RESEARCH ARTICLE

Estimation of Superoxide Dismutase and Glutathione Peroxidase in Oral Submucous Fibrosis, Oral Leukoplakia and Oral Cancer - A Comparative Study

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Abstract

Present study was undertaken to estimate and compare erythrocyte superoxide dismutase (E-SOD) and Glutathione peroxidase (GPx) levels in oral submucous fibrosis, oral leukoplakia and oral cancer patients and age/sex matched healthy subjects, 25 in each group. Statistically significant (P<0.001) decrease in E-SOD and GPx levels were observed in OSF, oral leukoplakia and oral cancer groups as compared to the control group. Oral leukoplakia group showed lower levels in comparison with OSF (P>0.05). Oral cancer group had the lowest levels amongst the study groups. Imbalance in antioxidant enzyme status may be considered as one of the factors responsible for the pathogenesis of cancer and may serve as a potential biomarker and therapeutic target to reduce the malignant transformation in oral premalignant lesions/conditions.

Keywords: Oral submucous fibrosis - oral leukoplakia - oral cancer - superoxide dismutase - glutathione peroxidase

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world, often preceded by specific premalignant lesions or conditions, the most common amongst them are the Oral Leukoplakia and Oral Submucous Fibrosis. Well known risk factors are consumption of tobacco, arecanut and alcohol, which result in increased free radicals production. Reactive oxygen species (ROS) and free radicals are conjectured to be involved in neoplastic transformation (Fiaschi., 2005).

ROS cause chemical modification in the cells by causing damage to proteins, lipids, carbohydrates and nucleotides. An imbalance between the production of ROS and the cell's oxidant capacity creates oxidative stress, which in turn may instigate or promote carcinogenesis in the cell by mutagenesis, cytotoxicity and changes in gene expression. Thus, free radicals are believed to play an elementary role in the disease progression (Lien et al., 2008).

A number of compounds and enzymes function, to overcome the consequences of ROS and protect cellular components from oxidative damage. Antioxidants are the first line of defence against free radical damage and are essential for maintaining optimum health and well being. Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase (CAT) are the three major enzymatic antioxidant defence system, responsible for scavenging free radicals and nascent oxygen (Manoharan et al., 2005) Antioxidant enzymes catalyze decomposition of ROS. Redox modulation is seen by distinctive changes in the activities of these enzyme systems in oxidative stress. Thus, an overall balance between production and removal of ROS may be more important in various cancers including OSCC (Yokoe et al., 2009)

Despite therapeutic and diagnostic advances, the rate at which Oral precancerous and cancerous lesions are spreading is alarming. This highlights the need for continued efforts to discover suitable biomarkers for early diagnosis. In spite of high prevalence of OSF and oral leukoplakia in India and their potential to undergo malignant transformation, the antioxidant status of these individuals has not been widely investigated. Moreover, to the best of our knowledge, literature on the antioxidant status in relation to premalignant lesion or condition is scarce. With this view in mind, this study was undertaken to investigate and compare the bio-chemical alterations in the sera of oral precancer, oral cancer and healthy subjects.

Materials and Methods

The study was designed with 25 newly diagnosed patients with oral submucous fibrosis, oral leukoplakia and oral cancer, who were not been previously treated

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for the same. A provisional diagnosis of leukoplakia was made when a predominantly white lesion at clinical examination cannot be clearly diagnosed as any other disease or disorder of the oral mucosa. Biopsy was performed and a definitive diagnosis was made when any etiological cause other than tobacco/areca nut use has been excluded and histopathology has not confirmed any other specific disorder (Warnakulasuriya, 2007). Oral leukoplakia lesion size ranged from 1x1 to 3x4cms. Control groups consisted of 25 healthy, age/sex matched subjects (Control A) for OSF and oral leukoplakia; and another control group (Control B) for oral cancer. Samples were randomly recruited based on the selection criteria, amongst the out-patients visiting the Oral Medicine and Radiology department of this Institute.

Inclusion criteria

Patients clinically and histopathologically diagnosed with oral submucous fibrosis, oral leukoplakia and oral cancer; Patients not on any treatment for the same; Patients who agreed for the biopsy and haematological examination; and Normal subjects without any oral lesions and systemic diseases.

Exclusion criteria

Patients below the age of 18 and above 65 years; Patients suffering from any systemic diseases like diabetes, hypertension, cardiovascular diseases, renal dysfunction or liver disorders; and Patients with previous history of treatment for the same conditions.

All subjects were interviewed before being clinically examined in the out-patient department. The questionnaire contained data on demographic factors, types of habits frequency duration of habits. All the study group patients, i.e. the OSF group patients were regular arecanut (gutka) chewers (average of about 3-5 years). Oral leukoplakia and oral cancer patients were regular and active smokers, tobacco chewers, and/or alcoholics. Oral leukoplakia lesions included in the study varied from 1x1cm to 3x4 cm in size. The clinical and pathological diagnosis was subsequently recorded (Table 1). This protocol was approved by the ethical committee of the Institutional Review Broad (IRB) of Rajiv Gandhi University of Health Sciences, to proceed with the research. Study protocol was explained and an informed consent was obtained the patients.

Under aseptic condition, 5 ml overnight fasting venous blood was obtained from the antecubital vein using sterile disposable syringe and was stored in heparinised vacutainer tubes. Serum was separated in 2.5 ml of blood by centrifugation (3000 rpm for 15 mins). The red blood cell (RBC) pellet was then washed three times with sterile saline to ensure complete removal of the plasma, leucocytes and platelets. The washed RBCs were haemolysed by the addition of sterile distilled water (1: 5). Then, the lysate was centrifuged at 3000 rpm for 15 min in order to make the lysate ghost free. The supernatant and remaining 2.5 ml of heparinised whole blood was stored at -70^{0}C until analysis. Estimation of both the enzymes E-SOD and GPx were determined by *Ransel anti-oxidant enzyme kit provided by RANDOX Laboratories Ltd* (Antrim, United Kingdom.) and samples were processed on *Bayer RA-50 chemistry analyzer for spectrometry.*

Superoxide Dismutase Assay

Total E-SOD cytosol and haemolysate was assayed based on the inhibition of a superoxide induced NADH oxidation. The decrease in the rate of NADH oxidation is dependent on the enzyme concentration and saturation levels were attainable by recording the corresponding readings, spectrophotometrically (520nm). Normal E-SOD level: 164-240 U/ml (Sun, 1988).

Glutathione Peroxidase Assay

Estimation of GPx activity in cytosol and haemolysate was based on the method of Paglia and Valentine using hydrogen peroxide and the rate of disappearance of NADPH at 37 ^{o}C and was recorded spectrophotometrically

Table 1. Clinical and Sociodemographic Details of the Subjects

Characteristics		Oral submucous fibrosis patients	Oral leucoplakia patients	Oral cancer patients
Sex	Male	n=20	n=20	n=20
	Female	n= 5	n=5	n=5
Age (Mean		32.33±9.01,	40.73±9.65	53.73±6.19
Habits		Areca nut chewers (gutka) (n=16)	Active smokers (n=9)	Smokers (n=6)
		Betel leaf + arecanut + tobacco (n=9)	Only tobacco chewers(n=5)	Tobacco chewers (n=7)
			Smoker & chewers (n=11)	Smoke+chewers+alcohol(n=12)
Sites affected		Buccal mucosa (n=25)	Buccal mucosa (n=14)	Buccal mucosa (n=17)
		Labial mucousa (n=21)	Labial mucosa (n=5)	Tongue (n=2)
		Soft palate & uvula (n=8)	Tongue (n=2)	Vestibule (n=5)
		Tongue (n=4)	Vestibule(n=4)	Floor of the mouth(n=1)
Clinical diagnosis		Stage I(n=15)*	Homogenous leukoplakia (n=16)	Stage I (T1N0M0) n=7**
		Stage II (n=10)	Speckled leukoplakia (n=9)	Stage II (T2N0M0) n=11
				Stage III (T3N0-1M0 or T1-2N1M0) n=8
Histopathological		Oral submucous fibrosis (n=25)	No dysplasia (n=2)***	SCC poorly diff (n=9)
			Mild dysplasia (n=8)	SCC moderately diff (n=5)
			Moderate dysplasia(n=9)	
			Severe dysplasia (n=6)	SCC well diff (n=11)

* Oral submucous fibrosis classification according to Pindborg (1989); ** TNM classification adopted from Burket's Oral Medicine 11th edition; *** According to WHO classification of grades of dysplasia-(Lindenblatt Rde C et al 2005).

Table 2. Comparison of mean Superoxide dismutase (SOD) levels between study and control groups.

Groups	Mean SOD (U/ml)	Std dev	t-value	p value	S
Oral Submucous Fibrosis group	104.35	27.42	13.117	0.000	HS
and Control group	199.35	17.23			
Oral Leukoplakia group	91.52	19.45	18.552	0.000	HS
and Control group A	199.35	17.23			
Oral Cancer group and	49.75	7.88	44.260	0.000	HS
Control group B	178.4	10.33			
Oral Submucous Fibrosis group	104.35	27.42	1.706	0.096	NS
and Oral Leukoplakia group	91.52	19.45			
Oral Submucous Fibrosis group	104.35	27.42	8.557	0.000	HS
and Oral Cancer group	49.75	7.88			
Oral Leukoplakia group	91.52	19.45	8.898	0.000	HS
and Oral Cancer group	49.75	7.88			

Table 3. Comparison of Mean Glutathione Peroxide (GPx) Levels between study and control groups

Groups	Mean GPx (U/g Hb)	Std dev	t-value	p value	S
Oral Submucous Fibrosis group	23.03	2.46	11.881	0.000	HS
and Control group A	60.46	13.87			
Oral Leukoplakia group	21.55	2.36	12.368	0.000	HS
and Control group A	60.46	13.87			
Oral Cancer group	11.37	1.47	14.764	0.000	HS
and Control group B	45.8	10.32			
Oral Submucous Fibrosis group	23.03	2.46	2.4	0.059	NS
and Oral Leukoplakia group	21.55	2.36			
Oral Submucous Fibrosis group	23.03	2.46	18.176	0.000	HS
and Oral Cancer group	11.37	1.47			
Oral Leukoplakia group	21.55	2.36	16.367	0.000	HS
and Oral Cancer group	11.37	1.47			

*HS - highly significant, NS – not significant, S - Significance

(340nm). Normal GPx level: 27.5-73.6 U/g Hb (Paglia et al., 1986).

Statistical Analysis

The quantified variables in the study (age, sex, superoxide dismutase and glutathione peroxidise levels) were subjected to statistical analysis. All these values were analyzed for mean, standard deviation, errors and range. The data were statistically analyzed using SPSS (Version 17) statistical software. Unpaired Student's 't' test was performed to compare the levels between control and study groups. P-value is less than 0.05 was considered significant.

Results

Demographics

The mean age in OSF, oral leukoplakia, oral cancer group was found to be 32.33±9.01, 40.73±9.65 and 53.73±6.19years, which reflects the subject population mostly being affected. All groups consisted of 20(80%) males and 5 (20%) females respectively (Table 1).

The mean E-SOD level of OSF, oral leukoplakia and oral cancer group was 104.35 ±27.42U/ml; 91.52 ±19.45U/ml and 49.75±7.88 U/ml respectively. The mean GPx level of OSF, oral leukoplakia and oral cancer group was 23.03±2.46U/gHb; 21.55±2.36U/gHb and 11.37±1.47U/gHb respectively. All patients in the control group had E-SOD (164-240 U/ml) and GPx (27.5-73.6 U/g Hb) levels within the normal range.

Table 2 and 3, shows a statistically significant ($p<0.001$) decrease of mean E-SOD and GPx levels in OSF, Oral leukoplakia and oral cancer patients when compared with the corresponding control groups. Lower mean E-SOD and GPx values were observed in oral leukoplakia group in comparison with OSF group but the difference observed was not statistically significant ($P>0.05$). A statistically significant difference was observed ($P<0.001$) with higher mean E-SOD and GPx values in OSF and oral leukoplakia in comparison with oral cancer group. Thus, oral cancer group showed the lowest mean E-SOD and GPx levels amongst the study groups.

Discussion

A male proclivity is observed in the present study groups consisting 20 males (80%) and 5 females (20%); who had tobacco, areca nut, betel quid chewing, alcohol consumption and other habits. Earlier studies have shown that these habits have clastogenic and carcinogenic effects (Patel et al., 2009). The fundamental hypothesis is, free radicals damage the cellular materials which would result in triggering or transforming normal cells into malignant ones. But, the magnitude of such damage is dependent on the body's defence mechanism, which is mediated by various cellular antioxidants. The two verified mechanisms favouring radical alteration of ROS metabolism in cancer cells are production of huge amounts of ROS compared with non-neoplastic cells and suppression of antioxidant system (Gokul et al., 2010)

Antioxidant enzymes such as E-SOD and GPx can directly counterbalance the oxidant attack and protect the cells against DNA damage. Superoxide dismutase, a decisive antioxidant enzyme in aerobic cells; which is responsible for the elimination of superoxide radicals. E-SOD converts two toxic species: superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) into water. This diminishes the toxic effects of superoxide radical and other radicals formed by secondary reactions. Glutathione peroxidise (GPx) is a selenocysteine – dependent enzyme. GPx in cells is the most important hydrogen peroxide (H_2O_2) scavenging enzyme (Hemalatha, 2006)

Three distinct isoforms of E-SOD have been identified in mammals, i.e. copper-zinc E-SOD (Cu/Zn-E-SOD), manganese E-SOD (Mn-E-SOD) and extracellular E-SOD; of which Cu/Zn-E-SOD and Mn-E-SOD are the major intracellular antioxidants and have generated great interest as potential targets in human carcinogenesis. Studies showed that E-SOD enzyme activity increases when the effectiveness of other enzymes decrease (Gokul et al., 2010). The induction of E-SOD in turn protects GPx inactivation by superoxide, resultant effect being a higher GPx activity. Considerable evidence suggests that antioxidant enzymes act to inhibit both initiation and promotion of carcinogenesis. The low activities of these enzymes play a key role in progression of lesion/ condition (Lindenblatt et al., 2012).

In the present study a statistically significant decrease in E-SOD and GPx levels were observed in OSF, oral leukoplakia in comparison with the corresponding control

group (p <0.001). This finding was in accordance with previous studies (Soma et al., 2004; Uikey et al., 2008). Oral leukoplakia patients had slightly lower levels of E-SOD and GPx than the OSF patients; but the difference was not statistically significant (p>0.05). Previous literature on comparison of antioxidant enzyme status between OSF and oral leukoplakia patients is scarce. Thus, this study forms an archetype; for it correlates the antioxidant enzyme status between patients with a premalignant condition and lesion.

Oral leukoplakia is caused due to tobacco; mainly by smoking. The sustained inhalation of ROS for a prolonged duration in the gas and tar phases of tobacco imposes an oxidative stress (Hemalatha et al., 2006). Studies have clearly showed the use of tobacco, suppressed the production of the antioxidant enzymes which was evident among the smokers than the non-smokers (Khanna et al., 2005; Hemalatha et al., 2006). Research works have showed risk of oral cancer development in habitual controls with lower antioxidant enzymes, lower oxidative stress markers, and higher lifetime tobacco exposure. Therefore, in patients having tobacco, betel quid and other addictive habits; the equilibrium between oxidative stress and antioxidant enzyme is adversely affected. A close inter-networking between genetic susceptibility, tobacco usage and oxidative stress can synergistically induced carcinogenesis in such patients (Sabitha et al., 1999; Patel et al., 2008).

In this study, oral cancer group showed a statistically significant (p<0.001) decrease in levels of mean E-SOD and GPx when compared to the control group and also the lowest levels amongst the study groups. This suggests that lower antioxidant enzymes activity in oral cancer patients might be due to the depletion of the antioxidant defence system that occurs as the consequence of overwhelming free radicals by the elevated levels of lipid peroxides. GPx levels were low suggesting that most cancer cell types cannot detoxify hydrogen peroxide (Sabitha et al., 1999). Baskar et al have reported altered temporal pattern in thiobarbituric acid reactive substance which was attributed to the circadian fluctuation in antioxidant enzymes in oral cancer patients (Baskar et al., 2004).

In conclusion, antioxidant enzyme levels are a subject of interest for their possible role in many cancerous conditions is time-honoured and serve as the backbone of cellular antioxidant defence mechanism. Thus, E-SOD and GPx may be a potential biochemical index for evaluating the disease process. This study adjoins and substantiates the E-SOD and GPx levels in oral precancerous lesion/ condition and cancer. These antioxidant enzymes might also serve as a therapeutic targets and a guide for prognosis in patients suffering from such a malady. Further elaborate studies with larger sample size of OSF and oral leukoplakia with different clinical stages; histopathological grading and follow-up are needed to ascertain the actual role of these biochemical parameters in the initiation and promotion of carcinogenesis.

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