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Antioxidant Changes in Precancerous and Cancerous Lesions of Oral Cavity

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Abstract: **Introduction: Reactive 0xygen species (ROS) have been implicated in the development of oral cell carcinoma in tobacco and betel quid chewers. However they are scavenged by enzymatic and non-enzymatic antioxidants. Oral cancer is generally preceded by some precancerous lesion e.g.: Leukoplakia or precancerous condition e.g.: Oral Sub mucosal Fibrosis (OSMF). We investigated the effect of ROS in OSCC, OSMF and Leukoplakia patients.**

Material and Methods: The study involved the analysis of various parameters using established standard methods. Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxide (GSH-Px) activities were determined in the blood lymphocytes of patients with precancerous lesions (OSMF & Leukoplakia) and biopsy proven well differentiated Squamous cell carcinoma, enrolled in Oral Radiology department of Nair Dental College & Hospital and from healthy age and sex matched control. Plasma levels of non-enzymatic antioxidants β- carotene, Retinol, α-tocopherol and vit C and plasma levels of TBARS expressed as MDA was determined to find the extent of lipid per oxidation.

Enzyme activity was represented as mean ± S.D. and the data was analyzed statistically. Student –t was applied to study the comparison of mean enzyme activity of healthy control group with patient groups and correlation coefficient (r) were computed using SPSS software. A highly significant decrease in the levels of SOD, CAT and GSH-Px (P<0.001) was noted in cancerous group. In precancerous cases all the enzymes showed moderate to significant fall in the activities. A moderate to significant fall in the plasma levels of β- carotene, retinol VIT c and α-tocopherol was noticed in both the groups as compared to the controls. MDA level expressed significant rise(P<0.001) in precancerous and cancerous group as compared to control.

The antioxidant enzyme activity was reduced and lipid per oxidation was increased in oral precancerous and cancerous group.

Keywords: **OSMF–Oral sub mucosal fibrosis, OSCC- Oral Squamous Cell Carcinoma ACD- Acid Citrate Dextrose. ROS-Reactive Oxygen Species**.

1. INTRODUCTION

The etiology of oral cancer is multifactorial and arises from life style, nutritional patterns and insults (1). Epidemiologic studies in India have established that the high incidence is due to widespread habits of tobacco chewing and smoking (2-4). Burst of reactive oxygen species has been implicated in the development of oral cavity cancer in tobacco chewers and smokers. Tobacco consumption in any form has been demonstrated to have carcinogenic, tetragenic and genotoxic effects and is positively correlated with DNA damages in the human oral cavity.

2. PRECANCERS MOST LIKELY TO UNDERGO MALIGNANT TRANSFORMATION

Oral cancer is generally preceded by some benign lesion or condition for varying length of time. Interestingly they share the etiology factors with oral cancer, particularly the use of tobacco, and exhibits the same site and habit relationships. Many of them show a high potential to become cancerous and therefore termed precancerous eg of precancerous lesion is leukoplakia and of precancerous condition is OSMF.

Leukoplakia is defined as white patch or plaque that cannot be characterized clinically or pathologically as any other disease and which is not associated with any physical or chemical agent except the use of tobacco. The term malignant transformation is used to denote the development of oral cancer from a preexisting leukoplakia such an observation confirmed by statistical test validates the precancerous nature of leukoplakia. It is the most common precancer representing 85% of such lesions. Histologically, over 95% of oral cancers are Squamous Cell Carcinomas. A vast majority of Squamous cell carcinoma arises from preexisting leukoplakia (5) .

The precancerous condition, defined as a "generalized state is associated with a significantly increased risk for cancer". eg is OSMF. It is generally a disease of long duration, the presence of oral cancer is a consequence of malignant transformation of the disease.

Although involvement of ROS in tobacco related oral carcinogenesis is speculated, little is known about the status of ROS scavengers or antioxidant enzymes and antioxidant vitamins A, C, E in precancerous and cancerous lesions of oral cavity. Taking into account the involvement of ROS in carcinogenesis, the enzymatic and non-enzymatic antioxidants were analysed from lymphocytes which are part of body's immune system and in particular are more sensitive to oxidative stress and plasma of patients with precancerous (OSMF& Leukoplakia) and cancerous lesions Squamous Cell Carcinoma) of oral cavity.

3. MATERIAL AND METHODS

Blood samples were collected from patients with precancerous lesions (OSMF n=30) & (Leukoplakia n=30) and biopsy proven well differentiated OSCC n=30, enrolled in oral radiology department of Nair Dental College & Hospital and from healthy age and sex matched controls, n=10. A clinical data sheet included history, nutritional status, habits, duration of disease clinical and histopathological data and the nature of treatment. 5 ml blood was drawn by venipuncture collected in an aACD bulb and diluted to1:1 with PBS, Ph7.4 .Sample was processed immediately for lymphocyte lysate preparation using (Ficoll-Urograffin) density gradient by the method of **Boyum et al** (6). Plasma layer was transferred to a separate tube and used to estimate TBARS using the method of **Yagi k et al** (7) ,Retinol by **Patterson&Wiggins** (8) ,Alpha-tocopherol by **Baker& Frank** (9) .Vit C by **Evelyn & Malloy**(10)and ß-carotene by **kaser M** (11) . Lymphocyte pellet was lysed using 0.2% solution of triton –x and was further used for estimation of enzymes SOD, CAT, GSH-Px and protein in order to express the enzyme activities per mg of protein.

4. STATISTICAL ANALYSIS

Enzyme activity was represented as mean ± S.D and the data was analysed statistically .Student –t test was applied to study the comparison of mean enzyme activity of healthy control group with patient groups. In order to study the relation between oxidative stress and antioxidant (enzymatic and non enzymatic), correlation coefficients(r) were computed using SPSS software.

5. RESULTS

The SOD activity in lymphocyte lysate of control OSMF& Leukoplakia and OSCC patients is depicted in **Table no 1.** It showed a significant rise of 95% ($p<0.001$) in OSCC patient group. This trend is in accordance with one of the research groups (12) in RBC hemolysate of OSCC patients, however the rise noted by them is much lower (29%).In contrast **Subapriya et al,** (13) have reported fall of 46% in the tumour tissue compared to the corresponding adjacent tissue and **Syed Sultan Beevi et al ,** (14) 52.8% respectively in the RBC hymolysate compared to the normal. leukoplakia group reported 41% ($p<0.001$) rise in the enzyme activity. while **khanna et al,** (15) measured the activity in serum of oral leukoplakia patients and reported fall of 76.0% compared to the control group. In OSMF group SOD activity was found overlapping with that of mean value of control. as a result a non-significant rise was noted. similar to our result , **Soma Gupta et al,** (16) reported a nonsignificant negligible rise in SOD activity of OSMF patients. On evaluating the correlation coefficient (r) value between SOD and MDA, a positive correlation $r = +0.668$ was obtained in the OSCC group, while the other two precancerous groups did not show any significant correlation.

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ANTIOXIDANT ENZYMES LEVELS IN LYMPHOLYSATE

	SOD(U/mg of prot	CAT(U/mg of prot)	GSH-Px(U/mg of prot)
Control (n=50)	15.36 ± 2.43	7.3 ± 0.94	0.11 ± 0.27
OSMF (n=30)	15.54 ± 2.41	6.13 ± 0.78*	0.08 ± .020*
Leukoplakia (n=30)	21.63 ± 3.93**	4.46 ± 0.84**	0.05 ± .013
OSCC (n=30)	29.27 ± 5.31**	4.19 ± 1.16**	0.04 ± .019**

** P<0.01 Significant ** P<0.001 Highly significant N.S. Non significant*

LEVELS OF ANTIOXIDANT VITAMINS AND PLASMA MDA

	β-Carotene (µg%)	Retinol (mg%)	α-Tocopherol (mg%)	Vit- C (mg%)	MDA (nmol/ml)
Control (n=50)	95. ± 15.77	0.100 ± 0.023	1.24 ±0.33	1.28 ± 0.31	13.94 ±2.51
OSMF (n=30)	83.28 ± 16.3*	0.097 ± 0.018	0.97 ± 0.23**	0.96 ± 0.21**	16.92 ± 2.6**
Leukoplakia (n=30)	76.36 ± 20.2**	0.085 ± 0.023*	0.78 ± 0.17**	0.76 ± 0.76**	19.27 ± 4.31**
OSCC (n=30)	58.45 ± 10.32**	0.057 ± 0.014**	0.45 ± 0.158**	0.53 ± 0.17**	20.35 ± 4.15**

** P<0.01 Significant ** P<0.001 Highly significant N.S. Non significant*

A consistently low cat activity 43% ($p<0.001$) as compared to control group in OSCC group compared to the control **Table no 1** is depicted. The decrease in CAT activity reflects the depletion of the antioxidant defense system. Our results are in line with similar findings with those of two research groups reported in the literature (17, 14). One research group (17) has reported fall in the enzyme activity as 24%, while the other (14) reported a drop by 57% in the RBC lysate of OSCC patients. Leukoplakia showed a marked decline of 40% ($p<0.001$) in the enzyme activity as also reported by **khanna et** al (15) in the serum of leukoplakia patients when compared with healthy control group. CAT activity in OSMF showed a decline of 17% on comparing with the control. None of the groups exhibited any significant correlation between CAT and MDA.

A similar downward trend in GSH-Px activity as tabulated **Table No 1** was seen in lymphocyte lysate of the patients of all the study groups. In OSCC group the activity was drastically impaired exhibiting a marked fall of 60 % ($p<0.001$). Similar results are put forward by two groups, **Syed Sultan Beevi et al,** (14) who have reported a decline of 76 % and **Subpriya et al**, (13) who have reported fall of 35 % in

GSH-Px activity in RBC haemolysate of OSCC patients compared to the healthy control group. Contradictory to these findings **krishnamurthy et al**, (12) noted a marginal increase of 9.0% in the GSH-Px activity in haemolysate of oral cancer patients. GSH-Px activity in leukoplakia group was decreased by 49% ($p<0.001$) and 28% in OSMF group when compared with control. Serum GSH-Px activity is reported in leukoplakia patients by **khanna et al**, (15) who have shown decreased activity on comparing with normal control group.

The significant fall in CAT and GSH-Px activities in lymphocyte lysate in our study clearly showed that the two enzymes could not cope up with the detoxification of excess H_2O_2 in the lymphocytes of these patients. No correlations between GSH-Px and MDA in all the groups were noted.

A moderate to significant fall in the plasma levels of ß-carotene, Retinol, vit C, and α-tocopherol was noted in both precancerous and cancerous groups as compared to the controls shown in **Table No 2**. In correlation study no association between any of the vitamins and MDA was seen in any of these groups. There are two reports (14, 12) on antioxidant vitamin levels such as vit A and E in OSCC patients. Both of them have reported lower levels in oral cancer patients compared to healthy control group. Few reports on vit A (16) vit C, (16,17), and vit E, (18) in leukoplakia .All of them showed lower levels as compared to the control subjects. ß-carotene a provitamin (16, 18) has also been measured in such patients and a fall has been noticed in its level. **Soma Gupta et al**, (19) measured carotene and vit E level in plasma of OSMF patients and have reported decreased level compared to healthy control group.

MDA level an indicator of oxidative stress was found to be significantly raised by 46 %($p<0.001$) in OSCC group showing a greater degree of oxidative stress as compared to control group, demonstrated in **Table no2.** TBARS / MDA has been measured in cancerous tissue of OSCC patients .Two reports , **S. Manoharan et al**, (20) and **Saroja M et al**, (21) have observed a decrease in TBARS levels in tumour tissue of OSCC patients when compared with normal tissue of disease free healthy subjects. One report in RBC and plasma (22), one in RBC (23), two others in serum (12,15) and one in plasma (14) have reported significant increase in the MDA level in OSCC patients when compared to normal control groups. Rise of 38 % in leukoplakia group was observed with the control group. Similar finding by **khanna et al,** (15) reported increased level in the serum of leukoplakia patients compared to control group. A moderate rise of 21% in OSMF group was noticed when compared with the control group. **Soma Gupta et al, (**19) have also studied MDA level in plasma of OSMF patients and have shown increased level compared to control group.

6. DISCUSSION

It is interesting to observe that superoxide dismutase (SOD) dismutase the toxic superoxide anion $O^{\cdot-}_2$ to H_2O and molecular oxygen. It showed a significant increase in lymphocyte lysate of leukoplakia patients and OSCC patient groups relative to the control group. The observation that the OSCC group revealed positive correlation between SOD and MDA leads to the fact that lymphocytes (immune cells) in particular are more susceptible to ROS induced lipid peroxidation in these patients.

It is well known that tobacco use predisposes to inflammatory conditions which may elicit oxidative responses via ROS produced by inflammatory cells (24). there is evidence that cancer cells produce large amounts of superoxide ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) ,hydroxyl(**OH**), radicals and singlet ($^1O_2$) (25-28). These facts indicate that there is persistent oxidative stress in cancer on account of imbalance between pro and antioxidants and cancer cells are more exposed to ROS than their surrounding normal tissue. Two mechanisms have been proposed by the researchers for ($O^{\cdot-}_2$) radicals based on their experimental findings .**B.P.Patel et al** (29) have reported a significant rise of SOD ($p=0.005$) in malignant tissues compared to adjacent normal tissue. However the overload of $O^{\cdot-}_2$ radicals exceeds the capacity of sod to quench all of them as a result there is increased superoxide $O^{\cdot-}_2$ radicals produced by tumour cells. On the other hand, **R.Subapriya et al**, (22) reported deficiency of SOD enzyme in tumour tissue. Although contradictory findings have been reported as regards SOD activity in malignant tissues, they agree on the fact that higher intracellular $O^{\cdot-}_2$ radical concentration persists in malignant tissues. The higher SOD activity in lymphocytes seen in the present study could be considered as the adaptive response of the cells to the entry of these excessive $O^{\cdot-}_2$ anions. As a consequence of increased SOD activity there could be higher concentration of H_2O_2 in the blood cells. Excess H_2O_2 generated in the blood cells is detoxified by Catalase (CAT) and Selenoprotein glutathione Peroxidase enzyme (GSH-Px) .The conventional view is that GSH-Px is more important at low physiological fluxes of H_2O_2 whereas CAT, with its higher km for H_2O_2 become more important at higher concentrations of H_2O_2 . Since H_2O_2 itself is not reactive , it may pass through cellular membrane and reach any cellular compartment including the nucleus and DNA resulting in greater DNA damage in tumour cells by production of **OH** · From reaction of H_20_2 in the lymphocytes of these patients . Reports suggest that $O^{\cdot-}_2$ itself affected directly the CAT (30) and GSH- Px (31). Blood 'se'levels are frequently reported to be lower in OSCC patients (32). The integrity of GSH-Px requires adequate intake of 'se' and its deficiency causes low activities of GSH-Px and reduction in GSH-Px protein (apo-enzyme) synthesis (33).Oxidative damage to cell membrane has been reported to inactivate GSH-Px. (34).

High levels of oxidative stress results in per oxidation of membrane lipids with the generation of peroxides that can decompose to multiple mutagenic carbonyl products. Lipid hydro peroxides and MDA are well characterized lipid peroxidation end products. They are considered to be mutagenic and carcinogenic (35).

The study demonstrated considerable fall in the non-enzymatic antioxidants in leukoplakia and OSCC patients while it was marginally lowered in OSMF patients. Our observations viewed regarding antioxidant vitamins namely retinol, ß-carotene α-tocopherol and vit C, particularly in leukoplakia and OSCC patients represented drained effective nutrient antioxidant scavengers creating pro-oxidant environment while in OSMF group the levels were almost same as that of the control group. This fall in the levels of vitamins could be due to dietary inadequacy , secondary malnourishment due to maldigestion or malabsorption ,caused by gastrointestinal complication (36), economic constraints , increased consumption in scavenging the ros (ß-carotene retinol , α-tocopherol ,ascorbic acid), decreased storage of vitamins(retinol and α-tocopherol in liver (37).

Lipid peroxidation such as TBARS exhibited considerable rise in their levels. A reason for this increased lipid peroxidation in the circulation may be a poor enzymatic and nonenzymatic antioxidant defense system. This may also be due to excessive generation of lipid peroxides produced into the circulation, indicating imbalance between pro-oxidants and antioxidants.

These observations of our study indicate poor antioxidant mechanisms probably accompanied by overwhelming free radicals as evidenced by higher lipid peroxidation noted in the present study. **Syed Sultan Beevi et al** (14) have attributed high lipid peroxidation to increased formation or inadequate clearance of free radicals by the cellular antioxidants. **Soma gupta et al**, (19), established that the lipid peroxidation increases with the severity of the disease reflecting the extent of tissue injury.

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