

Significance of Serum Nitric Oxide and Superoxide Dismutase in Oral Submucous Fibrosis and Squamous Cell Carcinoma: A Comparative Study

Abstract

Introduction: This study aimed at comparative analysis of serum nitric oxide (NO) and superoxide dismutase (SOD) levels as therapeutic and prognostic biomarkers in patients with oral submucous fibrosis (OSMF) and squamous cell carcinoma (SCC). **Materials and Methods:** Eighty-seven patients were grouped into Group I ($n = 29$, OSMF), Group II ($n = 29$, oral SCC), and Group III ($n = 29$, controls). Two ml of venous blood was collected from patients after overnight fast to avoid any dietary influence on the serum beta-carotene. Standard protocols were followed in transfer, storage, and processing of blood. Modified copper-cadmium reduction method for rapid assay to estimate the serum NO and Enzychrom™ SOD assay kit to determine SOD levels were used. **Results:** The mean level of NO level in Group I, Group II, and Group III was 42.49, 50.08, and 32.81, respectively, and mean level of SOD in Group I, Group II, and Group III were 207.65, 196.93, and 226.57, respectively. The P values were calculated and were statistically significant (<0.001). **Conclusion:** An increase in level of oxidant NO in OSMF followed by SCC and decrease in level of antioxidant SOD in OSMF followed by SCC were noted. These levels of NO and antioxidant SOD can be used as prognostic and therapeutic biomarkers.

Keywords: Nitric oxide, oral submucous fibrosis, serum antioxidant, squamous cell carcinoma, superoxide dismutase

Introduction

Oxidative stress has emerged as one of the leading causes for cancer. Oxidative stress is caused by an imbalance between the production of reactive oxygen and the ability of the biological system to readily detoxify the reactive intermediates or easily repair the resulting damage. It has been more distinctly defined as "An imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage."^[1] This usually results in the production of free radicals that can damage cell membranes through the production of lipid peroxides.^[2] Aerobic life is connected with continuous production of free radicals, particularly reactive oxygen species (ROS). Antioxidant enzymes present in the body help in scavenging these ROS, thus protecting the body from the harmful effects of ROS. Antioxidant imbalance resulting in excessive

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accumulation of ROS is considered to play a key role in tissue damage and promotion of various pathological processes including cancer.^[3] The primary target of ROS is the polyunsaturated fatty acids present in the membrane lipids, resulting in production of end products such as 4-hydroxynonenal, which serve as a marker of cellular damage caused by free radicals. Nitric oxide (NO) interacts with oxygen or other free radicals and generates a potent oxidant, peroxynitrite. Thus, NO is involved in cancer promotion.^[4] Antioxidant enzymes such as superoxide dismutase (SOD) protect the cells against ROS. Excessive production of ROS or deficient antioxidant system may also lead to malignant transformation.^[5] Thus, the serum NO and SOD can act as prognostic and therapeutic biomarker in oral premalignancy and malignancy. Keeping this in mind, this study was conducted to estimate the serum level of oxidant (NO) and antioxidant (SOD) in oral submucous fibrosis (OSMF) and squamous cell carcinoma (SCC) patients.

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Materials and Methods

The study was approved by the Institutional Ethical Committee. The study population included a total of 87 patients, who reported to the Department of Oral Medicine and Radiology, with age range of 20–60 years and were divided into three groups of 29 patients each. Inclusion criteria include Group I which consisted of patients with clinically diagnosed OSMF with different grades, Group II which consisted of patients with histologically proven oral SCC, and Group III which had controls free of any habits and systemic diseases. Patients who were treated in any manner for OSMF or SCC before the study, patients with systemic diseases, patients under aspirin and antioxidants, pregnant patients, and postmenopausal women were excluded from the study.

Informed consent was obtained from every patient, and they were subjected to routine blood investigation and habit cessation counseling in our institution before and during the study. Complete medical history and clinical findings of all the cases were recorded in the structured pro forma prepared for the study. Diagnosis of all the cases of OSMF was done on clinical grounds. Diagnostic criteria for OSMF were the presence of burning sensation, restricted mouth opening, mucosal blanching, restricted tongue protrusion, and the presence of palpable fibrous bands. The mouth opening was measured interincisally. The cases were classified into three stages based on mouth opening according to the functional staging of OSMF given by Haider *et al.*^[6] Stage A stood for mouth opening >20 mm, Stage B for mouth opening of 11–19 mm, and Stage C for mouth opening <10 mm. The patients were also classified into three stages based on the site of involvement according to the clinical staging of OSMF given by Haider *et al.*^[6] Patients with Stage I had the presence of faucial bands alone, Stage II had faucial and buccal bands, and Stage III had faucial, buccal, and labial bands.

Patients with ulceroproliferative growth were clinically diagnosed as malignant growth, and incisional biopsy was performed for these patients [Figure 1]. Final diagnosis was established based on clinical and histopathological findings. Once the clinical diagnosis was confirmed, patients were subjected to the next procedure of the study.

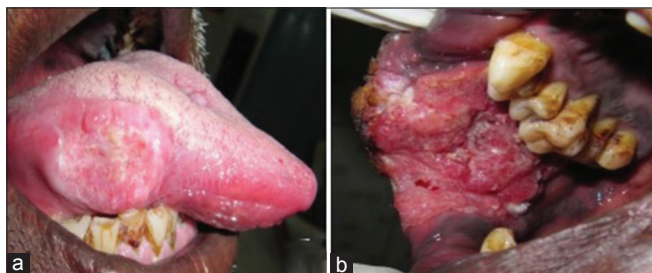


Figure 1: (a) Ulceroproliferative lesion on the lateral border of the tongue and (b) ulceroproliferative lesion on the right buccal mucosa

Blood was collected in the morning after overnight fast to avoid any dietary influence on the serum NO and SOD level. About 2 ml of venous blood collected from left cubital fossa was transferred to a plain 10 ml vacutainer test tube. Once the blood had coagulated, the test tube containing the blood was subjected to centrifugation for about 4–5 min at 2500 rpm. The test tube was then removed from the centrifuge, and the serum layer was pipetted into a vial. Serum was then transferred to Eppendorf tube and stored at –20°. Modified copper-cadmium reduction method for rapid assay of total NO was used to estimate the serum NO. Quantitative colorimetric determination using Enzychrom™ SOD assay kit was utilized to estimate serum SOD [Figure 2].

Statistical analysis

The software used for the statistical analysis was Statistical Package for Social Sciences (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 19.0. Chicago: SPSS Inc.) Chi-square test was used to find the level of significance (*P* value), where *P* < 0.001 was considered to be highly significant.

Results

Our study was aimed at the estimation of levels of oxidant NO and SOD in patients with OSMF, SCC, and controls. The study comprised of 87 individuals who were categorized into three groups of 29 patients each in OSMF, SCC, and healthy controls. Twenty-one males and 8 females constituted the OSMF group, 19 male and 10 female patients constituted the cancer patients group, and 20 male and 9 females constituted the healthy controls. In our study, the mean of NO level in Group I patients was 42.4950 µmol/l; in Group II, it was 50.0850 µmol/l; and in Group III, it was 32.8150 µmol/l [Chart 1 and Table 1]. The mean of SOD level in Group I patients was 207.65U/ml; in Group II, it was 196.93U/ml; and in Group III, it was 226.57U/ml [Chart 2 and Table 2]. Hence, mean value of NO and SOD for control group were 32.8150 µmol/l and 226.57U/ml, respectively. The result of the study is that the level of NO (oxidant level) increased in cancer and OSMF patients when compared to the healthy controls [Chart 3] and level of SOD (antioxidant level) decreased in cancer and OSMF patients when compared to the healthy controls [Chart 4].

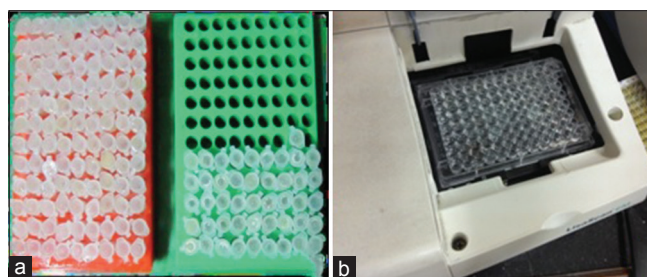


Figure 2: (a) Samples collected. (b) Erba Lisa Scan EM machine

Table 1: Nitric oxide values of groups

No OSMF	No cancer	No healthy controls
39.24	48.01	29.57
40.51	48.05	29.59
40.68	48.06	29.66
41	49.01	29.69
41.01	49.03	29.7
41.02	49.06	29.79
41.03	49.1	29.87
41.04	49.11	29.96
41.08	49.31	30.13
41.14	49.67	30.19
41.18	49.9	30.21
41.19	49.93	30.32
41.28	50.02	30.41
41.66	50.02	30.44
41.7	50.03	30.87
41.73	50.08	30.93
41.78	50.09	30.93
41.83	50.13	31.33
42.1	50.13	31.68
42.14	50.13	33.42
42.17	50.14	33.95
42.65	50.23	35.44
43.42	51.37	35.93
44.11	50.66	36.78
45.4	50.79	37.63
46.19	51.23	38.31
46.49	53.65	40.72
46.84	52.35	41.78
50.47	52.97	42.32
Mean=42.4950 $\mu\text{mol/l}$	Mean=50.0850 $\mu\text{mol/l}$	Mean=32.8150 $\mu\text{mol/l}$

OSMF: Oral submucous fibrosis

Discussion

Various ROS, either oxygen derived or nitrogen derived, are formed in our body as a result of metabolic reactions in the form of free radicals or nonradicals.^[7] These oxidants damage certain molecules including protein, DNA, and lipid, causing cellular/tissue damage. To counteract their effect, the body has compounds called antioxidants. The antioxidants are produced either endogenously or exogenously. Enzymes such as SOD, catalase, glutathione peroxidase, and glutathione reductase, minerals such as Se, Mn, Cu, and Zn, and vitamins such as Vitamin A, C, and E are some of the examples. Other compounds with antioxidant activity include glutathione, flavonoids, bilirubin, and uric acid.^[7,8]

In a healthy human, the balance is maintained between oxidants and antioxidants, but in an abnormal condition, a shift in this ratio toward pro-oxidants gives rise to oxidative stress.^[9] This oxidative stress may be either mild or severe depending on the extent of shift and remains the cause of several diseases such as cardiovascular diseases,

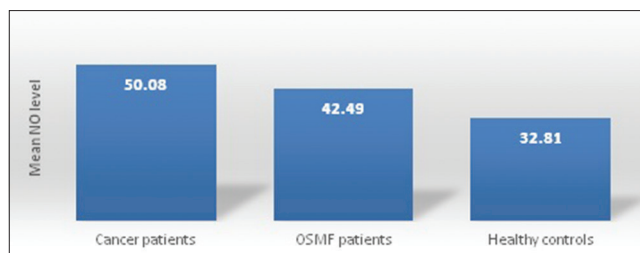


Chart 1: Comparison of mean serum nitric oxide levels in oral submucous fibrosis, oral squamous cell carcinoma, and healthy control groups

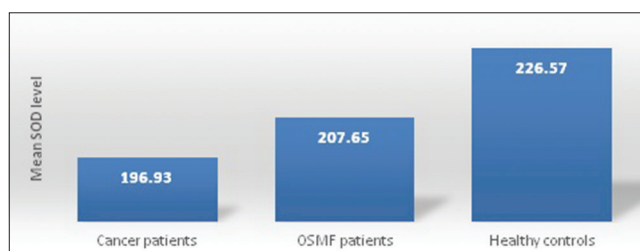


Chart 2: Comparison of mean serum superoxide dismutase levels in oral submucous fibrosis, oral squamous cell carcinoma, and healthy control groups

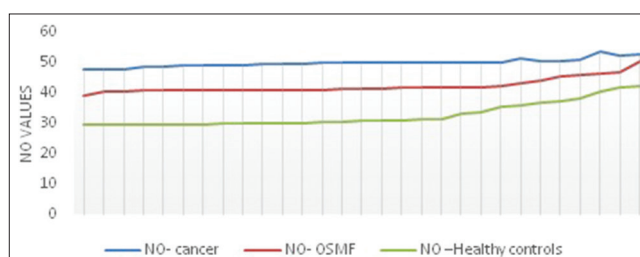


Chart 3: The plot of nitric oxide values shows an increased nitric oxide level for oral submucous fibrosis patients and cancer patients when compared to healthy controls due to the oxidative stress (imbalance between the oxidants and antioxidants)

neurological diseases, malignancies, renal diseases, diabetes, inflammatory problems, skin diseases, aging, respiratory diseases, liver diseases, and different types of viral infections.^[10]

A free radical is a molecular species capable of independent existence that has an unpaired electron in the outer shell and is unstable and highly reactive.^[11] The important free radicals responsible for important diseases are hydroxyl radical (OH⁻), superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, NO radical, and peroxynitrite radical.^[12] The production of free radicals in our body can be either from a normal metabolic process or an external source such as X-ray, ozone exposure, cigarette smoking, pollutants, pan masala chewing, and various industrial chemicals.^[13] Internally generated sources of free radicals are mitochondria, xanthine oxidase, inflammation, phagocytosis, arachidonate pathways, exercise, and ischemia reperfusion injury.^[14] Age, genetics, and environmental factors are expected to produce adverse changes in the free radicals that accumulate in our body.^[15]

Table 2: Superoxide dismutase values for groups

SOD OSMF	SOD cancer	SOD healthy controls
203.1397445	199.1594	213.3888
203.1727063	192.2385	213.8364
204.6852537	197.9842	215.1042
204.7326261	190.1694	215.8759
204.7326261	190.7152	217.7029
205.4199677	191.1616	217.8793
205.4644509	191.707	219.4055
205.6513682	191.9548	221.037
205.8096813	192.599	221.3281
206.3001446	192.6485	221.7161
206.3031878	195.0738	222.0556
206.5339554	195.2221	222.4434
206.8482607	195.7659	223.1703
206.9407158	196.0624	221.21
207.2348595	196.9517	223.8484
207.6759569	196.9517	224.7681
207.7739593	197.6429	227.6199
207.8878071	197.6429	228.73
208.263866	198.5804	229.1159
208.2842111	198.6791	230.7066
208.4509778	199.3201	236.2829
208.4597796	200.158	236.9063
210.1239132	200.2263	243.0293
211.1996262	201.2415	242.1741
211.4300625	201.5861	242.2185
211.7860213	201.7982	243.551
211.9788622	202.3114	234.8324
212.6651409	202.6194	234.4256
212.9669255	202.8653	226.2761
Mean=207.65 U/ml	Mean=196.93 U/ml	Mean=226.57 U/ml
SOD: Superoxide dismutase; OSMF: Oral submucous fibrosis		

The three partially reduced intermediate species between O_2 and H_2O are derived from enzymatic and nonenzymatic reaction. Superoxide (O_2^-) anion may be generated by direct auto-oxidation of O_2 using mitochondrial electron transport reaction. Alternative O_2^- is produced enzymatically by xanthine oxidase and cytochrome P_{450} in the mitochondria or cytosol. O_2^- so formed is catabolized to produce hydrogen peroxide (H_2O_2) by SOD. H_2O_2 is reduced to water enzymatically by catalase in the peroxisomes and glutathione peroxidase (both in the cytosol and mitochondria). OH^- : OH^- radical is formed by 2 ways in biologic processes: by radiolysis of water and by reaction of H_2O_2 with ferrous ions; the latter process is termed as Fenton reaction.^[16]

The antioxidants are radical scavenger, hydrogen donor, electron donor, peroxide decomposer, enzyme inhibitor, and metal chelating agent.^[17] These actions are due to two principle mechanisms. One is chain breaking mechanism in which antioxidants donates an electron to the free radicals. Other is removal of ROS by quenching chain initiating catalysts.^[12,18]

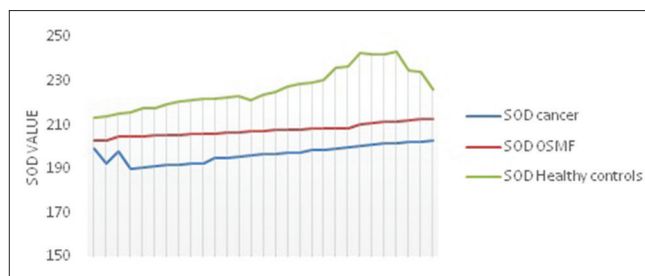


Chart 4: The plot of superoxide dismutase values shows increased values of superoxide dismutase enzyme in healthy controls when compared to the superoxide dismutase level in oral submucous fibrosis and in cancer patients. In oral submucous fibrosis patients, the level of superoxide dismutase slightly decreased. In cancer patients, a drastic decrease in the superoxide dismutase level was noticed

Cancer occurs due to the failures of the mechanisms that usually control the growth and regulation of the cell. The loss of cellular regulation that give rise to most or all cases of cancer are due to genetic damage that is often accompanied with by influences of tumor promoting chemicals, hormones, and sometime viruses.^[19]

Free radicals damage the cellular materials which would result in triggering or transforming normal cells into malignant ones. The magnitude of such damage is dependent on the body's defence mechanism, which is mediated by various cellular antioxidants. The mechanisms favoring radical alteration of ROS metabolism in cancer cells are production of ROS compared with nonneoplastic cells and suppression of antioxidant system.^[20]

Several studies have showed that the NO level is significantly increased in the OSMF and cancer patients. Previous study has reported significantly elevated NO levels in oral cancer patients as compared to normal healthy controls. Studies have also proven increased level of oxidants stress in oral precancer patients.^[21-25]

Choudhari *et al.* in their review have mentioned the diverse roles NO seem to play in various human cancers. As cause of head and neck cancer components of tobacco could be responsible for the generation of ROS/reactive nitrogen species that may lead to lipid peroxidation, enhanced NO products, and deranged antioxidant defense system in tobacco users. Raised levels of NO₂ and NO₃ are noted in patients with oral precancer and in healthy individuals with tobacco habit. Alcohol intake is related to stimulation of NO production by ethanol which plays an important role in the etiology of some cancers, including head and neck cancer, which preferentially rely on NO signaling.^[21] Feng *et al.* have shown in their study that NO was correlated well with lymph node metastasis, increased expression of vascular endothelial growth factor, and p53 protein accumulation which was related with TNM stages and carcinoma differentiation.^[22] Connelly *et al.* in their study have shown that increased NO synthase which leads to increased synthesis of NO is associated with the development of oral SCC^[23] Beevi *et al.* also have proven association of

increased oxidative stress in relation to cancer.^[24] Patel *et al.* have illustrated a potential involvement of NO and antioxidant enzymes in the pathogenesis of oral cancer as evident from enhanced NO products with deranged SOD and catalase antioxidant defense system.^[25]

NO has scavenging action at low concentration but it has opposite action at high concentration; it forms peroxynitrite a potent oxidant to cause cancer and other precancerous lesion. NO pathway appears to play a key role in angiogenesis and spread in patients with head and neck cancer. Generation of high NO levels might have role in oral SCC development. Thus, NO in cancer will have therapeutic implications for the diagnosis and treatment of disease.^[4,12] One of the important causes of pain in patients with precancerous lesions and cancer is higher concentration of NO.^[26] Hence, our treatment should be to reduce the level of NO in patients with precancerous and cancerous conditions.

Evidences from the literature also show that the antioxidant enzymes which inhibit both the initiation and promotion of carcinogenesis are considerably lower in these patients. This is also supposed to be cause for the progression of the cancerous condition.^[27-29] SODs are enzymes that catalyze the dismutation of superoxide into O_2 and H_2O_2 . SOD converts two toxic species: superoxide (O_2^-) and hydrogen peroxide (H_2O_2) into water. This diminishes the toxic effects of superoxide radical and other radicals formed by secondary reactions. They are an important antioxidant defense in all cells exposed to O_2 . The three major families of SOD are Cu/Zn, Fe/Mn, and Ni type.^[30]

In this study, NO level is found to be in increasing order in normal, OSMF, and cancer patients, respectively. This shows that elevated level of NO is involved in carcinogenesis and tumor progression. The mean NO level [Chart 1] in cancer patients is 50.08, OSMF 42.49, and in healthy controls, the value was found as 32.81. Chart 3 shows an increased NO level for OSMF patients and cancer patients when compared to healthy controls due to the oxidative stress.

A statistically significant decrease in SOD was observed in OSMF and OSCC in comparison with the corresponding control group ($P < 0.001$). The mean value of SOD [Chart 2] in cancer patients is 196.93, OSMF patients is 207.65, and in healthy controls, it was found as 226.57. 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. This finding was in accordance with previous studies.^[21-25] The plot of SOD values [Chart 4] shows increased values of SOD enzyme in healthy controls when compared to the SOD level in OSMF and in cancer patients.

Conclusion

This study substantiates that during carcinogenesis and tumor progression, the level of NO activity increases and the level of SOD decreases. These NO and SOD levels might also serve as therapeutic targets and a guide for prognosis in patients suffering from such a malady. Further elaborate studies with larger sample size of OSF and OSCC 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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Conflicts of interest

There are no conflicts of interest.

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