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Crevicular fluid glutathione levels in periodontitis and the effect of non-surgical therapy

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Abstract

Aim: To quantify reduced and oxidized glutathione (GSH and GSSG) levels in gingival crevicular fluid (GCF) of periodontitis patients pre-therapy (*versus* periodontally healthy controls) and ascertain whether successful non-surgical therapy alters glutathione levels.

Materials and Methods: Thirty-second GCF samples (6/subject) were collected on Periopaper™ strips from starved, non-smokers ($n = 20$; mean age 43.6 years) with chronic periodontitis, before and 3 months after non-surgical therapy, and periodontally healthy, age- and gender-matched controls ($n = 20$). GSH and GSSG levels were determined using reversed-phase high-performance liquid chromatography with fluorescence detection.

Results: Lower concentrations of GSH ($p < 0.01$) and GSSG ($p < 0.05$) were detected in GCF from patients (pre- and post-therapy) than controls and treatment had no significant effect. Amounts per 30-second sample did not differ between patients and controls. However, the amount of GSSG per 30-second sample decreased in patients after therapy ($p < 0.05$). Consequently, therapy increased the GSH:GSSG ratio ($p < 0.05$) in patients compared with the controls ($p = 0.8$).

Conclusion: These data demonstrate high concentrations of GSH within GCF, which are compromised in chronic periodontitis. While therapy does not appear to fully restore GSH concentrations in GCF, it does restore the redox balance (GSH:GSSG ratio), suggesting that the abnormal redox balance arises secondary to oxidative stress resulting from periodontal inflammation.

Keywords: antioxidant; chronic periodontitis; gingival crevicular fluid; glutathione; oxidative stress; redox imbalance

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Periodontitis is an inflammatory disease characterized by a non-resolving inflammation, generated in response to virulence factors elaborated by periodontal pathogens, and which is ineffective at controlling the infection (Gaffen & Hajishengallis 2008). Evidence exists for both exaggerated monocyte (Shapira et al. 1994) and neutrophil activity (Matthews et al. 2007) as consistently reported biological features of the periodontitis phenotype, consistent with a host response that is ''hyper-inflammatory'' in nature. Significantly, neutrophils are the predominant inflammatory cells within diseased periodontal tissues, and therefore the release of their lysosomal enzymes and the generation of extracellular reactive oxygen species (ROS) are thought to be major factors in the aetiology of local tissue damage.

Oxidative stress may result in periodontal tissue damage either directly, by oxidation of important biomolecules, or indirectly, by activating redox-sensitive transcription factors [e.g. nuclear factor-κB (NF-κB)] leading to downstream expression of pro-inflammatory genes (Chapple & Matthews 2007). Recent reports support the concept that oxidative stress is an important factor in the pathogenesis of periodontitis and have shown non-specific markers of lipid peroxidation, a hallmark of oxidative stress, are higher in gingival crevicular fluid (GCF) and saliva in periodontitis patients compared with controls (Akalin et al. 2007).

The role of oxidative stress in periodontitis is also suggested from reports showing that patients have compromised antioxidant defences. Case–control studies

Conflict of interests and source of funding

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have demonstrated that patients with periodontitis have reduced total antioxidant capacity (TAOC) within the blood and, locally, within the GCF (Brock et al. 2004). This has also been confirmed by large-scale association studies (Chapple et al. 2007b; Linden et al. 2009), which demonstrated that patients with periodontitis have compromised peripheral blood levels of vitamin C and bilirubin, both of which are important in antioxidant defence (Chapple et al. 2007b), as well as a reduced plasma TAOC. Importantly, current data suggest that compromised local antioxidant activity in GCF results from the periodontal inflammation, rather than predisposing to it as traditional, non-surgical therapy, aimed at plaque removal and reducing inflammation, restores the TAOC of GCF to levels comparable with those of healthy control patients (Chapple et al. 2007a).

The principal intracellular antioxidant is the tri-peptide thiol-reduced glutathione (GSH), which also exists in its oxidized form (GSSG). The maintenance of a high and stable GSH:GSSG ratio is essential for cellular survival and governs how efficiently cells can recover from oxidative insult. It is also a critical factor in the activation of pro-inflammatory, redox-sensitive transcription factors, such as NF-κB (Haddad 2002) and activating protein-1 (AP-1) (Klatt et al. 1999). Indeed, there is evidence that the intracellular GSH:GSSG ratio is one of the principal drivers of key gene transcription factors such as NF-κB and AP-1 that regulate the pro-inflammatory response of the innate immune system (Lentsch & Ward 2000, Chapple & Matthews 2007). While extracellular levels of glutathione are usually very low, GCF has been shown to contain unusually high GSH levels (Chapple et al. 2002), similar to those detected in the alveolar lining fluid (ALF) of the lungs (200–400 μM; Morris & Bernard 1994). The preliminary studies on GSH content of GCF also suggested that fluid levels were lower in periodontitis patients than controls (Chapple et al. 2002). The high levels of GSH in alveolar lining fluid and GCF has led to the proposal that GSH may represent and/or reflect an innate defence strategy at exposed and vulnerable epithelial surfaces (Chapple 1996). GCF is regarded as the most appropriate fluid in which to investigate pathobiological reactions within the periodontal tissues rather than saliva or serum which poorly reflect certain periodontal tissue processes, and which have a markedly different antioxidant composition to GCF (Brock et al. 2004).

Glutathione is poorly detected in the enhanced chemiluminescence TAOC assay used to demonstrate changes in antioxidants within GCF in health and disease (Chapple et al. 2002, Brock et al. 2004, Chapple et al. 2007a). Thus, while studies indicate that reduced levels of GCF TAOC appear secondary to inflammation (Chapple et al. 2007a), this may not be the case with the periodontitis-associated reduction in GSH as suggested by preliminary data (Chapple et al. 2002). Therefore, this case-control, intervention study was performed to determine, for the first time, GSH and GSSG levels, and GSH:GSSG ratios, in GCF of periodontitis patients pre-therapy (*versus* periodontally healthy controls) and whether successful non-surgical therapy alters these parameters.

Materials and Methods

Study groups and design

Forty patients were enrolled into this case–control study, including 20 (12 females; eight males) with chronic mild/moderate periodontitis and 20 age- and gender-matched periodontally healthy controls. All volunteers were never-smokers and otherwise in good health, as confirmed by a detailed medical history questionnaire. Chronic periodontitis was defined as the presence of at least two non-adjacent sites per quadrant with probing pocket depths $\geqslant$5 mm, which bled on probing and which demonstrated radiographic bone loss $\geqslant$30% of the root length (non-first molar or incisor sites). Control patients had no evidence of attachment loss, no probing pocket depths $>$3 mm and whole-mouth bleeding scores $<$10%. Inclusion criteria included the complete absence of vitamin supplements, no use of anti-inflammatory or antibiotic medication in the previous 3 months, no pregnancy, mouthwash use or special dietary needs (Brock et al. 2004). All volunteers provided written informed consent and ethical approval for the study was obtained from the south Birmingham local research ethical committee (number LREC 0405).

After enrolment, all volunteers were re-appointed for collection of baseline GCF samples, which were taken after an overnight fast and before recording clinical measures (bleeding on probing and probing pocket depth). Patients received oral hygiene instruction and conventional non-surgical therapy, in the form of scaling, and root surface debridement (RSD) was performed under local analgesia on a quadrant-by-quadrant basis within 2 months. Patients were recalled 3-months post-therapy to re-sample GCF and for repeat clinical measures. A 3-month recall was chosen to allow for initial healing and to reduce the risk of re-infection/disease re-activation (Chapple et al. 2007a, b).

GCF collection and preparation for high-performance liquid chromatography (HPLC) analysis

As the patient (periodontitis or control) formed the unit of analysis, GCF samples were collected from index sites without reference to clinical or radiological data. Samples were collected from a mesio-buccal and disto-lingual site on each of three teeth (a molar, pre-molar and canine or incisor) in the upper left (right-handed subjects), or upper right (left-handed subjects) quadrant, providing six samples, and potentially controlling for any quadrant-specific differences due to dexterity. The GCF samples were collected for 30 s on Periopaper™ strips (Oraflow, Plainview, NY, USA) from starved (overnight), non-smoker volunteers with chronic periodontitis and matched controls and GCF volumes were measured using a pre-calibrated Periotron 8000™ (Oralflow), as described previously (Brock et al. 2004). GCF samples from six sites per individual were pooled and eluted into 1 mM EDTA, 5 mg/l cresol red, 0.2 M boric acid in 3.5% (v/v) perchloric acid stabilizing medium (300 μl) to prevent oxidation of labile antioxidant species, snap frozen and stored under liquid nitrogen. GSH and GSSG were measured by HPLC using a fluorimetric detector after derivatization with dansyl chloride as described previously (Chapple et al. 2002). Concentrations of GSH and GSSG were determined by reference to standard curves acquired from a parallel measurement of external standards and adjustment for variations in derivatization and sample delivery to the column using an internal standard (10 mM γ-glu–glu).

Data analysis

Data were analysed using Minitab (version 9.0). Significant differences between groups were determined by the χ^2-test for

bleeding on probing and by the Mann–Whitney *U*-test for all other comparisons, to account for lack of normal distribution of some data.

Results

Clinical and GCF volume data

Two patients were unavailable for follow-up and one set of post-therapy GCF samples was contaminated with saliva, and therefore not analysed. The success of non-surgical therapy was confirmed by the observed reductions in whole-mouth probing pocket depth ($p<0.0001$) and percentage of sites bleeding on probing ($p<0.0001$) (Table 1). Similar significant decreases in these clinical measures were also demonstrated at sites chosen for GCF sampling. Crevicular fluid volumes decreased significantly after treatment to levels that were not significantly different from those obtained from sites in periodontally healthy controls (Table 1).

Glutathione levels in GCF

In all groups, mean GSH and total glutathione levels were detected in the millimolar range (range: 0.43–4.29 mM). Total glutathione, GSH and GSSG concentrations were significantly lower in the GCF from periodontitis patients before and after treatment, compared with those detected in control GCF (Fig. 1). Although treatment increased both GSH and total glutathione GCF concentrations, these changes did not attain statistical significance. However, the GSH:GSSG ratio in patients GCF was significantly increased after treatment from 7.16:1 to 8.98:1 ($p<0.05$), a value not significantly different from that found for the GCF from the matched control group (9.28:1; Fig. 1). This rebalancing of the GSH:GSSG ratio after treatment was supported by the data on amounts per 30 – second sample, which demonstrated that although GSH and total glutathione levels were not significantly changed by therapy, GSSG was significantly reduced ($p<0.05$; Fig. 2).

Discussion

The data presented confirm our previous studies suggesting that GCF contains millimolar concentrations of glutathione and that these concentrations are lowered in periodontitis (Chapple et al. 2002). In addition, the current data also show that although successful non-surgical therapy does not appear to restore GSH and total glutathione concentrations to the levels seen in health, the GSH:GSSG ratio, which is lower in diseased sites, can be returned to a value similar to that in the healthy control sites by therapy. It is difficult to know to what degree the anaerobic nature of the flora at the pocket base influences the redox state of the pocket, or vice versa, because strict anaerobes survive within shallow pockets where oxygen tensions are higher, and indeed ROS have very short half-lives of less than a microsecond (Chapple & Matthews 2007). It is interesting that despite the capacity of certain periodontal bacteria to metabolize GSH (Carlsson et al. 1993, Chu et al. 2003) biofilm removal via successful non-surgical periodontal therapy does not restore total GSH levels or GSH concentrations within GCF, implying a negligible impact of the microbial environment upon *in vivo* GCF glutathione levels.

Glutathione is the main regulator of intracellular redox status and important in detoxification reactions, both functions requiring cellular levels of GSH to be maintained at millimolar levels. Intracellular GSSG levels normally constitute $<5\%$ of the total glutathione (GSH predominating), but in the extracellular environment GSSG levels are elevated (Meister & Anderson 1983). Cellular export of glutathione (GSH, GSSG and glutathione conjugates) is not only necessary for detoxification and elimination of foreign chemicals but also to allow intracellular synthesis and turnover, as degradation occurs exclusively in the extracellular compartment (Ballatori et al. 2009). Thus, although large quantities of GSH are exported from cells, plasma levels are relatively low ($\sim 10\,\mu$M) owing to the rapid catabolism of the tripeptide by membrane-bound γ-glutamyl transpeptidase and dipeptidases (Meister & Anderson 1983). By contrast to the low levels of glutathione found in plasma, many mucosal surfaces of the body that are exposed to the external environment such as the lungs, are coated with epithelial lining fluid (ELF) containing concentrations of GSH >100-fold higher than in plasma (~ 0.4 mM; Cantin et al. 1987). This high level is reduced in chronic lung diseases (Rahman & MacNee 1999, Biswas & Rahman 2009) and parallels our data demonstrating reduced GCF glutathione in periodontitis. Both the lungs and gingival crevice are lined

Table 1. Demographics, probing pocket depths, percentage sites bleeding on probing and GCF volumes (means ± SD or range)

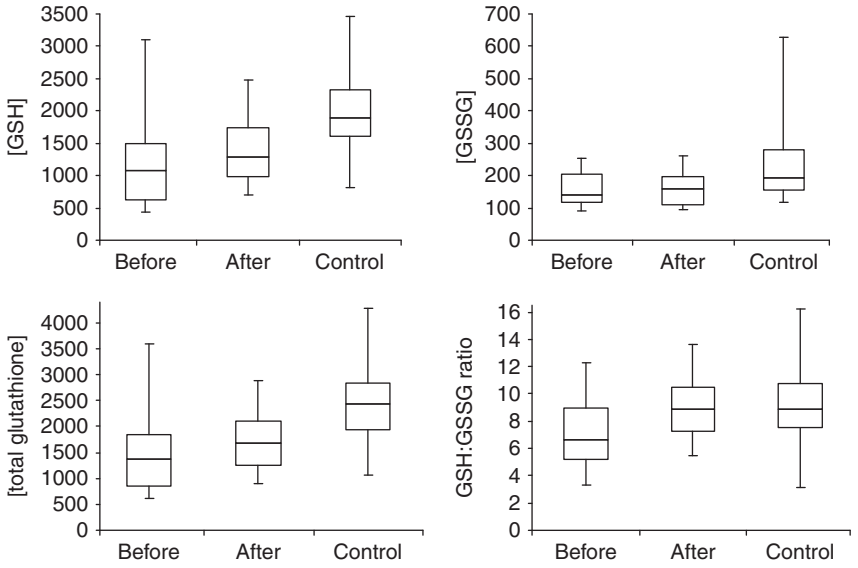
Age	Chronic periodontitis patients ($n=20$) 43.6 ± 2.1		Controls ($n=20$) 44.3 ± 2.3
	pre-treatment ($n=20$)	post-treatment ($n=17$)	
Whole mouth			
Probing pocket depth (mm)	3.05 ± 0.54	2.07 ± 0.21 ($p<0.0001$)*	⩽2 mm
% of sites bleeding on probing (range)	22.6 (9–36.5)	4.2 (0–16) ($p<0.0001$)	⩽10%
Sampled sites			
Probing pocket depth (mm)	3.75 ± 1.85	2.48 ± 0.91 ($p<0.0001$)	⩽2 mm
% sites bleeding on probing (range)	28.9 (0–3)	11.8 (0–3) ($p<0.01$)	⩽10%
Number of sites bleeding on probing	1.7 ± 0.9	0.7 ± 0.17 ($p<0.01$)	<0.6
GCF volume (μl) (mean of six sites from three index teeth)	0.24 ± 0.08	0.18 ± 0.07 ($p<0.05$)	0.15 ± 0.04 ($p<0.001$)#

*p values in parenthesis are comparisons with chronic periodontitis patients before treatment.

#p values in parenthesis is comparison between chronic periodontitis (pre-treatment) patients and controls.

The ''whole-mouth'' clinical data has been described previously (Chapple et al. 2007a), and is included in Table 1 only for completeness. GCF volumes of 0.24 μl reflect moderate levels of inflammation at sampled sites.

GCF, gingival crevicular fluid; SD, standard deviation.



	Chronic periodontitis patients		Controls (n=20)
	Before Treatment (n=20)	After Treatment (n=17)	
GSH	1182 ± 711 (*P*<0.001)*	1424 ± 554 (*P*<0.01)	1953 ± 566
GSSG	157 ± 48 (*P*<0.01)	161 ± 52 (*P*<0.05)	237 ± 123
Total glutathione	1497 ± 790 (*P*<0.001)	1746 ± 630 (*P*<0.005)	2427 ± 701
GSH:GSSG ratio	7.16 ± 2.56 (*P*<0.05)	8.98 ± 2.41 (*P*=0.8)	9.28 ± 3.28

*Mann Whitney *P* values, comparison with control.

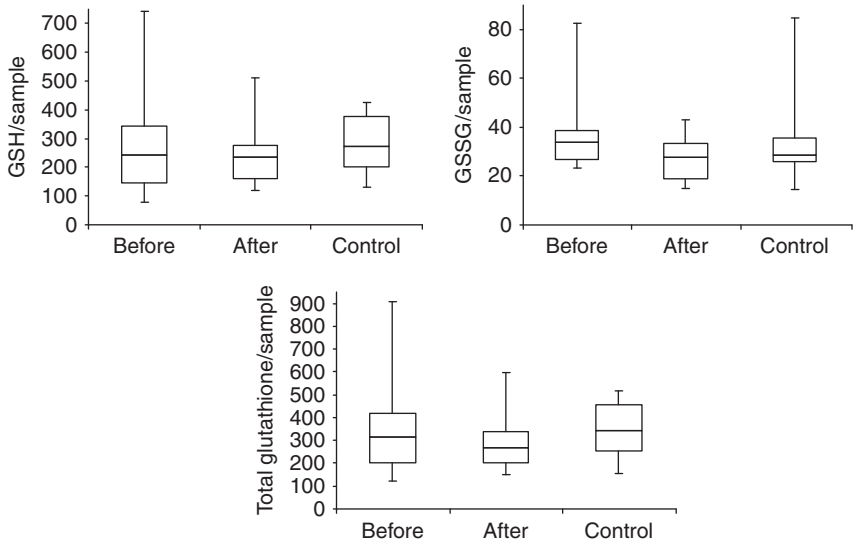
Fig. 1. Glutathione concentrations (μM) and GSH:GSSG ratio in gingival crevicular fluid from periodontitis patients (before & after therapy) and controls. Box and whisker plots show median, inter-quartile range, maximum and minimum. Table shows means ± SD.*Mann–Whitney *p* values, comparison with control.

by epithelium, exposed to external sources of infection, oxidants, pollutants and toxins and are subject to an intense neutrophilic inflammation within the underlying connective tissues. Both are also associated with a fluid exudate containing polymorphonuclear leucocytes (PMNLs), antimicrobial peptides and other defence factors that include millimolar concentrations of GSH, which can directly inactivate ROS, exogenously (smoking) or endogenously (PMNLs) derived by forming GSSG. Interestingly, sputum levels of GSSG are elevated in patients with chronic obstructive pulmonary disease (COPD) and the increase is associated with neutrophilic inflammation (Beeh et al. 2004).

In the current study, although treatment resulted in non-significant increases in the concentrations of GSH and total glutathione to levels that were still below those of the controls, the redox balance within the crevice in terms of the GSH:GSSG ratio increased to that of periodontally healthy controls. These data suggest that this change in thiol-redox status is due to a reduction in the amount of GSSG and maintenance of GSH levels within GCF after treatment. Given that levels of glutathione (GSH and GSSG) in GCF are most likely dependent upon export from crevicular epithelium, as plasma glutathione has a half-life of the order of seconds to minutes (Meister & Tate 1976, Meister & Anderson 1983), the reduced GSSG level could be a reflection of an increase in the intracellular GSH:GSSG ratio. This, in turn, would indicate that the crevicular/pocket epithelium is not being subjected to oxidative stress and that redox-sensitive pro-inflammatory gene transcription factors (e.g. NF-κB, AP-1) are not being activated. This molecular scenario is supported by the improvement in clinical markers of disease and the reduction of GCF flow indicating that inflammation has been resolved at these sites. Mechanistic studies are currently underway in our laboratories to investigate the impact of elevating the intracellular GSH:GSSG ratio of epithelial cells upon NF-κB activation and downstream pro-inflammatory cytokine production.

Clearly, utilizing the patient as the unit of analysis in our study is an accepted approach which prevents site-based variability confounding resulting data. However, it should be recognized that the GCF volumes (mean = 0.24 μl) recorded at sampled sites reflect moderate levels of inflammation and our data may underestimate the redox balance at deeper sites, where the inflammatory burden may be greater. This may have limited the sensitivity of this specific study design in detecting differences in GSH:GSSG ratios pre-therapy.

There are alternative explanations of our findings. The first relates to levels of proteolytic activity within the gingival crevice/periodontal pocket. *In vitro* studies of epithelial cell cultures report the release of GSH from its intracellular location upon exposure to proteolytic enzymes, where it may be subsequently catabolized or oxidized to GSSG (Reiners Jr. et al. 2000). It is plausible that the reductions in protease activity within the periodontal tissues following biofilm removal and resolution of periodontal inflammation may also result in lower levels of GSH release, and thus deplete subsequent extracellular GSSG formation. Secondly, post-therapeutic reductions in levels of inflammation results in lower levels of exogenously and endogenously derived oxidants in GCF reducing the amount of GSH being oxidized to GSSG. If this were the case, an increased amount of GSH should be seen but this is unlikely to be detected because GSH is always present in substantial excess over GSSG. In reality, glutathione levels detected in GCF, like those detected in ALF, are likely to depend upon a variety of factors including the rate of synthesis and export by epithelium (Fig. 3 demonstrates synthesis and fates of intracellular GSH), redox state of intracellular glutathione, size of the glutathione pool able to undergo redox cycling, the presence of GSH-reactive species in the crevice (Ballatori et al. 2009, Biswas & Rahman 2009) and the metabolism of GSH by certain bacteria (certain *Fusobacteria*, *Peptostreptococcus micros* and *Treponema denticola*) to hydrogen sulphide (Persson et al. 1990, Carlsson et al.



	Chronic periodontitis patients		Controls (n=20)
	Before Treatment (n=20)	After Treatment (n=17)	
GSH	263 ± 151	236 ± 94	283 ± 98
GSSG	35.6 ± 12.7	26.7 ± 8.3 (P<0.05)*	32.8 ± 14.5
Total glutathione	334 ± 173	289 ± 107	349 ± 108

*Mann Whitney *P* value, comparison with before treatment.
All other comparisons not significant.

Fig. 2. Glutathione (pmoles) per 30-second gingival crevicular fluid samples from periodontitis patients (before & after therapy) and controls. Box and whisker plots show median, inter-quartile range, maximum and minimum. Table shows means ± SD. *Mann–Whitney *p* value, comparison with before treatment. All other comparisons not significant.

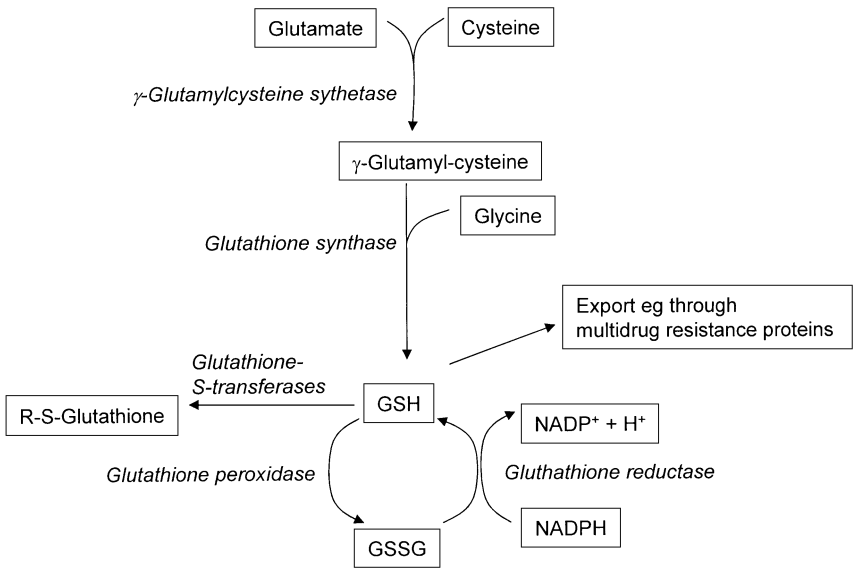


Fig. 3. Metabolism and fate of intracellular glutathione. γ-Glutamylcysteine synthase is the rate-limiting step in GSH synthesis. R, target of S-glutathionylation.

1993, Makinen & Makinen 1997, Chu et al. 2003, Zappacosta et al. 2007). Indeed, distinct metabolic pathways underlying this latter process in *T. denticola* have been reported (Chu et al. 2002).

Although the precise role(s) of glutathione in the maintenance of periodontal health are yet to be determined, recent evidence indicates that smoking, a major risk factor for periodontitis (Palmer et al. 2005), can have major effects on GSH levels within the lung. While elevated levels of GSH have been detected in the ALF of chronic smokers, cigarette smoke acutely lowers intracellular levels (Li et al. 1994, Rahman et al. 1995). This has recently been shown not to be due to oxidation of GSH to GSSG but to the formation of non-reducible glutathione-aldehyde derivatives, thus depleting the GSH pool (van der Toorn et al. 2007). These authors suggest that the development of COPD, known to be associated with reduced levels of GSH in ALF (Rahman & MacNee 1999) in smokers, may result from a genetic predisposition restricting their ability to synthesize sufficient GSH. Whether a similar situation exists in the periodontal tissues and periodontitis remains to be investigated, but given that total glutathione levels remained significantly below those of controls, both pre- and post-successful periodontal therapy, this is one plausible explanation.

These data confirm our preliminary findings that in non-smokers, GCF contains millimolar concentrations of GSH, which are significantly reduced in mild periodontitis (Chapple et al. 2002). In addition and for the first time, we have demonstrated that successful, non-surgical therapy does not fully restore GSH concentrations in GCF but does restore the redox balance (GSH:GSSG ratio), suggesting that these changes are secondary to oxidative stress resulting from periodontal inflammation. However, GSH, GSSG and total glutathione concentrations in GCF remain lower than control patients, implying a reduced buffering capacity against ROS activity in periodontitis patients, even following successful therapy. This may constitute a deficiency in innate immunity in periodontitis patients, rendering them more susceptible to oxidative stress and its sequelae, but further research is needed to confirm such a thesis. If demonstrated, then such findings open up the potential to develop novel therapeutic approaches based upon elevating the GSH buffering capacity within tissues using pharmacological interventions, such as the use of the GSH-promoting drug *N*-acetyl-cysteine (Chapple 1996), an approach currently under investigation in the management of rheumatoid arthritis (Kelly & Saravanan 2008). Alternatively, given that GSH is the

principal chain-breaking antioxidant in the extracellular environment, micronutritional approaches to boost tissue antioxidant concentrations, may preserve GSH and create an anti-inflammatory tissue redox state (Johnston et al. 1993). Such approaches are being actively pursued in the preventive management of other chronic inflammatory diseases where oxidative stress underpins their pathology (Ueno et al. 2002), and may also have a role to play in the management of periodontitis.

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Clinical Relevance

Scientific rationale for the study: Oxidative stress is implicated in the pathogenesis of periodontitis and alters the redox balance of the tissues and also the cells of the innate immune system. Glutathione is reported to be the most important redox regulator and controls inflammatory processes.

Principal findings: GCF and GSH concentrations are 1000-fold those of plasma and are lower in periodontitis. Successful non-surgical therapy restores the GSH:GSSG balance.

Practical implications: It appears that the periodontal redox balance is affected by periodontal inflammation, but can be restored to healthy levels by successful therapy.