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Estimation of Serum Malondialdehyde and Superoxide Dismutase Levels in Oral Submucous Fibrosis Patients: A Biochemical Study

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ABSTRACT

Background: Oral submucous fibrosis is the most common precancerous condition affecting the oral cavity. Early detection and knowledge of the dysplastic changes occurring in OSMF are helpful in prevention or progress of such lesions turning into oral cancer. Hence the aim was to estimate the serum superoxide dismutase (SOD) and serum Malondialdehyde (MDA) levels in patients with OSMF.

Materials and Method: A total of 120 patients were included in the present study ranging between 18 to 75 years. Sixty OSMF cases which were clinically diagnosed and histopathologically confirmed were divided into different stages and grades respectively. Sixty healthy subjects without any habits and clinically normal appearing oral mucosa were included as controls. Serum MDA will be measured using the method of Ohkawa et al. and Serum Superoxide will be measured using the method of Fridvich and Mishra.

Result: Serum MDA and serum SOD were compared within the clinical stages and histopathological groups of OSMF patients, analyzed by analysis of variance (ANOVA) test. Results showed a very high significant difference. The MDA and SOD levels were compared between the clinical stage and histopathological grades of OSMF patients, analyzed by student 't' test.

Conclusion: The present study showed significant increase in the serum MDA levels in all the clinical stage and histopathological grades, the maximum being in stage IV and Grade III. It was also observed a significant decrease in SOD levels in all the clinical stages and histopathological grades of OSMF patients, the maximum being in stage IV and grade III.

Keywords: MOD, oral cancer, oral precancerous, SOD.

INTRODUCTION

Oral submucous fibrosis (OSMF) is the most common precancerous condition affecting the oral cavity. Oral submucous fibrosis has a prevalence of 2.01% and a malignant transformation rate of 2.3 to 7.6% reported in the literature. Oral cancer is the eighth most common form of cancer in the world with a 5-years survival rate of less than 50%. In India oral cancer is the most common cancer in males and 3rd most common cancer in females. In

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India, the incidence rate of oral cancer is 12.6 per 100,000 population. Studies have shown that between 1% and 18% of oral potentially malignant lesions turn into malignant lesions.[1, 2]

The most expected culprit of dysplastic changes in these lesions include the free radicals and reactive oxygen species that lead to lipid peroxidation. These reactive free radicals may cause profound alteration in the structural integrity and the function of cell membrane resulting in damage to the cell. It is known that free oxygen radicals are probably mediators for tissue damage in neoplastic disease. Although the histopathological findings are the mainstay of dysplasia, some of the biochemical alterations in serum have found to be indicative of such changes. Therefore, identification of suitable biomarkers which are simple, rapid, convenient, inexpensive and reliable is need of the time.[2, 3]

The enzyme Superoxide dismutase (SOD) has many variants. The predominant among these are copper-zinc containing enzymes found in the cytoplasm, and manganese SOD located in the mitochondria. Dismutation reaction, hydrogen peroxide, which helps to conduit in transmission of the injury caused by free radicals. The human body produces an incredible number of reactive oxidants such as hydrogen peroxide, superoxide and hydroxyl radicals. The hydroxyl radical is the most catastrophic to the tissue causing destruction of the adjacent cells.[4, 5]

Superoxide dismutase (SOD) an antioxidant, inactivates the superoxide ion (O2-)by transforming it into hydrogen peroxide (H2O2). The latter is then quickly catabolized by catalase and peroxidases into dioxygen (O2) and water (H2O). When free radicals are generated in excess or when the cellular antioxidant defence system is defective, they can stimulate the chain reactions by interacting with proteins, lipids and nucleic acids causing cellular dysfunction and even cellular death.[6]

Malondialdehyde (MDA) a product of lipid peroxidation is a toxic compound that reacts with the DNA to form covalently-bonded adduct with deoxyadenosine and deoxyguanosine, an event that can cause a mutagenic transformation within the DNA. MDA can interact with several functional groups on proteins and lipoproteins, altering their chemical behaviour and possibly contributing to carcinogenesis and mutagenesis.[7]The clinically diagnosed and histopathologically confirmed OSMF cases were involved in the study with various clinical stages and histological grades and their outcome on prognosis of individuals. Hence the present study aims to evaluate the serum levels of SOD and MDA and the correlation of these parameters in OSMF Patients.

MATERIALS & METHODS

Patients clinically diagnosed and histopathologically confirmed Oral submucous fibrosis were selected for the present study after obtaining an informed consent. The ethical committee was informed about the study and the ethical clearance certificate was obtained from them prior to the start of the study.

The study included 60 subjects with clinically and histopathologically conformed Oral Submucous Fibrosis, and 60 healthy individuals as control group. The subjects included in the study age ranged from 20 to 70 years. The study group was grouped into 4 stages based on the clinical classification given by Ranganathan K et al (2004)29 which is as follows:

- Group I: Only symptoms with no demonstrable restriction of mouth opening.
- Group II: Limited mouth opening 20 mm and above.
- Group III: Mouth opening less than 20 mm.
- Group IV: OSMF advanced with limited mouth opening. Precancerous or cancerous changes are seen throughout the mucosa.

The study group was classified into 3 groups based on the histopathological features suggested by Pindborg J.J.[7]which is as follows:

Group I: Stomatitis includes erythematous mucosa, vesicles, mucosal ulcers,melanotic mucosal pigmentations and mucosal petechiae.

Group II: Fibrosis occurring in the healing vesicles and ulcers is the hallmark of the stage. Early lesions demonstrate blanching of the oral

mucosa. Older lesions include vertical and circular palpable fibrous bands in the buccal mucosa and around the mouth opening or lips resulting in mottled marble like appearance of the mucosa because of the vertical thick fibrous bands in association with blanched mucosa. Specific findings include reduction of mouth opening, stiff and small tongue, blanched and leathery floor of the mouth, fibrotic and depigmented gingiva, rubbery soft palate with decreased mobility, blanched and atrophic tonsils, shrunken bud like uvula and sunken cheeks, not commensurate with age or nutritional status.

Group III: Sequelae of OSMF as follows: Leukoplakia is found in more than 25 % of the individuals with OSMF. Speech and hearing defects may occur due to involvement of the tongue and eustachian tubes.

Inclusion criteria:

Patients of ages between 20 to 70 years with clinically and histopathologically diagnosed as OSMF and consisted of both the genders were included in the study.

Exclusion criteria:

Patients with known malignancies, pkost-surgical phase of treatment, postchemotherapy or post-radiotherapy, patients with surgical illness and or serious medical conditions like liver diseases i.e., obstructive jaundice, hepatitis, cirrhosis, malignancy or metastasis in the liver, myocardial infarction or pregnancy, Scleroderma etc were excluded from the study

Collection of Blood sample:

Five ml of venous blood was collected from both the groups and transferred to a vacutainer. Serum was separated by centrifuging the blood sample at 3000 rpm for 5 min, following which serum Malondialdehyde was measured using the method of Ohkawa ey al. (1979),[8] and serum superoxide was measured using the method of Fridvich and Mishra (1972).[9]

Serum level of MDA was measured according to the method of Ohkawa et al (1979)[8]. MDA level was determined by thiobarbituric acid reactive substances (TBARS) in serum, based on reaction between MDA and thiobarbituric acid. 1.5 ml of 0.8% thiobarbituric acid was added to 1 ml of serum sample. Then 0.4 ml of 8.1% dodecyl sulfate and 1.5 ml of acetic acid were added. The mixture was finally made upto a volume of 5 ml with addition of distilled water and placed in a hot water bath at 95 0 C for 1 h (Figure : 4). After cooling, 1.0 ml of distilled water and 5 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added. The mixture was vortexed and after centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer (upper layer) was measured by the spectrophotometer at 532 nm against blank using distilled water. Thiobarbituric acid when allowed to react with MDA aerobically formed a colored complex [MDA - (TBA)2 complex] which was measured by the spectrophotometer. MDA concentration (measured as TBARS) was calculated as nmol/ml. Serum MDA levels were estimated before undergoing treatment. Lipid Peroxidation (MDA) Assay Kit (sigma-aldrich) was used in our study.

Estimation of superoxide dismutase (sod) in serum fridvich and mishra method[10]

The supernatant sample (1 mL) was added into test tubes containing 0.5 ml of carbonate buffer (0.1M, pH 10.2). Distilled Water was added to this mixture to a final volume of 2.5 ml. This mixture was pipetted into a cuvette and 0.5 mL of epinephrine was added to it to initiate the reaction. The reaction was monitored at 12-second intervals for 1 minute at 480 nm and 25°C, using a UV-visible spectrophotometer. A suitable control that lacked the enzyme was run simultaneously. The change in the absorbance due to the inhibition of the conversion of epinephrine to adrenochrome was measured. The enzyme unit was calculated as the amount of the enzyme required to inhibit the auto-oxidation of epinephrine by 50%. 15 SOD Assay Kit-WST (sigmaaldrich) was used in our study.

Statistical data analysis.

Statistical data was analyzed by SPSS 16.0 version software, t-test, (chisquare) X2 and ANOVA test were used for significance and ($p<0.05$) is considered as significant.

RESULTS

A total of 120 patients were included in the study. The age range was between 20 to 70 years. Sixty OSMF cases which were clinically and histopathologicaly diagnosed were divided into different stages based on clinical and histopathological parameters. Sixty healthy subjects between the age of 20 to 70 years, without any habits and clinically normal oral mucousa were included as controls.

In our study maximum numbers of cases belong to the age group of 41-50 years (30.0%), followed by cases in the age group of 31-40 years (26.7%) and cases in the age group of 21-30 years (25.0%). Statistically analyzed by student 't' test, we found no statistically significant difference among the OSMF and control group with respect to the age (P>0.05).

As per the clinical classification of OSMF in our study group of 60 cases, 24 cases belonged to stage II followed by 16 cases belonging to stage I and 12 cases to stage IV and 8 cases to stage III. As per the histopathological classification in our study group of 60 cases, 20 cases belonged to grade I, 16 cases belonged to N group, 14 cases belonged to grade II and 10 cases belonged to grade III. There was no statistically significant difference of clinical stages and histopathological groups in OSMF group (P>0.05) analyzed by chi-square test.

In the present study when serum MDA and serum SOD were compared between OSMF cases and control group which were statistically analysed by student 't' test, we found statistically very highly significant difference (P<0.001) The mean MDA was significantly higher in the OSMF group as compared to control group. The mean SOD was significantly lower in the OSMF group as compared to control group.

We compared the serum MDA and serum SOD in OSMF patients within the clinical stages. It was statistically analyzed by ANOVA-test. There was very high significant difference (P<0.001). The mean MDA was significantly increased as clinical stages were increased. This was statistically significant (P = 0.001). We compared the serum MDA and serum SOD in OSMF patients within the histopathological groups, statistically analyzed by ANOVA-test, there was very high significant difference (P<0.001).

Table 1: Comparison of MDA and SOD among OSMF and Control groups

Serum variables	Study group	Control group	T test value	P value
MDA	18.28 ± ± 4.82	3.27 ± 0.25	17.34	0.002
SOD	3.28 ± 0.39	5.40 ± 0.90	8.02	0.003

Table 2: Comparison of clinical stages within the stages of MDA and SOD in OSMF group

Clinical stages	MDA	SOD
Stage I	12.65 ± 2.78	4.10 ± 0.52
Stage II	16.42 ± 1.10	3.20 ± 0.62
Stage III	19.58 ± 2.14	2.38 ± 0.32
Stage IV	23.56 ± 4.25	1.34 ± 0.23
P value	0.003	0.002

Table 3: Comparison of histopathological groups within the groups of MDA and SOD in OSMF group.

Histopathological stages	MDA	SOD
Stage N	-	4.36 ± 0.89
Stage I	15.42 ± 2.36	3.45 ± 0.48
Stage II	16.523 ± 1.58	2.95 ± 0.32
Stage III	20.23 ± 3.87	1.90 ± 0.09
P value	0.003	0.002

DISCUSSION

There are some biochemical parameters which get modified in oral submucous fibrosis. These alterations can be used as a tool for disease progression and avert malignant transformation. A number of biochemical compounds and enzymes may function to protect cellular components from oxidative damages.[11] The major antioxidant defence system comprises of SOD and guaiacol peroxidase. These are responsible for scavenging free radicals and oxygen. In normal cells, there is an intricate pro-oxidant and

antioxidant balance. In oxidative stress conditions, this balance shifts toward pro-oxidants. If the oxidant species production is increased with concomitant prolonged and massive stress, it can result in serious cell damage.[12]

Antioxidants play an important role in carcinogenesis. Superoxide dismutase is an enzyme with a generalized presence in the body which catalyses the dismutation of superoxide. As a by-product of this reaction, hydrogen peroxide is produced which helps to conduit in transmission of the injury caused by free radicals. The human body produces an incredible number of reactive oxidants such as hydrogen peroxide, superoxide and hydroxyl radicals. The hydroxyl radical is the most catastrophic to the tissue causing destruction of the adjacent cells.[13, 14]

The enzyme Superoxide dismutase (SOD) has many variants. The predominant among these are copper-zinc containing enzymes found in the cytoplasm, and manganese SOD are located in the mitochondria. Malondialdehyde, a product of lipid peroxidation is a toxic compound that reacts with the DNA to form covalently-bonded adducts with deoxyadenosine and deoxyguanosine, an event that can cause a mutagenic transformation within the DNA.[15]

Malondialdehyde can interact with several functional groups on proteins and lipoproteins, altering their chemical behaviour and possibly contributing to carcinogenesis and mutagenesis. In our study the coloured complex was measured by photo spectrometer. This method is of precise significance because of its procedural simplicity and nanomolar sensitivity.[16]

Our study consisted of 60 subjects with oral submucous fibrosis and 60 healthy individuals as control group of patients. In the OSMF group the largest number of patients belonged to the age group of 41-50 years. The total numbers of patients in this age group were 20 out of the 60 study patients, (33.3%). These findings of our study correlate with the study reports of **Kumar chandan Srivastava et al.[17]**

Our study group of OSMF patients revealed at least to have chewed one of the arecanut based products. Similar observations were also reported of the studies by **Gupta S et al[18]** reported the incidence of OSMF of 0.12% and 1.81% of control group of patients respectively, who were otherwise, without any pernicious habits. In our study, we observed that none of the control group of the patients presented with the symptoms of the OSMF.

In our study we attempted to evaluate the levels of oxidative stress in different stages of OSMF. Mean serum MDA levels in stage I OSMF was [12.65 ± 2.78nmol/ml], serum MDA levels in stage II OSMF was found to be [16.42 ± 1.10 nmol/ml], in stage III OSMF it was [19.58 ± 2.14 nmol/ml] and in stage IV OSMF it was [23.56 ± 4.25 nmol/ml]. This shows that the oxidative stress increases as the disease progresses, reflecting the amount of tissue damage. Our study results also showed statistically significant difference in MDA level of stage I and stage II OSMF cases ($p < 0.032$), as well as stage II and stage III OSMF cases ($p < 0.030$), stage III and stage IV disease ($p = 0.047$), which is in correlation with the study conducted by **Soma Gupta et al,[19]** where plasma mean MDA levels were found to be increased in all stages of OSMF (mean = 4.6 ± 0.3 nmol/ml) compared to healthy controls (mean= 3.8 ± 0.8 nmol/ml) and the increase was statistically significant ($p < 0.001$). It is established that the lipid peroxidation increases with severity of the disease reflecting the extent of the tissue injury. Their results also showed that mean MDA levels were more in OSMF stage II than in stage I. However it did not vary much between grade II and grade III. In our study it is observed that mean serum MDA levels increased proportionally with increased clinical staging of OSMF patients.

Generally, it is observed that Superoxide dismutase levels are reported low in patients with OSMF, and a steady decrease in the levels were found with the progression of clinical stage and histopathologically it is even observed and reported there is a inverse relation of disease progression and SOD levels. **Uikey *et al*.[20]**have shown that SOD levels were decreased in oral submucous fibrosis patients when compared with controls. Thisobservation was statistically

significant. Our findings of mean SOD levels in OSMF patients and mean SOD levels incontrols are inconsistent with their study results.

Our study group of OSMF patients were histopathologically graded in to three groups. The mean SOD values of group I patients was 3.45 ± 0.48 nmol/ml, group II patients was 2.95 ± 0.32 nmol/ml, and group III patients was 1.90 ± 0.09nmol/ml with p value (p= 0.041) which was significant. These results are in accordance with **Shalu Rai et al,**[21] where the serum SOD levels were highest in group I patients and gradually decreased in groups II and III, and the results were found to be statistically significant (P < 0.001).

Comparing our data, we even affirmed that SOD to be a decisive antioxidant enzyme in aerobic cells that which is thought to be responsible for the elimination of superoxide radicals. **Yuthicka sirohi et al,**[22] reported reduced levels of SOD in clinical stages of OSMF patients in comparison to control group of patients. According to their statistics, in stage I, the level of SOD was 0.8343u/ml, and in stage II, 0.6642 u/ml in stage III, 0.46 u/ml. In our present study, the OSMF cases were divided into four clinical stages. The mean SOD values values of stage I were, 4.10 ± 0.52 nmol/ml, stage II, 3.20 ± 0.62 nmol/ml, stage III, 2.38 ± 0.32 nmol/ml and stage IV, 1.34 ± 0.23 nmol/ml, and p value was (P=0.000), which was very highly significant, reflecting an inverse relation of SOD to clinical progressive stage of OSMF patients. These findings of our study are in consistence with the study of **Yuthicka sirohi et Al**

Mean MDA levels were increased with the clinical stages and histopathological grades, whereas SOD levels decreased with increased progressive stage of OSMF in our study.

But our study with similar analysis of parameters on statistical analysis by ANOVA test, reveal a significant difference (p<0.001), we are of the view that, different types of processed areca nut, betel nut, tobacco consumed with slacked lime, and other additive substance, the duration of the individuals chewing these substances and also the intensity and interval or frequency of use of these products may have had an additive influence synergistically on cellular damage leading to the observation of significant difference of MDA and SOD levels in our present study.

CONCLUSION

From this study, we conclude that mean serum MDA level increases in oral submucous fibrosis cases as compared to normal healthy individuals. Increase serum levels may indicate a change from normal to high-risk cases of OSMF but, yet a non-cancerous state. It may serve as a marker for prevention, clinical intervention, as an essential early diagnostic indicator for treatment planning and prognosis of the disease. Our study also revealed steady decline in the levels of mean SOD with increase in clinical stages of OSMF patients and in higher histopathological grades indicating a reduced antioxidant defence mechanism in the study group patients.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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