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ORIGINAL ARTICLE

# Oxidant–antioxidant status in blood and tumor tissue of oral squamous cell carcinoma patients

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**BACKGROUND AND OBJECTIVE: Increased oxidative and nitrosative stress associated with disturbances in antioxidant defense system have been implicated in the pathogenesis of several diseases, most notably oral cancer. The aim of this study was to evaluate the oxidant–antioxidant status in blood samples and tumor tissue in oral squamous cell carcinoma (OSCC) patients in comparison with the healthy controls.**

**METHODS: Blood and tumor tissue samples from the diseased individuals and the normal controls were analyzed for malondialdehyde (MDA) and nitric oxide (NO) as indicators of oxidative stress and nitrosative stress respectively; superoxide dismutase (SOD) and catalase enzymes as indicators of antioxidant defense by UV visible spectrophotometer.**

**RESULTS: Malondialdehyde and NO levels were significantly elevated in the blood and tissue samples of OSCC patients as compared with the healthy controls. The antioxidant enzymes SOD and catalase were significantly reduced in tissue samples of OSCC group than in the control group while in the erythrocytes, catalase levels were significantly reduced and the SOD levels were higher in OSCC group in comparison with the healthy controls.**

**INTERPRETATION AND CONCLUSION: Increased levels of MDA and NO indicate an increase in the oxidative stress in OSCC patients associated with a deficient antioxidant defense mechanism. This oxidant–antioxidant imbalance may be considered as one of the factors responsible for pathogenesis of cancer. Future studies regarding assessment of oxidant–antioxidant status in OSCC patients in view of selecting appropriate mode of therapy and the effectiveness of such therapy in limiting the tumor progression and recurrence is to be carried out.**
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**Keywords:** oral squamous cell carcinoma; oxidative stress; nitric oxide; antioxidants

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## Introduction

Aerobic life is connected with continuous production of free radicals, particularly reactive oxygen species (ROS) (Van Wijk *et al*, 2008). Antioxidant defense which deals with the ROS produced as a consequence of aerobic respiration and substrate oxidation also concomitantly exist. Oxidant- antioxidant imbalance resulting in excessive accumulation of ROS is defined as oxidative stress. Oxidative stress is considered to play a key role in tissue damage and promotion of various pathological processes including cancer (Topdag *et al*, 2005).

Substantial evidence has established the carcinogenic role of ROS in initiation and promotion of cancer. ROS can cause DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of protooncogenes. The primary targets of peroxidation by ROS are the polyunsaturated fatty acids in the membrane lipids. The decomposition of these lipids yields a variety of end products such as lipid hydroperoxides (LHP) and malondialdehyde (MDA). The levels of these end products indicate the extent of lipid peroxidation and serve as a marker of cellular damage caused by free radicals (Beevi *et al*, 2004).

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy and a major cause of morbidity and mortality (Nagpal and Das, 2003). It is the most common form of cancer affecting males and account for 50–70% of all cancers diagnosed in India (Park, 2005). Tobacco consumption is a well known risk factor for oral cavity cancer. Tobacco consumption is positively correlated with DNA damage and could provide direct damaging effects on cellular DNA in human oral cavity. Burst of ROS have been implicated in the development of oral carcinoma in tobacco and betel quid chewers (Kolanjiappan *et al*, 2003).

In the recent past, evidence provides a critical role for nitric oxide (NO) and ROS in the pathogenesis of cancer. NO is a key signaling molecule which is involved in numerous regulatory function. It is known for its role in anti-tumor and anti-pathogen host response (Wink and Mitchell, 1998). However, under appropriate conditions NO interacts with oxygen or other free radicals thus generating a potent oxidant, peroxynitrite (reactive nitrogen species, RNS). Peroxynitrite and its

degradation products have been linked to interaction with a variety of compounds including DNA and thereby thought to be involved in pathogenesis of cancer (Davies *et al*, 1995; Rasheed *et al*, 2007).

Cells have an elaborate defense system against active oxygen in the form of certain antioxidant enzymes which includes superoxide dismutase (SOD) and catalase. These enzymes protect cells against ROS during normal metabolism and after an oxidative insult by preventing accumulation of ROS (Beevi *et al*, 2004). Excessive production of ROS or deficient antioxidant system leads to cellular damage and ultimately leads to malignant transformation.

As it is well known that an interaction between ROS and NO is a crucial determinant in the etiology of cancer, the study was performed to evaluate both ROS and RNS as indicators of oxidative and nitrosative stress, simultaneously with the determination of antioxidant enzymes SOD and catalase in both blood and tissue samples of OSCC patients and also compared with normal healthy controls.

## Materials and methods

*Patients*

A total of 18 new cases (15 male and three female) of histologically proven OSCC (well to moderately differentiated) patients with clinical stage III/IV were included in the study. All the patients were either tobacco chewers or smokers ( > 10 years) with mean age being 49.33 ± 9.6 years and free of any systemic illness. The control group included 25 healthy controls free of any habits and systemic illness with a mean age of 40.4 ± 2.31 years. Informed consent was obtained from all the patients prior to the study.

*Collection of samples*

Blood and tumor tissue samples were obtained from both the groups. The tissue samples that comprise the control group were obtained from the adjacent uninvolved normal mucosa of OSCC patients. Fresh tissue samples were collected at the time of surgery. The tissues were thoroughly washed to remove blood stains and any necrosed tissue using 0.9% HCL and were then blotted dry and weighed. 1 g of the sample was homogenized in 10 ml of 0.1 M cold phosphate buffer of pH 7.4 with 1 mM EDTA for 10 min and the obtained homogenate was centrifuged at 3000 rpm for 15 min. The clear supernatant obtained was used for analysis. Blood samples (5 ml) were collected from the subjects by venous arm puncture under aseptic precautions and transferred into a presterilized EDTA vials. The collected blood samples were then centrifuged at 3000 rpm for 10 min to separate plasma and erythrocytes. Plasma samples were used for NO analysis while erythrocyte samples were utilized for analysis of MDA, SOD and catalase. For MDA 1:1 RBC hemosylate was used and for SOD and catalase, the hemosylate was diluted to 1:50 with 0.9% normal saline. Analysis was performed using UV visible spectrophotometer (Shimadyu Scientific Instruments, USA).

*Biochemical measurement*

Malondialdehyde levels in erythrocytes and tissue homogenate were measured by the method of Ohkawa *et al* (1979). MDA is estimated as thiobarbituric acid reactive substances. MDA reacts with thiobarbituric acid at 100°C in acidic medium to form a pink colored complex which was measured at 535 nm. MDA concentration is calculated using the molar extinction co-efficient of MDA-TBA complex ($1.5 \times 10^5$l mol$^{-1}$ cm$^{-1}$). The result was expressed as nmol g$^{-1}$ Hb for erythrocytes and nmol mg$^{-1}$ protein for tissue samples.

Estimation of NO was performed using the method of Miranda *et al* (2001) by measuring the end product namely nitrite and nitrate. The sample was deproteinized by ethanol (1:2 V/V). In this assay, reduction of nitrate and measurement of nitrite is performed in a single step. Reduction is achieved by vanadium (III). Nitrite is immediately trapped by Griess reagent (Napthyl Ethylene Diamine Dihydrochloride + Sulphanilic acid) to give a pink colored complex which is measured at 540 nm. To 1 ml of protein free filtrate, 1 ml of saturated vanadium chloride ($Vcl_3$) (400 mg dissolved in 50 ml of 1 M HCL), 0.5 ml of 0.1% (W/V) NEDD and 0.5 ml of 2% (W/V) sulphanilic acid in 5% $H_3PO_4$ were added. Blank, standard and sample blank were taken separately. All tubes were incubated at 37°C for 45 min and optical density was read at 540 nm. The results were expressed as $\mu$mol l$^{-1}$ for plasma and $\mu$mol g$^{-1}$ protein for tissue samples. A series of $KNO_3$ standards (20–100 $\mu$mol l$^{-1}$) were run to obtain the standard curve. NO concentration in the sample was obtained according to the standard concentration taken.

Superoxide dismutase was measured using the method of Marklund and Marklund (1974). The principle involves the inhibition of autoxidation of pyrogallol by SOD which was determined as an increase in absorbance at 420 nm. One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation. The obtained results were expressed as U g$^{-1}$ Hb for erythrocytes and U mg$^{-1}$ protein for tissue samples.

Catalase estimation was performed using the method of Aebi (1983). Catalase decomposes $H_2O_2$ to form $H_2O$ and $O_2$. The decomposition of $H_2O_2$ by catalase was measured at 240 nm and expressed as mM g$^{-1}$ Hb for blood and mM g$^{-1}$ protein for tissue samples. One unit of catalase activity is expressed as mM of $H_2O_2$ decomposition mg$^{-1}$ Hb min$^{-1}$. The molar extinction co-efficient of $H_2O_2$ is 71 l mol$^{-1}$ cm$^{-1}$.

Tissue protein was estimated utilizing the method of Lowry *et al* (1951). This method is based on biuret reaction followed by reaction of aromatic amino acid tyrosine and tryptophan in proteins, with Folin Ciocalteau's phenol reagent to form blue color. The end products such as tungsten blue and molybdenum blue are measured at 750 nm.

*Statistical analysis*

The findings are expressed as mean ± s.d. Statistical analysis was performed using unpaired *t*-test. *P* value $< 0.05$ was considered statistically significant, $P < 0.01$

as highly significant and $P < 0.001$ as very highly significant.

## Results

The results obtained of the assessed parameters in OSCC patients and healthy controls are shown in Tables 1 and 2 for blood and tissue samples respectively.

Table 1 shows that the levels of MDA is significantly elevated in erythrocytes of OSCC group than in the healthy controls ($P < 0.05$). Plasma NO levels were elevated in OSCC group than in the controls and the difference was statistically significant ($P = 0.001$). Among antioxidant enzymes evaluated in erythrocyte samples, it was found that while catalase levels were significantly reduced in OSCC group ($P < 0.05$), the levels of SOD were, however, found to be non-significantly high among the oral cancer group ($P > 0.05$).

Table 2 shows that the levels of MDA and NO levels were significantly elevated in tumor tissue than in the control tissue ($P < 0.05$ and $P < 0.01$ respectively) and the antioxidant enzyme levels of SOD and catalase were significantly reduced in tumor tissue ($P < 0.01$ and $P < 0.05$ respectively).

## Discussion

Highly ROS and RNS are implicated in the pathogenesis of oral cavity cancer. The basic supposition is that the free radicals damage cellular materials which would result in triggering or transforming normal cells into malignant ones (Seven *et al*, 1999). The magnitude of this damage is also dependent on the body's defense mechanisms against free radicals which are mediated by various cellular antioxidants. The metabolism of ROS in cancer cells is drastically altered with evidence favoring at least two mechanisms; cancer cells produce large amounts of ROS compared with non-neoplastic cells and suppression of antioxidant system in cancer cells (Van Wijk *et al*, 2008).

The ultimate step resulting in carcinogenesis is DNA damage. ROS and RNS are two important agents of DNA damage. They are found to be involved in both initiation and promotion of multistep carcinogenesis (Beevi *et al*, 2004).

Several investigators have revealed that the cellular responses to external stimuli are mediated by lipid peroxidation caused by increased oxidative stress and these have been implicated in tumor development (Kolanjiappan *et al*, 2003). LHP and MDA are well characterized end products of lipid peroxidation. The levels of MDA reflects the extent of peroxidation and are considered as an indicator of the extent of prevailing oxidative stress (Beevi *et al*, 2004).

The results obtained from our study revealed that MDA levels were significantly elevated in erythrocytes and tumor tissue of OSCC group than in the control group ($P < 0.05$).

Our findings correlated with several studies that have found significantly increased levels of MDA in erythrocytes of oral cancer groups (Sabitha and Shyamaladevi, 1999; Beevi *et al*, 2004; Manoharan *et al*, 2005; Rasheed *et al*, 2007). The increased levels could be attributed to the decomposition products of polyunsaturated fatty acids of biomembranes (Sabitha and Shyamaladevi, 1999). Others stated that the increased MDA in tissues occurs as a result of the lipoxidative damage and these are released into the plasma; thus plasma MDA level is considered to reflect tissue lipid peroxidation on whole body basis (Beevi *et al*, 2004).

Tumor cells through their transformation process produce a high level of ROS in OSCC patients. Lipid peroxides can further initiate lipid peroxidation through prostaglandin synthesis, which is a well recognized pathobiological alteration in cancer cells. Besides, lipid peroxidation products possess cytotoxic and mutagenic

**Table 1** Comparison of blood levels of MDA, NO, SOD, and catalase between the normal controls and OSCC groups (all values expressed in mean ± s.d.)

| *Groups* | *MDA (nmol $g^{-1}$ Hb)* | *NO ($\mu$mol $l^{-1}$)* | *SOD (U $g^{-1}$ Hb)* | *Catalase (mM $g^{-1}$ Hb)* |
|---|---|---|---|---|
| Group I: controls ($n = 25$) | 139.44 ± 22.32 | 87.59 ± 21.61 | 33.52 ± 8.69 | 1.71 ± 0.31 |
| Group II: OSCC ($n = 18$) | 159.83 ± 36.39 | 173.18 ± 98.21 | 39.42 ± 10.1 | 1.5 ± 0.33 |
| *P*-value | 0.04 | 0.001 | 0.053 | 0.04 |
| Significance | S | HS | NS | S |

S, significant; HS, highly significant; NS, not significant.

**Table 2** Comparison of tissue levels of MDA, NO, SOD, and catalase between the normal tissue and OSCC tumor tissues (all values expressed in mean ± s.d.)

| *Groups* | *MDA (nmol $mg^{-1}$ protein)* | *NO ($\mu$mol $g^{-1}$ protein)* | *SOD (U $mg^{-1}$ protein)* | *Catalase (mM $g^{-1}$ protein)* |
|---|---|---|---|---|
| Group I: controls ($n = 18$) | 0.68 ± 0.33 | 38.12 ± 30.89 | 4.15 ± 2.31 | 19.45 ± 16.92 |
| Group II: OSCC ($n = 18$) | 1.12 ± 0.76 | 67.1 ± 35.13 | 2.45 ± 1.21 | 10.16 ± 7.46 |
| *P*-value | 0.03 | 0.01 | 0.01 | 0.04 |
| Significance | S | HS | HS | S |

S, significant; HS, highly significant; NS, not significant.

properties and are capable of modulating cell growth and tumor promotion by activating signal transduction pathways (Rasheed *et al*, 2007).

Increased levels observed in our study were similar to the findings obtained in certain lung and laryngeal cancers (Inci *et al*, 2003; Topdag *et al*, 2005) . The significantly elevated lipid peroxidation end products in the tumor tissue could reflect a state of increased oxidative stress.

Plasma and tissue NO levels were significantly increased in OSCC group than the control group ($P < 0.01$). The findings in plasma levels were in accordance with few other studies (Beevi *et al*, 2004; Rasheed *et al*, 2007). The tissue levels of increased NO levels in tumor samples were in line with the findings obtained from similar studies performed in gastric cancer (Bakan *et al*, 2002). Immunohistochemical studies performed in oral cancer tissue samples demonstrated increased NOS expression in oral cancer (Connelly *et al*, 2005).

The reactive nitrogen oxide species (RNOS) formed from the reaction of NO either with oxygen or superoxide can mediate either nitrosative or oxidative stress. At lower concentration NO acts as antioxidant by scavenging superoxide anion, while at higher concentration it forms peroxynitrite ($ONOO^-$) which is a potent oxidant known to play a key role in oral cancer development (Rasheed *et al*, 2007). Maximum lipid peroxidation occurs when the fluxes of superoxide and NO are equivalent.

Dinitrogen trioxide ($N_2O_3$) is the predominant RNOS formed from autoxidation of NO in biological systems. Carcinogenic nitrosamines can be formed from the same secondary amines by nitrozation mediated by $N_2O_3$ and can have deleterious consequences (Wink and Mitchell, 1998). As NO acts as cellular signal for angiogenesis, it is also thought to be involved in carcinoma growth and invasion (Connelly *et al*, 2005).

A possible side effect of high concentration of NO in OSCC tumor microenvironment is pain caused by activation of primary afferent nociceptor. This could be considered as a probable factor for causation of pain noticed in majority of our patients (67%). Targeting therapy toward inhibiting inducible NOS or decreasing NO levels on tumor microenvironment may be a novel approach in treating pain in OSCC in the near future (Connelly *et al*, 2005).

Considerable evidence suggests that antioxidant enzymes act to inhibit both initiation and promotion of carcinogenesis. The low activities of these enzymes play a key role in progression of cancer. Our results revealed decreased SOD and catalase levels in the tumor tissue which was statistically significant ($P < 0.01$ and $P < 0.05$ respectively). Similar findings were noted in several other studies (Nagini *et al*, 1998; Sabitha and Shyamaladevi, 1999; Subapriya *et al*, 2002; Manoharan *et al*, 2005; Rasheed *et al*, 2007). The decreased catalase levels might be caused by the increased endogenous production of superoxide anion or increased NO end products or decreased activity of SOD or all of the above (Beevi *et al*, 2004). The decrease in catalase activity in oral tumor tissue may also be caused by the exhaustion of these enzymes in catalyzing the overproduction of hydrogen peroxide by tumor cells (Kolanjiappan *et al*, 2003). The reduced catalase in the erythrocytes may have occurred as a consequence of overwhelming free radicals in the circulation of oral cancer patients (Beevi *et al*, 2004).

The SOD levels of erythrocytes in OSCC patients were found to be elevated than in the control group which correlated with studies obtained in breast cancer patients (Surapaneni and Chandra Sada Gopan, 2007). This was in contrast to the findings of several studies where SOD levels were reduced in erythrocytes of oral cavity cancer. The overexpression of SOD might be an adaptive response to the increased lipid peroxidation end products in erythrocyte samples and it results in dismutation of superoxide to $H_2O_2$.

Studies showed that SOD enzyme activity increases when the effectiveness of other enzymes decrease (Topdag *et al*, 2005). SOD is reported to be the first induced enzyme. Its higher activity could be due to its induction by increased superoxide anion production as seen in our study (RBC). The induction of SOD in turn leads to the protection of glutathione peroxidase (GPx) against inactivation by superoxide, the net effect being a higher glutathione peroxidase activity. The constitutive levels of antioxidant enzymes and their inducibility vary among different tissues. GSH and GPx levels also carry importance as studies have suggested that GSH dependent system modulates sensitivity of tumor to cytokines, chemotherapeutic drugs or to radiation therapy. Depletion of GSH may provide an approach to sensitize tumors to chemotherapy and radiotherapy (Inci *et al*, 2003). Thus with regard to these facts, the assessment of antioxidant status both in tumor tissue and the cancer free adjacent tissue might prove beneficial for cancer patients prior to radiotherapy and chemotherapy.

In conclusion, the observations made in the present study shows a significant increase in the oxidative and nitrosative stress with concomitant decrease in the antioxidant enzymes in OSCC patients. There is increased production of ROS by cancer cells and also suppression of antioxidant system. This oxidant–antioxidant imbalance is thought to be one of the factors which may be responsible for carcinogenesis and tumor growth and invasion. Thus maintenance of balance between the oxidant and antioxidants by appropriate therapy may be of some help to limit the progression of precancerous condition toward malignancy. Further studies are needed to assess the role of oxidant–antioxidant status on response to chemotherapy, radiotherapy and preventive strategies in OSCC.

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