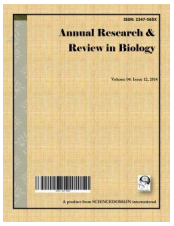

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Oxidative Stress and Antioxidant Defenses: With Special Reference to Oral Lesions

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Authors' contributions

This work was carried out in collaboration between all authors. Author AS designed the study performed the statistical analysis and decided the protocol. Author PT managed the analysis, literature searches of the study and wrote the first draft of the manuscript. Author AY managed the sample collection for the study. All authors read and approved the final manuscript.

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ABSTRACT

Background: Oxidative stress is involved in the pathogenesis of various lifestyle-related diseases, including malignancies. The body naturally produces antioxidants as a means of defending itself against these free radicals which neutralize them, thereby rendering them harmless to other cells. There is a close relation between oxidative stress and all aspects of cancer, from carcinogenesis to the tumor-bearing state, from treatment to its prevention.
Aim: The present study was aimed to estimate the plasma levels of antioxidant enzymes and molecules in cases of oral lesion patients.
Study Design and Methodology: A case control study was designed in Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow campus. A total of fifty histopathologically proven oral lesion cases (leukoplakia, erythroplakia, lichen planus and oral submucous fibrosis patients) were taken for the study. Their blood samples were collected and plasma was subjected to

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evaluation of oxidative stress markers. Control group consisted of equal number of healthy subjects. The data is expressed as mean±SD. Student -t test was applied for significance of the biochemical parameters.

Results: The results have demonstrated that levels of catalase, myeloperoxidase, reduced glutathione glutathione reductase and glutathione peroxidase are decreased whereas those of malondialdehyde and nitric oxide have increased in the oral lesions patient group as compared to controls.

Conclusion: Oxidative stress has been shown to be an important indicator in case of oral cancer. Similar findings in pre-malignant oral lesions can be correlated in establishing the role of oxidative stress in initiation and conversion of premalignant lesions into malignant ones.

Keywords: Oxidative stress; free radicals; oral lesions; oxidative stress markers; oral submucous fibrosis; leukoplakia; erythroplakia; lichen planus.

1. INTRODUCTION

Cells of the body undergoing aerobic metabolism are always in danger from reactive nitrogen species (RNS) and reactive oxygen species (ROS). But, powerful and highly efficient antioxidants neutralize such free radicals. These antioxidants include various free-radical 'scavenger' molecules and antioxidant enzymes. When there is a disturbance in the balance between production of ROS/RNS and antioxidant defense-system, it leads to oxidative stress, resulting in damage of cells, tissues and organs of the body. Peroxidation of lipids, DNA damage and oxidation of proteins are the most common outcomes of this oxidative stress and they lead either towards abnormal functioning of a cell or its untimely death [1]. The ROS/ RNS- mediated diseases include various conditions such as gastro-intestinal ulcers, neurodegenerative disease, cardiovascular diseases, rheumatoid arthritis, metabolic disorders and cancer [2]. Recent researches have established the association between oxidative-stress with pre-cancer and cancer [3,4]. Free radical generation results in damage to the biological molecules and hence constructs the main basis for development of cancer [5].

Cancer of the oral cavity comes under head and neck cancers and comprises approximately 85% of this category. It is reported to be one of the most widespread and the sixth most common malignancy in the world [6]. Around 275,000 people are diagnosed with oral cancer annually worldwide [7]. It is the most important form of cancer in India. On the other hand, a pre-cancer or pre-malignant state can be defined as the condition of disordered cell-morphology that has its association with an elevated risk of cancer development. If proper treatment is not administered, it may cause cancer. The most common type of oral potentially malignant lesions includes leukoplakia, erythroplakia, erythroleukoplakia, proliferative verrucous leukoplakia, lichen planus and oral submucous fibrosis.

ROS/ RNS can cause breakage of DNA strands, alterations of its bases, increased proto-oncogene expression and damage to tumor suppressor genes. They are known to cause damage to proteins and deteriorate membrane lipids resulting in their peroxidation that leads to the formation of products like malondialdehyde (MDA). Antioxidant enzymes viz., superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), myeloperoxidase (MPO) and non-enzymatic antioxidants viz., reduced glutathione (GSH) are found to act in synergism with one another to detoxify the harmful effects of lipid peroxidation [8]. The present study was planned to evaluate the oxidative stress level in four oral lesion patients by measuring the plasma levels of five antioxidant enzymes (Catalase, GPx, GR, MPO and SOD) and three molecules (GSH, MDA and NO).

2. MATERIALS AND METHODS

2.1 Study Group and Its Characteristics

Fifty patients admitted with oral lesion problem (leukoplakia, erythroplakia, lichen planus and oral submucous fibrosis) in outpatient department of Saraswati Dental College and Hospitals, Lucknow from both rural and urban areas were enrolled for the study. The patients were of both sexes and within the age group of 21-50 years. The ethical committee of the dental college has approved the study. Equal number of age, sex and habit matched healthy subjects were included in the study as control group. A proforma directed history was taken and examination performed on admission. A detailed

case history of the patients with emphasis on their habits (chewing Gutka and/or Tobacco and/or betel nut, smoking Cigarettes and/or Bidi, taking gulmanjan or alcohol) was taken and recorded on a standard proforma along with thorough clinical examinations. Histopatho-logically OSMF patients were divided into grade 1/2/3 on the basis of staging system.

Inclusion criteria for the study was patients having any one of the four above mentioned clinically confirmed oral lesions, of 18 years and above, without any other predisposing disease and willing to participate in the study. Patients diagnosed/treated for any malignancy in the past, having any other condition like hypertension, diabetes etc. or not willing to participate were excluded from the study.

2.2 Blood Sample Collection

Blood sample, (3-5 ml.) were collected from both the study and control group by venous arm puncture under aseptic precautions and transferred into presterilized EDTA vials. The collected blood was then subjected to centrifugation at 3000 rpm for 10 min to segregate plasma and erythrocytes. All biochemical estimations were carried out in plasma on the same day.

2.3 Biochemical Estimation

2.3.1 Estimation of catalase activity

The catalase activity in the plasma samples was measured using the protocol of Luck et al., (1974) with some modifications [9]. Hydrogen peroxide phoshate buffer (H_2O_2-PO_4) and plasma sample were mixed properly and its absorbance was recorded immediately at 240nm against a blank. The time taken for decrease in absorbance by 0.05 unit was noted down. If the time exceeded 60 sec then the assay was repeated by using less diluted plasma. The catalase levels were calculated and values were presented as units of catalase/ml of plasma (units/ml).

2.3.2 Estimation of glutathione peroxidase (GPx) activity

The protocol of Rotruck et al. with some modifications was used to assess the glutathione peroxidase levels in plasma samples [10]. 0.3 M phosphate buffer, 0.2 ml of sample, 0.2 ml of EDTA, 0.2 ml of H_2O_2 and 0.2 ml of 4 mM reduced glutathione were mixed properly in a test tube. The final volume was made up to 2ml with double distilled H_2O. This tube was incubated at 37ºC for 90 min. 1 ml of 10% TCA was added to the mixture and centrifuged. The supernatant was separated and 0.3 M Na_2HPO_4 solution was added to it. 1.0 ml of DTNB reagent was added to the reaction mixture just before monitoring the absorbance at 412 nm. GPx activity was calculated and presented as units of GPx / ml of plasma (units/ml).

2.3.3 Estimation of glutathione reductase (GR) activity

Measurement of the glutathione reductase activity in the plasma samples was done according to the modified protocol of Carlberg et al. [11]. 0.1M phosphate buffer, 0.4ml of sample was mixed with 0.2mM NADPH, 0.1ml of GSSG and 1.8ml of double distilled H_2O. Its absorbance was measured at 340nm. The decrease in absorbance was monitored for 2 min.

2.3.4 Estimation of reduced glutathione (GSH)

The protocol given by Moron et al. along with some modifications was used to assess the reduced glutathione levels in plasma samples [12]. 0.3 ml of TCA and 0.3ml of plasma sample were mixed properly and refrigerated for 10 min followed by centrifugation. 0.1ml of the supernatant obtained was mixed with 0.2M phosphate buffer and 2ml of DTNB reagent. It was incubated for 15 min at 37ºC and absorbance was measured at 412nm.

2.3.5 Estimation of malondialdehyde (MDA)

Measurement of malondialdehyde levels in the plasma samples were done with the help of the protocol of Ohkawa, along with some modifications [13]. 0.4ml of plasma sample and 0.1 ml of 10% SDS were mixed with 1.3 ml H_2O followed by incubated for 5 min at room temperature. After this, 0.6ml of CH_3COOH was added to the reaction mixture and incubated again for 2 min at room temperature. 0.8% TBA was added which was followed by a final incubation of 1hr in a boiling water bath followed by centrifugation at 4ºC for 10 min at 10,000 rpm. The supernatant was separated and absorbance was recorded at 532 nm. The MDA levels in blood plasma were calculated as units of MDA / ml of plasma (nmol/ml).

2.3.6 Estimation of myeloperoxidase (MPO) activity

The myeloperoxidase activity in the plasma samples was measured with the help of the protocol of Suzuki et al. along with some modifications [14]. 0.01 ml of plasma sample, 0.08 ml of H_2O_2 and 0.11ml of TMB solution were mixed properly and incubated for 5 min at 37ºC. 0.05 ml of 2 M H_2SO_4 was added to this mixture to stop the reaction and absorbance was measured at 450 nm.

2.3.7 Estimation of nitric oxide (NO)

The protocol of Griess et al. with some modifications was used to assess the nitric oxide levels in plasma samples [15]. Equal volumes of sample and Griess reagent were incubated for 10 min at room temperature and absorbance was measured at 540 nm.

2.3.8 Estimation of superoxide dismutase (SOD) activity

Measurement of the superoxide dismutase activity in the plasma samples was done by means of the modified protocol of Nishikimi et al. [16]. 0.5 ml of NBT, 0.5 ml of 0.47 mM NADH, 0.5 ml of 0.10 M sodium pyrophosphate buffer and 0.1ml of plasma sample were added in a glass cuvette. The reaction was initiated by addition of 0.1 ml of freshly prepared PMS and the increase in absorbance was measured at 560 nm.

2.4 Statistical Analysis

The parametric data are expressed in the form of mean±SD. Graphical representation of the results were carried out for both cases as well as controls. All the variables and results from the study were statistically analyzed for the mean values, standard deviation and P value. T test was performed and P value <0.05 was considered as statistically significant; P value >0.05 was taken as not significant; P value <0.01 as highly significant and p value <0.001 as very highly significant. The data were analysed using Microsoft excel 2010.

3. RESULTS AND DISCUSSION

A total of fifty patients having one or more indicators of oral lesions, along with equal number of normal healthy individuals as controls were included in the study. Of the four oral lesions, eleven patients were of Leukoplakia, six of Erythroplakia, ten from Lichen planus and twenty three from Oral submucous fibrosis. Thirty five patients were males (70%) and 15 (30%) were females, with a mean age of 33.96±8.43 years; whereas, in the control group 32 (64%) were male and 18 (36%) were female, with a mean age of 32.23±8.98 years. This study was planned to estimate the plasma levels of antioxidant enzymes and molecules related to oxidative stress. Levels of five antioxidant enzymes (Catalase, GPx, GR, MPO and SOD) and three molecules (GSH, MDA and NO) were estimated in both groups i.e. patient samples and healthy controls.

In the present study, higher level of oxidative stress was observed in oral lesion patients when compared with controls. Table 1 demonstrates the comparison between the two groups, which indicates that antioxidant enzymes were significantly decreased in the test group. Plasma catalase activity was almost more than six times reduced in the patients, similarly MPO level was also decreased in the patient's plasma when compared with healthy controls. This finding was consistent with that of Oberley et al. [17]. Various researches have reported increased

Table 1. Mean plasma levels of enzymes and molecules in case and control groups

S. No.	Enzyme/molecules	Case (n=50)	Control (n=50)
1	Catalase (U/ml)	0.52±0.117	3.43±2.26
2	Glutathione peroxidase (U/ml)	20.03±9.8	41.3±6.5
3	Glutathione reductase (U/ml)	4.45±0.92	7.23±2.1
4	Myeloperoxidase (nmol/min/ml)	12.4±3.7	39.3±13.06
5	Superoxide dismutase* (U/ml)	13.4±1.01	12.4±1.1
6	Reduced glutathione (µm/ml)	9.9±2.9	20.7±6.13
7	Malondialdehyde (nmoles/ml)	28.1±3.5	17.24±4.3
8	Nitric oxide (µmol/ml)	7.2±1.4	1.6±0.5

*Data is expressed as mean±SD (*p<0.05)*

oxidative damage and decreased antioxidant levels in oral pre-cancerous state as well as in smokers and tobacco-chewers. This may become helpful to predict the risk of oral carcinogenesis. In such cases, the significance of prognostic markers that are in association with various stages of the disease can make an important contribution towards predicting the rate of recovery and prognosis.

According to Zhang et al. increased oxidative stress results in the process of membrane lipid peroxidation. It generates peroxides which can decompose to multiple mutagenic products. MDA is one such end product which is carcinogenic [18]. Thus, higher the oxidative stress, higher will be the amount of MDA produced. Increased plasma levels of malondialdehyde (MDA) in the patient samples was found in the present study where the mean MDA concentration was 28.1 nmoles/ml in test group as compared to 17.24 nmoles/ml in controls. This observation was also reported by Shilpashree et al. [19]. Studies have established that increased levels of MDA are seen in precancerous states and also in healthy people with smoking and tobacco chewing. This aspect can be crucial to predict the risk of oral cancer in healthy smokers and tobacco chewers [20-22]. There was not much difference in SOD values with test group showing mean SOD level of 13.4±1.01 U/ml as compared to control 12.4±1.1 U/ml. However when student-t test was applied for all the enzymes and molecules, it was found to be highly significant for SOD only with a value of 0.0006. Das et al. has demonstrated that plasma of a pre-cancer patient possesses relatively decreased amounts of SOD which is known to quench the superoxide anion [23].

The water soluble antioxidant, glutathione is responsible for directly neutralizing ROS such as lipid peroxides. Glutathione system includes glutathione, glutathione reductase, glutathione peroxidase and glutathione S transferase. An increased GSSG (oxidized state) to GSH (reduces form) ratio is considered as indicative of oxidative stress. The study also indicated that plasma glutathione reductase, glutathione peroxidase and reduced glutathione levels in healthy controls were higher than the study group. In another set of experiments, plasma nitric oxide levels in the patients were found to be more than four times higher than controls. The NO level was derived/noted to be 1.6 µmol/ml in controls and 7.2 µmol/ml in patient samples. The present study thus reports decreased reduced glutathione (GSH) and increased nitric oxide (NO) plasma levels in cases of oral lesion patients. These findings are consistent with those of John et al. [24]. Glutathione is a major constituent for the detoxification of carcinogens while the reverse is true in case of NO. Therefore, increased glutathione levels and decreased NO levels in the plasma of healthy controls are associated with a decreased risk for oral pre-cancer and hence cancer.

In a review on oxidative stress, inflammation and cancer, Reuter et al have explained the link amongst ROS and RNS with chronic inflammation leading to cancer, diabetes, cardiovascular, neurological and pulmonary disorders [25]. Free radicals are known to accumulate and cause DNA damage which leads to the development of cancer. However, carcinogenesis may also be the result of low values of antioxidants in the body. Studies have reported the role of oxidative stress in case of oral pre-cancer and cancer by estimation of various antioxidant enzymes, molecules and lipid peroxidation in plasma [26,27] saliva [28] and tumor tissue [29].

4. CONCLUSION

The assay of plasma oxidant-antioxidant parameter has indicated oxidative stress in pre-malignant lesions. Their determination in plasma can be a useful marker to assess the severity of disease. However further studies in larger samples are needed to evaluate the transformation of these lesions into OSCC.

CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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