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Original Article

Dentistry Section

Assessment of Lipid Peroxides in Multiple Biofluids of Leukoplakia and Oral Squamous Cell Carcinoma Patients- A Clinico- Biochemical Study

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ABSTRACT

Background: Oral pre cancer and oral cancer results in lipid peroxidation, and assessment of lipid peroxides in body fluids may give insights into the role of anti oxidants in its management.

Aim: The study was conducted to discern the varying levels of lipid peroxides in saliva, serum and tissue in oral pre cancer and oral cancer and also various forms of tobacco usage with sex as an added parameter.

Materials and Methods: The levels of lipid peroxides were measured in saliva, serum and tissue in a total of 50 patients, 20 belonging to control, and 30 study group in which 10 with oral leukoplakia and 20 with histologically proven oral squamous cell carcinoma (OSCC). The mean value of malondialdehyde (MDA) were also recorded in males and females among the patients with oral leukoplakia and OSCC. Among the study group patients, the levels of MDA were also recorded in habits of smoking and chewing tobacco.

Statistical analysis used: Student's independent t-test, one way ANOVA, Tukey HSD procedure.

Results: Significantly elevated levels of lipid peroxides were seen in saliva, serum and tissue in oral leukoplakia and OSCC when compared to control patients. Among the study group, there were statistically significant increased levels of MDA in OSCC when compared to oral leukoplakia. There was also increase in MDA level in patients with smoking and chewing, but the variations seen in males and females were not very significant.

Conclusion: The results clearly indicate the increase in lipid peroxidation in oral pre cancer and oral cancer with no significant difference between gender groups. The role of saliva as a relatively risk free and reliable, easy to obtain biofuid for diagnostic purposes has been highlighted. Also, since the levels of antioxidants are drastically decreased in carcinogenesis, the importance of anti oxidant supplements in the early stages of the disease has also been elucidated.

Keywords: Anti oxidants, Lipid peroxidation, Malondialdehyde, Oral cancer, Oral leukoplakia

INTRODUCTION

Oral cancer is one of the major forms of cancer worldwide and is one of the most common malignancies in India. It accounts for 30-40% of all cancers [1]. The age standardized incidence rate of patients with oral cancer in India is about 12.6/100,000 population and prevalence of oral cancer in India is high and is upto 4 times higher than in other countries [2]. The important promoters/initiators of oral cancer in India are tobacco, chewing with betel quid or tobacco smoking and alcohol consumption [3]. Use of tobacco leads to oral pre-cancerous lesions and leukoplakia is among the important pre-cancerous lesion with 0.13%-10% malignant transformation in India [4]. Among oral cancers 90% are histologically proven to be oral squamous cell carcinoma (OSCC) [5].

Even though various theories have been established in the mechanism of carcinogenesis free radicals play a very important role. Free radicals are produced in vivo from various biochemical reactions and also from respiratory chain as a result of occasional leakage [6]. Free radicals can be an atom or molecule with one or more unpaired electron. Free radicals have short half life, high reactivity and have damaging activity towards micromolecules like proteins and lipids. These free radicals can be oxygen derived and are called reactive oxygen species. The oxygen derived species include O_2 (superoxide), HO (Hydroxyl), HO_2 (Hydroperoxyl), ROO (peroxyl), RO (alkoxyl) [7]. Antioxidants are natural defense mechanisms and they are capable of scavenging the free radicals. The deleterious effects of free radicals are kept under check by a delicate balance between the rate of their production and the rate of their elimination by different defense mechanism and any shift in this balance leads to cellular damage [6].

If there is excess of free radicals, the lipids in the cells and cell membrane are affected leading to death of cells called lipid peroxidation [8]. Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane poly unsaturated fatty acid (PUFA). MDA is mutagenic, genotoxic agent and potential carcinogen in mammalian system, which readily reacts with deoxy nucleosides to produce adducts causing DNA damage [9]. So, lipid peroxidation has gained importance because of its involvement in various diseases including cancer [6]. Thus, our study was undertaken to discern the varying levels of lipid peroxides in saliva, serum and tissue in oral pre cancer and oral cancer and also amongst various forms of tobacco usage as in smoking and in chewing, with sex as added parameter.

MATERIALS AND METHODS

1 Subject

In the present study, total number of 50 patients were involved Group I - 20 patients belonging to control, Group II- study group consisting of 10 patients with oral pre-cancer (oral leukoplakia) (sub group A) and 20 patients with oral carcinoma oral squamous cell carcinoma-OSCC- subgroup B.

The subject patients were selected and physical examination with routine investigation including complete blood examination and reports were done and were declared free from any other systemic diseases. The mean age of the patients was between 45.1y to 52.8

Medium	Group	Mean ± S.D.	p-value*	Significant Groups at 5% Level #	Significant / Non Significant
Saliva	Control Group (I) Oral leukoplakia (II) Oral Squamous cell carcinoma (III)	0.349±0.09 0.651±0.08 1.007±0.16	<0.0001	III vs I, II II vs I	Significant
Serum	Control Group (I) Oral leukoplakia (II) Oral Squamous cell carcinoma (III)	0.712±0.13 1.346±0.26 1.824±0.55	<0.0001	III vs I, II II vs I	Significant
Tissue	Control Group (I) Oral leukoplakia (II) Oral Squamous cell carcinoma (III)	0.59±0.13 0.79±0.09 1.115±0.12	<0.0001	III vs I, II II vs I	Significant

[Table/Fig-1]: Comparison of mean lipid peroxides (mda) between different study group in each medium
*One -way-ANOVA was used to calculate the p-value
Multiple Range Test by Tukey – HSD Procedure was employed to identify the significant groups All MDA values expressed in nmol/ml

Medium	Group	Males	Females	p-value*
		Mean ± S.D.	Mean ± S.D.	
Control Group	Saliva Serum Tissue	(n=13) 0.347±0.10 0.717±0.12 0.576±0.15	(n=7) 0.353±0.08 0.703±0.15 0.611±0.11	0.90 (Non Significant) 0.81 (Non Significant) 0.59 (Non Significant)
Oral leukoplakia	Saliva Serum Tissue	(n=7) 0.661±0.08 1.273±0.17 0.794±0.09	(n=3) 0.629±0.08 1.515±0.41 0.780±0.11	0.60 (Not Significant) 0.20 (Not Significant) 0.84 (Not Significant)
Oral Squamous cell carcinoma	Saliva Serum Tissue	(n=13) 0.964±0.16 1.814±0.46 1.104±0.12	(n=7) 1.087±0.14 1.844±0.74 1.135±0.12	0.11 (Not Significant) 0.91 (Not Significant) 0.59 (Not Significant)

[Table/Fig-2]: Comparison of mean lipid peroxides (mda) between males and females
* Student's independent t-test was used to calculate the p-value
All MDA values expressed in nmol/ml

y. Signed informed consent on an institutionally approved document was obtained from all the participants. The study was approved by the human ethics committee of India. The patients selected for oral leukoplakia or OSCC had a habit of smoking, chewing or both habits together. The patients who had come for dental examination of carious tooth were selected for control group and for selecting the patients with oral leukoplakia, patients were clinically examined, and criteria for selection was evidence of white lesion in the oral mucosa more than 5mm in diameter, non scrapable and the lesion is raised, firm, mildly indurated and on histopathological examination the leukoplakia showed dysplastic changes in the epithelium. The patients suffering from oral carcinomas were clinically and histologically confirmed as squamous cell carcinoma.

2. Methodology

a) Collection of blood sample

Estimation of lipid peroxides was done in control and study groups including oral leukoplakia and OSCC. A needle puncture was made with the help of 2ml sterile disposable syringe and 2ml of venous blood from ante cubital vein were collected. The blood was transferred to sterile test tube and left for clotting. The clotted blood sample was centrifuged at 3000rpm for 5min and obtained serum sample was used.

b) Collection of saliva sample

Unstimulated saliva was collected and the sample was then centrifuged at 3000rpm at 4°C for 5min and saliva free of large particle debris were used for estimation of lipid peroxide in saliva.

Medium	Group	Smoking - Yes	Smoking - No	p-value*
		Mean ± S.D.	Mean ± S.D.	
Oral leukoplakia	Saliva Serum Tissue	(n=6) 0.682±0.07 1.299±0.17 0.820±0.06	(n=4) 0.605±0.08 1.416±0.39 0.744±0.12	0.13 (Non Significant) 0.53(Non Significant) 0.21 (Non Significant)
Oral Squamous cell carcinoma	Saliva Serum Tissue	(n=8) 1.027±0.12 1.647±0.43 1.120±0.10	(n=12) 0.994±0.19 1.943±0.61 1.112±0.13	0.67 (Non Significant) 0.25 (Non Significant) 0.88 (Non Significant)

[Table/Fig-3]: Comparison of mean lipid peroxides (mda) between smokers and non smokers
*Student's independent t-test was used to calculate the p-value
All MDA values expressed in nmol/ml

Medium	Group	Chewing - Yes	Chewing - No	P-Value*
		Mean ± S.D.	Mean ± S.D.	
Oral leukoplakia	Saliva Serum Tissue	(n=5) 0.643±0.11 1.370±0.35 0.766±0.11	(n=5) 0.659±0.04 1.321±0.18 0.813±0.07	0.77 (Non Significant) 0.79 (Non Significant) 0.43 (Non Significant)
Oral Squamous cell carcinoma	Saliva Serum Tissue	(n=15) 0.989±0.18 1.823±0.60 1.114±0.12	(n=5) 1.061±0.10 1.830±0.44 1.116±0.11	0.41(Non Significant) 0.98 (Non Significant) 0.97 (Non Significant)

[Table/Fig-4]: Comparison of mean lipid peroxides (mda) between chewers and non chewers
* Student's independent t-test was used to calculate the p-value
All MDA values expressed in nmol/ml

c) Collection of normal and pathological tissue sample

Normal tissues were collected from gingival biopsy from normal persons free from disease who accompanied the patients who came for treatment. For the study group patients, toluidene blue (1%) staining of the lesion was done and areas of maximum staining were chosen for biopsy in order to specifically limit the incision to areas of dysplasia [4]. The tissues which were collected were made to two sections and transferred in bottles containing normal saline and the other one containing 10% formalin and sent for histopathological examination using routine staining procedure. The fresh tissue samples were homogenized with phosphate buffer and processed immediately.

d) Biochemical estimation

Lipid peroxidation was estimated as evidenced by formation of thiobarbituric acid reactive substances TBARs. TBARs were assessed and described by Okhawa et al., [10]. The pink coloured chromogen formed by the reaction of thiobarbituric acid with MDA malondialdehyde breakdown products of lipid peroxidation was read at 532 nm [8]. All the MDA values were expressed in nmol/ml.

STATISTICAL ANALYSIS

Descriptive statistics included mean and standard deviation which were estimated from the sample for each study group. Mean values were compared among different study groups by students independent t-test of one-way-ANOVA approximately. Multiple range tests by Tukey-HSD procedure was employed to identify the significant groups, if p-values in one-way-ANOVA was significant. Students paired t-test was used to test the significance of difference in mean values between mediums, within each study group.

In the present study, $p<0.05$ was considered as the level of significance.

RESULTS

I Comparison between control and study group [Table/Fig-1]

The levels of lipid peroxidation were recorded with reference to the released MDA and the results in saliva, serum and tissue were compared with control group and study groups with oral leukoplakia and OSCC.

Study group A-Leukoplakia

The control values of mean lipid peroxides in saliva were 0.349± 0.09 but the values in saliva were increased in patients with leukoplakia which was 0.651±0.08. This increase was statistically significant with p-value <0.0001.The control values of mean lipid peroxides in serum was 0.712±0.13, which were increased in patients with leukoplakia as 1.346±0.26 which was statistically significant (p<0.0001). The control values of mean lipid peroxides in tissue were 0.59±0.13 and the values were increased in patients with leukoplakia as 0.79±0.09 which was also statistically significant (p<0.0001).

Study group B- OSCC

In saliva, the mean lipid peroxides (MDA) values in patients with OSCC was 1.007±0.16, which was increased when compared to control group which was statistically significant (p<0.0001). In serum, the mean lipid peroxide value in patients with OSCC was 1.824±0.55, which was also increased when compared to control group and was statistically significant (p<0.0001). Similarly, in tissue also the values were increased in patients with OSCC which was 1.115±0.12 which was also statistically significant. Thus, lipid peroxidation in all the mediums (saliva, serum and tissue) was more in patients suffering with oral leukoplakia and OSCC when compared with control group.

II Comparison between Males and females [Table/Fig-2]

The present study also included the evaluation of mean lipid peroxides in males and female patients. The value of mean lipid peroxides were 0.347±0.10 in saliva, 0.717±0.12 in serum and 0.576±0.15 in tissue in males of the control group. In females of the control group, values were recorded and were 0.353±0.08 in saliva, 0.703±0.15in serum and 0.611±0.11 in tissue.

Oral leukoplakia

(i)The mean saliva lipid peroxides in males in patients suffering with oral leukoplakia was 0.661±0.08 and mean value in females in the same study group was 0.629±0.08. On comparison, it was not statistically significant as there was only a slight variation between males and females.

(ii) In serum, the mean lipid peroxides in males in patients with oral leukoplakia was 1.273±0.17 and in females it was 1.515±0.41. In this also there was a mild variation which was not statistically significant.

(iii) In tissue, the mean lipid peroxides in oral leukoplakia patients were 0.794±0.09 in males and 0.780±0.11 in females which was also statistically not significant.

OSCC

(i)In saliva, in male patients with OSCC, the mean MDA values were 0.964±0.16 and in female patients it was 1.087±0.14.

(ii) In serum of male patients with OSCC, the mean MDA values were 1.814±0.46 and in female patients, the values were 1.844±0.74.

(iii) In tissue of male patients with OSCC, the values were 1.104±0.12 and in female patients, it was 1.134±0.12

The mean values of lipid peroxides in saliva, serum and tissue in male and female patients with OSCC were found to be only a slight variation which was not statistically significant. Also in the mean value of MDA in males with oral leukoplakia and OSCC compared to control and female patients with oral leukoplakia and OSCC compared to control, there was slight variation which was not statistically significant.

III Comparison between Smokers and chewers [Table/Fig-3&4]

(i)Smokers

In leukoplakia, the mean value of lipid peroxides in smokers in saliva, serum and tissue were compared with non smokers in all the medium and was found to be statistically non significant. Also, in OSCC, the mean values of lipid peroxides among smokers were compared with non smokers in all the mediums and was found to be non significant.

(ii) Chewers

In leukoplakia the patients with chewing habit showed mild variation between patients with non chewing habit in the entire medium. In OSCC also, the patients with chewing habit showed slight variation between patients with non-chewing habit. In both study groups, the values were statistically non significant. On comparison of mean values of lipid peroxides among smokers and chewers the values were increased in patients with OSCC than in patients with leukoplakia

The results show that the lipid peroxide mean values in saliva, serum and tissue is more in patients with leukoplakia and OSCC compared to the control group. The mean lipid peroxide values among study group is more in OSCC than oral leukoplakia in saliva, serum and tissue. The lipid peroxide value in saliva, serum and tissue in males and female patients with oral leukoplakia and OSCC were not much significant when compared with control, but the values were higher in OSCC compared to oral leukoplakia in both males and females. In smoking and chewing habit also, the mean lipid peroxide values were increased in patients with OSCC than oral leukoplakia.

DISCUSSION

The aerobic life cycle, oxygen free radicals are formed in normal cell metabolism from molecular O_2, even though body has anti oxidant defenses, oxygen free radicals can cause constant damage to oxidizable molecules which are repaired or replaced in a dynamic equilibrium. When there is oxidative stress either in the form of over production of oxygen free radicals or from the deficiency of anti oxidant defense or repair mechanism, there can be irreversible tissue injury [11], (rheumatoid arthritis, ischemic heart disease, several auto immune disorders and cancer) [12].

The important exogenous cause of oxidative stress associated with oral pre-cancerous and cancer is the use of tobacco. Continuous exposure to carcinogenic benzopyrene and nitrosamine present in tobacco and areca nut predisposes the mucosal surface to malignant transformation. Tobacco smoking has been reported to stimulate H_2O_2 and hydroxyl radicals and chewing of betel quid with generate DNA maging oxygen free radicals causing chromosomal damage [13]. In the present study, patients with oral pre-cancer(oral leukoplakia) has been taken as oral leukoplakia is the best known precursor lesion and most common potentially transforming malignant lesion of the oral cavity. The risk of malignant transformation of leukoplakia with dysplasia has been reported as high as 43% [14].

Free radical chain reaction leads to lipid peroxidation which causes degeneration of cell membrane. Lipid peroxides are disintegrated quickly and forms reactive carbon compounds. Among these MDA is an important reactor carbon compound which is used as an indicator of lipid peroxidation [15].

The levels of MDA were evaluated in saliva, serum and tissue in this study. Saliva , a heterogenous fluid, apart from cleaning , lubricating, and buffering properties also acts as a first line of defense against free radical mediated oxidative stress involving a variety of reactions including lipid peroxidation [16]. So, saliva was used as a biological sample in monitoring lipid peroxidation in the present study. The MDA values were higher in patients with oral leukoplakia and oral cancer compared to normal patients. Interestingly, this clearly shows the relationship between free radical metabolism and malignancy [7].

The increase in lipid peroxidation in abnormally proliferating cells leads to an increase in serum lipid peroxides in precancer and cancer patients. Increase in MDA in oral cancer patients might be due to decomposition products of polysaturated fatty acids of biomembrane which are released in the blood. Such results have been reported in breast cancer patients also [17]. Similar statistically significant MDA levels were observed in tissue samples which is in contrast to many studies [13,18,19] wherein the inverse relationship of TBARS to tumour progression was attributed to cellular proliferation [13], decreased concentration of free fatty acids In tumour tissue and decreased levels of phospholipids [18]. However, in the present study, the positive correlation between TBARS and tumour tissue as compared to normal mucosa could be attributed to greater cyclooxygenase activity resulting from inflammatory mediators involved in tumour progression [20].

Thus, the patients with oral cancer showed increased levels of MDA when compared to patients with oral pre-cancer. This could be attributed to the initiation and promotion of multistage carcinogenesis induced by free radicals which were increased in oral cancer [11]. Free radicals and radical mediated lipid peroxidation reaction can alter according to sex, as per the study by Bast et al., [21], but in our study, these were not much variations in sex, which was similar to the study by Bulent et al., [22].

In patients with tobacco chewing and smoking, the mean MDA values are increased as tobacco consumption is positively co related with accumulation of free radicals leading to lipid peroxidation [23]. In the present study, increase in salivary concentration of MDA is co related with increase in serum. These findings reinforce the concept that saliva is a viable, easy to obtain and relatively risk free diagnostic biofluid for assessing biomarkers.

CONCLUSION

As there is an imbalance in the body defense mechanism, the anti oxidant activity is reduced in patients with oral cancer and pre cancer. Various studies have proved the role of vitamins and other anti oxidants in primary prevention of oral cancer. So, further emphasis should be given in various plant based anti oxidants which can detoxify the deleterious free radicals and may be also beneficial in chemoprevention of oral cancer. The role of saliva as a viable and relatively risk free biofluid warrants more attention and research into finding out more biomarkers for assessing biochemical changes of various chronic ailments like cancer, which affects the human body.

CLINICAL SIGNIFICANCES

The results of this study highlight the already established role of anti oxidants in the chemotherapeutics of oral pre cancer and oral cancer. However, the role of saliva as an easily available and non invasively extractable biofluid, which is highlighted in this study assumes significance in the light of the fact that the salivary values of lipid peroxides obtained corresponded with the values obtained in serum and tissue. Therefore, future studies aimed at standardizing values of salivary MDA would provide clinicians an easily available biofluid to prognostically assess results of anti oxidant cancer therapy.

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