

# Local and systemic total antioxidant capacity in periodontitis and health

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## Abstract

**Background:** The involvement of reactive oxygen species (ROS) in periodontal pathology is unclear but will be modulated by in vivo antioxidant defence systems. The aim of this cross-sectional study was to determine both local (saliva and gingival crevicular fluid (GCF) and peripheral (plasma and serum) antioxidant capacity in periodontal health and disease.

**Materials and Methods:** Twenty non-smoking volunteers with chronic periodontitis were sampled together with twenty age- and sex-matched, non-smoking controls. After overnight fasting, saliva (whole unstimulated and stimulated) and blood were collected. Total antioxidant capacity (TAOC) was determined using a previously reported enhanced chemiluminescence method.

**Results:** GCF antioxidant concentration was significantly lower ( $p < 0.001$ ) in periodontitis subjects compared to healthy controls. Although mean levels of peripheral and salivary TAOC were also lower in periodontitis the difference was only significant for plasma ( $p < 0.05$ ). Healthy subjects' GCF antioxidant concentration was significantly greater than paired serum or plasma ( $p < 0.001$ ). Data stratified for gender did not alter the findings and a male bias was revealed in all clinical samples except GCF.

**Conclusions:** These findings suggest that the antioxidant capacity of GCF is both qualitatively and quantitatively distinct from that of saliva, plasma and serum. Whether changes in the GCF compartment in periodontitis reflect predisposition to or the results of ROS-mediated damage remains unclear. Reduced plasma total antioxidant defence could result from low-grade systemic inflammation induced by the host response to periodontal bacteria, or may be an innate feature of periodontitis patients.

Key words: antioxidant; gender; gingival crevicular fluid; periodontitis; plasma

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Reactive oxygen species (ROS) not only play an important role in cell signalling and metabolic processes (Bogdan et al. 2000) but are also thought to be implicated in the pathogenesis of a variety of inflammatory disorders (Davies 1995, McCord 2000). Indeed, novel therapies are being developed, specifically aimed at reducing oxidative stress at the tissue and cellular level (McCord 2000, MacNee & Rahman 2001). Oxidative stress arises within tissues when the normal balance between ROS generation and antioxidant defence shifts in favour of the former, a situation arising

from either an excess of ROS and/or a depletion of antioxidants (MacNee & Rahman 2001).

A variety of ROS (e.g. superoxide and hydroxyl radicals, hydrogen peroxide, hypochlorous acid) are well characterised and to a greater or lesser extent are able to cause direct damage to proteins, DNA carbohydrates and lipids (Babior 2000). In addition, the production of ROS and subsequent disturbance in tissue redox status, can modulate the expression of a variety of immune and inflammatory molecules via redox-sensitive transcription factors (e.g. NF- $\kappa$ B,

AP-1), thereby causing indirect tissue damage and exacerbating inflammation (Rahman & MacNee 1998, Mates et al. 2000). A defined role for ROS in the tissue destruction that characterises periodontitis has been described (Bartold et al. 1984, Gustafsson & Åsman 1996), but the precise contribution to the periodontal destructive process remains unclear (Halliwell 2000) and the role of antioxidant species has received scant attention (Chapple 1997).

Within the gingival crevice, neutrophils perform an innate cellular host defence role and contribute half of the

leukocytes infiltrating the junctional epithelium and 90% of the leukocytes isolated from crevicular fluid (Miyasaki 1991). The combination of bacterial phagocytosis, and secretion of proteolytic enzymes and immuno-modulatory compounds that assist in the killing and digestion of bacteria, is accompanied by a "respiratory burst" – the sudden increase in non-mitochondrial oxidative metabolism, producing superoxide radicals and a battery of other ROS via the leucocyte NADPH-oxidase complex (Babior 1999). Unfortunately, during the course of this upregulated neutrophil activity, ROS may cause excessive and indiscriminate "collateral" host-tissue damage when the ROS-antioxidant balance is upset (McCord 2000).

Control and modulation of ROS activity is normally achieved by the synthesis and accumulation of antioxidants which are substances that, when present at low concentrations compared with those of an oxidisable substrate, significantly delay or prevent oxidation of that substrate (Halliwell 1995). Antioxidants may be classified according to their mode of action (Chapple 1997); extracellular antioxidants likely to be of importance in periodontal disease are the chain-breaking or radical scavenging antioxidants such as ascorbate,  $\alpha$ -tocopherol, carotenoids, metal binding proteins and compounds with oxidisable -SH (thiol) groups (Chapple 1996, Waddington et al. 2000).

The investigation of disease-related oxidant-antioxidant imbalance is problematic due to the limited availability of specific biomarkers of oxidative stress, and the fact that measurement of individual antioxidants may give a misleading picture because antioxidants work in concert through chain-breaking reactions. For example, ascorbic acid will regenerate  $\alpha$ -tocopherol from the tocopherol radical and reduced glutathione (GSH) will regenerate ascorbate from its radical (Niki 1989, Winkler et al. 1994). Therefore, analysis of total antioxidant capacity (TAOC) may be the most relevant *initial* investigation (Woodford & Whitehead 1998). However, it is important to remember that decreased antioxidant capacity may indicate either inherently low basal antioxidant defence status or may result from an increase in oxidative stress.

There have been few published studies investigating the antioxidant capacity of fluids local to the oral cavity. The ability of gingival crevicular

fluid (GCF) washings to inhibit cytochrome *c* reduction by the superoxide radical was examined in one study (Guarnieri et al. 1991). Results showed no significant difference in antioxidant capacity between healthy and periodontitis subjects, although samples were stored under conditions incompatible with the preservation of low molecular weight antioxidants. Moore et al. (1994) investigated the antioxidant capacity of saliva using an alternative assay. No significant change in antioxidant status was reported in periodontal disease, although the authors suggested that a local decrease in salivary antioxidants might be compensated for by an increased GCF flow. Pilot study data generated in our laboratory during the validation of an enhanced chemiluminescence (ECL) assay (Chapple et al. 1997) and to investigate GCF (Chapple et al. 2002) TAOC in periodontitis subjects and controls, indicated that periodontitis could potentially be associated with a reduced local antioxidant defence. Based on these preliminary observations, we hypothesise that differences in total antioxidant levels exist between periodontitis patients and healthy controls and that reduced TAOC may be a feature of both local and peripheral extracellular fluids in periodontitis. Therefore, the aim of this cross-sectional case-control study was to examine both local (saliva and GCF) and peripheral (plasma and serum) TAOC, in subjects with chronic periodontitis and age- and sex-matched healthy controls, using the previously reported ECL assay. A rigorous clinical sampling protocol and subject selection criteria were employed to eliminate known confounders of antioxidant capacity, such as smoking and short-term dietary variables.

## Materials and Methods

### Subject recruitment

Twenty subjects with chronic periodontitis were recruited from new patient consultation clinics at Birmingham Dental Hospital's Periodontal Department. Chronic periodontitis was defined as the presence of at least two non-adjacent sites per quadrant with probing pocket depths (PPDs)  $\geq 5$  mm, which bled upon gentle probing, demonstrated radiographic bone loss  $\geq 30\%$  of the root length, and were not first molar or incisor sites. Control subjects ( $n = 20$ )

were age- and sex-matched, had no evidence of attachment loss, no PPDs  $> 3$  mm at any sites and whole mouth bleeding scores less than 10%.

All subjects were systemically healthy, with no medical condition that would affect their participation in the study. Exclusion criteria applied were a course of anti-inflammatory or antimicrobial therapy within the previous 3 months, pregnancy, a history of previous or current smoking, regular use of mouthwashes, use of vitamin supplementation within the previous 3 months, and any special dietary requirements (e.g. Coeliac disease).

Following informed consent, a detailed medical questionnaire was completed and subjects who fulfilled the study requirements were enrolled. Ethical approval was obtained from South Birmingham Regional Ethics Committee (LREC 0405).

### Periodontal assessment

All assessments were carried out following clinical sample (GCF, saliva and blood) collection. PPDs were recorded in duplicate at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-palatal, mid-palatal, disto-palatal), using a Hu-Friedy (PCP UNC15) pocket-measuring probe. Sites that bled upon gentle probing were also recorded (dichotomously) at all index sites around all standing teeth. Bleeding scores were expressed as the percentage of positive sites per subject. The same operator (G.R.B.) recorded all clinical data, which was double entered onto a Microsoft Excel 97 spreadsheet.

### Clinical samples

All samples were obtained the morning following an overnight fast. Subjects were asked not to drink (except water) or chew gum for the same period and abstinence was checked prior to biological sample collection. One subject was lost to the study, having consumed a cup of tea within 2 h of their appointment time.

Unstimulated saliva samples were collected by subjects allowing saliva to passively flow into a 20 ml sterile polypropylene container for 5 min in an area away from the main clinic. After measurement of the volume, aliquots were dispensed into cryogenic vials (Appleton Woods, Birmingham, UK) and stored immediately in liquid nitrogen. Follow-

ing a brief rinse with sterile water and a 3 min rest period, a stimulated saliva sample was collected over a five minute period by rolling a sterile marble around the mouth and expectoration into a sterile receptacle (Chapple et al. 1997). The volume of stimulated saliva was measured and samples aliquotted and stored in liquid nitrogen. In both cases, salivary flow rates were calculated by dividing the volume collected by time.

GCF samples were collected from a mesio-buccal and disto-palatal site on each of three teeth (molar, premolar, canine/incisor) in the upper left (right-handed subjects), or upper right quadrant (left-handed subjects), providing six samples per subject. No attempt was made to specifically select sites with deep pockets, because samples were pooled per subject to ensure sufficient assay sensitivity and the patient was used as the unit of analysis. Site-specific differences were therefore not analysed. Sites were isolated using cotton wool rolls and gently air dried prior to sampling. The same operator performed all sampling. Periopaper™ strips were placed in the gingival sulcus until mild resistance was felt. After a 30 s collection period, volumes were measured on a pre-calibrated Periotron 8000™ (Chapple et al. 1999) and all six samples pooled in 600 µl of PBS/BSA (50mg/L). Samples were eluted for 30 min at room temperature (Chapple et al. 1997) prior to removing Periopaper strips and storage of the eluate in liquid nitrogen.

Venous blood was collected in two Vacutainer™ tubes (lithium heparin and plain). Both tubes were initially stood at room temperature for 30 min. Lithium heparin tubes (for plasma) were then centrifuged at 1000 × g for 30 min (4°C), whilst plain tubes (for serum) were kept at 4°C for a further 30 min prior to centrifugation at 5000 × g for 3 min (room temperature). Serum and plasma samples were aliquotted into cryogenic vials and stored in liquid nitrogen.

#### Total antioxidant assay

The TAOC of clinical samples was measured using the previously described enhanced chemiluminescence (ECL) assay (Chapple et al. 1997). Briefly, light emission from the reaction depends on the constant production of free radical intermediates and is therefore sensitive to interference by scavenging water-soluble antioxidants. The time period of light suppression, in-

duced by the addition of antioxidant containing solutions, is directly proportional to the amount of antioxidant added to the reaction. In this way, the antioxidant capacity of biological samples can be related to an external standard calibrant (a water soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid – Trolox™). TAOC of samples is therefore expressed in Trolox equivalents (µM).

Standard curves were constructed using Trolox standards (20–80 µM), which were run prior to, and on completion of assaying clinical samples from each patient and the matched control subject. Samples were defrosted and assayed immediately to ensure minimal deterioration; plasma and saliva were diluted prior to assay (1:10 and 1:5, respectively) with PBS containing 50mg/L bovine serum albumin. Twenty µl volumes were routinely used for assaying standards and diluted plasma or saliva samples, whereas 100 µl volumes of GCF eluate were used without dilution in combination with matched volumes of Trolox standards for assay calibration. Each patient's samples were assayed at the same time as the matched control samples.

#### Statistical analysis

Between-subject samples were compared using the Wilcoxon test. Within subject paired data were analysed by paired *t*-test. In all cases, Graphpad Instat™ version 2.04a was used and a 95% confidence interval taken as significant.

## Results

#### Clinical and demographic data

Twenty patients were recruited to the study. One subject was subsequently rejected due to consumption of a cup of tea 2 h prior to sampling and a further two did not fit the recruitment criteria

upon closer questioning, 17 patients and 17 age- and sex-matched control subjects therefore completed this cross-sectional study, with 10 female and 7 male subjects and 15 right handed and 2 left handed in both the test and control groups (Table 1). Although there were no significant differences in salivary flow rates (stimulated or unstimulated) between periodontitis subjects and controls ( $p > 0.1$ ), GCF volumes were significantly greater in the disease group ( $p < 0.001$ ; Table 1). As expected, there were significant differences between stimulated and unstimulated saliva flow rates in both groups ( $p < 0.005$ ). Mean PPDs of sampled sites was 3.91 mm (range 1–9 mm) and whole-mouth pocket depths were  $3.98 \pm 1.08$  mm (range 1–9 mm). Whole mouth percentage of sites bleeding on probing in the periodontitis group was 22.6% (range 9%–36.5%), and 41.67% (range 0%–100%) for the 6 sampled sites. GCF volume showed a significant positive correlation with PPD in the periodontitis group ( $p < 0.0001$ ;  $r = 0.38$ ).

#### Laboratory data

The total antioxidant concentration in GCF was significantly lower in periodontitis patients than in healthy control subjects ( $p < 0.001$ ; Fig. 1). Although mean TAOC per 30-second sample was lower in periodontally diseased subjects than in age- and sex-matched healthy control subjects, despite the larger volumes collected in the former group, the difference did not reach significance ( $p > 0.1$ ; Table 1). Furthermore, GCF total antioxidant concentration was significantly greater than paired serum or plasma samples in healthy subjects ( $p < 0.001$ ) whilst in patients with periodontal disease no such difference was seen ( $p > 0.1$ ; Fig. 1).

There were no relationships between TAOC per 30-second sample and either PPD ( $r = -0.12$ ) or fluid volume

Table 1. Subject demographics ( $n = 34$ ), salivary flow rates (ml/min), GCF volumes (µl) and AO capacity (nmoles Trolox per sample) (mean ± SD)

	Periodontitis	Health
sex	7♂ and 10♀	7♂ and 10♀
mean age (range)	43.5 (23–60)	44.7 (24–63)
unstimulated saliva flow rate	$0.31 \pm 0.37$	$0.39 \pm 0.29$
stimulated saliva flow rate	$0.91 \pm 0.87$	$0.87 \pm 0.44$
GCF volume/30 s sample	$0.27 \pm 0.12$	$0.14 \pm 0.03$
AO capacity/30 s sample	$0.14 \pm 0.06$	$0.18 \pm 0.08$

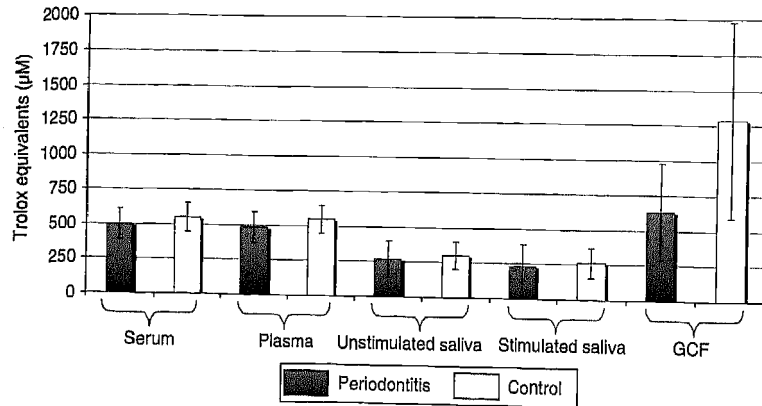


Fig. 1. Total antioxidant concentrations (mean  $\pm$  SD) of serum, plasma, saliva and GCF.

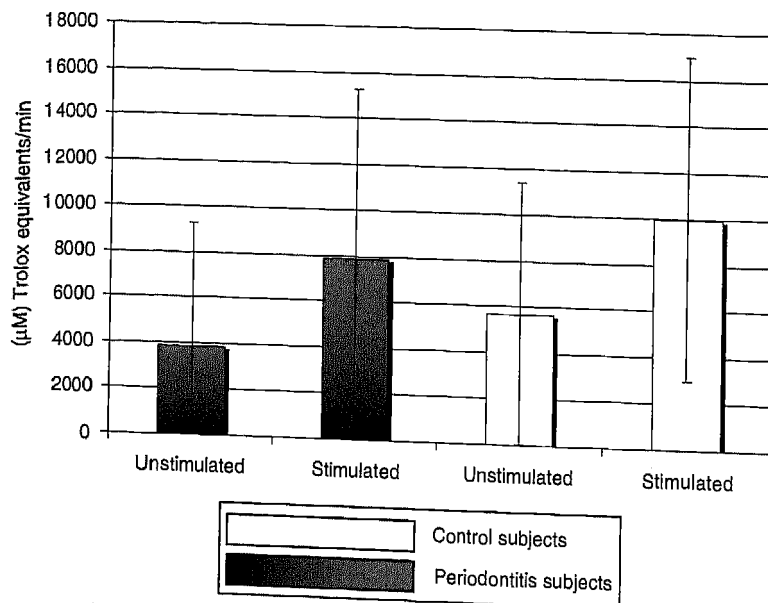


Fig. 2. Effects of stimulation on the rate of production of salivary total antioxidants.

( $r = 0.08$ ). Although a significant negative correlation was found between antioxidant concentration and PPD ( $p = 0.048$ ;  $r = -0.49$ ), this correlation is invalidated by the demonstrable dependence of fluid volume on pocket depth.

Both unstimulated and stimulated saliva samples from periodontitis patients contained non-significantly lower mean concentrations of total TAOC than controls ( $p > 0.1$ ; Fig. 1). Furthermore, total antioxidant concentrations of unstimulated and stimulated saliva were generally low and significantly lower than paired GCF, serum or plasma samples from any subject, regardless of their periodontal status (unstimulated,  $p < 0.0005$ ; stimulated,  $p < 0.04$ ). When the total salivary antioxidant capacity was examined with respect to flow rate, a significantly

lower rate of antioxidant production was noted in patients with periodontal disease compared with healthy matched controls for unstimulated saliva ( $p < 0.04$ ) but not for stimulated saliva ( $p > 0.1$ ; Fig. 2). The total antioxidant delivery in both subject groups was significantly greater in stimulated than in paired unstimulated samples ( $p < 0.005$ ; Fig. 2). There were no significant correlations between unstimulated or stimulated total antioxidant concentration and age or flow rate regardless of periodontal status.

The mean total antioxidant concentrations of serum and plasma from patients with periodontal disease were lower than healthy control samples (serum,  $p > 0.1$ ; plasma  $p < 0.048$ ). No significant correlations were found between serum or plasma antioxidant

concentration and age in healthy or diseased subjects ( $p > 0.1$ ). Interestingly, plasma antioxidant concentrations were marginally, yet significantly lower than paired serum levels in all study subjects ( $p < 0.02$ ). However, data stratified for periodontal status showed that this significant difference was a feature of patients with periodontal disease ( $p < 0.02$ ) and not controls.

Because uric acid is thought to be responsible for gender bias (in favour of males) in assays of serum antioxidant capacity (Woodford & Whitehead 1998), data was also stratified for gender (Figs. 3 and 4).

Serum and plasma samples from male subjects possessed significantly greater antioxidant concentrations than those from females, whether or not periodontal status was considered ( $p < 0.04$ ; Fig. 3). Similarly, unstimulated and stimulated saliva antioxidant concentrations in male control subjects were significantly greater than those in female controls ( $p < 0.04$ ), although there was no significant gender bias in patients with periodontal disease ( $p > 0.5$ ; Fig. 4). Nevertheless, all the samples from males, other than GCF, possessed greater mean total antioxidant concentrations than those from females regardless of periodontal status (Figs. 3 and 4). By contrast, GCF samples showed no significant gender bias and mean total antioxidant concentrations in samples from female subjects (with or without periodontitis) were greater than those from males ( $p > 0.2$ ). No significant differences in peripheral or salivary TAOC were detected when gender-stratified data were used for comparisons between patients and controls.

## Discussion

This is the first report that has simultaneously quantified local (GCF, stimulated and unstimulated saliva) and peripheral (serum and plasma) TAOC in periodontal health and disease. The results are in broad agreement with preliminary observations (Moore et al. 1994, Chapple et al. 1997, 2002) and demonstrate that, while mean levels of serum and salivary TAOC are non-significantly lower in periodontitis compared to age- and sex-matched healthy controls, GCF and plasma antioxidant concentration is significantly reduced. However, the data also show, for the first time that, in health, antioxidant

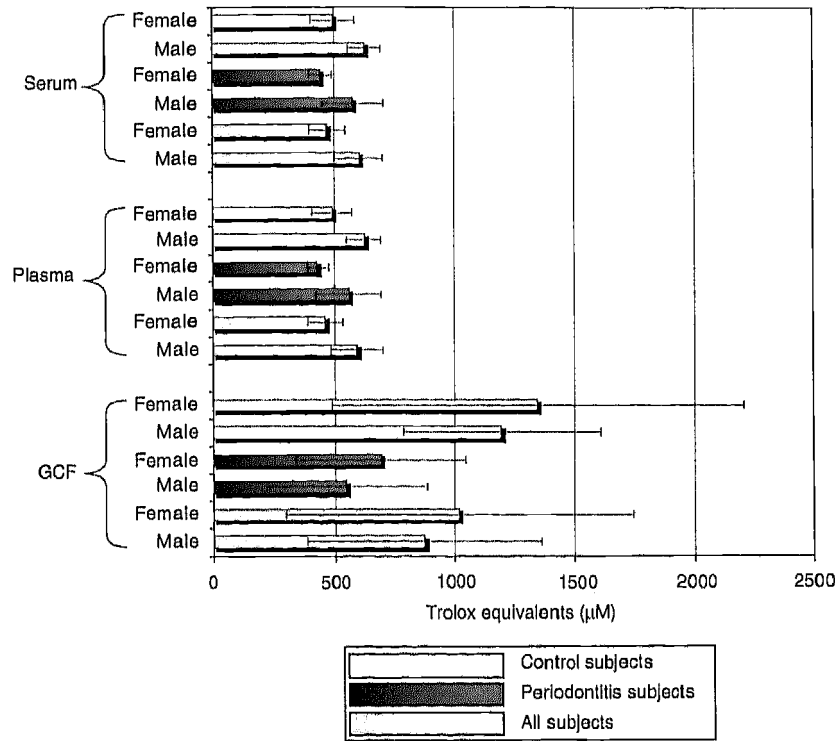


Fig. 3. Total antioxidant concentrations in serum, plasma and GCF: stratified for gender.

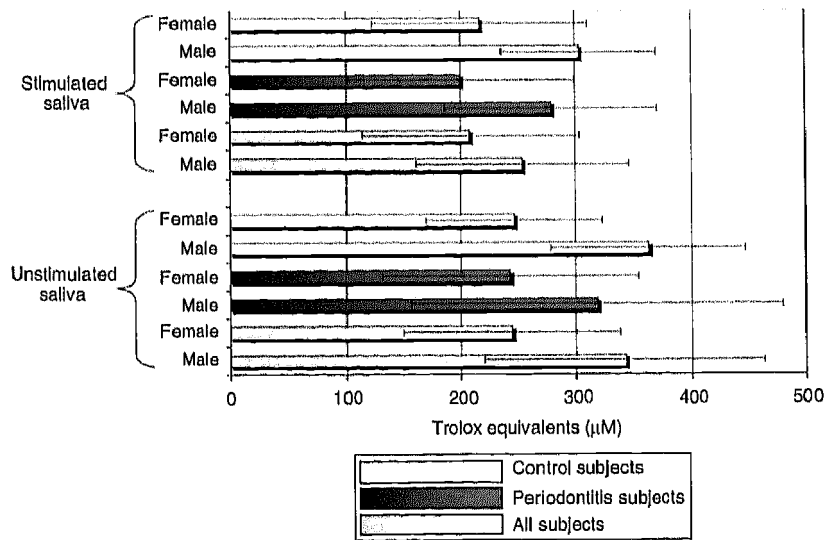


Fig. 4. Total antioxidant concentrations in stimulated and unstimulated saliva: stratified for gender.

concentrations in GCF are significantly greater than those of serum and plasma indicating local synthesis or storage within the periodontium, and are independent of gender, indicating that uric acid is not a major antioxidant in the gingival crevice (Woodford & Whitehead 1998).

The current results do not demonstrate that serum or salivary (stimulated and unstimulated; concentration or rate of production) antioxidant capacities are

significantly lower in periodontitis and agree with the report of Moore et al. (1994) on saliva (stimulated and unstimulated). However, they do differ from our previous data from a less well controlled pilot study where a reduction in salivary antioxidant concentration was detected in samples from periodontitis patients (Chapple et al. 1997). This initial work was not controlled for smoking, a known source of ROS, "reducer" of antioxidants (Pryor & Stone 1993)

and risk factor for periodontal disease (Haber et al. 1993), whereas the current study only invited "never smokers" to participate and included a rigorous pre-sampling protocol. Interestingly, both serum and plasma TAOC were reduced in periodontitis subjects relative to controls, though this did not reach significance in the former case. Given the established role for ROS in cardiovascular pathology and the recently established links between periodontal disease and cardiovascular disease, the reduced plasma total antioxidant capacity in periodontitis subjects warrants further investigation. One may speculate that reduced plasma antioxidant defences are a common risk factor for both diseases, but it is also possible that the chronic low-grade release of ROS from peripheral hyper-responsive neutrophils in periodontitis subjects, whether innate or induced by periodontal bacteria, may predispose to the development of atheromatous vascular pathology.

Peripheral and salivary antioxidant concentrations were greater in males than females, irrespective of periodontal status. Such differences have been reported previously (Woodford & Whitehead 1998) and are thought to be due to males having higher levels of urate, a major antioxidant in these fluids. No significant differences in TAOC were found when these gender differences were taken into account reinforcing the idea that serum and salivary antioxidant levels do not reflect disease status. However, the lower mean TAOC values obtained in the periodontitis group remained after removing the confounding influence of gender. It is possible that cross-sectional studies on larger numbers of men or women, or longitudinal investigations, might detect disease-associated alterations in serum and/or salivary TAOC confirming the plasma data. An indication that such studies should be pursued comes from reports finding hyperreactive neutrophils (Gustafsson & Åsman, 1996) and monocytes (Offenbacher et al. 1993) in the peripheral blood of patients with periodontitis. Theoretically such cells, in combination with an oral plaque-derived bacteraemia, could generate ROS and cause a reduction in peripheral antioxidant capacity in periodontitis subjects.

In contrast to the data obtained for peripheral and salivary TAOC, the antioxidant concentration of GCF from periodontitis patients compared with controls was greatly reduced. This lack

of concordance in results between the TAOC of the different fluids sampled and disease status was also evident when data were stratified for gender. Thus, there was no gender bias associated with the TAOC of GCF indicating that gender is not a confounder in analyses of TAOC in GCF and that urate is not a major antioxidant in this fluid (Woodford & Whitehead 1998). The former conclusion is supported by the fact that the difference in antioxidant concentrations between diseased and healthy groups remained when results were stratified for gender, and the latter, by recent studies demonstrating that about 75% of the TAOC of GCF detected using the ECL assay is due to thiol-containing compounds (Chapple et al. 2002).

Taken together, these findings suggest that the gingival crevice is bathed in a fluid that has antioxidant characteristics that differentiate it from both peripheral and salivary compartments. It seems logical that plasma will provide the basic antioxidant profile of gingival tissue and crevicular fluids, as there is a constant flow of fluid from the blood into the gingival crevice (Kowashi et al. 1980). However, that profile obviously undergoes significant modification, both qualitatively and quantitatively, within the tissues, such that the emergent GCF has a different antioxidant composition. Some of these modifications appear to be independent of periodontal status as the lack of gender bias in all GCF samples indicates that urate is not a major component of GCF in health or disease. Furthermore, the finding that GCF total antioxidant concentrations in health are significantly greater than those in paired samples of plasma and serum suggests local antioxidant synthesis and/or storage within the periodontium. Recent data supports this concept and indicates that thiol-containing antioxidants, in particular reduced glutathione, are present at high concentrations in GCF (Chapple et al. 2002) and are comparable to those found in the lungs (Cantin et al. 1987). In the lungs reduced glutathione is thought to be an important component of extracellular defence and is thought to be produced and exported by alveolar epithelium (Rahman & MacNee 1999). Preliminary *in vitro* studies from our laboratory indicate that oral epithelial cells contain high levels of reduced glutathione (44 nmol/10<sup>6</sup> cells; unpublished observations) and could be the

source of this antioxidant component within GCF.

While the data clearly demonstrate lower local, GCF concentrations of total antioxidants in periodontal disease, the mechanisms underlying the difference and whether the difference is a cause or effect of the disease are unclear. The simplest explanation, that increased GCF volume as a consequence of local inflammation has diluted the antioxidants and reduced the concentration, is not supported by the data which show that the amount of TAOC sampled in 30 s is unrelated to the volume of fluid collected ( $r=0.08$ ). Indeed, the summary data (Table 1) indicate that sites from periodontitis patients yielded lower overall amounts of antioxidants even though the volume of fluid collected was nearly double. Thus it would appear that there is a true reduction in TAOC at sites in periodontitis patients compared with controls, as well as a lower concentration. Whether the changes in GCF TAOC reported in the current study result from, or predispose to, periodontal tissue breakdown remains to be determined. However, the potential consequences of a compromised local antioxidant defence for the progression of the periodontal lesion are significant.

In conclusion, this study has demonstrated that the TAOC of GCF is both qualitatively and quantitatively distinct from that of saliva, plasma and serum. Furthermore, sites within periodontitis patients are characterised by displaying a lower TAOC. Whether this reduced antioxidant defence reflects inherent deficiencies predisposing to periodontitis or results from the inflammatory lesion remains unclear. Longitudinal studies and investigations involving sampling of healthy and diseased sites within periodontitis patients are in progress to more clearly define the role of antioxidants in the pathobiology of periodontitis. Finally, the reduced plasma total antioxidant status in periodontitis subjects warrants further investigation as it may provide a mechanistic link between periodontal disease and cardiovascular disease and help shed light upon the temporal relationship between these two free radical-associated chronic diseases.

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