Evaluation of Oxidative Stress and Nitric Oxide Levels in Patients with Oral Cavity Cancer

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Objective: The aim of this study was to evaluate the magnitude of oxidative stress and levels of nitric oxide in patients with oral cavity cancer by analyzing the levels of lipid peroxidation products, antioxidants and nitric oxide products.

Methods: This prospective study was conducted on 15 patients with biopsy proven squamous cell cancer of the oral cavity with clinical stage III/IV and an equal number of age and sex matched healthy subjects. The levels of lipid peroxidation products, antioxidants and nitric oxide products were determined by colorimetric methods.

Results: Lipid peroxidation products like lipid hydroperoxide (LHP) and malondialdehyde (MDA) and nitric oxide products like nitrite (NO₂⁻), nitrate (NO₃⁻) and total nitrite (TNO₂⁻) were significantly elevated, whereas enzymatic and non-enzymatic antioxidants were significantly lowered in oral cavity cancer patients when compared to normal healthy subjects.

Conclusions: Enhanced lipid peroxidation with concomitant decrease in antioxidants is indicative of oxidative stress that provides evidence of the relationship between lipid peroxidation and oral cavity cancer. Increased nitric oxide production represents a general mechanism in its pathogenesis.

Key words: oral cavity cancer – nitric oxide – lipid peroxidation – antioxidants

INTRODUCTION

Oral cavity cancer is an important cancer globally and is one of the ten most frequent cancers worldwide (1). Tobacco is the primary etiological factor in its development, other factors being alcohol, genetic predisposition and a diet lacking in micronutrients.

The proposal that reactive oxygen species (ROS) such as superoxide radicals (O₂⁻), hydroxyl radicals (OH⁻) and hydrogen peroxide (H₂O₂) play a key role in human cancer development has gained much support recently. They have been shown to possess several characteristics of carcinogens (2). ROS can cause DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of protooncogenes (2). ROS-induced mutation could also arise from protein damage and attack on lipids, which then initiate lipid peroxidation.

It should be noted, however, that the development of human cancer is multifactorial, depending on several factors including the extent of DNA damage, effectiveness of antioxidant defense, DNA repair system and growth promoting effect of ROS (3).

The burst of ROS has been implicated in the development of oral cavity cancer in tobacco chewers and smokers (4). Tobacco consumption in any form has been demonstrated to have carcinogenic, teratogenic and genotoxic effects and is positively correlated with accumulation of DNA damage. Tobacco is therefore believed to directly induce cellular DNA damages in the human oral cavity (5). Oxidative modification of nucleic acids by ROS could result in the transformation of normal cells into malignant cells (6). ROS induced lipid peroxidation has been implicated in malignant transformation (7). The prime targets of peroxidation by ROS are the polyunsaturated fatty acids (PUFA) in the membrane lipids. Furthermore, the decomposition of these peroxidized lipids yields a variety of end products, including lipid hydroperoxides (LHP) and malondialdehyde (MDA). The levels of these lipid peroxides...
indicate the extent of lipid peroxidation in general and serve as markers of cellular damages due to free radicals.

Enzymatic and non-enzymatic antioxidant defense systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamins E, C and A and β carotene. They protect cells against ROS produced during normal metabolism and after an oxidative insult. Antioxidant defense systems work cooperatively to alleviate the oxidative stress caused by enhanced free radical production. Any changes in one of these systems may break this equilibrium and cause cellular damages and ultimately malignant transformation.

Nitric oxide (NO) is a free radical, an uncharged molecule with an unpaired electron. NO plays multiple roles in both intracellular and extracellular signaling mechanisms (8). This highly reactive, yet simple molecule is produced in the body by the isoenzyme nitric oxide synthase (NOS) using L-arginine as a substrate. Three isoforms of NOS have been characterized. Two of them are constitutive NOS (cNOS) and the third is inducible (iNOS) by endotoxins and cytokines (9).

Reaction of NO with oxygen or other free radicals generates reactive nitrogen species (RNS), which cause multiple biological effects (10). NO is either cytostatic or cytotoxic, interacting with a number of molecular targets within cells. Cells within different tissues display varying responses to NO, which may relate to the presence of cellular antioxidants such as GSH, GPx, CAT and SOD (11).

Overexpression of NOS in chronic inflammation can lead to genotoxicity. NO may mediate DNA damage through the formation of carcinogenic nitrosamines, generation of RNS and inhibition of DNA damage repair mechanism. It can thus be considered as a tumor initiating agent (12). However, NO may also have an impact on other stages of cancer development. These effects of NO are broad, with its involvement ranging from cellular transformation and formation of neoplastic lesions to the regulation of various other aspects of tumor biology (11).

NO plays an important role in host defense and homeostasis when generated at a low level for a brief period of time, but becomes genotoxic and mutagenic when generated at higher concentrations for prolonged periods of time. Thus, the biological outcome of the NO mediated effects is complex and depends on the internal and external environment of the target and generation sites of the cells as well as the concentration of NO generated.

The aim of this study was to evaluate the extent of oxidative stress and the levels of nitric oxide in oral cavity cancer patients by analyzing the levels of lipid peroxidation products, antioxidants and nitric oxide products.

**SUBJECTS AND METHODS**

**PATIENTS**

This prospective study was conducted on 15 patients (12 males, 3 females) with biopsy proven squamous cell carcinoma of the oral cavity with clinical stage III/IV, registered at the Department of Oncology, Government Royapettah Hospital, Chennai. All the patients included in the investigation were either smokers or tobacco chewers. The control groups consisted of 15 age and sex matched non-smoking healthy volunteers (12 males, 3 females) from similar socioeconomic backgrounds. The age range was 33 to 72 years (mean ± SD 56.2 ± 11.8 years) for both patients and controls. The clinical characteristics of patients (sex, age, disease localization and stage) are shown in Table 1. Tumors were classified according to the UICC criteria (13). Two patients were in stage III and 13 patients in stage IV. All the carcinomas were graded as well-differentiated squamous cell carcinoma. The ethical committee of the institution approved the study, and informed consent of all the subjects was obtained. None of the patients and control subjects had concomitant diseases such as diabetes mellitus, liver disease and rheumatoid arthritis. All the patients and the control subjects included in this study were not on any medical treatment including supplementation of antioxidants. All the subjects underwent a thorough dental checkup. The subjects who had poor oral hygiene had dental scaling, removal of caries-affected teeth and were treated with antibiotics and mouth cleaning agents for a period of one week. In such cases, blood samples were collected 15 days after the completion of the dental treatment.

**COLLECTION OF SAMPLES**

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 was collected with 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 sera were separated by centrifugation at 1000 g for 15 min.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
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<tbody>
<tr>
<td>Patients</td>
<td>15</td>
</tr>
<tr>
<td>Male/Female</td>
<td>12/3</td>
</tr>
<tr>
<td>Age (years) (range)</td>
<td>56.2 (33–72)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>13</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>I (well differentiated SCC)</td>
<td>15</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
</tr>
<tr>
<td>Anterior 2/3rd of the tongue</td>
<td>4</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>7</td>
</tr>
<tr>
<td>Soft palate</td>
<td>1</td>
</tr>
<tr>
<td>Alveolus</td>
<td>2</td>
</tr>
<tr>
<td>Retromolar area</td>
<td>1</td>
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Table 1. Clinical characteristics of patients
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 estimations were carried out immediately.

A portion of the oral mucosa was excised from oral cancer cavity patients by punch biopsy and washed immediately in physiological saline. Tissues were preserved in 0.9% formalin saline. Thin tissue sections were fixed and stained with eosin–hematoxylin mixture. The stained tissue smear was observed through medical microscope under 100× magnification.

**BIOCHEMICAL MEASUREMENTS**

The estimation of MDA in plasma was done by the method of Draper and Hadley (14). The color produced by the reaction of thiobarbituric acid with MDA was measured colorimetrically at 533 nm. The results were expressed as nmoles/ml plasma.

LHP in plasma was estimated by the method of Jiang et al. (15). This method is based on the ability of H2O2 to oxidize ferrous ion under acidic condition in the presence of xylenol orange. The resultant chromophore was measured colorimetrically at 560 nm. The LHP level was expressed as μmoles/ml plasma.

SOD was assayed in RBC hemolysate by the method of Misra and Fridovich (16). This method is based on the ability of SOD to inhibit autoxidation of epinephrine under specific conditions. One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of epinephrine peroxidation as evidenced by plasma LHP and MDA was

<table>
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<th>Table 2. Levels (µmol/ml) of LHP and (nmol/ml) of MDA in plasma of oral cancer patients and control subjects (mean ± SD)</th>
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<tr>
<td></td>
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<tr>
<td>Control group (n = 15)</td>
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<tr>
<td>Patient group (n = 15)</td>
</tr>
<tr>
<td>P value</td>
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</tbody>
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*P < 0.001, when compared with control group

Table 3. Activities (units/100 mg protein) of SOD, (units/mg protein) of GPx and CAT in erythrocytes of oral cancer patients and control subjects (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>SOD</th>
<th>GPx</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 15)</td>
<td>21.35 ± 2.80</td>
<td>13.80 ± 1.22</td>
<td>33.63 ± 2.59</td>
</tr>
<tr>
<td>Patient group (n = 15)</td>
<td>10.07 ± 2.93*</td>
<td>3.33 ± 0.80*</td>
<td>14.44 ± 1.63*</td>
</tr>
<tr>
<td>P value</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

*P < 0.001, when compared with control group

Our findings on the assessed parameters in oral cavity cancer patients and healthy control subjects are shown in Tables 2, 3, 4 and 5.

Table 2 shows the levels of plasma LHP and MDA in control subjects and oral cavity cancer patients. The extent of lipid peroxidation as evidenced by plasma LHP and MDA was

**STATISTICAL ANALYSIS**

The findings were expressed as the mean ± standard deviation. The data were analyzed with Student’s independent t test. All statistical analyses were performed with the program Statistical Package for the Social Science (SPSS for Windows, Version 10.0). A P value of <0.05 was accepted as statistically significant.

**RESULTS**

Our findings on the assessed parameters in oral cavity cancer patients and healthy control subjects are shown in Tables 2, 3, 4 and 5.
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significantly increased in the oral cavity cancer patients ($P < 0.001$), compared to control subjects.

The enzymatic antioxidants profile in the circulation of oral cavity cancer patients and control subjects is presented in Table 3. A decrease in the activities of SOD, GPx and CAT in the erythrocyte lysate was seen in the oral cavity cancer patients as compared to control subjects. The difference approached a statistical significance of $P < 0.001$.

Table 4 presents the non-enzymatic antioxidants in the control subjects and oral cavity cancer patients. The levels of GSH and vitamin C in plasma and vitamin E in serum were significantly lower in the oral cavity cancer patients ($P < 0.001$), when compared to the control subjects.

Table 5 shows the levels of NO in terms of NO$_2^-$, NO$_3^-$ and TNO in the control subjects and oral cavity cancer patients.

The levels were found to be significantly elevated in the oral cancer patients group ($P < 0.001$), as compared to the control subjects.

Histopathology reports are shown in Figs 1, 2 and 3. Figure 1 shows oral mucosa from a control subject, which reveals stratified squamous epithelium with normal cellular architecture. Figure 2 shows well-marked inflammatory regions, characterized by accumulation of lymphocytes.

DISCUSSION

Cancer is essentially an event occurring at the gene level and the ultimate step resulting in carcinogenesis is DNA damage. Multiple factors such as viruses, chemicals, irradiation and the
genetic makeup of the individual play a role in carcinogenesis. ROS and RNS are two important agents of DNA damage. Damage to the DNA by ROS/RNS is believed to occur naturally as well, since low steady-state levels of base damage products have been detected in human nuclear DNA. The magnitude of this damage (called oxidative stress or oxidative damage) depends not only on ROS/RNS levels but also on the body’s defense mechanisms against them mediated by various cellular antioxidants. Disruption of this delicate oxidant/antioxidant balance in the body seems to play a causative role in carcinogenesis.

There are increasing evidences to support the role of oxidative stress in several human pathological conditions such as rheumatoid arthritis, ischemic heart disease, several autoimmune disorders and cancer. ROS/RNS are found to be involved in both initiation and promotion of multistep carcinogenesis. They can cause DNA damage, activate procarcinogens, initiate lipid peroxidation, inactivate enzyme systems and alter the cellular antioxidant defense system (24).

High levels of oxidative stress result in peroxidation of membrane lipids with the generation of peroxides that can decompose to multiple mutagenic carbonyl products. LHP and MDA are well-characterized lipid peroxidation end products. They are considered to be mutagenic and carcinogenic (25). They can also modulate the expression of genes related to tumor promotion (26). The level of LHP and MDA reflect the extent of lipid peroxidation.

In our study, we observed increased systemic levels of LHP and MDA in patients with oral cavity cancer, which could be attributed to increased formation or inadequate clearance of free radicals by the cellular antioxidants. Elevated levels of lipid peroxidation products support the hypothesis that the cancer cells produce large amount of free radicals (2) and that there exists a relationship between free radical activity and malignancy (27). Observations similar to our findings have been reported in studies on various human cancers (28–30).

Antioxidant enzymes have been shown to inhibit both initiation and promotion in carcinogenesis and counteract cell immortalization and transformation (31). Activities of different antioxidants show different patterns during neoplastic transformation and tumor cells exhibit abnormal activities of antioxidant enzymes, when compared to their appropriate normal cell counterparts (24).

Cellular antioxidant enzymes and free radical scavengers protect a cell against toxic oxygen radicals. GSH, an important non-protein thiol, in conjugation with glutathione transferase (GST) and GPx, plays a significant role in protecting cells by scavenging ROS (32). A significant depletion of plasma GSH observed in our study reflects enhanced pro-oxidant milieu of the cells and correlates with the increased lipid peroxides in the circulation of oral cavity cancer patients.

Antioxidant enzymes such as SOD, CAT and GPx provide the first line of cellular defense against toxic free radicals. These enzymes react directly with oxygen free radicals to yield non-radical products. Selenium dependent GPx removes both H$_2$O$_2$ and LHP using GSH. This prevents H$_2$O$_2$ mediated intracellular DNA damage, which is thought to be a prerequisite for carcinogenesis (33). Oxidative damage to the cell membrane has been reported to inactivate GPx (34). SOD metabolizes free radicals and dismutates superoxide anions (O$_2^\cdot$) to H$_2$O$_2$ and protects the cells against O$_2^\cdot$ mediated lipid peroxidation. CAT acts on H$_2$O$_2$ by decomposing it, thereby neutralizing its toxicity. It has been reported that superoxide radicals inhibit catalase activity and H$_2$O$_2$ suppresses SOD activity in the cell (35). Gupta et al. (36) demonstrated that reduction in several antioxidant defense mechanisms correlates with the emergence of the malignant phenotype. The low activities of these antioxidant enzymes observed in our study might be due to the depletion of the antioxidant defense system. This could occur as a consequence of overwhelming free radicals, as evidenced by the elevated levels of lipid peroxides in the circulation of oral cavity cancer patients. Reports on CAT activity in cancer are contradictory. Both increase (37) and decrease (28,29,38) in CAT activity have been reported previously. 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 increased nitric oxide end products, or decreased activity of GPx and 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 a large tumor burden.

A strong synergism exists between GSH, vitamin E and vitamin C. Vitamin E is an important antioxidant in the lipid domain. It is present in both erythrocyte membrane and plasma. It is readily exchanged between erythrocytes and plasma, with a balance in favor of plasma. Vitamin C is an important extracellular antioxidant that disappears faster than other antioxidants when plasma is exposed to oxygen free radicals. 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, diminished level of plasma GSH in oral cavity cancer patients might be responsible for the low levels of these antioxidants.

The role of nitric oxide (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. Chronic inflammation can lead to the production of NO$, which in turn has the potential to mediate DNA damage directly, or indirectly through the generation of more persistent RNS (12).

Serum levels of nitrite (NO$_2^-$) and nitrate (NO$_3^-$) are used to estimate the level of NO$^\cdot$ formation, since NO$^\cdot$ is highly unstable and has a very short half-life. Evidence of the role of NO$^\cdot$ in carcinogenesis is provided by the fact that both cNOS and iNOS are detected in various human cancers (39,40). Biopsy samples of human breast cancer show the presence of greater expression of iNOS in a high-grade tumor, which tends to be more invasive (9). Our study is the first to undertake the evaluation of serum nitric oxide levels in human oral cavity cancer. We observed significantly higher NO$^\cdot$ end products in the
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serum of oral cavity cancer patients. This could result from a generalized increased NO synthesis throughout the body of the cancer patient or reflect increased NO degradation promoted by oxidative stress. Observations similar to ours have been reported previously in gastric, colorectal, hepatocellular and breast cancer (41–44).

Histopathological examinations of the oral mucosa revealed necrosis and inflammatory changes. The oxidative stress might have resulted in the abnormal changes evidenced in the histological examinations. Lipid peroxidation and oxidative stress have been reported to be among the causes of tissue damage and inflammation (30).

From this study, it could be concluded that oxidative stress is increased (as evidenced by elevated levels of lipid peroxidation products and nitric oxide products) and antioxidant defenses are compromised (as evidenced by depletion of enzymatic and non-enzymatic antioxidants) in patients with oral cavity cancer. A weak antioxidant defense system makes the mucosal cells more vulnerable to the genotoxic effect of ROS. This creates an intracellular environment more favorable for DNA damage and disease progression. Studies have shown that a diet rich in antioxidants may alleviate oxidative stress to some extent (45). However, the degree of effectiveness with which the antioxidant system can be restored with dietary modifications and nutritional supplements, so that cancer patients can actually be benefited remains to be elucidated. Since the role of NO in tumorigenesis is multidimensional, an elaborate study actually be benefited remains to be elucidated. Since the role of

Acknowledgment

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References


