A biochemical research focused on the association of the levels of oxidative stress and nitric oxide with premalignant disorders and oral squamous cell carcinoma

ABSTRACT

Context: The reactive oxygen species such as superoxide radicals (O2 •), hydroxyl radicals (OH•), and hydrogen peroxide play a vital role in the pathogenesis of human cancer development and have become one of the areas of key interest in the field of biochemical analysis.

Aims: The present study was designed to determine the significance of oxidative stress and levels of nitric oxide (NO) in patients with premalignant disorders and oral squamous cell carcinoma (OSCC), by evaluating the levels of lipid peroxidation products, antioxidants, and NO products.

Settings and Design: The present study was conducted on 280 patients for 2 years. These patients were divided into 4 groups, Group I (n = 70, control), Group II (n = 70, oral submucous fibrosis), Group III (n = 70, OSCC), and Group IV (n = 70, OL).

Subjects and Methods: The levels of lipid peroxidation products, antioxidants, and NO products were determined by colorimetric methods.

Statistical Analysis Used: Paired t-test was used to compare the mean.

Results: Lipid peroxidation products such as lipid hydroperoxide and malondialdehyde and NO products such as nitrite (NO2–), nitrate (NO3–), and total nitrite (TNO2–) were significantly elevated, whereas enzymatic and nonenzymatic antioxidants were significantly lowered in OSCC, oral submucous fibrosis, and oral leukoplakia when compared to normal healthy participants. The P values were calculated and came as statistically significant (<0.05).

Conclusions: Antioxidant enzyme impairment and NO status may be considered as one of the factors responsible for oral cancer pathogenesis and may serve as a promising biomarker and therapeutic target for minimizing malignant transformation in oral premalignant disorders.

Keywords: Antioxidants, lipid peroxidation, nitric oxide, oral squamous cell carcinoma, oral submucous fibrosis, psychological stress

INTRODUCTION

Oral cancer in India considers as number one among all cancers and is often preceded by specific premalignant lesions or conditions such as oral submucous fibrosis (OSMF) and Oral Leukoplakia (OL). Well-known risk factors are the consumption of tobacco, areca nut, and alcohol, which result in increased free radicals production. Reactive oxygen species (ROS) and free radicals are conjectured to be involved in neoplastic transformation. Oxidative stress is induced by a disparity between reactive oxygen supply and the biological system’s capacity to quickly detoxify the reactive intermediate or restore the subsequent damage.
This usually results in the production of free radicals that can damage cell membranes through the production of lipid peroxides. An antioxidant deficiency that results in excessive ROS accumulation is known to play a key role in tissue harm and in fostering multiple pathological processes like cancer. The primary target of ROS is the polyunsaturated fatty acids present in the membrane lipids, resulting in the production of end products such as lipid hydroperoxides (LHPs) and malondialdehyde (MDA), which serve as a marker of cellular damage caused by free radicals. Enzymatic and antioxidant defense systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), Vitamins E, C and A, and β-carotene. Nitric oxide (NO) reacts with oxygen or other free radicals and releases a strong oxidant, peroxynitrite which may in effect induce malignancy.

Considering developments in the therapy and treatment, the pace at which precancerous oral lesions and cancerous lesions spread appears concerning. It illustrates the need for ongoing attempts to identify appropriate biomarkers for early detection. Despite the high prevalence of OSMF and oral leukoplakia in India and their potential to undergo malignant transformation, the antioxidant status of these individuals has not been widely investigated. In particular, the literature on antioxidant status concerning premalignant lesions or conditions is minimal, to the best of our insight. Bearing this in mind, this study was performed to quantify antioxidants stress and serum NO levels in patients with premalignant disorders such as OSMF/oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC) patients among Kanpur community visiting private dental college.

SUBJECTS AND METHODS

The present study was conducted in the dental outpatient department of a private dental college in Kanpur City for 2 years from January 2018 to February 2020. The ethical clearance obtained from the Institutional Ethical Committee with reference no. 01/EC/RDCHRC/2018-19, dated on 2.2.2018. The study participants included a total of 280 patients selected from the general patients reporting to the Department of Oral Medicine and Radiology, with age range of 20–60 years and were further divided into four groups of 70 patients each.

- **Group I** consisted of control group, these patients were free of any deleterious habits such as tobacco, guthka, or pan masala chewing and were not suffering from any systemic diseases
- **Group II** consisted of patients with clinically diagnosed OSMF with different grades
- **Group III** which consisted of patients with histologically proven OSCC
- **Group IV** consisted of patients with clinically diagnosed oral leukoplakia (OL).

The exclusion criteria included those patients treated for OSMF; OSCC, or OL in any manner, patients with systemic diseases, patients under aspirin and antioxidants, pregnant women, and postmenopausal females were excluded from the study.

A preinformed consent form was filled by all the patients included in the study and was advised to routine blood investigation and was followed by tobacco cessation counseling before and during the study in our institution. A questionnaire was used to collect the data regarding demographic factors, medical history, the form in which the patient is consuming tobacco/pan masala/gutkha, frequency of consumption, and duration of the habit. The diagnostic criteria for OSMF included the presence of burning sensation, restricted mouth opening, mucosal blanching, restricted tongue protrusion, and the presence of palpable fibrous bands. The interincisal distance was measured to measure the mouth opening of the OSMF patients. The OSMF patient was divided into 3 groups depending on the stages based on mouth opening according to the functional staging of OSMF given by Haider et al.

- **Stage A** – patients with mouth opening > 20 mm
- **Stage B** – patients with mouth opening of 11–19 mm
- **Stage C** – patients with mouth opening < 10 mm.

Those patients with ulcer proliferative growth were subjected to an incisional biopsy. Based on the clinical and histopathological findings, a final diagnosis of OSCC was given. Oral leukoplakia patients were divided into Stages I–IV based on the Oral Leukoplakia (OL) staging system.

Taking aseptic precautions, blood samples (approximately 10 ml) were collected in appropriate sterile vials by venous arm puncture after overnight fasting. Five milliliters of blood were collected with Ethylenediamine tetraacetic acid (EDTA) as an anticoagulant for erythrocyte preparation and plasma. Another 5 ml of blood was collected without anticoagulant for the separation of serum. Plasma and serum were separated by centrifugation at 1000 g for 15 min. After the separation of plasma, theuffy coat was removed and the packed cells were washed thrice with 0.89% saline. A known volume of erythrocytes was lysed with deionized water. The hemolysate was separated by centrifugation at 2500 g for 15 min at 2°C.

Biochemical analysis

Biochemical estimations were carried out for all the samples. The estimation of MDA in plasma was done...
by the method of Draper and Hadley and LHP in plasma was estimated by the method of jiang et al.[14,15] SOD was assayed in red blood cell (RBC) hemolysate by the method of Misra and Fridovich; GPx and CAT were assayed in RBC hemolysate by the method of Flohe and Gunzler, Clairborne, respectively.[16‑18] GSH was estimated in plasma by the method of Thomas and Skrinska. Vitamin E was estimated in serum by the method of Baker and Frank meanwhile Vitamin C in plasma was analyzed by the method of Jacob.[19‑21] Serum NO was measured in terms of its products, nitrite, and nitrate, by the method of Griess modified by Fiddler.[22]

Statistical analysis
The software used for the statistical analysis was Statistical Package for the Social Sciences (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 19.0. Chicago, IBM Corp). The paired t-test was used to compare the mean and to find the level of significance (P value), where P < 0.05 was considered to be highly significant.

RESULTS
The present study comprised of 280 individuals who were categorized into four groups of 70 patients each: Group I – controls (no tobacco/pan masala/gutkha habit associated lesions), Group II – OSMF; Group III – OSCC, Group IV – OL and was aimed at the estimation of levels of oxidative stress and NO in the patient’s in these four groups.

Table 1 represents the levels of plasma LHP and MDA in all study participants. The extent of lipid peroxidation as evidenced by plasma LHP and MDA was significantly increased in the precancerous and oral cancer patients (P < 0.05), as compared to control participants.

Table 2 represents the enzymatic antioxidants profile in the circulation of patients with OSMF, oral squamous carcinoma, and oral leukoplakia patients and controls. A decrease in the activities of SOD, GPx, and CAT in the erythrocyte lysate was seen in the OSMF and oral squamous carcinoma patients as compared to control participants. This difference was statistically significant (P < 0.05).

Table 3 represents the nonenzymatic antioxidants in the control participants and OSMF, OSCC, and oral leukoplakia patients. The levels of GSH and Vitamin C in plasma and Vitamin E in serum were significantly lower in the precancerous and cancer patients (P < 0.05) when compared to the control participants.

Table 4 represents the levels of NO• in terms of NO2−, NO3−, and TNO in the control participants and OSMF, OSCC, and oral leukoplakia patients. The levels were found to be significantly elevated in Groups II, III, IV patients (P < 0.05), as compared to the control participants.

DISCUSSION
Oral precancer and cancer are an event occurring at the gene level, and the result of carcinogenesis is DNA damage. Many factors such as chemicals, irradiation, and the genetic makeup of the individual play a key role in carcinogenesis. Damage to the DNA by reactive oxygen species/reactive nitrogen species ROS/RNS is believed to
The low levels of Vitamin C in the plasma of oral cancer patients might be due to the depletion of the antioxidant defense system, similar to work done by Bhat et al. and Sadaksharam and Beevi et al. Reduction in CAT activity as observed in this study might be due to increased endogenous production of the superoxide anion, as evidenced by increased LHP and MDA, or decreased activity of SOD or all of these factors. Furthermore, it might also be due to a higher magnitude of oxidative stress, since all our patients were in advanced clinical stages (Stage III/IV) with large cancer masses.

Sadaksharam and Beevi et al. demonstrated a strong synergism exists between GSH, Vitamin E, and Vitamin C.[11,29] Vitamin E is an important antioxidant present in both the erythrocyte membrane and plasma. Vitamin C is an important extracellular antioxidant that disappears faster than other antioxidants when plasma is exposed to oxygen-free radicals.[32] Vitamin C spares GSH and together with Vitamin E prevents the oxidation of GSH. Since regeneration of both Vitamin E and Vitamin C requires GSH, a decreased level of plasma GSH in oral cavity cancer patients might be responsible for the low levels of these antioxidants similar to the results of the present study.[29,32]

The role of NO (NO•) is multidimensional. It functions as an intracellular messenger and is also implicated as a deleterious agent in various pathophysiological conditions including cancer, inflammatory conditions, and autoimmune diseases. Serum levels of nitrite (NO2−) and nitrate (NO3−) are used to estimate the level of NO• formation since NO• is highly unstable and has a very short half-life. Evidence of the role of NO• in carcinogenesis is provided by the fact that both constitutive nitric oxide synthase (NOS) and inducible NOS are detected in various human oral cancers.[31,34] Sadaksharam and Beevi et al. also reported raised in NO and end product levels in oral cancer and precancer patients in their respective studies.[11,29] We observed significantly higher NO• end products in the serum of the oral cavity in oral cancer and precancer patients. This could be attributed to the generalized increased NO• synthesis throughout the body of the oral cancer patient or reflect increased NO• degradation promoted by oxidative stress as well as hyperplastic and/or dysplastic epithelium are associated with the progression to malignancy. Tumor–stroma interactions provide a niche to potentiate cancer stem cell behavior, and the tumor-associated stroma has been shown to revive basal stem cell activity by initiating a vicious cycle between epithelial andstromal stem cell compartments.[32]

CONCLUSIONS
Antioxidant enzyme levels have long been recognized because of their potential relevance in a variety of cancers.

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**Table 4: Level (μmol/dl) of NO2−, NO3−, and total nitrite in serum of oral submucous, oral squamous cell carcinoma, and oral leukoplakia patients with control participants (mean±standard deviation)**

| Variables        | NO2−     | NO3−     | TNO−     |
|------------------|----------|----------|----------|
| Control group (n=70) | 0.194±0.068 | 0.257±0.043 | 0.53±0.119 |
| OSMF group (n=70)   | 0.865±0.232 | 0.71±0.154 | 1.02±0.074 |
| OSCC group (n=70)   | 0.843±0.248 | 0.76±0.133 | 0.998±0.089 |
| OL group (n=70)     | 0.718±0.277 | 0.84±0.112 | 1.07±0.041 |
| P                 | 0.0003*   | 0.0007*  | 0.0067*  |

*P<0.05 significant when compared with control group. OSMF: Oral submucous fibrosis, OL: Oral leukoplakia, OSCC: Oral squamous cell carcinoma, TNO−: Total nitrite

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In the present study, we observed increased systemic levels of LHP and MDA in patients with OSMF and OSCC and oral leukoplakia, similar to the study done by Beevi et al., and it could be attributed to the increased formation or inadequate clearance of free radicals by the cellular antioxidants.[29,30]
They also serve as the backbone of the cellular antioxidant defense mechanism. Thus, the antioxidants and NO can play a potential biochemical marker for evaluating the disease process. The present study substantiates that the level of NO activity and ROS increases along with carcinogenesis and oral cancer growth. Such levels of NO and ROS could also function as therapeutic interventions. Since the role of NO• in tumorigenesis is multidimensional, further elaborate studies with a larger sample size of OSMF, OSCC, and OL with different clinical stages, histopathological grading, and follow-up are necessary to determine the actual role of these biochemical parameters in triggering and facilitating carcinogenesis.

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Conflicts of interest
There are no conflicts of interest.

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