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Oxidant-antioxidant status in tissue samples of oral leukoplakia

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ABSTRACT

Background: Imbalances between the oxidant-antioxidant status have been implicated in the pathogenesis of several diseases, including oral cancer. Majority of oral cancer are preceded by a well-recognized group of pre-malignant lesions. However, only a small fraction of those lesions, undergo malignant transformation. Hence, there is a great need to identify biological markers, which will assist in identifying lesion carrying high-risk. This study aims to evaluate and compare the status of oxidative stress and antioxidant enzymes in tissue samples of patients with various clinicopathological stages of oral pre-malignancy.

Materials and Methods: A case control study consisting of 20 new histopathologically proven leukoplakia patients and equal number of age, sex, and habit matched healthy subjects were recruited for this study. Their tissue samples were subjected to evaluation of lipid peroxidation product, thiobarbituric acid reactive substances and antioxidant enzymes, namely, superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GPx) using spectrophotometric methods. The data are expressed as mean ± standard deviation. The statistical comparisons were performed by independent Student's unpaired *t*-test and one-way analysis of variance. Pearson's correlation was performed for the biochemical parameters within the group and between the groups. For statistically significant correlations, simple linear regression was performed. P- value < 0.05 was considered statistically significant.

Results: Significant reduction in lipid peroxidation ($P < 0.001$) SOD and CAT ($P < 0.001$) was observed in the tissue of leukoplakia patients as compared to the healthy controls. On the other hand, GSH and GPx were significantly increased in tumor samples.

Conclusion: Reduced lipid peroxidation and increased activity of GSH and GPx provides the suitable environment for the tumor growth and malignant transformation in the later stages. Among the antioxidant enzymes, glutathione reductase appears to have a profound role in carcinogenesis.

Key Words: Antioxidant enzymes, lipid peroxidation, oral pre-cancer, oxidative stress

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INTRODUCTION

According to the international classifications of disease, cancers of the oral cavity include all the malignancies that are originating from the oral tissues such as lip, tongue, gum, floor of the mouth, and unspecified parts of mouth. More than 90% of oral cancers are mostly squamous cell carcinoma.[1] It is more common in the Southeast Asian countries as the most attributed risk factors such as usage of tobacco/ betel quid and alcohol habits are more prevalent in these regions. The incidence and prevalence of oral cancer in India constitutes about 12% of all cancers in men and 8% in women.[2] The most unfortunate aspect of oral cancer is its high morbidity and mortality rates, despite the availability of varied treatment options.

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The concept of a two-step process of cancer development in the oral mucosa that is the initial presence of a pre-cursor (pre-malignant, pre-cancerous) lesion subsequently

developing into cancer is well-established. Oral leukoplakia (OL) is the best known potentially malignant disorder of the oral mucosa.[3] It has recently been redefined as “a white plaque” of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer.[4]

OL, except the non-homogenous type, are asymptomatic in nature and thus poses a difficulty in its early detection. Diagnosis and prediction of neoplastic transformation of these lesions are primarily based on its histological grading (dysplasia). The risk of malignant transformation of leukoplakia varies from 0.3% to 25%. The presence of dysplasia in OL lesion increases the malignancy incidence by over 30%.[5] The programs in cancer control are based on the premise that the earlier the cancer is diagnosed, the better the outcomes in terms of increased survival and reduced mortality.[6] Thus, the search for molecular biological markers for predicting malignant transformation of oral pre-malignant lesions becomes increasingly important.

Substantial evidence indicates that tobacco consumption increases the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Either directly or through activation of inflammatory cascade, ROS and RNS are proposed to play a key role both as initiators and promoters in carcinogenesis.[7] Low levels of ROS are required in various homeostatic mechanisms and are effectively counterbalanced by an array of antioxidant enzymes. When there is an excess of ROS or a state of depleted antioxidant enzymes (known as oxidative stress), lipid peroxidation is initiated and thus can cause DNA damage later.[8]

ROS has been implicated in the development and progress of various oral conditions such as, lichen planus, recurrent aphthous ulcer, and periodontitis.[9,10] Recent studies have also demonstrated the development of oxidative stress in the circulation of patients with oral cancer.[11] Even in this state, the malignant cells had shown an uninterrupted growth. This had laid the foundation for the studies assessing the tumor biology in response to the ROS insult. Reduced peroxidation levels with altered antioxidant enzymes are observed at the tissue level and presumed to be the reason for continuous growth of the tumor.[12] Thus, it reflects that serum and tissue behave differently to ROS.

Only a few studies were performed on serum samples of potentially malignant disorder like oral sub-mucous fibrosis. They have depicted increased lipid peroxidation with compromised antioxidants in the patient’s circulation.[13]

To the best of our search, no studies have been undertaken to evaluate the tissue behavior to ROS in leukoplakia. The present study also outscores the previous investigators in terms of comparing parameters such as thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPx), and catalase (CAT) in various clinicopathological stages of leukoplakia. This study is undertaken in an attempt to evaluate the relationship between the biochemical parameters and dysplasia, which in turn will reflect their impact on malignant transformation. The usage of statistical tools also appears to be limited in comparable studies.

Therefore, this study aims to evaluate and compare the status of oxidative stress and antioxidant enzymes in tissue samples of patients with various clinicopathological stages of oral pre-malignancy.

MATERIALS AND METHODS

Patients

This study was conducted at the Department of Oral Medicine & Radiology, Rajah Muthiah Dental College and Hospital, Annamalai University, Annamalai Nagar, India. Twenty, newly diagnosed, biopsy proven cases of leukoplakia were recruited for the study (group 1). All the patients in this group had a history of tobacco usage. Twenty, age and sex matched, healthy volunteers with tobacco chewing habit were included in the control group (group 2). The Institutional Ethical Committee approved the study and written informed consent was obtained from all the participants of the study. None of the subjects included in the study had any concomitant disease such as diabetes, hypertension, liver or kidney disorders or other systemic diseases. Leukoplakia patients, who had previously undergone any treatment, were excluded from the study. All the subjects were interviewed before a thorough clinical examination. A questionnaire was used to collect the data regarding demographic factors, type of habits, frequency, and duration of habits. The group 1 patients were divided into stages I-IV on the basis of the Oral Leukoplakia (OLEP) staging system.[14]

Tissue sample collection

Surgically resected tumor tissues were obtained. Later, the samples were homogenized in phosphate

buffer saline, pH 7.4. The cytosols were separated by centrifugation at 20,000 (rpm) in cooling centrifuge. Normal uninflamed tissues were taken from disease free, healthy subjects who underwent surgical removal of impacted teeth or vestibuloplasty.

Biochemical assays

Lipid peroxidation in tissue is analyzed by the method of Ohkawa *et al*.[15] The reaction of thiobarbituric acid with breakdown products of lipid peroxidation will result in pink color chromogen, to be read calorimetrically at 532 nm.

GSH was estimated by the method of Beutler and Kelly.[16] This method was based on the development of yellow color, read at 412 nm spectrophotometrically, when 5,5'-dithiobis 2-nitrobenzoic acid reacts with the supernatant. SOD was assayed by the method of Kakkar *et al*.[17] It is based on the 50% inhibition of the formation of Nicotineamide-adenine-dinucleotide (NADH) NADH-phenazine methosulphate nitroblue tetrazolium formazan at 520 nm. The activity of CAT was assayed by the method of Sinha,[18] based on the utilization of H_2O_2 by the enzyme. The color developed was read at 620 nm. GPx activity was estimated by following the utilization of hydrogen peroxide according to the method of Rotruck *et al*.[19]

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD), whereas qualitative data in numbers and percentiles. Tabulation of the results was carried out for leukoplakia and the control group. All the variables of the study were statistically analyzed for the mean values, SD, and *P* value. The statistical comparison of biochemical parameters between the case and the control group was performed by unpaired Student's *t*-test. Analysis of variance (ANOVA) was used to compare parameters in various OLEP stages. Pearson's correlation was used to evaluate whether any correlation exists between TBARS and antioxidant enzymes. A similar correlation analysis was carried out among antioxidant enzymes. For statistically significant correlations, Simple linear regression was performed. The data were analyzed using the Statistical Package for Social Sciences (SPSS) 13.0 package. In all the above tests, the *P* value <0.05 was considered statistically significant; *P* value >0.05 was taken to be statistically not significant; *P* value <0.01 was taken to be statistically highly significant and *P* value <0.001 as very highly significant.

RESULTS

In our study, the average age of patients with leukoplakia was 46.20 ± 11.08 to a maximum percentage (60%) of patients falling in the range of 35-50 years. The study sample showed male predominance (75%) with a male to female ratio of 3:2. The distribution of the lesions seen was minimum at the lateral border of tongue (5%) and maximum at the buccal mucosa (70%). Alveolus and vestibule contributed to 15% and 10% respectively. We found that all our patients used tobacco with or without additives with an average duration of habit as 21.55 ± 10.70 years and average frequency of 7.75 ± 3.32 times/day [Table 1].

Table 1: Clinicopathological characteristics of leukoplakia patients (group 1) and control group (group 2) participated in the study

Variable	Subcategories of variable	Statistic values (%)
Sample size (*n*)	Group I (leukoplakia)	20
	Group II (control)	20
Age	Group I	
	Range (in years)	22-70
	Mean and SD	46.20±11.08
	SEM	2.47
	Group II	
	Range (in years)	25-60
	Mean and SD	39.55±9.22
	SEM	2.06
Gender	Group I	
	Male	15 (75)
	Female	5 (25)
	Group II	
	Male	15 (75)
	Female	5 (25)
Duration of habit (years) group I - displayed in class intervals	0-10	2 (10)
	11-20	7 (35)
	>20	11 (55)
	Mean and SD	21.55±10.70
	SEM	2.39
Frequency of habit (times/day) - group I in class intervals	0-5	2
	6-10	13
	11-15	5
	Mean and SD	7.75±3.32
	SEM	0.74
Site of the lesion (for group I)	Buccal mucosa	14 (70)
	Alveolus	3 (15)
	Vestibule	2 (10)
	Tongue	1 (5)
Clinical stage (OLEP) - for group I	I	5 (25)
	II	5 (25)
	III	5 (25)
	IV	5 (25)

SEM: Standard error of mean; SD: Standard deviation; OLEP: Oral Leukoplakia Staging

Table 2 shows a comparison of lipid peroxidation end products TBARS and various antioxidant enzyme profiles, between leukoplakia patients and control subjects. The extent of lipid peroxidation was significantly ($P < 0.001$) decreased in leukoplakia patients, as compared to control subjects. Among the antioxidant enzymes, SOD and CAT were significantly ($P < 0.001$) decreased, whereas GSH and GPx were significantly increased, when compared to the normal controls.

Lipid peroxidation and antioxidant enzymes were also evaluated according to tumor staging. TBARS showed a significant ($P < 0.05$) decrease only in respect to stage IV of leukoplakia patients. Among antioxidant enzymes, only GSH and GPx showed significant ($P < 0.001$) decrease along the stages of the disease [Table 3].

In Pearson's correlation analysis, TBARS showed a significant ($P < 0.001$) positive correlation with SOD and CAT, and a significant ($P < 0.001$) negative correlation with GSH and GPx [Table 4].

In an attempt, to study the relationship between the antioxidant enzymes, it was found that GSH had a significant negative correlation with SOD ($P < 0.001$) and CAT; and significant positive correlation with GPx ($P < 0.001$) [Table 4]; thus, laying stress on the crucial role played by GSH enzyme. Henceforth, exploration of GSH was performed with a simple linear regression model [Table 5]. With the presumption of GSH being an independent variable, a reliable prediction of the dependent variables such as GPx, SOD, CAT, and TBARS ($P < 0.001$) can be made. A significant proportion of variance ($R^2 = 0.79$; 79%) in the GPx is thought to be brought by GSH. Regression equation (GPx = 23.27 + 1.05GSH) will be able to predict the value of GPx, for a unit change in GSH.

DISCUSSION

We observed significant decrease ($P < 0.001$) tissue levels of TBARS in patients with leukoplakia as compared to the control subjects [Table 2]. This is suggestive of decreased activity of lipid peroxidation at the tumor tissue level. Similar results were reported in the tissues of oral cancer as well.[12] Continuous cell proliferation is the essence of carcinogenesis. For such uninterrupted growth of tumor cells, lipid peroxidation has to be at a very low level. Inverse relationships have been observed between the levels of lipid peroxidation and the rate of cell proliferation.[20]

Reduced lipid peroxidation at the tissue level can be attributed to the altered lipid profile at the circulation and tissue level.

Table 2: Comparison of tissue levels of TBARS, SOD, GSH, GPx, and CAT between the normal controls and leukoplakia (all values are expressed in mean±SD)

Study groups	Parameters				
	TBARS (nM/mL)	SOD (U/g Hb)	GSH (mg/dl)	GPx (U/g Hb)	CAT (U/g Hb)
Group I: Leukoplakia (*n*=20)	91.99±2.97	14.48±1.05	30.43±2.90	22.99±3.43	6.36±1.10
Group II: Control (*n*=20)	127.93±2.97	18.54±0.54	22.90±1.10	15.16±0.48	10.46±0.79
P value	0.000*	0.000*	0.000*	0.000*	0.000*
Inference	VHS	VHS	VHS	VHS	VHS

Student's unpaired *t*-test, **P* < 0.001. VHS: Very high significance; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; CAT: Catalase

Table 3: Comparison TBARS, SOD, GSH, GPx, and CAT among clinical stages of leukoplakia group (all values are expressed in mean ± SD)

Parameters	Study groups				
	Group II: Control (n = 20)	Group I			
		Stage I	Stage II	Stage III	Stage IV
TBARS (nM/mL)	127.93±2.97	95.92±1.45$^{a,\#}$	92.90±1.07$^{a,\#}$	90.52±0.99$^{a,\#,b,‡}$	88.63±0.76$^{a,\#,b,\#,c,*}$
SOD (U/g Hb)	18.54±0.54	15.46±0.42$^{a,\#}$	14.72±0.50$^{a,\#}$	14.38±0.83$^{a,\#}$	13.36±1.12$^{a,\#,b,\#,c,*}$
GSH (mg/dl)	22.90±1.10	26.80±1.12$^{a,\#}$	29.56±1.06$^{a,\#,b,‡}$	31.22±0.96$^{a,\#,b,\#}$	34.17±0.89$^{a,\#,b,\#,c,\#,d,‡}$
GPx (U/g Hb)	15.16±0.48	18.60±0.55$^{a,\#}$	22.40±1.09$^{a,\#,b,\#}$	23.66±2.37$^{a,\#,b,\#}$	27.30±0.85$^{a,\#,b,\#,c,\#,d,\#}$
CAT (U/g Hb)	10.46±0.79	7.26±0.74$^{a,\#}$	6.72±0.75$^{a,\#}$	6.36±0.83$^{a,\#}$	5.12±0.95$^{a,\#,b,‡}$

One-way ANOVA test. [a]Compared to control subjects, [b]Compared to stage I, [c]Compared to stage II, [d]Compared to stage III, [#]*P*<0.001, [‡]*P*<0.01, [*]*P*<0.05. TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; CAT: Catalase

Because of the increased rate of peroxidation of polyunsaturated fatty acids in the plasma, there is a greater utilization of lipids leading to its reduced levels.[11] Depleted levels of blood lipid have been associated with various cancers including oral cancer and pre-cancer[21] During the course of peroxidation, there is a release of peroxide radicals. They attack upon the essential constituents of the cell membrane like cholesterol. Under physiological conditions, it maintains the structural and functional integrity of the biological membrane along with the stabilization of DNA helix[20,21] Thus, low serum cholesterol has also found to be related with the increased risk of cancer occurrence and mortality[22]

In order to maintain the pace of cellular proliferation, cells sequester lipids including total cholesterol, lipoproteins, and triglycerides from the plasma, for the neogenesis of cellular membranes. Raised cholesterol level inside the cell had brought rigidity in the biomembrane, thus making it less assessable for attack by oxygen radical species[21] Hence, tumor cells have raised cholesterol or cholesterol/ phospholipids ratio.

Table 4: Correlation among antioxidant enzymes in leukoplakia group

Parameters	TBARS	SOD	GSH	GPx	CAT
TBARS	1	*r*=0.843 *P*=0.000# PC; VHS	*r*=−0.963 *P*=0.000# NC; VHS	*r*=−0.854 *P*=0.000# NC; VHS	*r*=0.814 *P*=0.000# PC; VHS
SOD	—	1	*r*=−0.799 *P*=0.000# NC; VHS	*r*=−0.694 *P*=0.023* NC; SS	*r*=0.907 *P*=0.000# PC; VHS
GSH	—	—	1	*r*=0.890 *P*=0.000# PC; VHS	*r*=−0.780 *P*=0.000# NC; VHS
GPx	—	—	—	1	*r*=−0.648 *P*=0.002‡ NC; HS
CAT	—	—	—	—	1

r: Pearson's correlation coefficient; NC: Negative correlation; PC: Positive correlation; VHS: Very high significance; HS: High significance; SS: Statistically significant; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; CAT: Catalase. #*P*<0.001, ‡*P*<0.01, **P*<0.05

Raised level of TBARS in plasma samples of pre-cancer[11] is in contrast to the result of the current study of tissue. This indicates that the tissue and serum are two different compartments with varied biological behavior. Mean TBARS level among the stages of OL was not of much statistical significance [Table 3].

In the current study, it has been observed that antioxidant enzymes display different patterns of activity at the tissue level. Statistically, significant ($P < 0.001$) decrease in SOD and CAT with increased GSH and GPx levels were recorded [Table 2].

As a matter of fact, SOD, CAT, and GPx are considered to be the first line of defense against the free radical attack. Increased lipid peroxidation in the serum will produce large quantities of free radicals including superoxide anion (O_2^-). This gets easily diffused across the membrane and gets accumulated in large volumes even inside the cell. SOD gets thoroughly utilized in scavenging superoxide anion via dismutation reaction. This reaction produces hydrogen peroxide (H_2O_2) as a byproduct, which is also a potentially reactive radical. In an attempt to eliminate hydrogen peroxide, CAT, then gets consumed. Apart from reaction's byproduct, hydrogen peroxide is also generated in a considerable amount within the cell[23]

With the increasing levels of H_2O_2 and depleting CAT, GPx emerges as a savior for the cell. It has a higher affinity for H_2O_2 than CAT or SOD and also has a wider range of action on various hydroperoxides produced during the process of lipid peroxidation. In situations of increasing ROS within the cell, neoplastic cell has shown to compensate the need for antioxidant enzyme by absorbing them from the serum[24] Previous reports have thus shown the reduced levels of all antioxidant enzymes in the serum samples from leukoplakia patients.[11]

Table 5: Simple linear regression the parameter TBARS, SOD, GPx, and CAT on GSH (regression model $y = a + b$GSH)

Parameters	*a* value	*b* value	R^2 value	*P* value	Regression line
TBARS	122.03	−0.98	0.927	0.000*	TBARS=122.03−0.98GSH
SOD	23.27	−0.28	0.638	0.000*	SOD=23.27−0.28GSH
GPx	23.27	1.05	0.793	0.000*	GPx=23.27+1.05GSH
CAT	15.42	−0.29	0.609	0.000*	CAT=15.42−0.29GSH

**P*<0.001. VHS: Very high significance; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; CAT: Catalase

Along with GPx, GSH levels were also higher in the current study. GSH has got high redox potential and thus it acts as potent antioxidant and a suitable cofactor for enzymatic reaction. Because of these properties, GPx utilizes it as a cofactor in the process of neutralizing H_2O_2. GSH is also known to have a prominent role in detoxification of chemical carcinogens and protection of cell against cytotoxic oxygen free radicals. Known carcinogens from tobacco smoke or quid have been found to be predominantly detoxified by glutathione dependent enzymes. Prolonged direct contact of the quid with the oral mucosa leads to the seepage of the carcinogens and finally gets concentrated in high volumes in the local environment of the tissue. This leads to the increased activity of GSH in the tumor tissue[25] Over expression of the GPx and GSH has been reported in a wide range of malignant conditions[26]

In the present study, only GSH and GPx have shown a highly significant increase from stage I to stage IV patients despite a small sample size in each category of stages I-IV (5 each) [Table 3]. These results also reflect the decisive role played by these enzymes. Thus, the tumor cells have a low availability of the substrate for lipid peroxidation. This, along with increased levels of GSH and GPx, facilitates the growth of the tumor.

The current study also made an attempt to explore the individual interactions among the various biochemical parameters under consideration. Correlation and regression statistical tools were used. TBARS have shown significant ($P < 0.001$) positive correlation with SOD and CAT, whereas negative correlation with GSH and GPx [Table 4]. On performing correlation analysis among the enzymes, GSH showed significant ($P < 0.001$) positive correlation with GPx [Table 4]. This observation, that both have interplay in the glutathione redox cycle operating for the purpose of detoxifacating H_2O_2 is widely documented in the literature[27] Another evidence for this observation comes from the significant ($P < 0.001$) regression analysis between GSH and GPx. Furthermore, Considering the R^2 value, the contribution of GSH in the variance seen in GPx was found to be 79% [Table 5]. Hence, according to our results, GSH and GPx have emerged as the most influencing parameter.

We hypothesize that serum interaction in comparison to tissue, poses an increased threat as it produces free radicals in a large amount which get readily diffused inside the cell to cause various mutations, favoring carcinogenesis. The tissue, on the other hand, is producing a relatively lesser amount of free radical and at the same time is capable of neutralizing them with the available enzymes. Therefore, it can be assumed that along with the internal factors, external environment also influences the selective growth of the tumor cells.

CONCLUSION

Reduced lipid peroxidation along with raised levels of GSH and GPx in the leukoplakia tissue is reported in the current study. This creates conducive internal environment for the tumor growth and thus predisposes it for malignant transformation. This altered oxidant-antioxidant status is more pronounced in the advanced clinical stages of leukoplakia. Thus, the critical levels (in tissue) of TBARS, GSH, and GPx have a potential to act as oxidative markers for the identification of high-risk lesions.

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