-surgical therapy & 3 months healing period



Plaque levels post therapy in the smokers (37.25%) and non-smokers with periodontitis group B (39.61%) were no longer significantly different (p>0.6) as they had been at baseline. Plaque levels were not assessed in the non-smokers with periodontitis group **A** at either baseline or post therapy.

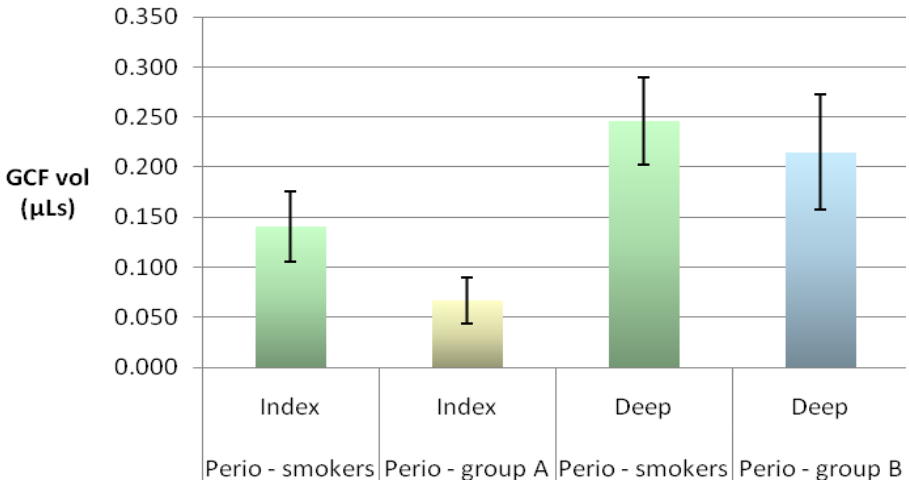
The mean probing pocket depths and GCF volumes of the sites sampled (per 30secs) were all seen to reduce following the non-surgical periodontal therapy (Table 4.3, Fig 4.6). The "index" (2.4.2.1.) sites sampled for GCF in both the smokers and non-smokers with periodontitis group **A** showed significant (p<0.0001) improvements in mean probing pocket depths following periodontal therapy. The non-smokers group **A** demonstrated a greater reduction in mean probing depths (1.35mm) at index sites compared to the smokers (1.05mm), but these improvements were not significantly different (p=0.26).

Table 4.3.: Clinical demographic data for Deep and Index sites that were sampled for GCF TAOC analysis, plus the TAOC nM Trolox equivalent per 30 second sample sampled sites pre- and post- non-surgical periodontal therapy

	Periodontitis (moderate) smokers		**Group A Periodontitis (mild) non-smokers**		**Group B Periodontitis (moderate) non-smokers**	
	Baseline	**3mth review**	**Baseline**	**3mth review**	**Baseline**	**3mth review**
Probing depths Index (mean ±SD)	4.63 ±1.01	3.58 ±0.93	3.91 ±1.82	2.63 ±1.27		
GCF vol µl Index (mean ±SD)	0.45 ±0.18	0.31 ±0.11	0.27 ±0.12	0.19 ±0.14		
TAOC nM/30sec Index (mean ±SD)	0.30 ±0.27	0.33 ±0.36	0.14 ±0.06	0.16 ±0.07		
Probing depths Deep (mean ±SD)	6.04 ±0.66	4.49 ±0.80			6.42 ±0.97	3.85 ±0.89
GCF vol µl Deep (mean ±SD)	0.59 ±0.21	0.36 ±0.18			0.44 ±0.22	0.22 ±0.09
TAOC nM/30sec Deep (mean ±SD)	0.22 ±0.17	0.39 ±0.44			0.28 ±0.13	0.23 ±0.14

The GCF volumes per 30sec samples at index sites also reduced in both the smokers (0.14µls, p<0.001) and the non-smokers with periodontitis group **A** (0.07µls, p<0.01). The reduction in GCF volume within the index sites following treatment in the smokers was slightly higher, but this was not significant (p=0.12). No correlation was seen in the reduction in pocket probing depth and GCF volume at index sites in the smokers (p=0.56, R^2=0.02, r=0.14). However a significant correlation was seen in the non-smokers with periodontitis group **A** between the reduction in probing pocket depths and the reduction in GCF volume (p=0.03, R^2=0.26, r=0.51).

Fig 4.6: Mean decrease in GCF volumes (SEM) in sites sampled following non-surgical therapy & 3months healing period



When comparing the disease matched groups, the non-smokers with periodontitis group **B** experienced a significantly (p=0.002) greater reduction in mean probing depth in "deep" (2.4.2.2.) pockets (2.56mm, p<0.0001) compared to the deep sites sampled in the smokers with periodontitis (1.54mm, p<0.0001). Although the non-smokers group **B** displayed a significant reduction in GCF volume (0.214µls, p=0.002), this reduction was not as great as that reported in the smokers (0.245µls, p<0.0001), but the difference between groups was not significant (p=0.6). Post therapy, despite the greater reduction in GCF volumes in the smokers, the GCF volume remained significantly higher in the smokers 0.36µls (±0.18µls) compared to

the non-smokers with periodontal disease group **B** 0.22µls (±0.09µls) (p=0.007), implying greater residual inflammation in the smokers.

A significant positive correlation was seen between the reduction in mean probing pocket depths and GCF volumes in the non-smokers with periodontitis group **B** (p=0.009, R^2=0.36, r=0.6) and a non-significant weak correlation was seen in deep sites sampled in the smokers with periodontitis (p=0.106, R^2=0.14, r=0.37) following periodontal treatment.

When the ex-smokers (four recently quit smokers) were removed from the analysis of the GCF volume changes for the smokers, it had little impact (0.251µls) nor was it a significant reduction compared to the non-smokers group B (p=0.6). The post treatment GCF volumes also remained significantly higher in the smokers (0.39µls +0.18µls) without the ex-smokers than the non-smokers group B (p=0.002).

4.3. Total antioxidant capacity (TAOC)

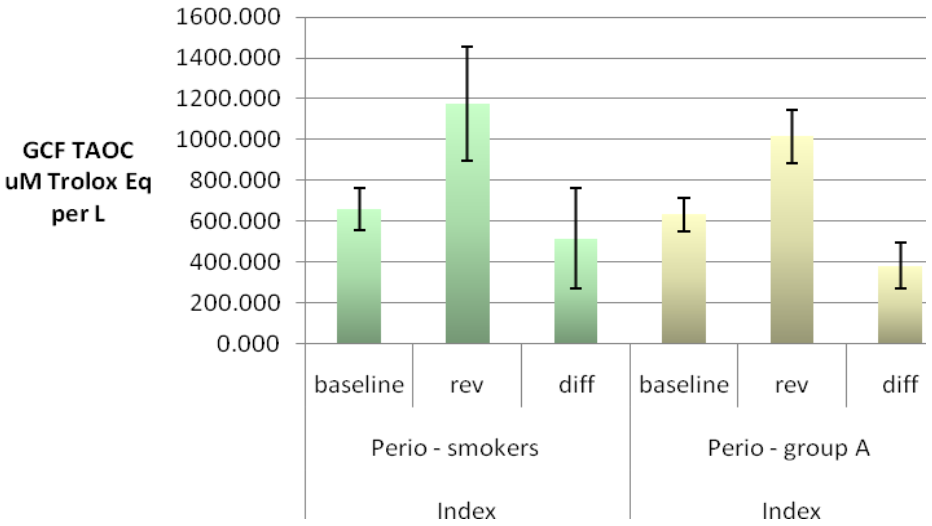
4.3.1. GCF TAOC per 30 second sample of smokers with periodontitis versus group A (patient as unit of analysis)

Due to a technical problem with the baseline assay of an index site sampled only nineteen of the smoker's samples were also run for longitudinal differences. Following non-surgical management of the periodontal disease and a three month healing period, the mean GCF TAOC per 30 second sample were seen to improve in both the smokers and the non-smokers with periodontitis group **A** at index sites, although these improvements were not significant (p>0.1) (Table 4.3). The greater improvement was seen in the smokers with periodontitis (0.03 ±0.05 nmoles/sample) compared with the non-smokers with periodontitis group **A** (0.02 ±0.01 nmoles/sample), although again this difference was not significant (p>0.8) between the groups.

4.3.1.2. GCF TAOC concentration of smokers with periodontitis versus group A (patient as unit of analysis)

The TAOC was also expressed as a GCF concentration (TAOC μM Trolox equivalents per litre) to allow for differences in volume collection. At the index sites the GCF TAOC concentration was seen to significantly improve in both the smokers with periodontitis (516μM, ±1078μM, p=0.05) and in the non-smokers with periodontitis group **A** (383μM, ±468μM, p=0.004) (Fig 4.7). However, the greatest improvement in the index sites GCF TAOC concentration was seen in the smokers, which was almost double, however, this was not significantly greater than the improvement seen in the non-smoking group **A** (p>0.6). The resultant increase in both the smokers with periodontitis to 1176.41μM (±122.57μM) and the non-smokers with periodontitis group **A** to 1014.90μM (±548.77μM) following treatment resulted in a higher TAOC concentration in the smokers, but this was not significant (p>0.6).

Fig. 4.7 Mean GCF TAOC μM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis group **A** at baseline and 3months review following non-surgical therapy plus differences



Both the smokers and non-smokers GCF TAOC concentration increased to similar levels of the non-smokers healthy control group **A** 1287.43μM, in the

smokers the GCF TAOC was non-significantly higher (p>0.2) and in the non-smokers group **A** it was significantly lower (p=0.04).

4.3.2. GCF TAOC per 30 second sample of smokers with periodontitis versus group B (diseased sites as unit of analysis)

The mean GCF TAOC per 30 second sample of the deep sites was seen to improve in the smokers with periodontitis (0.18 ±0.09 nmoles/sample, p=0.62), although not significantly. There was no change in the TAOC per 30sec sample in disease matched non-smokers with periodontitis group **B** (-0.05 ±0.16 nmoles/sample, p=0.2) following non-surgical management and 3 months healing time (Table 4.3).

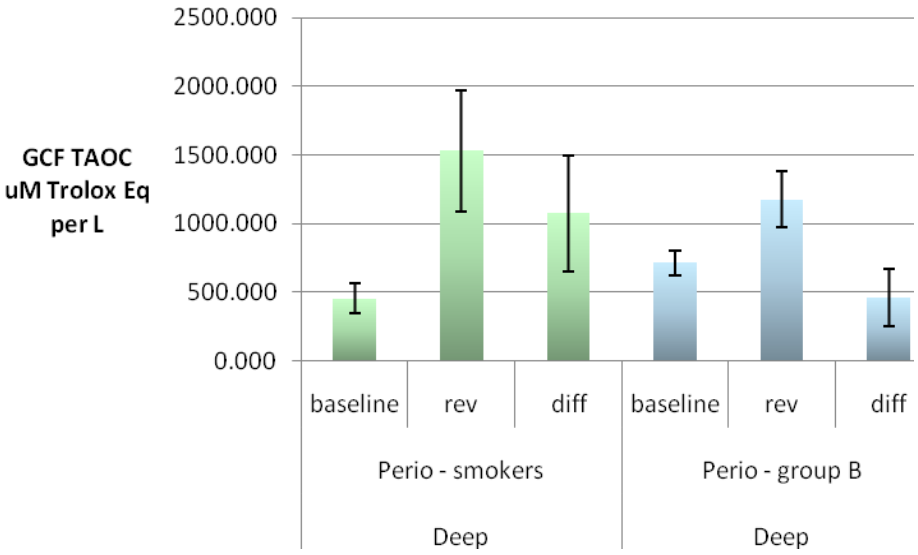
4.3.2.1. GCF TAOC concentration of smokers with periodontitis versus group B (diseased sites as unit of analysis)

When the GCF TAOC was expressed as a concentration (μM Trolox equivalents per litre) at the deep sites following treatment (and a 3 month healing period), there was a significant improvement in both the smokers with periodontitis (1076μM ±1896μM, p=0.02) and non-smokers with periodontitis group **B** (461μM, +894μM, p=0.04) (Fig 4.8). The greater improvement in GCF TAOC concentration was seen in the smokers but this was not significant compared with the improvement seen in the non-smokers group **B** (p>0.2). However, the post-healing increase in GCF TAOC concentration in the smokers with periodontal disease (1528.85μM, ±1966.27μM), was threefold that of the baseline concentration, but was not significantly different to the post-therapy GCF TAOC concentration in the non-smokers with periodontal disease group **B** (1177.20μM, ±847.24μM) (p=0.4).

Both the smokers and non-smokers GCF TAOC concentration increased to similar levels of the non-smokers healthy control group **B** 1215.44μM, in the smokers the GCF TAOC was non-significantly higher (p>0.4) and in the non-smokers group B it was non-significantly lower (p>0.6).

Removal of the ex-smokers from the analysis had no bearing on the GCF TAOC concentrations data for any comparisons performed.

Fig. 4.8 Mean GCF TAOC µM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis group B at baseline and 3 months review following non-surgical therapy plus differences

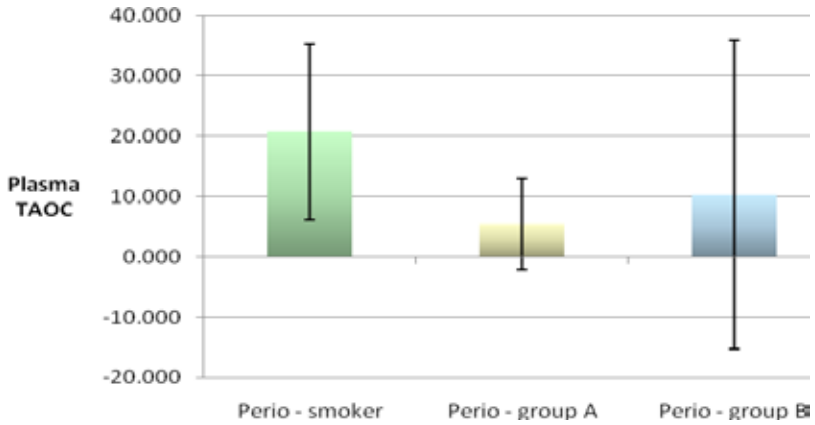


4.3.3. Plasma TAOC

In all three study groups a small and insignificant increase in plasma TAOC concentration was seen following periodontal therapy and a three month healing period; smokers with periodontal disease (20.79µM ±65.09µM, p>0.1), non-smokers with periodontitis group **A** (5.49µM ±31.00µM, p>0.4) and non-smokers with periodontitis group **B** (10.35µM ±109.01µM, p>0.6).

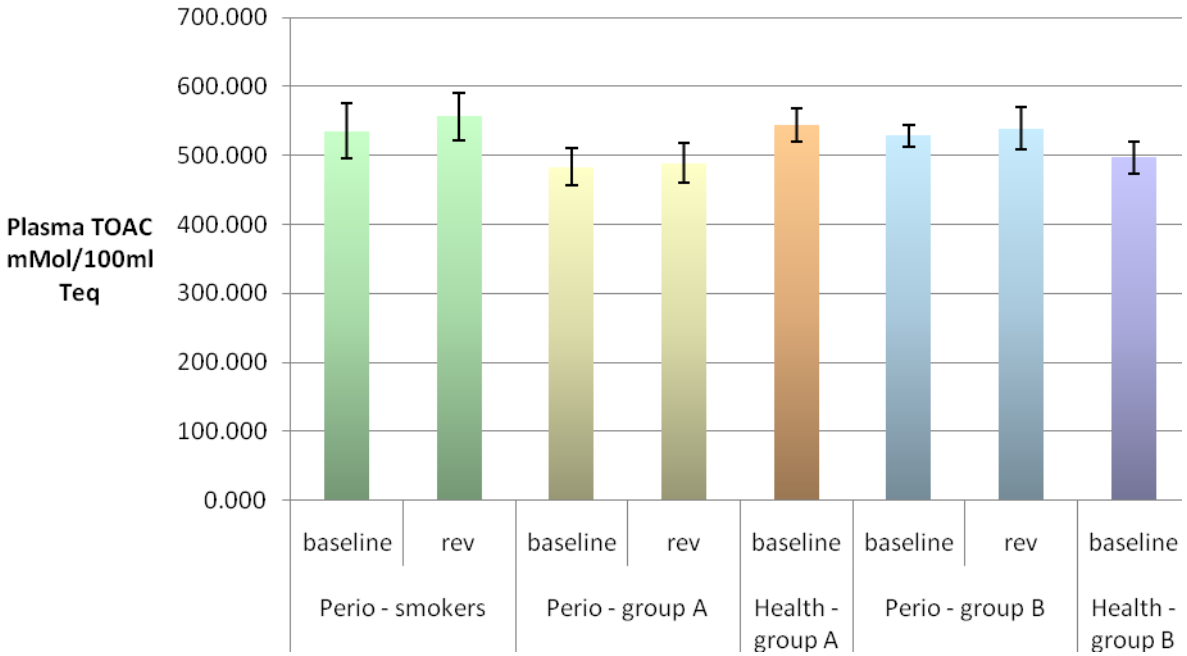
The greatest increase in plasma TAOC concentration was seen in the smokers with periodontitis, while the smallest increase was seen in the non-smokers with periodontitis group **A**, but the differences between the groups were not significant (p>0.2, Fig 4.9). When the plasma concentrations were calculated without the ex-smokers, the difference between all groups still failed to reach significance (P>0.4).

Fig. 4.9 Mean change in Plasma TAOC µM Trolox equivalents per litre (SEM) from smokers and non-smokers with periodontitis following non-surgical therapy



Post therapy and healing, the plasma TAOC of all the periodontitis groups were seen to reach levels similar to the healthy control groups (p>3, Fig 4.10). The non-smokers with periodontitis group **A**'s plasma TAOC was no longer significantly lower than the control group A (p>0.1).

Fig. 4.10 Mean baseline & post non-surgical management with 3months healing period Plasma TAOC µM Trolox equivalents per litre (SEM) from smokers and non-smokers compared to age- and gender-matched health controls



4.4 Summary of findings

Following non-surgical management of the periodontitis and a 3 month healing period: -

- Reductions in BOP, PPD and CAL gain were seen in the smokers with periodontitis compared to the non-smokers
- GCF volumes remained higher in smokers with periodontitis than the non-smokers with periodontitis post-therapy
- The GCF TAOC concentrations of all periodontitis groups recovered to levels similar to those of the non-smoking periodontally healthy control groups
- Little difference was seen in plasma TAOC between the periodontitis groups pre- and post-therapy

4.5. Discussion

This is the first longitudinal study to investigate how local (GCF) and peripheral (plasma) total antioxidant capacity in smokers with periodontal disease is influenced by a reduction in periodontal inflammation following non-surgical therapy.

The longitudinal study demonstrated healing following non-surgical management of the periodontitis in all three volunteer groups; these results were in broad agreement with previous research (reviewed by Cobb 1996 & 2002). The lesser reduction in probing pocket depth and clinical attachment gain following treatment within the smokers also agrees with previous research, which concludes that smoking influences healing and regeneration, and that, although improvements are seen following non-surgical management, they are reduced in extent relative to non-smokers (Kinane & Chestnutt 2000). The four subjects, from within the smokers with periodontitis group, who quit their tobacco habit during the healing phase had no influence on the resultant clinical attachment gain in this group.

Bleeding on probing, a clinical sign of inflammation, was seen to significantly reduce in all three groups as was expected. The limited reduction in mean BOP in non-smokers with periodontitis group **B** compared to the other periodontitis groups likely reflects the milder disease status seen in that group. Although the BOP in the smokers with periodontitis following non-surgical management reduced significantly ($p>0.0001$), at review it remained higher than both of the non-smoking periodontitis groups, which may be explained by residual inflammation as a result of a poor response to treatment (Palmer *et al* 2005).

The mean PPDs of the smokers did not reduce as much as the other periodontitis groups following therapy and a three month healing period. Indeed this difference between the disease matched groups (the smokers and non-smokers with periodontitis group **B** at deep sites) reached significance ($p=0.002$).

The mean full mouth CAL gain was seen to be greater in the non-smokers with periodontitis group **B** compared with the smokers but this did not reach significance. A large variance in the smokers CAL gain was reported and a negative correlation was seen between CAL gain and number of cigarettes smoked ($R^2= 0.14$, $r=0.37$) and with pack years ($R^2=0.11$, $r=0.33$), which almost reached statistical significance ($p=0.08$). Studies have shown a dose dependent relationship with attachment loss in the amount of cigarettes smoked and pack years (Grossi *et al* 1994, Alpagot et *al* 1996). Within the current study, 30% of the group smoked 10 or fewer cigarettes per day (light smokers) and if the group had consisted of large numbers of volunteers the variance may well have reached significance.

The reduced clinical healing response, including BOP, PPD and CAL gain, found in the current study within the smokers is in line with previous research which suggests that the detrimental effects of smoking on clinical healing following non-surgical therapy for periodontitis has a multi-factorial biological basis, as smoking affects the vasculature, revascularization, the inflammatory response and fibroblast function (Palmer *et al* 2005). Periodontal ligament

fibroblast attachment following root planing is seen to be significantly reduced in heavy smokers compared with non-smokers and healthy controls (Gamal & Bayomy 2003). Smoking also negatively influences the capacity of the periodontal tissues to regenerate, particularly bone (Kinane & Chestnutt 2000).

GCF volumes in the smokers were higher at baseline and remained higher following therapy (and the healing period) despite a non-significant greater reduction in volume compared to both the non-smokers with periodontitis groups **A** & **B** ($p>0.1$). Cessation of smoking has been shown to have an impact on gingival blood flow rate, which is seen to significantly increase over the first 3 days, and further smaller increases are seen to occur over the following 4-8 weeks. This in turn influences GCF flow rate, which is seen to be greater at 5 days post cessation (Morozumi *et al* 2004). However, despite a 20% quit rate in the smokers with periodontitis group during the healing phase, when these ex-smokers were removed from the statistical analysis, the GCF volumes in the smokers remained significantly higher post healing than the disease matched group **B** ($p=0.002$). The higher GCF volumes seen in the smokers with periodontal disease following therapy may therefore also be attributed to residual inflammation, as a result of a poor response to healing following the non-surgical management, which is in line with the other parameters of clinical healing discussed above.

GCF TAOC concentrations were seen to increase in all periodontitis groups following non-surgical management and the 3 months healing period. As discussed in the cross-sectional study, the simplest explanation for this would be due to the decrease in GCF volume following resolution of the inflammation. However, this increase in concentration is not entirely explained by the reduction in GCF volume in the unmatched diseased groups. No significant correlation was found in the smokers ($R^2=0.001$, $r=0.04$, $p>0.8$) between the amount of GCF TAOC sampled in 30 seconds and the GCF volumes collected at index sites. Furthermore, GCF TAOC per 30 second sample actively increased in the smokers and in the non-smokers group **A** (non-significantly), demonstrating an increase in TAOC irrespective of

volumes collected. Thus the results show a true increase in antioxidant levels following treatment in the periodontitis groups, irrespective of smoking status.

Within this study the GCF TAOC concentration in all groups, with the exception of the non-smokers with periodontitis group **A**, increased to similar levels found in the periodontally healthy control groups **A** & **B,** suggesting that the total antioxidant compromise seen at baseline may result from the inflammatory lesion, rather than predispose to it. These data are consistent with the literature (Chapple *et al* 2007).

At baseline the GCF TAOC concentrations in the smokers with periodontitis were significantly lower than the non-smokers with periodontitis group **B** (p=0.02) and the control group **B** (p=0.007). It is logical to contend that the further reduction in the smokers compared with the non-smokers with periodontitis, is due to the local impact of the additional ROS created through their use of tobacco both chronically and acutely (Pryor *et al* 1983, Palmer *et al* 2005). However, the GCF TAOC concentration in the smokers recovered to the same level as the healthy control group, implying that the main contributor for the reduced local TAOC was the periodontal inflammation and that the increased ROS exposure locally did not impact on the recovery of the local antioxidant status. It may therefore be that smokers may have a compensatory protective mechanism to deal with the additional local oxidative stress, whereby antioxidant systems are up-regulated. The removal of the inflammation and associated oxidative stress, results in a stronger recovery of TAOC locally in smokers, despite apparently greater residual inflammation post-therapy.

Care must be taken when interpreting the results as the enhanced chemiluminescence assay used within the current study has been shown to be sensitive to uric acid, vitamins A, C and E with proteins having a low influence on derived TAOC (Maxwell *et al* 2006). The importance of the compartmental nature of the body's antioxidant defences must also not be over looked. Brock et al (2004) highlighted that the most influential antioxidants in GCF were not the same as in serum or saliva, with uric acid

predominating in the latter, while reduced glutathione predominates in the former (Chapple *et al* 2002, Brock *et al* 2004). The temptation is to investigate the potential protective effect of individual antioxidant species, but the analysis of isolated antioxidants from the TAOC could lead to misinterpretation of results (Chapple *et al* 2006). The use of assays assessing TAOC are advantageous as they analyse the combined effectiveness of the contributing species and may take into account the activities of hitherto undiscovered antioxidants or antioxidants that are difficult to measure, whilst being more efficient, cheaper and less time consuming to perform (Maxwell *et al* 2006). However, their failing is their sensitivity to individual species and they may therefore not reflect the activities of certain antioxidants within a particular body system, for example the ECL assay used in the current study is poorly sensitive to GSH (Maxwell *et al* 2006) a major antioxidant in GCF.

The plasma TAOC concentrations were similar pre-treatment and post-treatment (p=NS). The periodontitis group **A**'s plasma TAOC concentration almost reached significantly lower levels than their respective healthy control group **A** at baseline, demonstrating a mild antioxidant compromise in plasma in the periodontitis cohort. Other studies by Pavlica *et al* 2004 and Baltacioğlu *et al* 2006, also demonstrated significantly lowered plasma or serum TAOC concentration, however these studies were possibly under powered as they only involved small numbers of volunteers and did not take gender into account, Baltacioğlu's study only involved female subjects (Chapple *et al* 2006). The small and insignificant changes seen in the current study are likely to lack clinical relevance, given their low concentrations relative to those of GCF in health.

In conclusion, the longitudinal data reported here suggest the reduced protective effect of local (GCF) antioxidant capacity in volunteers with periodontal disease, particularly those who use tobacco, results from the periodontal inflammatory lesion, rather than predisposing to it, and following successful therapy GCF TAOC concentrations returns to levels seen in healthy controls. Smokers may in fact have an up-regulated antioxidant

defence system to act as a compensatory protective mechanism to deal with the additional local oxidative stress. However these data can not preclude the possibility that constitutional deficiencies in individual antioxidant species not assessed by the ECL assay may underpin damage in periodontitis, nor can it determine whether boosting levels of specific antioxidant may afford protection against tissue damage medicated by oxidative stress in the periodontal inflammatory lesion.

5. Site specific longitudinal investigations: Local total antioxidant capacity

5.1. Context

The longitudinal data in chapter 4 demonstrated a possible compensatory mechanism, up-regulation of antioxidant defences, in smokers to protect against the additional oxidative stress caused by the periodontal inflammatory response compared to non-smokers with periodontitis. Due to the site specific nature of periodontitis further investigation was warranted into the differences in antioxidant capacity between initially deep versus initially shallow sites following a course of non-surgical periodontal therapy within the smokers group. The environment of deep pockets is more anaerobic than shallow pockets. Research also suggests that smokers are exposed to more free radicals from their smoking habit (Pryor *et al* 1983) as well as possessing reduced antioxidant defences (Alberg 2002).

5.2. Clinical and demographic data

The twenty smokers with periodontitis (2.2.2.1) from the longitudinal study were assessed to analyse differences in TAOC in sites with deeper periodontal probing depths relative to clinically shallow and healthy sites. This volunteer group contained 14 females and 6 males, with a mean age of 44.6yrs (±8.3) and a range of 23 – 65 yrs.

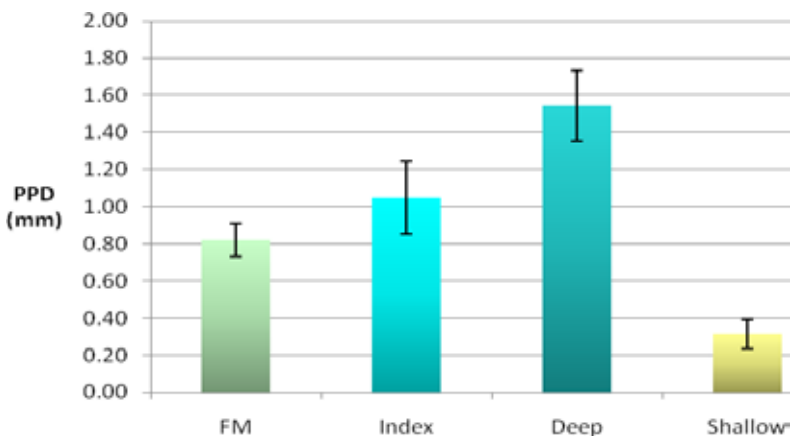
Significant changes were seen in all the clinical parameters measured in the smokers as a result of the non-surgical periodontal therapy after a three month healing period (table 5.1). All probing depth measurements were seen to significantly decrease; 0.82mm ±0.40 Full mouth ($p<0.0001$), 1.05mm ±0.86 Index sites (2.4.2.1, $p<0.0001$), 1.54mm ±0.84 Deep sites (2.4.2.2, $p<0.0001$) and 0.31mm ±0.35 Shallow sites (2.4.2.3, $p=0.001$). The Deep sites showed a significantly greater resolution in probing pocket measurements, as

would be expected, compared to the Full mouth measures (p=0.004), Index sites (p=0.069) and Shallow sites (p<0.0001) (Fig 5.1).

Table 5.1.: Clinical demographics for the smokers with periodontitis plus the TAOC nM Trolox equivalent per 30second sample for sites sampled

	Full Mouth		Index sites		Deep sites		Shallow sites	
	Baseline	Review	Baseline	Review	Baseline	Review	Baseline	Review
Probing depths (mm) mean ±SD	3.83 ±0.71	3.01 ±0.48	4.63 ±1.04	3.58 ±0.93	6.04 ±0.68	4.49 ±0.80	2.58 ±0.28	2.26 ±0.43
Recession (mm) mean ±SD	0.72 ±0.50	0.94 ±0.55	0.45 ±0.51	0.73 ±0.68	0.56 ±0.63	0.93 ±0.82	0.96 ±0.80	1.11 ±0.91
CAL (mm) mean ±SD	4.08 ±0.85	3.34 ±0.88	5.08 ±1.11	4.32 ±1.07	6.61 ±0.78	5.42 ±0.92	3.56 ±0.87	3.39 ±0.96
GCF vol (µl) mean +SD	-	-	0.45 ±0.18	0.31 ±0.11	0.61 ±0.21	0.36 ±0.18	0.16 ±0.08	0.16 ±0.07
TAOC (nM Trolox/30sec) mean +SD	-	-	0.30 ±0.27	0.33 ±0.36	0.22 ±0.17	0.39 ±0.44	0.16 ±0.10	0.27 ±0.37

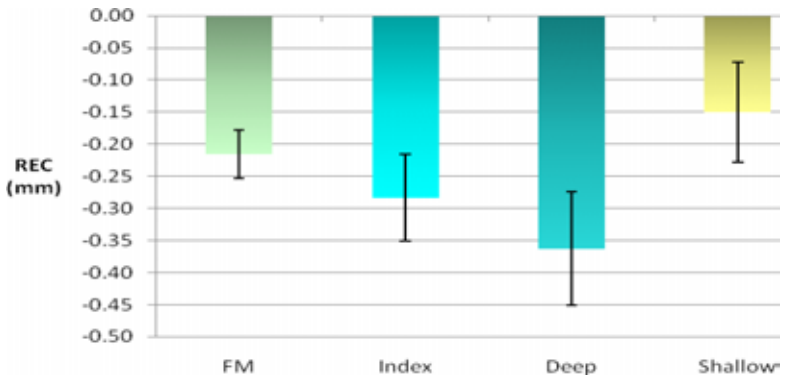
Fig 5.1.: Mean reduction in probing pocket depth (PPD, ±SEM) following non-surgical therapy & 3 months healing period



The recession measurements were seen to significantly increase at all sites measured (table 5.1, fig 5.2); 0.21mm ±0.16 Full mouth (p<0.0001), 0.28mm

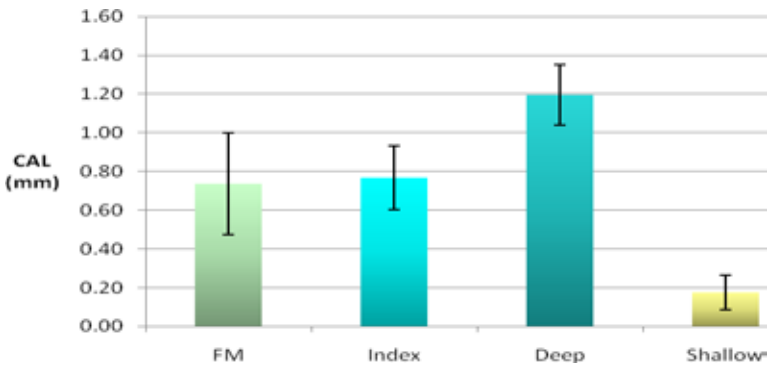
±0.30 Index sites (p<0.0001), 0.36mm ±0.39 Deep sites (p<0.001) and 0.15mm ±0.34 Shallow sites (p<0.07). The deep sites showed non-significantly greater recession following treatment compared to the full mouth means (p=0.126) and the shallow sites (p=0.65), but the recession increase seen in these sites was not significantly different to the index sites (p=0.65).

Fig 5.2: Mean changes in recession (REC, ±SEM) following non-surgical therapy & 3 months healing period



When probing pocket depths and recession were examined in combination as "clinical attachment loss", significant improvements were seen in all sites measured as would be expected following successful non-surgical therapy (table 5.1, fig 5.3); 0.73mm ±1.18 full mouth (p=0.012), 0.76mm ±0.73 index sites (p<0.0001), 1.19mm ±0.69 deep sites (p<0.0001) and 0.17mm ±0.39 shallow sites (p=0.064).

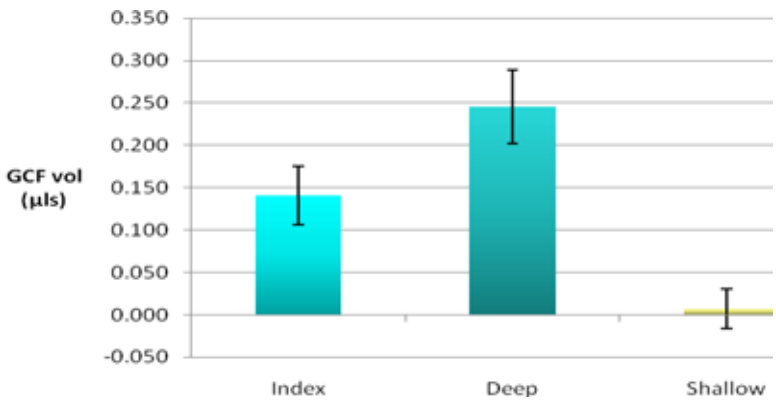
Fig 5.3: Mean gain in clinical attachment loss (CAL, ±SEM) following non-surgical therapy & 3 months healing period



The greatest improvement in clinical attachment level was seen at deep sites, as expected, this improvement exceeded that for full mouth means ($p<0.015$), index sites ($p=0.063$) and shallow sites ($p<0.0001$).

The GCF volumes per 30 second sample were seen to significantly reduce in both the index sites 0.14µls ±0.15 ($p<0.001$) and the deep sites 0.24µls ±0.19 ($p<0.0001$), whereas no change was seen in the shallow sites, despite the small but significant reduction in probing pocket depth within these sites. The reduction in GCF volumes was however not significantly different between the deep sites compared to the index sites ($p=0.68$).

Fig 5.4: Mean reduction in GCF volumes per 30 seconds (±SEM) following non-surgical therapy & 3 months healing period



No correlation was seen between the reduction in pocket probing depth and GCF volumes at the index sites ($p=0.56$, $R^2=0.02$, $r=0.14$) or at the deep sites ($p=0.106$, $R^2=0.14$, $r=0.37$).

5.3. Total antioxidant capacity (TAOC)

5.3.1. GCF TAOC per 30 second sample

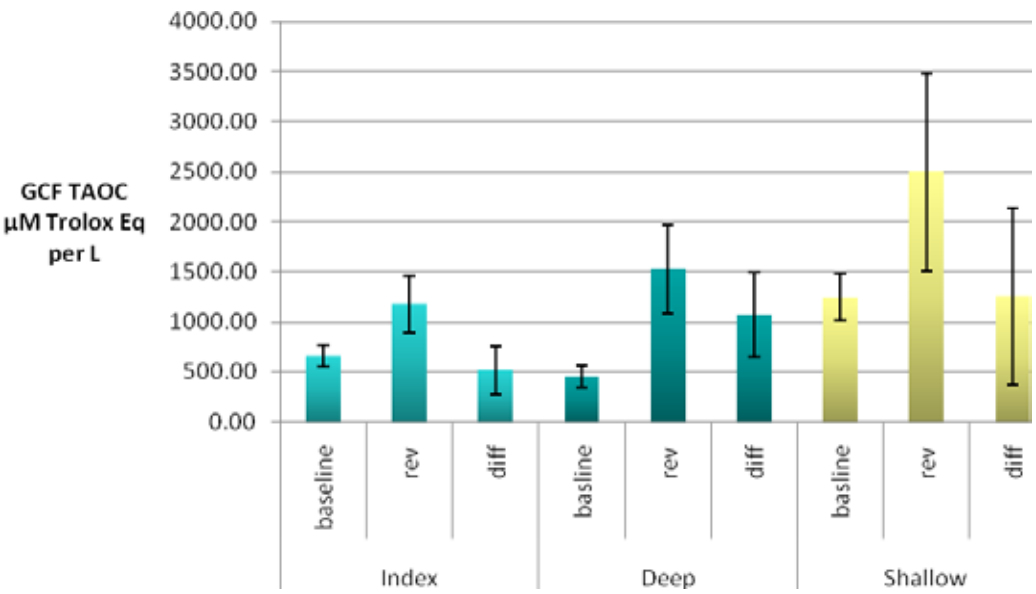
All sites sampled for GCF were assayed for TAOC, with the exception of one of the volunteer's index sites due to a technical problem with the baseline

assay. Following non-surgical management of the periodontal disease and a three month healing period, the mean GCF TAOC per 30 second sample values were seen to increase insignificantly for all groups; index sites 0.01 ±0.23 nmoles/sample (p>0.5), deep sites 0.18 ±0.40 nmoles/sample (p>0.6) and shallow sites 0.11 ±0.38nmoles/sample (p>0.2). The greatest improvement was seen in the deep sites but this increase was not significantly greater than the index or shallow sites sampled (p>0.4).

5.3.2. GCF TAOC concentration

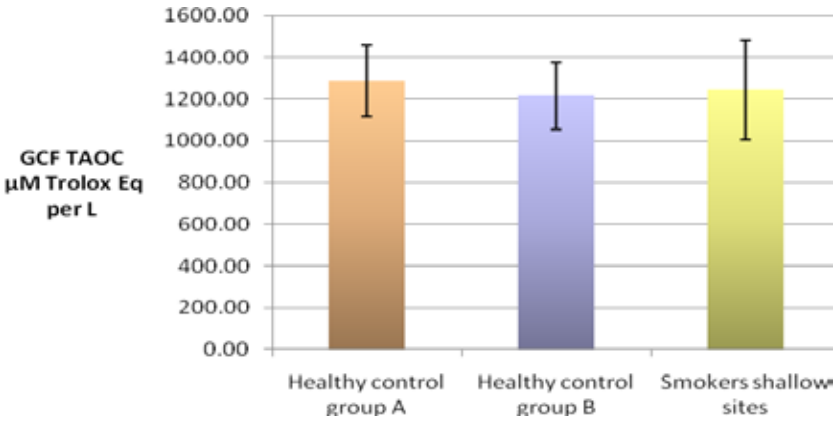
When the TAOC was expressed as a GCF concentration (TAOC µM Trolox equivalents per litre) to allow for differences in volume collection, all sites sampled were seen to increase in GCF TAOC concentration, however these only reached significance in the index 516µM (±1078µM, p=0.05) and deep sites 1076µM (±1896µM, p=0.02). While the shallow sites appeared to have the greatest increase in GCF TAOC 1253µM (±3949µM), the difference following treatment did not reach significance (p>0.1), nor was the improvement significantly greater than the other sampled sites (p>0.2).

Fig. 5.5 Mean GCF TAOC µM Trolox equivalents per litre (SEM) from smokers with periodontitis at baseline and 3 months following non-surgical therapy



The GCF TAOC concentration for the shallow sites sampled in the smokers with periodontitis at baseline were not significantly different from those of the non-smoking healthy control groups **A** and **B** ($p>0.9$). Following non-surgical management of the periodontal disease the GCF TAOC doubled in the shallow sites of the smokers, but this was still not significantly greater than the healthy control groups **A** and **B** ($p>0.2$).

Fig. 5.6 Mean GCF TAOC µM Trolox equivalents per litre (SEM) from healthy control groups **A** and **B** plus smokers with periodontitis (baseline)



The GCF TAOC concentration of the smokers with periodontitis at index sites was significantly lower than the healthy control group **A** ($p<0.0001$) and returned to similar levels to those of the control group following non-surgical management of the periodontal disease. The GCF TAOC concentrations at deep sites were significantly lower at baseline than both the non-smokers with periodontitis group **B** and the healthy control group **B** ($p<0.02$). These deep sites also returned to levels similar as the control groups following non-surgical management.

Removal of the four ex-smokers, who quit during the healing phase, from the analysis had no bearing on any of the GCF TAOC concentration data for any of the comparisons.

5.4. Discussion

This is the first longitudinal study to investigate how local (GCF) total antioxidant capacity in smokers with periodontal disease is influenced by a reduction in periodontal inflammation following non-surgical therapy with particular attention to the site specific nature of the disease.

All clinical parameters assessed were seen to improve in the smokers with periodontal disease following non-surgical management of the periodontitis but to a lesser extent than the non-smokers with periodontitis as would be expected (Kinane & Chestnutt 2000). The four subjects that quit smoking during the healing phase of treatment had no influence on the resultant clinical improvements. The 20% cessation rate within the smokers with periodontal disease group is greater than normally seen in the dental setting with brief interventions and exceeds figures normally achieved by the intensive support of the NHS Stop Smoking Service (West *et al* 2000). This may be explained by the close and regular contact with the volunteers throughout treatment and the nature of the secondary care referral for their periodontal disease, which may equate to a greater level of motivation.

Within this study the GCF TAOC concentration at deep or index sites in the smokers with periodontitis increased to similar levels found in the periodontally healthy control groups **A** & **B,** suggesting that the total antioxidant compromise seen at baseline may result from the inflammatory lesion, rather than predispose to it. These data are consistent with the literature (Chapple *et al* 2007). Furthermore, the significantly lower GCF TAOC concentration seen in smokers at baseline compared to disease matched non-smokers with periodontitis group **B** ($p=0.02$), indicated a further compromise of GCF TAOC possibly due to the local impact of the additional ROS created through their use of tobacco both chronically and acutely (Palmer *et al* 2005, Pryor *et al* 1983). As the GCF TAOC concentration in the smokers recovered to the same level as the healthy control groups, it is proposed that the main contributor for this antioxidant reduction was the additional oxidative stress from the periodontal lesion and that the recovery of

local antioxidant status was not influenced by the increased ROS exposure locally created from their tobacco habit. It was hypothesised in chapter four that smokers may have a compensatory protective mechanism to deal with the additional local oxidative stress, whereby antioxidant systems are up-regulated. This theory may be further strengthened as the shallow sites sampled within the smokers displayed similar GCF TAOC concentrations to the healthy control groups and, following non-surgical management, levels increased to non-significantly higher levels. This type of compensatory factor is seen in individuals with chronic obstructive pulmonary disease (COPD), where smoking is a major factor in the pathogenesis of the disease, yet increased levels of glutathione (GSH) as seen in the lung epithelial lining fluid in chronic smokers, whereas in acute smoking levels are depleted (Rahman & MacNee 1999).

In conclusion, the longitudinal data reported here suggest the reduced protective effect of local (GCF) antioxidant capacity in volunteers with periodontal disease who use tobacco results from the periodontal inflammatory lesion. Smoker's antioxidant defences return to the levels seen in healthy individuals following successful therapy and suggest a possible up-regulation of antioxidant defence systems as a compensatory protective mechanism to deal with the additional local oxidative stress inflicted by their smoking habit.

GENERAL DISCUSSION AND CONCLUSION

6. General discussion and conclusions

In periodontitis the host response to microbial plaque is designed to protect the individual; however some collateral tissue damage is unavoidable. The persistence of plaque at or below the gingival margin results in a chronic inflammatory response, which in the susceptible host, results in tissue damage if it remains unresolved. A central feature of the inflammatory process is the recruitment of phagocytic cells, such as neutrophils and macrophages. The activity of these cells results in production of superoxide and other reactive oxygen species (ROS) during a non-mitochondrial respiratory burst via NADPH oxidase. It follows that most inflammatory diseases are accompanied by an increase in ROS (Halliwell 1991), nonetheless the presence of ROS in inflamed tissues does not mean they play a role in the pathogenesis and disease progression, but they may simply be a consequence of the inflammatory process. However, research suggests that excessive or prolonged release of neutrophil enzymes and ROS are believed to be responsible for the majority of host-tissue destruction in periodontitis (Gustafsson *et al* 1997). While more recent studies have demonstrated that peripheral neutrophils are both hyper-reactive to FCγ-receptor stimulation and hyper-active even when unstimulated, releasing excessive ROS in chronic periodontal disorders (Gustafsson *et al* 2006, Matthews *et al* 2006, Matthews *et al* 2007).

To combat disproportionate ROS production the body possesses a variety of antioxidant defence mechanisms, which act in concert. Their role is to protect vital cell and tissue structures and bio-molecules from host-derived ROS as well as those of parasitic origin (Chapple *et al* 1996), by removing them as they form and repairing the damage they cause. A delicate balance exists between antioxidant defence and repair systems and pro-oxidant mechanisms of tissue destruction, and if the balance is tipped in favour of ROS activity, significant tissue damage ensues (Chapple *et al* 2002).

Smoking has long been recognised as a risk factor for periodontal disease and a great deal of research into the detrimental effects of tobacco smoking have concluded that it has widespread systemic effects, many of which may provide mechanisms that increase the individual patients susceptibility to periodontal disease and affect their response to treatment, by stimulating destructive/inflammatory responses and impairing protective/reparative responses (Ryder 2007). Combustion of tobacco creates great numbers of ROS in the oral cavity and may also affect systemic production from inflammatory cells. The research on the effects of tobacco products on neutrophil expression of ROS is rather inconsistent and some researchers have suggested that tobacco constituents can exacerbate aspects of the respiratory burst, enhancing the production of ROS (Iho *et al* 2003). An increased priming effect has also been demonstrated increasing the generation of oxygen radicals as well as up-regulation of other neutrophil functions (Gustafsson *et al* 2000). Several studies have demonstrated lower plasma concentrations of antioxidants in smokers *in vivo* (Alberg 2002) and a decrease in serum antioxidant concentration has been negatively associated with the prevalence of inflammatory periodontitis (Chapple *et al* 2006).

This thesis attempted to follow on from recent research into antioxidant defences in patients with periodontal disease that demonstrated both a reduced peripheral (plasma) and local (GCF) total antioxidant capacity (Brock *et al* 2004). It utilises the same ECL assay (2.2.6.) to quantify TAOC both in GCF and plasma to discover whether the compromised antioxidant defence systems predispose to chronic periodontitis or result from the inflammatory process, in both smokers and non-smokers with periodontitis.

The increased oxidative stress may be a primary result of ROS production or a secondary consequence of decreased antioxidant protection. The cross-sectional data presented here suggested a compromised GCF TAOC seen in subjects with periodontitis, regardless of smoking status. These results were comparable with the preliminary work carried out by Chapple *et al* (2002) and Brock *et al* (2004), who both utilised the same sampling, storage and ECL assay techniques as the current study. Only two other studies have

investigated the GCF TAOC GCF, while the research by Pavlica *et al* (2004) on a breed of miniature dogs confirmed the above data, finding a significant correlation between serum and GCF TAOC and gingival inflammation. The work by Guarnieri and colleagues (1991) demonstrated spontaneous generation of superoxide in GCF of periodontal subjects, but could not demonstrate a difference in antioxidant scavenging capacity between subjects with chronic adult periodontitis and healthy controls. Their collection method for GCF, by crevice washing, plus storage of the samples at -20°C, both oxygenated and allowed for rapid loss of scavenging antioxidants (Chapple *et al* 1997), which may explain their difference in findings compared to the subsequent research.

No studies have been carried out on GCF TAOC in smokers with periodontitis, while minimal research has been carried out on some individual antioxidants in GCF samples and reductions have been reported. Seri *et al* (1999) demonstrated lower levels of vitamin C and non-significantly vitamin A in periodontally healthy smokers. A single study by Bunduneli *et al* (2006) on smoking and gingival inflammation on salivary antioxidant capacity, found no change in salivary status and they suggested that analysis of GCF TAOC would better address any imbalance in oxidants-antioxidants within the periodontal tissues. The cross-sectional data presented here suggest that smokers with periodontitis have a further compromised GCF TAOC compared to age-, gender- and disease-matched non-smokers with periodontitis. This further reduction in GCF TAOC may be explained by the additional local and systemic ROS production associated with the tobacco habit, both chronically and acutely (Pryor *et al* 1983, Palmer *et al* 2005).

The longitudinal data presented within this thesis suggest that the compromised GCF TAOC concentration seen in periodontitis, regardless of smoking status, is likely to be a secondary effect of the inflammatory lesion, rather than predisposing to it, as following non-surgical management of the periodontal inflammation GCF TAOC concentrations were seen to return to levels similar to that of healthy controls. This work is in broad agreement with the longitudinal data by Brock (2005) and Chapple *et al* (2007) on non-

smokers with periodontitis. Furthermore, the current data suggests an up-regulated antioxidant defence system to act as a compensatory protective mechanism in the smokers with periodontitis, as following therapy their GCF TAOC was seen to recover and exceed that of the healthy controls despite greater residual inflammation (as a poorer healing response was achieved as would be expected due to their continued smoking habit). Though no studies have been carried out on GCF TAOC longitudinally, parallels can be drawn between the fluid lining the gingival crevice and that of the alveolar epithelial lining fluid in the lungs, where increased levels of the antioxidant GSH are seen in chronic smokers with COPD (Rahman & MacNee 1999).

The data for the peripheral (plasma) TAOC displayed a mild (insignificant) reduction in antioxidant concentration within the non-smokers with periodontitis. This is broadly in agreement with those studies that analysed individual antioxidant scavengers in plasma, which indicate only mildly compromised levels in periodontitis subjects compared to healthy control subjects, with the exception of vitamin C in smokers which is further compromised (Chapple & Matthews 2007). The limited studies on plasma TAOC also indicate a mild antioxidant compromise in periodontitis subjects (Brock *et al* 2004, Pavlica *et al* 2004 and Baltacioğlu *et al* 2006). However, no research has been carried out on the plasma TAOC of smokers with periodontitis. While individual antioxidants have been seen to be reduced, in the current study the smokers with periodontitis were seen to have a non-significantly higher plasma TAOC concentration than the control groups. Following non-surgical management the small and insignificant changes seen in the current study are likely to lack clinical relevance, given their low concentrations relative to those of GCF in health.

The choice of total antioxidant assay within the current study must also be considered, as there is a complex array of global antioxidant assays, all with different specificities for different biological molecules in different tissue or fluid compartments. While all assays assess either *in vitro* oxidative stress, it's onset or the inhibition by antioxidants within the test sample, they all produce different measures and differ in sensitivity to the known major

antioxidants. The ECL assay used within the current study is sensitive to uric acid, vitamin A, C and E with proteins having a low influence on the derived TAOC (Maxwell *et al* 2006). This sensitivity may lead to misinterpretation of results as it may not reflect the activities of certain antioxidants within a particular body system. GSH is a major antioxidant in GCF (Chapple *et al* 2002, Brock *et al* 2004) and the ECL assay used in the current study is poorly sensitive to GSH (Maxwell *et al* 2006), therefore the choice of the ECL assay may have impacted on our understanding of the pathogenic process induced by tobacco smoking and the potential for possible future therapeutic strategies.

In conclusion, the longitudinal data reported within this thesis indicate the compromised local (GCF) antioxidant capacity seen in volunteers with periodontal disease, principally those who use tobacco, results from the periodontal inflammatory lesion and following successful therapy GCF TAOC concentrations return to levels seen in healthy controls. It also indicates that smokers with periodontitis may have up-regulated antioxidant defence systems to act as a compensatory protective mechanism to deal with the additional local oxidative stress. However, these data can not preclude the possibility that constitutional deficiencies in individual antioxidant species not assessed by the ECL assay may underpin damage in periodontitis, nor can it determine whether boosting levels of specific antioxidants with therapeutic interventions may afford protection against tissue damage mediated by oxidative stress with the periodontal tissues.

APPENDICES:

Periotron calibration – volumes “look-up” table

Publications arising directly from this thesis:

Brock *et al* 2004 *Journal of Clinical Periodontology*

Chapple *et al* 2007 *Journal of Clinical Periodontology*

Publications arising indirectly from this thesis:

Matthews *et al* 2007 *Journal of Dental Research*

Wright *et al* 2008 *Journal of Immunology*

Periotron Calibration - volume "look-up" table

Periotron Reading	Working Volume µl	Periotron Reading	Working Volume µl	Periotron Reading	Working Volume µl	Periotron Reading	Working Volume µl
1	0.004	**51**	0.27	**101**	0.688	**151**	1.09
2	0.008	**52**	0.28	**102**	0.7	**152**	1.1
3	0.012	**53**	0.287	**103**	0.707	**153**	1.243
4	0.016	**54**	0.293	**104**	0.714	**154**	1.386
5	0.02	**55**	0.3	**105**	0.721	**155**	1.529
6	0.026	**56**	0.303	**106**	0.728	**156**	1.672
7	0.033	**57**	0.306	**107**	0.735	**157**	1.815
8	0.039	**58**	0.309	**108**	0.742	**158**	1.985
9	0.04	**59**	0.312	**109**	0.749	**159**	1.2
10	0.046	**60**	0.315	**110**	0.756	**160**	1.23
11	0.052	**61**	0.318	**111**	0.763	**161**	1.26
12	0.058	**62**	0.32	**112**	0.77	**162**	1.3
13	0.06	**63**	0.34	**113**	0.777	**163**	1.33
14	0.064	**64**	0.35	**114**	0.784	**164**	1.36
15	0.068	**65**	0.36	**115**	0.791	**165**	1.4
16	0.072	**66**	0.37	**116**	0.798	**166**	1.45
17	0.076	**67**	0.38	**117**	0.8	**167**	1.5
18	0.08	**68**	0.39	**118**	0.807		
19	0.086	**69**	0.4	**119**	0.814		
20	0.092	**70**	0.42	**120**	0.821		
21	0.098	**71**	0.427	**121**	0.828		
22	0.1	**72**	0.437	**122**	0.835		
23	0.103	**73**	0.44	**123**	0.842		
24	0.106	**74**	0.447	**124**	0.849		
25	0.109	**75**	0.454	**125**	0.856		
26	0.112	**76**	0.46	**126**	0.863		
27	0.115	**77**	0.47	**127**	0.87		
28	0.12	**78**	0.48	**128**	0.877		
29	0.13	**79**	0.487	**129**	0.884		
30	0.14	**80**	0.494	**130**	0.891		
31	0.144	**81**	0.5	**131**	0.898		
32	0.148	**82**	0.51	**132**	0.9		
33	0.152	**83**	0.52	**133**	0.91		
34	0.156	**84**	0.53	**134**	0.92		
35	0.16	**85**	0.54	**135**	0.93		
36	0.17	**86**	0.55	**136**	0.94		
37	0.18	**87**	0.56	**137**	0.95		
38	0.184	**88**	0.57	**138**	0.96		
39	0.188	**89**	0.58	**139**	0.97		
40	0.192	**90**	0.6	**140**	0.98		
41	0.196	**91**	0.608	**141**	0.99		
42	0.2	**92**	0.616	**142**	1		
43	0.21	**93**	0.624	**143**	1.01		
44	0.22	**94**	0.632	**144**	1.02		
45	0.226	**95**	0.64	**145**	1.03		
46	0.232	**96**	0.648	**146**	1.04		
47	0.24	**97**	0.656	**147**	1.05		
48	0.246	**98**	0.664	**148**	1.06		
49	0.253	**99**	0.672	**149**	1.07		
50	0.26	**100**	0.68	**150**	1.08		

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