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Levels of lipid peroxides and antioxidants in smokers and nonsmokers

N. Garg[1], R. Singh[2], J. Dixit[1], A. Jain[2], V. Tewari[2]
Departments of [1]Periodontics and [2]Microbiology, King George's Medical University, Lucknow, Uttar Pradesh, India

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Background and Objective: The aim of the study was to evaluate the relationship between cigarette smoking and periodontal damage in terms of the levels of free radicals and antioxidants.

Material and Methods: Thirty-five healthy subjects in the age group 25–56 yr and with chronic moderate inflammatory periodontal disease (attachment loss of 3–4 mm) were selected. All subjects were matched with respect to the clinical parameters plaque index, gingival index and attachment loss. Of the 35 subjects, 25 were smokers (smoking a minimum of 15 cigarettes/day) and 10 were nonsmokers. Smokers were subdivided into three subgroups: group I (10 subjects smoking 15–20 cigarettes/day); group II (10 subjects smoking 21–30 cigarettes/day) and group III (five subjects smoking > 50 cigarettes/day). Gingival tissue (obtained during Modified Widman surgery) and blood samples were collected from each of the subjects and analyzed for the following parameters: lipid peroxide, superoxide dismutase, catalase, glutathione and total thiol.

Results: The level of lipid peroxide was lowest in nonsmokers (2.242 ± 0.775 in tissue and 1.352 ± 0.414 in blood) and highest in smokers smoking > 50 cigarettes/day (6.81 ± 1.971 in tissue and 4.96 ± 0.890 in blood), both in tissue and in blood. The increase was statistically significant in all groups, except in tissue of group I smokers. Catalase showed a similar trend, where the levels increased from 0.245 ± 0.043 in controls to 0.610 ± 0.076 in group III smokers for tissue, and from 0.231 ± 0.040 in controls to 0.568 ± 0.104 in group III smokers for blood. The increase was statistically significant for all groups. Total thiol levels were also higher in smokers than in controls (0.222 ± 0.050 in controls vs. 0.480 ± 0.072 in group III smokers in tissue; 0.297 ± 0.078 in controls vs. 0.617 ± 0.042 in group III smokers in blood). Except for group I in both tissue and blood, the increase was statistically significant. The superoxide dismutase (SOD) level was higher in nonsmokers (2.406 ± 0.477 in tissue and 2.611 ± 0.508 in blood) than in group III smokers (1.072 ± 0.367 in tissue and 0.938 ± 0.367 in blood), both in tissue and in blood, but this was significant only in the case of blood and for group III smokers in tissue. The glutathione level in tissue was consistently lower in smokers than in contols, showing a decrease from 121.208 ± 37.367 in controls to 46.426 ± 14.750 in group III smokers, but the decrease was not significant in group I smokers. In the case of blood, the glutathione level dropped from 262.074 ± 68.751 in controls to 154.242 ± 51.721 in group III smokers, but was statistically significant only for group III smokers.

Conclusion: The study results show that smoking increases the level of free radicals in periodontal tissues, which in turn may be responsible for the destruction seen in periodontal diseases.

Dr Nimit Garg F-69 Bali Nagar, New Delhi-110015, India
Tel: +91 989 1647510
e-mail: nimit_garg@yahoo.com

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Cigarette smoking accounts for approximately half of the cases of periodontitis diagnosed in young adults (1). Smokers are likely to show almost three times more severe periodontal disease compared with nonsmokers (2). Moreover, 30% of the incidence of periodontal disease in nonsmokers is caused by exposure to environmental, second-hand smoke (3). Thus, cigarette smoking is the single modifiable environmental factor responsible for the high prevalence of periodontal disease in the population. Cases of periodontal disease attributed solely to smoking are far greater than those caused by other important factors, such as diabetes mellitus (4). Accordingly, cigarette smoking has been demonstrated to fit all of the nine 'Bradford Hill Criteria' for causation and, as such, is proposed as a causal factor in severe periodontal disease (5).

In recent years, increasing evidence has supported the involvement of free oxygen radicals in several human diseases, such as atherosclerosis, rheumatoid arthritis, ischemic heart disease, inflammatory disorders and cancer. These free radicals are generated in many physiological processes, such as mitochondrial oxidation, oxygen transportation by haemoglobin (Hb) and cytochrome P_{450} activity. Normally, there is a delicate balance between the production of reactive oxygen species (free radicals) and tissue concentrations of antioxidants in the body. These free radical scavengers may take the form of enzymes [e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase], or low-molecular-weight free radical scavengers (e.g. vitamin E, total thiol, glutathione, etc.). However, when there is an excess of free radicals from exogenous (outside the body) sources, or an increased endogenous (within the body) production of these reactive oxygen species, the available tissue antioxidant systems may become overwhelmed, leading to oxidative damage to tissues.

A major exogenous source of free radicals is cigarette smoke (6–9). Cigarette smoke contains two classes of free radicals: one in the 'gas phase' and the other in 'tar'. There are $> 10^{18}$ organic free radicals per puff in the gas phase of cigarette smoke, while the tar phase has 10^{19} free radicals per gram (10). The obligatory use of the body reserve of antioxidants to detoxify the excess of these free radicals in smokers therefore results in an alteration in the level of different antioxidants. In addition, the antioxidant disturbance in smokers may be further enhanced by their generally lower intake of both supplemental and dietary antioxidants.

Despite the fact that the epidemiological evidence linking cigarette smoking with periodontal disease is overwhelming, the precise components of cigarette smoke responsible for this relationship, and the mechanisms by which they exert their effect, have not yet been clearly elucidated. The present study was therefore planned with the following aims and objectives.

1 To assess the level of lipid peroxidation product [malondialdehyde (MDA)] in blood and inflamed gingival tissue of smokers and nonsmokers.
2 To evaluate the levels of enzymatic antioxidants (SOD and catalase) and nonenzymatic antioxidants (glutathione and total thiol in blood and inflamed gingival tissue) in smokers and nonsmokers.

Material and method

The present study was conducted on 35 systemically healthy subjects (25–56 yr of age) with chronic inflammatory periodontal disease. Smokers were matched with nonsmokers with respect to the baseline characteristics of certain clinical parameters, namely gingival index (11), plaque index (12) and attachment loss. All exhibited chronic moderate periodontitis (1–3 mm of clinical attachment loss). Twenty-five of the subjects were smokers who smoked a minimum of 15 cigarettes/day at the time of initial examination. Smokers were further subdivided into three subgroups: group I subjects ($n = 10$), smoking 15–20 cigarettes/day; group II subjects ($n = 10$), smoking 21–30 cigarettes/day; and group III subjects ($n = 2$), smoking > 50 cigarettes/day. This contrasted with the study of Kaldahl *et al.* (13), who classified smokers as heavy smokers (≥ 20 cigarettes per day), light smokers (≤ 19 cigarettes a day), past smokers, and those who had never smoked. In the present study, as no smoker reported smoking 31–50 cigarettes/day, smoking criteria in group II were limited to 21–30 cigarettes/day. In group III, four subjects out of the total of five smoked 51–60 cigarettes/day. There was a single smoker who smoked 70 cigarettes/day; therefore, in group III, smoking criteria were based on smoking > 50 cigarettes/day. The remainder of the 10 subjects were nonsmokers who acted as a control.

Patients who had received active periodontal treatment within the 12 months before study entry, or who had received any drug therapy 30 d before study selection, were excluded from the investigation.

Gingival tissue

After the obtaining the subjects' consent to participate in the study, a gingival tissue sample was obtained under local anesthesia (lignocaine 2% with 1/80,000 adrenaline), while performing modified Widman Flap surgery. The excised tissue was thoroughly washed with chilled normal saline to remove blood and then stored in vials containing 0.15 M KCl.

Blood

Venous blood was obtained from the antecubital vein using a 2-ml syringe and placed in vials containing 3% citric acid.

Preparation of lysate

The blood was centrifuged at 2000 *g* for 20 min at 25°C. The plasma was removed aseptically. The red blood cell (RBC) pellet was washed three times with sterile saline (0.85 g/100 ml) to ensure complete removal of the plasma, leucocytes and platelets. The washed RBCs were haemolyzed by the addition of sterile distilled water (1 : 5). Then, the lysate was centrifuged at 800 *g* for 15 min at 4°C in order to make the lysate ghost free. The supernatant was used

as a source of various antioxidants (enzymatic/nonenzymatic).

Preparation of tissue homogenate

The gingival sample was weighed and a minimum of 400 mg of tissue sample was obtained. Tissue sample was then homogenized (10% w/v) in cold 0.15 M KCl using a tissue homogenizer.

Estimation of lipid peroxides

Lipid peroxide content in the gingival tissues and plasma was estimated according to the modified method of Ohkawa *et al*. (14). A 0.2-ml sample of plasma/tissue homogenate was mixed with 0.2 ml of 8% aqueous sodium dodecyl sulphate. Subsequently, 1.0 ml of 20% acetic acid was mixed in the above reaction mixture, and the pH of the mixture was adjusted to 4.0 using concentrated sodium hydroxide. After adjusting the pH, 1.5 ml of 0.8% 2-thiobarbituric acid, pH 7.2, and sufficient amounts of distilled water, were added to make the final volume 4.0 ml. The reaction mixture was incubated in a boiling water bath for 1 h and then centrifuged at 10062 ***g*** for 15 min. The clear supernatant obtained after centrifugation was used for measuring the absorbance at 532 nm. An appropriate standard of MDA (2.5 nm) was run simultaneously. A molecular extinction coefficient of 1.56×10^5/M/cm was used to calculate nmoles of MDA formed per mg of protein in plasma.

Estimation of the SOD level

SOD was estimated in the erythrocytic lysate/tissue homogenate by using the method of Misra & Fridovich (15). The assay system consisted of 2.45 ml of carbonate buffer (0.05 M, pH 10.2), a suitable aliquot of the lysate/tissue homogenate and water to make up the volume. Three hundred microlitres of epinephrine was added to give a final concentration of 2 mM to start the reaction. The reaction was monitored at 12-s intervals for 1 min, at 480 nm and 25°C. Suitable control lacking enzyme was run simultaneously. The enzyme unit expressed was calculated as the amount of the enzyme required to inhibit the auto-oxidation of epinephrine by 50%.

Estimation of catalase activity

The catalase activity was estimated in erythrocytic lysate/tissue homogenate by using the method of Aebi (16), with minor modifications. The reaction system, in a total volume of 3 ml, comprised 2.8 ml of phosphate buffer (50 mM, pH 7.0) and 100 µl of haemolysate. The reaction was started by quickly pipetting 0.1 ml of hydrogen peroxide (30 mmoles) at 25°C, and the absorbance was recorded at 240 nm, at 12-s intervals for 1 min. The rate constant of the first order reaction (K), related to the haemoglobin content (K/g of Hb), served as a measure of the specific activity of erythrocyte catalase.

Estimation of glutathione (GSH) level

The GSH level was measured in plasma/tissue homogenate according to the method of Ellman (17). The GSH level was measured in erythrocytic lysate/tissue homogenate after the precipitation of proteins with prechilled 10% (w/v) trichloroacetic acid (TCA). After 30 min, the mixture was centrifuged at 906 ***g*** for 10 min, and a 0.5-ml aliquot was mixed with 2 ml of 0.3 M disodium hydrogen phosphate solution. A 0.25-ml volume of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB reagent) in 100 ml of 1% (w/v) sodium citrate) was added just before measuring the optical density of the samples at 412 nm. The GSH solution of known concentrations (10–50 µg) was simultaneously processed to create a standard curve. The amount of GSH in the sample was measured by reference to the standard curve. The standard curve was obtained by plotting a line graph between a range of predefined concentrations of GSH (10–50 µg) and the absorbance obtained at 412 nm.

Estimation of total thiol group

The total thiol level was estimated in tissue/plasma by the method of Hu (18). A 0.2-ml sample of 10% plasma/tissue homogenate was mixed with water to make the volume up to 0.5 ml. Two milliliters of 0.3 M disodium hydrogen phosphate was added to each sample and 0.25 ml of DTNB reagent was added just before measuring the absorbance at 412 nm. Total thiol groups were calculated using an absorptivity of 13,600/cm/M.

Protein estimation was performed according to the method of Lowry *et al*. (19).

Statistical methodology

Data were entered into MS Excel, a component of MS Office software. Primary data analysis was carried out to check and clean the data. Data were analyzed using the statistical software package, STATA 6.0. A difference between the two values was considered to be significant only if the '*p*' value was found to be < 0.05. The following statistical tests were used to establish the difference between the two groups, as described below.

1. The two-sample *t*-test was used to determine the difference between the mean of two different groups, if data were normally distributed. The normal distribution of the data was checked by Shapiro Wilk's Statistics and if the *p*-value was < 0.05, data were considered to be nonnormally distributed.
2. If data were not found to be normally distributed, a nonparametric equivalent of two sample *t*-test, the Mann–Whitney test, was used to test the level of significance between two values.

Results

In the present study, smokers were matched with nonsmokers with respect to the baseline characteristics of clinical parameters (i.e. gingival index, plaque index and attachment loss). This ensured that any change in the level of lipid peroxide or antioxidants between smokers and nonsmokers could be solely attributed to cigarette smoking because all subjects selected for the study were systemically healthy.

The levels of lipid peroxide and antioxidants in tissue and blood are presented in Tables 1 and 2,

Table 1. Levels of lipid peroxide and antioxidants in tissue

Parameter	Group	*n*	Mean ± SD	Range	*p*-value (vs. control)
Lipid peroxide	Control	10	2.242 ± 0.775	1.09–3.85	
(nmole MDA/mg protein)	Exp. Gp I	10	2.51 ± 0.787	1.49–4.21	0.4528
	Exp. Gp II	10	3.249 ± 1.051	1.31–4.53	0.0253
	Exp. Gp III	5	6.81 ± 1.971	4.89–9.73	0.0022*
SOD	Control	10	2.406 ± 0.477	2–3.38	–
(units/ml tissue suspension)	Exp. Gp I	10	2.402 ± 0.346	2–2.67	0.8646*
	Exp. Gp II	10	2.268 ± 0.346	2–2.67	0.5469*
	Exp. Gp III	5	1.072 ± 0.367	0.67–1.34	0.0001
Catalase	Control	10	0.245 ± 0.043	0.1813–0.2971	–
(K/mg protein)	Exp. Gp I	10	0.288 ± 0.039	0.2213–0.3678	0.0298
	Exp. Gp II	10	0.363 ± 0.046	0.2982–0.4273	0.0000
	Exp. Gp III	5	0.610 ± 0.076	0.5285–0.7218	0.0022*
Glutathione	Control	10	121.208 ± 37.367	59.6–181.41	–
(μg/mg protein)	Exp. Gp I	10	103.246 ± 29.098	52.63–145.2	0.2459
	Exp. Gp II	10	89.631 ± 15.718	63.15–119.18	0.0297
	Exp. Gp III	5	46.426 ± 14.750	25.19–63.19	0.0010
Total thiol (mM)	Control	10	0.222 ± 0.050	0.1543–0.3289	–
	Exp. Gp I	10	0.251 ± 0.039	0.1998–0.3182	0.1794
	Exp. Gp II	10	0.281 ± 0.046	0.1963–0.3517	0.0138
	Exp. Gp III	5	0.480 ± 0.072	0.3914–0.5713	0.0022*

*p-value, as determined by the Mann–Whitney test.
Exp. Gp, experimental group; MDA, malondialdehyde; SD, standard deviation; SOD, superoxide dismutase.

Table 2. Levels of lipid peroxide and antioxidants in blood

Parameter	Group	*n*	Mean ± SD	Range	*p*-value (vs. control)
Lipid peroxide	Control	10	1.352 ± 0.414	0.8621–2.18	
(nmole MDA/mg protein)	Exp. Gp I	10	2.286 ± 0.583	1.56–3.12	0.0006
	Exp. Gp II	10	3.589 ± 0.952	2.51–5.73	0.0000
	Exp. Gp III	5	4.96 ± 0.890	3.82–5.94	0.0022*
SOD	Control	10	2.611 ± 0.508	2–3.38	–
(units/ml RBC suspension)	Exp. Gp I	10	2.003 ± 0.543	1.34–2.67	0.0186
	Exp. Gp II	10	1.802 ± 0.319	1.34–2	0.0005
	Exp. Gp III	5	0.938 ± 0.367	0.67–1.34	0.0000
Catalase	Control	10	0.231 ± 0.040	0.1812–0.2783	–
(K/g Hb)	Exp. Gp I	10	0.320 ± 0.041	0.2521–0.3841	0.0001
	Exp. Gp II	10	0.351 ± 0.085	0.1996–0.4893	0.0015
	Exp. Gp III	5	0.568 ± 0.104	0.4568–0.6892	0.0022*
Glutathione	Control	10	262.074 ± 68.751	164.74–373.41	–
(μg/mg protein)	Exp. Gp I	10	257.463 ± 63.322	150.41–349	0.8778
	Exp. Gp II	10	233.036 ± 38.799	169.47–285.12	0.2599
	Exp. Gp III	5	154.242 ± 51.721	95.62–222.62	0.0088
Total thiol	Control	10	0.297 ± 0.078	0.1899–0.4215	–
(mM)	Exp. Gp I	10	0.343 ± 0.110	0.1892–0.4892	0.2962
	Exp. Gp II	10	0.385 ± 0.064	0.2991–0.4612	0.0127
	Exp. Gp III	5	0.617 ± 0.042	0.5615–0.6772	0.0000

*p-value, as determined by the Mann–Whitney test.
Exp. Gp, experimental group; MDA, malondialdehyde; RBC, red blood cell; SD, standard deviation; SOD, superoxide dismutase.

respectively. The level of lipid peroxide was consistently lower in nonsmokers (2.242 ± 0.775 in tissue and 1.352 ± 0.414 in blood) than in smokers smoking > 50 cigarettes/day (6.81 ± 1.971 in tissue and 4.96 ± 0.890 in blood), both in tissue and in blood. The increase was statistically significant in all groups, except in the tissue of group I smokers. The SOD level fell throughout, from nonsmokers (2.406 ± 0.477 in tissue and 2.611 ± 0.508 in blood) to group III smokers (1.072 ± 0.367 in tissue and 0.938 ± 0.367 in blood) both in tissue and blood, but the decrease was statistically significant only in the case of blood and for group III smokers in the case of tissue. Catalase levels were consistently higher in smokers than in controls (0.245 ± 0.043 in the control group vs. 0.610 ± 0.076 in group III smokers, in the case of tissue; and 0.231 ± 0.040 in the control vs. 0.568 ± 0.104 in group III smokers, for blood) and the increase were found to be statistically significant for all groups. The

glutathione level in tissue was higher in the controls (121.208 ± 37.367) than in group III smokers (46.426 ± 14.750), but the decrease was not statistically significant in group I. In the case of blood, the glutathione level was higher in controls (262.074 ± 68.751) than in group III smokers (154.242 ± 51.721), but statistically significant only for group III smokers. For total thiol, the levels were higher in smokers than in the controls (0.222 ± 0.050 in controls vs. 0.480 ± 0.072 in group III smokers in tissue; 0.297 ± 0.078 in controls vs. 0.617 ± 0.042 in group III smokers in blood). Except for group I in both tissue and blood, the increase was statistically significant for the remaining groups.

Discussion

In this study, a deliberate effort was made to estimate certain clinical parameters (i.e. gingival index, plaque index and attachment loss), both in gingival tissue and in blood samples, as a reflection of the destructive effects of tobacco smoke, both locally (in periodontal tissues) and systemically.

The increase in the mean lipid peroxide level in smokers was caused by an increase in free radical production, which damages the cell membrane lipids. The increase in free radicals might be attributed to cigarette smoke, which by itself is a rich source of free radicals (7), and to polymorphonuclear neutrotrophil activation by cigarette smoke (20) which again, in turn, increases the free radical activity in the body. The difference in mean lipid peroxide level between control subjects and group I smokers in tissue was not significant, possibly because the subject smoked only a few cigarettes (15–20/d), and the increase in free radical activity might be adequately scavenged by the antioxidant defense system of the body.

The decrease in SOD level in smokers may be explained as follows: it is a well-known fact that hydrogen peroxide inactivates the enzyme SOD (21) and therefore it is possible that the low levels of SOD are caused by inactivation by hydrogen peroxide. Moreover, the decrease in mean SOD level was found to be significant in blood, but not in tissue, for smokers smoking < 30 cigarettes/day (groups I and II). This may be because in such smokers the free radicals, particularly hydrogen peroxide, generated by the direct interaction between smoke and periodontal tissues, may not be present in sufficient quantity to produce a significant change in the level of the antioxidant enzyme, SOD.

The increase in catalase activity might be caused by the fact that for a given concentration of catalase, the initial rate of hydrogen peroxide removal is directly proportional to the hydrogen peroxide concentration (22). The enzyme catalase comes into action only after a particular concentration of hydrogen peroxide has been obtained. Below that threshold concentration, catalase has no role to play. Thus, an increase in the catalytic activity in smokers may be caused by high levels of hydrogen peroxide formation.

GSH is a scavenger of hydroxyl radicals and singlet oxygen. It functions as a substrate for the hydrogen peroxide-removing enzyme, glutathione peroxidase (GPx). Exposure of the tissues to a large flux of hydrogen peroxide and hydroxyl radicals might result in an imbalance of the GSH/GSSG (reduced/oxidized GSH) ratio (23). GSSG accumulates in and contributes to various inactivating enzymes, which may have led to the lower levels of GSH found in smokers.

The fall in mean GSH level was not significant in smokers smoking < 20 cigarettes/day (group I) in either tissue or blood samples, probably because smoking up to 20 cigarettes/day may not upset the balance between oxidants and antioxidants sufficiently to cause a significant fall in the GSH level. On the other hand, in smokers of group II (21–30 cigarettes/day) a significant difference was found in the mean GSH level in tissue, but not in blood. This difference might be caused by the shielding of GSH in blood samples. GPx is one of three enzymes which functions as a potent antioxidant. There is a possibility that GSSG accumulation in blood inhibits GPx. As GPx is inhibited, GSH, which is a substrate for this enzyme, might not be utilized for the breakdown of hydrogen peroxide, to a certain extent. Thus, the decrease in GSH level was determined.

The increase in total thiol level in smokers may be explained as follows: essentially all of the plasma thiol groups are protein associated (24). In smokers, because of oxidative activity, there may be some structural modifications in proteins, which results in the exposure of protein-linked thiol groups to react freely with Ellman's reagent and thus depict a significantly higher total thiol level.

The insignificant increase in total thiol, both in tissue and blood in smokers smoking 15–20 cigarettes/day (group I), may be attributed to increased free radical production but in amounts that are scavenged by antioxidants, such that the damage is limited to few protein molecules.

The clinical importance of the present results suggests that free radical production mediated by cigarette smoke is highly toxic, and impaired oxidant-antioxidant balance is a risk factor in periodontal disease. As the severity of periodontal disease is affected both by the quantity and duration of smoking, as stated by Haber & Kent (25), further studies must be undertaken to establish the differences between analysis parameters of smokers and nonsmokers, as this parameter was not included in the present study.

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