J Oral Pathol Med (2005) 34: 7–12

www.blackwellmunksgaard.com/jopm

The levels of plasma and salivary antioxidants in the patient with recurrent aphthous stomatitis

Yelda Karincaoglu[1], Kadir Batcioglu[2], Tamer Erdem[3], Muammer Esrefoglu[1], Metin Genc[4]

Departments of [1]Dermatology, Faculty of Medicine, [2]Biochemistry, Faculty of Pharmacy, [3]Otorhinolaryngology, Faculty of Medicine and [4]Public Health, Inonu University, Faculty of Medicine, Turgut Ozal Medical Center, Malatya, Turkey

BACKGROUND: Despite plenty of research, the cause of recurrent aphthous stomatitis (RAS) remains obscure. It has been proposed that, the aetiological factors such as local trauma, smoking, vitamin deficiencies and viral infections lead to aphthae formation via final common pathway based on increased oxidative stress. The aim of this investigation was to evaluate the antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) alterations in plasma and saliva, and in addition uric acid (UA) in saliva, in patients with RAS and healthy controls.

METHODS: Thirty-two patients with RAS and 30 healthy controls were included into the study. The SOD, CAT, GSHPx and UA levels were measured in plasma and saliva in study and control groups.

RESULTS: In the RAS group, although the mean SOD ($P < 0.001$) and CAT ($P < 0.05$) levels of plasma were lower, GSHPx ($P < 0.001$) levels were higher than control group. The salivary concentrations of the SOD ($P < 0.001$), CAT ($P < 0.05$) and GSHPx ($P < 0.001$) in RAS group were entirely opposite to plasma concentrations. UA were not significant between RAS group and controls.

CONCLUSION: Since we found salivary SOD and CAT levels were high whereas plasma levels were low, it has been thought that, salivary defence mechanisms via antioxidant agents may be stimulated against to the ulcerous lesion. We consider that the organism might mobilize the antioxidant potential to the sites where they were needed. At this point, decrease of SOD and CAT levels in the plasma may be related to this shift. It is also thought that GSHPx secretion in the saliva may also be increased but the increase in its turnover may be responsible for the diminished activity.

J Oral Pathol Med (2005) **34**: 7–12

Keywords: antioxidants; recurrent aphthhous stomatitis; salivary

Correspondence: Yelda Karincaoglu, Inönü Universitesi, Tıp Fakültesi, Turgut Özal Tıp Merkezi, Dermatoloji AD, 44300 Malatya, Turkey. Tel: +90 422 341 06 60, Fax: +90 422 341 10 58, E-mail: ykarincaoglu@inonu.edu.tr
Accepted for publication May 21, 2004

Introduction

Recurrent aphthous stomatitis (RAS) is one of the most common oral disorders occurring between 25 and 35% of the population (1, 2). The clinical features of RAS are recurrent attacks of one or several, discrete, rounded, shallow, painful oral ulcers at intervals of a few days to a few months (3, 4). The exact aetiology of RAS still remains unclear, however, various factors such as local trauma, smoking, vitamin deficiency (folate, vitamin B12 and thiamine) and iron deficiency, virus [human herpes virus 6 (HHV-6), human cytomegalovirus (HCMV) and varicella zoster virus (VZV)], bacteria (*Streptococcus sanguis*), stress, hormonal status, genetics, nutrition, drug, allergy, immunology may contribute to the pathogenesis of this clinical entity (4–15). It has also been associated with a variety of underlying systemic diseases, including chronic inflammatory bowel diseases, gluten enteropathy, agranulocytosis, cyclical neutropenia (4). All the factors mentioned above that are thought to have influences in the aetiology of the disease are in relation with the oxidant–antioxidant balance of the organism directly or indirectly and are factors that trigger free radical formation (16–19).

Reactive oxygen species (ROS) are involved in the aetiology of ageing and the pathogenesis of a variety of diseases. ROS are generated *in vivo* by multiple mechanisms, including the respiratory redox chain in mitochondria, the respiratory burst of phagocytes and the activity of various oxidases. Oxidative stress occurs when the intracellular concentrations of ROS increase over the physiological values. Several conditions, including viral and bacterial infections, hyperthermia, ionizing and UV irradiation, as well as environmental pollutants, cause oxidative stress. The pathology associated with ROS is derived from their ability to modify cellular and extracellular macromolecules, such as protein, lipid, and DNA, and to disrupt cellular function. Mammalian cells have developed elaborate antioxidant defence systems to prevent oxidative damage and to allow survival in an aerobic environment. This system includes enzymatic activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase

(GSHPx) as well as non-enzymatic antioxidants including vitamins E, C, A, melatonin, uric acid (UA) and glutathione (20–22).

Since the mouth is the first entrance of the body for the food, drink and inhalants, saliva is the first milieu for those environmental materials. It has been shown that, saliva includes many defensive mechanisms such as secretory IgA and protein-enzymatic defence system, histatin, lysosin and lactoferrin. On the contrary, nowadays, another salivary defence mechanism, which is called salivary antioxidant system including UA, SOD, CAT, GSHPx is known. UA is the major component of the salivary antioxidant system constituting the 70% of the total antioxidant capacity (23, 24). To our knowledge, there is only one study concerning plasma antioxidant levels with RAS (25), but not in saliva in English literature. Therefore, the aim of this study was to evaluate both plasma and saliva antioxidant status with active RAS and healthy controls.

Materials and methods

Patients and controls

The study involved on 32 patients with RAS of whom 14 were men (14–44 years; 31.8 ± 10.0, mean ± SD) and 18 were women (17–46 years; 29.5 ± 8.6, mean ± SD) and 30 healthy people of whom 12 were men (19–40 years; 29.6 ± 3,4, mean ± SD) and 18 were women (16–45 years; 24.5 ± 3.0, mean ± SD).

The RAS patients had oral ulcer attack recurring at least three times a year (8, 25). However, despite taking this definition as the basis, the incidence of RAS in our patients was six times or more in a year. They also had active lesions when examined by the dermatologist during the study. The following laboratory tests were performed; erythrocyte sedimentation rate, blood cell counts and indexes; glucose, electrolytes, kidney and liver function tests, UA, calcium, phosphorus, globulin, albumin, iron in blood, vitamin B12 and folic acid. The patients with normal biochemical analysis mentioned above were included into the study. The patients were otherwise healthy. They were not under a therapeutic regimen for the previous 3 months and had not received drugs containing iron and/or vitamins. Patients with Behçet's disease, chronic diarrhoea, trauma history, and any systemic disease were not included in the study. The control group consisted of 30 self-admitted healthy individuals that were either medical students or hospital staff, who denied having RAS or using medications or vitamins. All individuals with any history of smoking and alcohol habits were excluded.

The informed consent was taken before the procedure. Fasting venous blood was collected within a heparin-coated injector and was processed immediately. Plasma was separated from all the subjects and stored at −40°C until analysis.

The unstimulated saliva samples were obtained by spontaneous flow after cannulation of the Stenon duct of the parotid gland to eliminate the contamination of the oral content. And no oral stimulus was permitted for 90 min prior to collection. The ductal cannulations were achieved by the 22 gauge intravenous cannulas under microscopic view for precise procedure. After sampling, the samples were stored at −40°C until analysis.

Analysis

Chemicals

All chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

Plasma preparation

Blood samples (5 ml) from 32 patients and 30 control individuals were drawn into vacutainers containing heparin as anticoagulant. Blood was centrifugated at 1000 *g* for 10 min at 4°C to obtain plasma, which was stored in small aliquots at −40°C.

Saliva preparation

Saliva samples were centrifugated 4000 *g* for 10 min at +4°C, the upper parts were drawn and stored in small aliquots at −40°C.

Determination of protein concentration

Protein determination in plasma and saliva samples were analysed according to Lowry method (26) using bovine serum albumin (BSA) as standard.

A Shimadzu 1601 UV/VIS Spectrophotometer (Shimadzu Co., Tokyo, Japan) with a connected personnel computer and Grand LTD 6G thermostability unit adjusted to 37 ± 0.1°C was employed for all spectrophotometric assays.

Assay of GSHPx activity

The GSHPx activity was measured according to Lawrence and Burk (27): 1.0 ml of 50 μM phosphate-buffered saline (PBS) solution (pH 7.4) including 5 μM ethylenediaminetetraacetic acid (EDTA), 2 μM nicotinamide adenine dinucleotide phosphate (NADPH), 20 μM GSH, 10 μM NaN_3 and 23 mU of oxidised glutathione (GSSG) reductase was incubated at 37°C for 5 min. Then 20 μl of 0.25 μM H_2O_2 solution and 20 μl sample (plasma or saliva) were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank with all ingredients except sample was also monitored. Specific activity was calculated as micromole NADPH consumed per minute per milligram protein (i.e. U/mg protein) using an appropriate molar absorption coefficient ($\varepsilon = 6220$).

Assay of SOD activity

The SOD activity was measured according to McCord and Fridovich (28). Solution A was prepared by mixing 100 ml of 50 mM PBS (pH 7.4) including 0.1 mM EDTA and 2 μmol cytochrome c with 10 ml 0.001 N NaOH solution including 5 μM xanthine. Solution B included 0.2 U xanthine oxidase/ml and 0.1 mM EDTA. Fifty microlitres of sample were mixed with 2.9 ml of solution A and the reaction was started by adding 50 μl of solution B. Change in absorbance was

monitored at 550 nm. A blank was run by substituting 50 μl ultra pure water for sample. SOD levels are expressed as U/mg protein with reference to the activity of a standard sample of bovine copper-zinc SOD under the same conditions.

Assay of CAT activity
The CAT activity was measured in samples by the method of Luck (29). The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as micromole substrate decomposed per minute per milligram of protein (i.e. U/mg protein).

Determination of uric acid concentration
Uric acid concentration was measured according to the method proposed by Fossati et al. (30). In this assay, UA was transformed by uricase into allantoin and hydrogen peroxide, which, under the catalytic influence of peroxidase, oxidized the chromogen (4-aminophenazone/*N*-ethyl-methylaniline propanesulfonate sodic) to form a red compound whose intensity of color was proportional to the amount of UA present in the sample; it was read at a wavelength of 546 nm.

Statistical analysis
All values were expressed as mean ± SD. Statistical analysis was performed by using Student's *t*-test (two-tailed) and Fisher's exact test. Significance was set at $P < 0.05$.

Results

Antioxidants levels
Table 1 shows the plasma and saliva SOD, CAT, GSHPx activities and levels of UA concentration in patients and controls. We found decreased SOD and CAT activities ($P < 0.001$ and $P < 0.05$ respectively) and increased GSHPx activity ($P < 0.001$) in plasma compared with control.

No saliva could be drawn in five patients and seven controls. In the saliva increased SOD and CAT activities ($P < 0.001$ and $P < 0.05$ respectively) and decreased GSHPx activity ($P < 0.001$) were detected. Furthermore, no statistically significant difference in saliva was found for UA levels between patients and controls ($P > 0.05$).

Discussion

Despite plenty of research, the cause of RAS remains obscure. Many factors thought to have been involved in the aetiology of RAS might have, at the same time, a direct or indirect impact upon oxidant/antioxidant system. Despite a great deal of effort spent on the subject, only one study was reported in the literature investigating the possible relation between RAS and oxidative stress (25).

The signs regarding both local and systemic immune response disorders exist in RAS has been determined in several studies. Lukac et al. showed in their study that there was a decrease in phagocytic activity and ingestion ability of polymorphonuclear neutrophils (PMNs) in saliva (31). Sistig et al. found the level of B-lymphocytes (CD19), total T-lymphocytes (CD3) and the ratio of CD4 lymphocytes in the peripheral blood of the RAS patients in active state to be low but T-suppressor cells remaining unchanged. In the same study, spontaneous migration and ingestion values were also found in patients with acute RAS and, they showed a significantly depressed natural killer cell activity in patients with acute RAS (32).

Stenman et al. stated that the histological and enzyme histochemical investigations were indicated the early degeneration of individual, suprabasal epithelial cells. The vacuolated cells without metabolic activity were surrounded by cells having normal enzyme activity. The inflammatory reactions in the connective tissue are not observed at this stage. The increased distribution of vacuolated cells within the oral epithelium is occurred at further stages of the disease without any inflammatory reaction in the connective tissue (33, 34). Following this stage but still in the pre-ulcerative phase, a dense lymphocytic and monocytic infiltrate is observed closely associated with damaged epithelial cells, and there is exocytosis of these cells into the epithelium (33). Preponderant cells are CD4 (helper) T lymphocytes, with shift to CD8 (suppressor) T lymphocytes in the following ulcerative phase. These results indicate that the immunohistological changes in oral lesions of RAS manifest an enhanced immune response in the epithe-

Table 1 Plasma and saliva SOD, CAT, GSHPx activities, and UA levels of patients and controls

	SOD (U/mg protein)	*CAT (U/mg protein)*	*GSHPx (U/mg protein)*	*UA*
Plasma				
RAS ($n = 32$)	1347.33 ± 22.16	208.81 ± 6.91	24.62 ± 1.27	–
Control ($n = 30$)	1567.31 ± 37.51	227.11 ± 4,62	19.01 ± 0.67	–
P-value	< 0.001	< 0.05	< 0.001	–
Saliva				
RAS ($n = 27$)	0.86 ± 0.04	0.90 ± 0.04	1.70 ± 0.14	6.31 ± 3.29
Control ($n = 23$)	0.56 ± 0.11	0.78 ± 0.03	2.88 ± 0.18	5.26 ± 2.87462
P-value	< 0.001	< 0.05	< 0.001	> 0.05

SOD, superoxide dismutase; CAT, catalase; GSHPx, glutathione peroxidase; UA, uric acid.

lium, keratinocytes express human leucocyte antigen (HLA)-class II antigen and increased number of Langerhans cells as well as in the lamina propria with a prominent infiltration of CD4, CD8 and macrophage-like cells (33, 34). Buno et al. found the level of interleukins (IL)-2, IL-4, IL-5, IL-10, interferon (IFN)-α and tumour necrosis factor (TNF)-α mRNA to be higher in lesional biopsy in comparison with non-lesional biopsies of patients with RAS (12). Ueta et al. showed that lymphokine-activated killer activity was decreased in RAS. Nevertheless, there were no great differences in phagocytosis or natural killer (NK) activity among patients and healthy controls, in spite of the fact that NK activity in controls was slightly higher than that in patients. A higher proportion to neutrophil superoxide generation in RAS was observed as well (35).

Potent free radicals' attack on the oral mucosa leading to various alterations in a wide spectrum from infection to lethal cancer has been suggested in the literature (23). Dayan et al. in their experimental study showed the anticanceriogenic impact of saliva. In this study, saliva was shown to be inhibiting significantly the initiation and progression of tongue cancer in animal model (36). Nishioka et al. observed that the mutagenicity of well-known oral cancer inducers such as cigarette smoking, 4-nitroquinoline 1-oxide and benzopyrene was inhibited by saliva (37). It was also reported that saliva inhibits the production of ROS (38).

Several studies point out the relation between the inflammatory process and free radical mechanisms (39). Enzymes of the oxidative system may be induced by free radicals, which are produced as a result of infiltration and activation of lymphocytes. Human peripheral blood lymphocytes have been shown to be more sensitive to such oxidant agents as H_2O_2, and exhibiting more damage (DNA strand breaks) compared with other cell types (40).

There is no study in the literature with regard to saliva oxidant/antioxidant system in patients with RAS. We found in the study that SOD and CAT activity in saliva of the patient with RAS increased while the activity of GSHPx decreased but there was no significant change in UA level. Indications regarding inflammation in RAS have been shown by many authors (32, 33, 41, 42). Infiltration of immune system defence cells into lesion area in RAS has been reported and killer activity emerged from these cells result in an increase in the concentration of free radicals. Dismutation of increased superoxide radicals, in particular, can be achieved by high SOD activity. The increased activity of SOD results in overproduction of H_2O_2 that is a product of dismutation reaction. GSHPx is the dominant antioxidant defensive element participating in getting rid of H_2O_2 in the site. CAT is accepted to be secondary antioxidant enzyme in peroxidative defence. During the detoxification of increased H_2O_2 by GSHPx, the consumption of reduced glutathione also increases and thus enough reduced GSH may not be supplied for the detoxification of excessive H_2O_2, which has been generated as a result of both dismutations of superoxide radicals by SOD and immune cells. During oxidative stress the ratio of GSH/GSSG and as a result of which that of NADPH/NADP has been reported to be changed since, on one hand, GSH is rapidly consumed by GSHPx, on the other hand, oxidative glutathione generated by glutathione reductase through NADPH should be provided as reductive glutathione to the site (43, 44). This mechanism may not occur fast enough in the existence of high concentration of H_2O_2 for a long time and because of enough unprovided GSH as well as due to oxidative damages of free radicals existing in the site on GSH and GSHPx, the activity of GSHPx may have decreased. On the contrary, the variations in antioxidant enzyme activity in oxidative stress are linked with genetic control mechanism. In case of low concentration of free radicals, some antioxidant enzymes are reported to stimulate mRNA expression (45, 46). However, which molecules actively participate in this mechanism has not yet been determined extensively. Molecular studies on the subject are still needed.

The results we had from RAS plasma samples were not consistent with, but rather totally opposite of, antioxidant composition in saliva. We found that the activity of SOD and CAT in RAS plasma increased while that of GSHPx decreased. Çimen et al. whose study was the only one on the subject we managed to get from literature determined that the activity of GSHPx and CAT in plasma of RAS patients had decreased while MDA level had increased. The results indicate that antioxidant system in case of RAS has an impact on blood level (25). The reason why the GSHPx activity in saliva was low in our study was due to, we believe, possible high concentration of H_2O_2 in lesion area. Diffusion of H_2O_2 thought to be in high concentration in lesion area into plasma will be more limited and GSHPx activity elevated in response to possible low concentration of H_2O_2 in plasma will be able to lead to the detoxification of H_2O_2 in plasma. Furthermore, the plasma antioxidant system elements in the organism are likely to be deported to area where oxidative stress is localized is a subject, which has yet to be studied. As the activity of SOD is high in saliva but low in plasma it led us to bear in mind those antioxidant molecules or molecules that may lead to synthesis of mRNA for antioxidants are likely to be transported from plasma to the body fluids in areas where ulcers occur. We plan to research this theory by means of antioxidant molecules labeled with radioactive isotopes.

The data obtained from the study show that in RAS, the equilibrium in antioxidant system in both plasma and saliva has varied and the variation is not the same in plasma and saliva, and antioxidant system has also been affected in the process of RAS. However, the relationship between free radical mechanism and RAS still needs to be extensively studied in many respects.

References

1. Embil JA, Stephens RG, Manuel FR. Prevalence of recurrent herpes labialis and aphthous ulcers among young adults on six continents. *Can Med Assoc J* 1975; **113**: 627–30.

2. Fahmy MS. Recurrent aphthous ulcerations in a mixed Arab community. *Commun Dent Oral Epidemiol* 1976; **4**: 160–4.
3. Ship JA. Recurrent aphthous stomatitis. An update. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996; **81**: 141–7.
4. Porter SR, Hegarty A, Kaliakatsou F, Hodgson TA, Scully C. Recurrent aphthous stomatitis. *Clin Dermatol* 2000; **18**: 569–78.
5. Haisraeli-Shalish M, Livneh A, Katz J, Dollman R, Sela BA. Recurrent aphthous stomatitis and thiamine deficiency. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996; **82**: 634–6.
6. Mcleod RI. Drug-induced aphthous ulcers. *Br J Dermatol* 2000; **143**: 1137–9.
7. Nolan A, Lamey PJ, Milligan KA, Forsyth A. Recurrent aphthous ulceration and food sensitivity. *J Oral Pathol Med* 1991; **20**: 473–5.
8. Piskin S, Sayan C, Durukan N, Senol M. Serum ferritin, folic acid, and vitamin B12 levels in recurrent aphthous stomatitis. *JEADV* 2002; **16**: 66–7.
9. Jaber L, Weinberger A, Klein T, Yaniv I, Mukamel M. Close association of HLA-B52 and HLA-B44 antigens in Israeli Arab adolescents with recurrent aphthous stomatitis. *Arch Otolaryngol Head Neck Surg* 2001; **127**: 184–7.
10. Ghodratnama F, Riggio MP, Wray D. Search for human herpesvirus 6, human cytomegalovirus and varicella zoster virus DNA in recurrent aphthous stomatitis tissue. *J Oral Pathol Med* 1997; **26**: 192–7.
11. Tuzun B, Wolf R, Tuzun Y, Serdaroglu S. Recurrent aphthous stomatitis and smoking. *Int J Dermatol* 2000; **39**: 358–60.
12. Buno IJ, Huff JC, Weston WL, Cook DT, Brice SL. Elevated levels of interferon gamma, tumor necrosis factor alpha, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. *Arch Dermatol* 1998; **134**: 827–31.
13. Hasan A, Shinnick T, Mizushima Y, van der Zeer R, Lehner T. Defining a T-cell epitope within HSP 65 in recurrent aphthous stomatitis. *Clin Exp Immunol* 2002; **128**: 318–25.
14. Boulinguez S, Reix S, Bedane C, et al. Role of drug exposure in aphthous ulcers: a case-control study. *Br J Dermatol* 2000; **143**: 261–5.
15. Wray D, Rees SR, Gibson J, Forsyth A. The role of allergy in oral mucosal diseases. *QJM* 2000; **93**: 507–11.
16. Hollan S. Free radicals in health and disease. *Haematologia (Budap)* 1995; **26**: 177–89.
17. Gate L, Paul J, Ba GN, Tew KD, Tapiero H. Oxidative stress induced pathologies: the role of antioxidants. *Biomed Pharmacother* 1999; **53**: 169–80.
18. Mccord JM. The evolution of free radicals and oxidative stress. *Am J Med* 2000; **108**: 652–9.
19. Peterhans E. Oxidants and antioxidants in viral disease: disease mechanisms and metabolic regulation. *J Nutr* 1997; **127**: 962–5.
20. Young IS, Woodside JV. Antioxidants in health and disease. *J Clin Pathol* 2001; **54**: 176–86.
21. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am J Med* 1991; **91**: 14–22.
22. Sardesai VM. Role of antioxidants in health maintenance. *Nutr Clin Pract* 1995; **10**: 19–25.
23. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick A. Characterization of the differentiated antioxidant profile of human saliva. *Free Radic Biol Med* 2002; **32**: 268–77.
24. Zappacosta B, Persichilli S, de Sole P, Mordente A, Goardoma B. Effect of smoking one cigarette on antioxidant metabolites in the saliva of healthy smokers. *Arch Oral Biol* 1999; **44**: 485–88.
25. Cimen MYB, Kaya TI, Eskandari G, Tursen U, Ikizoglu G, Atik U. Oxidant/antioxidant status in patients with recurrent aphthous stomatitis. *Clin Exp Dermatol* 2003; **28**: 647–50.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *Biol Chem* 1951; **193**: 265–75.
27. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 1976; **71**: 952–58.
28. Mccord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969; **244**: 6049–55.
29. Luck H. *Methods of enzymatic analysis*, 2nd edn. New York: Academic Press, 1963; 885–88.
30. Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin Chem* 1980; **26**: 227–31.
31. Lukac J, Mravak-Stipetic M, Knezevic MA, et al. Phagocytic functions of salivary neutrophils in oral mucous membrane diseases. *J Oral Pathol Med* 2003; **32**: 271–4.
32. Sistig S, Cekic-Arambasin A, Rabatic S, Vucicevic-Boras V, Kleinheinz J, Piffko J. Natural immunity in recurrent aphthous ulceration. *J Oral Pathol Med* 2001; **30**: 275–80.
33. Stenman G, Heyden G. Premonitory stages of recurrent aphthous stomatitis: I. Histological and enzyme histochemical investigations. *J Oral Pathol Med* 1980; **9**: 155–62.
34. Poulter LW, Lehner T. Immunohistology of oral lesions from patients with recurrent oral ulcers and Behcet's syndrome. *Clin Exp Immunol* 1989; **78**: 189–95.
35. Ueta E, Umazume M, Yamamoto T, Osaki T. Leukocyte dysfunction in oral mucous membrane diseases. *J Oral Pathol Med* 1993; **22**: 120–5.
36. Dayan D, Hirshberg A, Kaplan I, Rotem N, Bodner L. Experimental tongue cancer in desalivated rats. *Oral Oncol* 1997; **33**: 105–9.
37. Nishioka H, Nishi K, Kyokane K. Human saliva inactivates mutagenicity of carcinogens. *Mutat Res* 1981; **85**: 323–33.
38. Nair UJ, Floyd RA, Nair J, Bussachini V, Friesen M, Bartsch H. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients. *Chem Biol Interact* 1987; **63**: 157–69.
39. Winrow VR, Winyard PG, Morris CJ, Blake DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br Med Bull* 1993; **49**: 506–22.
40. Pahlavani MA, Harris MD. Effect of in vitro generation of oxygen free radicals on T cell function in young and old rats. *Free Radic Biol Med* 1998; **25**: 903–13.
41. Splettstoesser WD, Schuff-Werner P. Oxidative stress in phagocytes – 'the enemy within'. *Microsc Res Tech* 2002; **57**: 441–55.
42. Duncan M, Grant G. Oral and intestinal mucositis-causes and possible treatments. *Aliment Pharmacol Ther* 2003; **18**: 853–74.

43. Aceto A, Dillio C, Angelucci S, et al. Glutathione related enzyme activities in testis of patients with malignant diseases. *Clin Chim Acta* 1989; **183**: 83–6.
44. Cotgreave IA, Gerdes RG. Recent trends in glutathione biochemistry –glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation? *Biochem Biophys Res Commun* 1998; **242**: 1–9.
45. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Toxicol* 1999; **39**: 67–101.
46. Janssen YM, van Houten B, Borm PJ, Mossman MT. Cell and tissue responses to oxidative stress. *Lab Invest* 1993; **69**: 261–74.