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Original Article

Dentistry Section

Comparative Evaluation of Serum Malondialdehyde (MDA) Level in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma

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ABSTRACT

Introduction: Lipid peroxidation, which is induced by reactive oxygen species, is involved in the pathogenesis of malignancy. This lipid peroxidation levels are indicated by Malondialdehyde (MDA), which is the most frequently, used biomarker for the detection of oxidative changes.

Aim: Comparative evaluation of serum MDA level in Oral Submucous Fibrosis (OSMF) and Oral Squamous Cell Carcinoma (OSCC) patients and comparison of their serum MDA levels with healthy controls.

Materials and Methods: The study included 150 patients comprising 50 apparently healthy controls without any tobacco chewing habits, 50 clinically diagnosed patients with OSMF which were also subgrouped according to Interincisal Opening (IIO) and 50 clinically diagnosed patients with OSCC, they were also subgrouped according to site, size and histopathological differentiation. Blood samples were obtained; serum was separated and evaluated for MDA levels, by using principle of spectrophotometry at 532 nm absorbance. Statistical analysis were conducted using independent t-test and one-wayanova test. Statistical package for social science (SPSS 16) was used for the analysis.

Results: The mean serum malondialdehyde level in the control group was found to be 10.50 nmol/mL, whereas it was 25.87 nmol/mL and 57.00 nmol/mL in OSMF and OSCC, respectively. Different grades of OSMF patients according to IIO showed $p<0.05$, which was statistically significant. Among the subgroups of OSCC patients with respect to site and histopathological differentiation the results were not significant whereas there was statistically significant increase in MDA levels with increase in primary tumour size.

Conclusion: The increased level of MDA reflects the extent of lipid peroxidation and is considered to be mutagenic as well as carcinogenic and can also modulate the expression of genes related to tumour promotion.

Keywords: Lipid peroxidation, Oral cancer, Oral potential malignant disorders, Oxidative stress, Reactive oxygen species

INTRODUCTION

OSCC is one of the major cause of morbidity and mortality and is the sixth most common malignancy known. It is the most common form of cancer affecting males and account for 50-70% of all cancers diagnosed in India [1]. India has always been cited as country with highest incidence of oral cancer with registration of over 1,00,000 cases every year [2]. Five year mean survival rate remains very low, despite improvements in diagnostic and treatment modalities. Two-thirds of oral cancer patients are diagnosed at advanced tumour stages, where survival drops to a little more than 30% and its prognosis is unpredictable [3].

Tobacco is an exogenous source of Reactive Oxygen Species (ROS) that subsequently leads to Oxidative Stress (OS). Tobacco products of Polynuclear Aromatic Hydrocarbons (PAH) and nitrosoamines cause increase in free radicals and ROS production, which have a pathognomonic role in multistep carcinogenesis. They initiate mutagenic events by causing DNA damage that ultimately leads to degeneration of cellular components [4]. This ROS and free radicals primarily target peroxidation of poly unsaturated fatty acids in membrane lipids. Lipid peroxidation produces many damaging or mutagenic aldehydes like MDA, propanedial, 4-hydroxynonenal (4-HNE), etc., [5].

Malondialdehyde is a major genotoxic carbonyl compound which is generated by lipid peroxidation. It is a three carbon dialdehyde compound that appears mostly in blood, saliva and urine and serves as an important biomarker for oxidative stress [6]. During intracellular oxidative stress and reaction with biological important macromolecules, MDA formation occurs endogenously and they form MDA-DNA adducts which is a suitable biomarker of endogenous DNA damage.

Oral submucous fibrosis is a potentially malignant and crippling condition of the oral mucosa in which the oral epithelium becomes atrophic and more vulnerable to carcinogens [7]. The risk of malignant transformation of patients with OSMF is high and hence it is very important to monitor these patients to identify early transformation into OSCC patients [8]. If identified at initial stages, the incidence of death rates due to OSCC can be reduced considerably.

Thus, there is a need to measure the oxidative stress in normal individuals, in oral potentially malignant diseases and in oral cancer. Previously many investigators have done various studies to measure oxidative stress by using different biomarkers such as C-reactive protein, oxidative stress markers using different group of potentially malignant disorders such as leukoplakia, oral lichen planus [9,10]. To our knowledge till date there is no data available in literature where comparison of serum MDA was done exclusively in OSMF in such a large sample within potentially malignant group.

There is overall limited data on serum MDA expression in OSMF and OSCC compared to high burden of these entities on society. Hence, the present study was carried out to evaluate and compare serum MDA level to assess the degree of oxidative damage caused in OSMF and OSCC patients and to establish the diagnostic efficacy of serum in evaluating serum levels of MDA in OSMF and OSCC patients.

MATERIALS AND METHODS

Source of Data

This was a prospective cross-sectional study. The study was independently reviewed and approved by Ethical Committee of Government Dental College and Hospital, Nagpur, Maharashtra, India (MUHS/PG/E-2/2240/2014). The study was conducted at the Outpatient Department of College, from January 2015 to October 2016 which included 150 patients divided into three groups: Group I: 50 apparently healthy controls not habituated to tobacco chewing and smoking, Group II: 50 clinically diagnosed cases of oral submucous fibrosis, they were not histopathologically confirmed because it is not possible to do the biopsy for each and every OSMF patients unless indicated. All the patients of OSMF were having habit of chewing areca nut and/or tobacco chewing habit in various forms (kharra, pan masala, gutkha etc.,) for over 5 years. The patients were subgrouped according to IIO based clinical grading as per Khanna and Andrade classification [11]. Grade I which is >35 mm IIO consisted of 3 patients, Grade II (26-35 mm IIO) of 17 patients, Grade III (16-25 mm IIO) of 20 patients and Grade IV A (<15 mm IIO) of 10 patients. Group III consisted of 50 clinically diagnosed and histopathologically confirmed cases of OSCC of which 37 had well differentiated, 10 moderately differentiated and 3 poorly differentiated OSCC. These patients were selected irrespective of tumour size, site and grade and all were regular tobacco chewers in various forms for over 10 years.

All individuals included in the study were aged from 18-60 years. The control group includedage and gender matched 50 healthy individuals from a similar socioeconomic background as that of the patient group. They did not have any tobacco habits, systemic or local illness and had visited the hospital for routine check-ups or other minor problems. Patients with HIV, chronic alcoholics, with underlying systemic diseases, cancer other than oral cancer were excluded from the study. Written informed consent was obtained from the patients, after the planned study was explained in detail. A detailed history with thorough clinical examination was performed, and the findings were recorded. The clinical characteristics of controls, OSMF and OSCC patients are shown in [Table/Fig-1].

	Control group (Group I)	OSMF (Group II)	OSCC (Group III)
Number of patients	50	50	50
Mean age	28.98 yrs	28.98 yrs	43.04 yrs
Gender	M= 25 F=25	M=47 F=03	M=39 F=11

[Table/Fig-1]: Clinical and sociodemographic details of patients.

Sample Collection

Five mL of Intravenous blood sample was collected from all 150 patients for estimation of MDA. It was collected using a disposable syringe with 23-gauge needle and was transferred into plain vacutainer. The blood was allowed to clot in an upright position for at least 30 minutes but not longer than 1 hour before centrifugation. These samples were centrifuged in Remi bench top centrifuge for 15 minutes at 3000 RPM within one hour of collection. Serum was seen separated as the top transparent layer. Serum was then transferred to a new test tube and stored in a deep freezer at -20°C for quantitative estimation of serum MDA. MDA forms a 1:2 adduct with thiobarbituric acid and produce coloured complex which can be measured by fluorometry or spectrophotometry. Mean±SD or SE must be established by each laboratory. Variations may occur in individual laboratories due to pipetting, geographic temperature change, incubator temperature etc. As oxidativestress increases Thiobarbituric Acid Reactive Substances (TBARS) which is present in biological specimens also increases. Depending upon the presence of anti-oxidants TBARS return to normal levels over time. TBARS was expressed in terms of MDA equivalents. In the assay a standard curve is constructed using MDA standard against unknown samples. The tests was performed by using the Oxitek TBARS Assay kit and is specially designed for researchers studying oxidative stress and anti-oxidant activity. Every reagent was prepared fresh for each analysis. Absorbance of supernatants was read at 532 nm with the help of BIO RAD SmartSpec Plus spectrometer.

STATISTICAL ANALYSIS

The data was collected, tabulated and analysed by SPSS 16© (Statistical package for Social Sciences) software. Appropriate test of significance was applied. For inter group comparison One-way ANOVA and Post-hoc test were applied and for comparison between two groups independent t-test was applied p-value <0.05 was considered as statistically significant and <0.001 as highly significant.

RESULTS

Comparative statistics for mean serum MDA levels between the healthy control group, OSMF and OSCC groups was done; results are shown in [Table/Fig-2]. The present study has revealed an intriguing aspect of tumour biochemistry. This study showed highest values of MDA in OSCC (57 nmol/mL), in OSMF (25.87 nmol/mL) and lowest (10.50 nmol/mL) in normal healthy control. Thus, a significant increase in serum MDA levels was found from normal healthy individuals to OSMF to OSCC. One-way ANOVA test was applied and p-value was <0.001 i.e., statistically highly significant. When post-hoc test was applied for further inter group comparison; the mean serum MDA level was significantly high in OSCC patients (Group III) as compared to OSMF patients (Group II) and control group (Group I). Estimation of mean serum MDA level of Group II patients with subgroups according to IIO was done, calculated and compared as shown in [Table/Fig-3].

Group	No. of patients	Mean MDA (nmol/mL)	Standard deviation	p-value	Post-hoc test
Group I	50	10.50	8.43	<0.001	**Group III>Group II>Group I**
Group II	50	25.87	13.36		
Group III	50	57.00	26.80		
Total	**150**	**31.85**	**26.24**		

[Table/Fig-2]: Comparison of mean serum MDA values in Group I (Healthy controls), Group II (OSMF patients) and Group III (OSCC patients).

Grade	Number of patients	Mean MDA (nmol/mL)	Std. deviation	p-value	Post-test
Grade I	3	23.31	1.01	**0.000000251 i.e., < 0.05**	**IVA=III>II>I**
Grade II	17	27.77	8.55		
Grade III	20	29.42	9.77		
Grade IVA	10	27.21	13.95		
Grade IVB	0	0	0		

[Table/Fig-3]: Comparison of mean serum MDA values in Group II (OSMF) patients according to IIO based clinical grading.

Group III patients were categorised based on site of lesion in oral cavity and estimation of serum MDA value was done based on site distribution and the results were tabulated as shown in [Table/Fig-4]. It was observed from the results that buccal mucosa was the predominant site for the occurrence of squamous cell carcinoma. Out of 50 patients, 26 patients were having lesion over buccal mucosa but the average serum MDA value was highest in patients involving alveolus and minimum with tongue lesions. When one-way ANOVA test was applied to compare these values, p-value was 0.77 i.e., statistically not significant.

Group III patients when subgrouped according to primary tumour size, for which TNMS classification of clinical grading was used

[12]. The results are shown in [Table/Fig-5]. It was observed that there was a significant increase in serum MDA value from T1 to T3 group. One-way ANOVA test was applied and p-value was <0.05 (i.e., statistically significant). This implies that, there was a significant difference in level of mean serum MDA in patients of OSCC with different tumour size.

Group III patients were also grouped according to histopathological grading for which Bryne's histopathological grading (1989, 1992) (ITF) which is based on InvasiveTumour Front Grading System was used [13]. Bryne M et al., suggested a hypothesis that at the invasive front area of several squamous cell carcinomas, the morphological characteristics reflects tumour prognosis far better than any other parts of the tumour [13]. Estimation of serum MDA value was done according to histopathological differentiation as shown in the following [Table/Fig-6]. Serum MDA level was evaluated according to histological grading but the measured parameter did not show significant changes. In Oral squamous cell carcinoma the level of lipid peroxidation was inversely proportional to the degree of differentiation. MDA levels in well-differentiated squamous cell carcinoma were greater as compared to moderately differentiated and poorly differentiated squamous cell carcinoma, and this difference was statistically not significant.

Group	No. of patients	Percentage	Mean MDA (nmol/mL)	S.D.	p-value
Alveolobuccal complex	9	18%	54.19	26.68	>0.05
Buccal mucosa	26	52%	55.57	27.25	
Alveolus	9	18%	70.50	30.46	
Alveololabial complex	1	2%	46.57	-	
Lip	2	4%	57.13	20.43	
Tongue	3	6%	40.71	14.82	
Total	**50**	100%			

[Table/Fig-4]: Distribution of Group III (OSCC) patients based on site and Comparison of mean serum MDA values in Group III (OSCC) patients based on site. (ANOVA TEST).

Size	Avg. Tumour size in (sq.cm)	No. of patients	Percentage	Avg. MDA (nmol/mL)	S.D.	p-value	Post-hoc test
T1	1.42	03	6%	29.04	8.08	<0.05	T3>T2>T1
T2	8.27	29	58%	44.94	17.7		
T3	18.34	18	36%	81.09	23.4		

[Table/Fig-5]: Distribution and comparison of Group III (OSCC) patients based on primary tumour size and serum MDA value.

Histopathological grade	No. of patients	Percentage	Mean MDA (nmol/mL)	S.D.	p-value
WDSCC	37	74%	59.81	26.9	>0.05
MDSCC	10	20%	53.55	28.13	
PDSCC	03	6%	33.79	1.7	

[Table/Fig-6]: Distribution and comparison of serum MDA in Group III (OSCC) patients based on histopathological differentiation.
p<0.05=Significant and p<0.001=Highly significant
WDSCC: Well differentiated squamous cell carcinoma; MDSCC: Moderately differentiated squamous cell carcinoma; PDSCC: Poorly differentiated squamous cell carcinoma

DISCUSSION

Areca nut is a known etiological factor for oral submucous fibrosis which contains arecoline, arecaidine, guvacolin and guacine which produces numerous free radicals and ROS. Free radicals can produce lipid peroxidation in membrane, Oxidative modification of proteins, and lesions in DNA which can directly and indirectly stimulate the carcinogenic effect in cells. Intracellular and extracellular antioxidant system neutralises the deleterious effects of ROS. Hence, estimation of the level of ROS in Oral submucuous Fibrosis patients may serve as important biomarker to analyse the progression and malignant transformation of the disease [14]. Nicotine which is present in tobacco causes pH changes during chewing which causes partial reduction of oxygen and produces highly reactive free radicals like hydrogen peroxide (H_2O_2), hydroxyl radical (OH*) and superoxide anion (O2-) in the body fluid, such as blood and enhance lipid peroxidation levels of biological molecules, hence serum MDA levels thus thereby increased, as seen in OSMF and OSCC patients [15].

Naturally occurring lipid peroxidationend product, MDA is highly mutagenic andtumourogenic. The MDA reacts with both the Deoxyadenosine and Deoxyguanosine in the DNA and produce DNA-MDA adduct [14]. Early detection of OSCC and OSMF transforming into malignancy can drastically improve the treatment outcomes and prognosis. In the search for possible causes of malignancies on one hand, and the need for the modality affording early diagnosis, there are various biochemical findings which can be useful in early diagnosis at an early stage of the disease [16]. The MDA value in blood is a measure of the ability of the body to handle the oxidative stress [17]. So, in the present study serum MDA level in OSMF and OSCC was estimated to assess the degree of oxidative damage of the disease so that it can be arrested in early stages to avoid the possible consequences of OSMF turning into malignancy.

There was a statistically significant increase in serum MDA level from group I (10.50 nmol/ml) to group II (25.87 nmol/ml) to group III (57.00 nmol/ml) with p-value < 0.001. The present study findings were in accordance with the study conducted by Chole RH et al., Korde SD et al., D'souza D et al., and Ganesan A et al., [17-20]. ROS-induced lipid peroxidation has been indicative in malignant transformation. The prime targets of peroxidation by ROS are the polyunsaturated fatty acids in membrane lipids. The decomposition of these peroxidised lipids yields a variety of end products which also includes MDA. Thus, the level of MDA indicates the extent of lipid peroxidation and serves as a marker of cellular damage due to free radicals. The increase in MDA may be attributed to excessive formation of free radical due to various tissue abuse habits and due to decomposition of polyunsaturated fatty acids present in membranes. It may also occur due to inadequate clearance of free radicals by poor cellular antioxidant system [21].

In group II patientsclinical grade wise analysis showed that mean MDA level gradually increased from grade I to grade IV and the result were statistically significant (p<0.05) and so there exists a direct correlation between clinical grading and MDA value. However, there was slight reduction in mean serum MDA value in grade IVA probably because of unequal distribution of sample size in each grade. It was established that the lipid peroxidation increases with the severity of disease thus indicating the extent of tissue injury [19]. These findings were similar to Metkari SB et al., and Paulose S et al., [22,23]. Contrasting results were reported by Tejasvi MA et al., [24]. Our findings were also not in accordance with the findings by Shakunthala GK et al., in which the difference in serum MDA levels among different clinical staging of OSMF patients were not statistically significant [25]. The present study results showed slight reduction in serum MDA level in grade IVA than in grade III could be due to self–defence mechanism by the proliferative tumour cells to resist deleterious effects of lipid peroxidation on their cell membranes or possible reason could be due to utilisation of MDA in crosslinking of collagen. Chojkier M et al., studied the effects of MDA on collagen production and they found that the addition of MDA (200 μM), to the cultured human fibroblasts incubated in MEM {Eagle's minimal essential medium} without fetal calf serum (to minimise MDA binding to media proteins), increased 2-fold the production of collagen without affecting non-collagen protein production. The concentration of MDA in the media was 80% of the initial concentration after 3 hour incubation [26]. Thus, it can be concluded that the addition of MDA to cultured fibroblasts increase collagen production by 2-3 times [27].

Group III patients were categorised based on site of occurrence of lesion, serum MDA level in patients involving alveolus was highest.

In contrast to the results obtained by Subapriya R et al., who found highest serum MDA level in buccal mucosa [28], the results of the present study revealed that the tumour site and blood comprise two separate metabolic compartments with respect to their susceptibility to lipid peroxidation levels.

Comparison of mean serum MDA level done based on tumour size, a statistically significant difference (P<0.05) was found with mean serum MDA value being highest in T3 patients. Therefore a positive relationship between tumour size and serum MDA was found. Similarly, Manoharan S et al., obtained results in oral cancer patients and found a significant increase in serum MDA level with increase in clinical staging [29]. Our findings were also in accordance with the findings of Srivastava KC et al., [30]. Our results were not in accordance with the study done by Rasheed MH et al., Gupta A et al., who showed no statistical difference in mean serum MDA level with increase in clinical staging [31,32]. Advance stage Head and Neck cancer patients are exposed to higher oxidative stress. Measurement of lipid peroxidation by product in circulation of oral cancer patients may thus be helpful in assessing the clinical stage of oral squamous cell carcinoma. Cell membranes of erythrocytes and other cells are mainly composed of Polyunsaturated Fatty Acid (PUFA) which is considered to be highly susceptible to oxidative attack and also become the major substrate for ROS mediated damage. Due to such damage the fluidity and permeability of the membranes are altered. Thus, large volumes of MDA levels in plasma could be attributed to its increased formation in erythrocytes and its consequent leakage into the plasma or due to inadequate clearance of free radicals by the cellular antioxidants.

A significant decrease in serum MDA level was observed from well differentiated to poorly differentiated squamous cell carcinoma and was not found to be significant statistically (P >0.05). Similar results were obtained by Metgud R et al., Patait MR et al., [15,33]. A complex relationship is anticipated in histopathological differentiation and serum MDA levels which is very poorly explored till date. In our study, there was no significant difference in serum MDA levels of Group III subjects based on histopathological differentiation, probably due to random selection of the patients and thereby unequal distribution of patients in each grade, hence; there is need to conduct further studies in this area, with equal number of patients in each group.

LIMITATION

This study is an effort to find out a cause and effect relationship between OSMF, OSCC and serum MDA levels. The patients were selected randomly for this study. In this study an attempt to correlate serum MDA levels with histopathological grading of OSMF patients was not done as it was not possible to obtain biopsy of these patients unless it is advised/indicated. For OSCC patients instead of the entire TNMS clinical staging only tumour size was considered for the study purpose since the patients were selected randomly and enrolled prior to the treatment at the institute. The disease was diagnosed based on their clinical, radiological, and histopathological examinations. Large scale studies with long term follow up and equal distribution of samples among different grades of OSMF and OSCC should be carried out in order to establish MDA as a potential biomarker for oxidative stress.

CONCLUSION

The estimation of serum MDA levels might be helpful to determine the possibility of malignant transformation of OSMF, assessing the status of OSCC and thereby possibly in clinical intervention of OSCC patients to some extent. The present study suggests a role of MDA as one of the diagnostic biomarkers and innovative tools to monitor oxidative stress and their impact on progression of oral potentially malignant disorders and epithelial malignancy. Serum can be used to determine the impact of redox imbalance on the progression of OSCC but it is difficult to prove cause-effect relationship or to predict its prognostic use with such observational study with small sample size.

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