

## Research Paper

# Lipid peroxidation and antioxidant status in head and neck squamous cell carcinoma patients

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**Abbreviations:** EDTA, ethylenediamine tetra acetic acid; DNA, deoxyribonucleic acid; DTNB: 2,2-dithiobisnitrobenzoic acid; KPS, karnofsky performance status; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; SH, sulfhydryl; TEP, 1,1,3,3-tetraethoxy propane

**Key words:** lipid peroxidation, superoxide dismutase, total thiols, ascorbic acid, oxidative stress, head and neck cancer

Oxidative stress, a consequence of an imbalance between the formation and inactivation of reactive oxygen species, may be involved in the pathogenesis of many diseases including cancer. To evaluate the magnitude of oxidative stress, a study on the plasma levels of superoxide dismutase, total thiols, ascorbic acid and malondialdehyde (MDA) has been done in head and neck squamous cell carcinoma patients before the start of any oncological treatment and compared with healthy controls. The specific activity of superoxide dismutase in cancer patients is decreased significantly when compared to the control ( $p < 0.05$ ). The total thiol and ascorbic acid levels are significantly reduced ( $p < 0.005$ ) whereas MDA levels are significantly increased in the patients ( $p < 0.00005$ ). Our findings show that the oxidative stress is elevated in cancer patients as evidenced by elevated levels of lipid peroxidation products and depletion of enzymatic and non-enzymatic antioxidants.

## Introduction

Carcinogenesis is a complex multisequential process leading a cell from a healthy to a precancerous state and finally to an early cancerous stage. Three distinct stages of carcinogenesis have been defined and these include initiation, promotion and progression.<sup>1</sup> It has been proposed that reactive oxygen species are associated with the different stages of carcinogenesis through structural DNA damage, interaction with oncogenes, tumor suppressor genes or immunological mechanisms.<sup>2</sup>

A substantial body of evidence has been produced that links the production of reactive oxygen species (ROS) and subsequently oxidative stress and damage to the pathogenesis of age related and

chronic diseases and cancer.<sup>3-6</sup> Oxidative stress has been defined as an imbalance between pro-oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of reactive oxygen species.<sup>7</sup> Reactive oxygen species such as  $O_2^-$ ,  $H_2O_2$  and  $OH^\cdot$ , are generated inevitably as a part of normal cell metabolism including oxidative phosphorylation, P450 metabolism, peroxisomes and inflammatory cell activation. ROS can interact with cellular macromolecules such as DNA, proteins and lipids and interfere with vital cellular functions. ROS can cause DNA base alterations, strand breaks, damage to tumor suppressor genes and enhance expression of proto-oncogenes.<sup>8</sup> These oxidative modifications could result in the transformation of normal cells into malignant cells.<sup>3</sup> The prime target of peroxidation by ROS are the polyunsaturated fatty acids (PUFA) in the membrane lipids leading to the formation of lipid peroxides such as malondialdehyde (MDA).<sup>9</sup> MDA itself, owing to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter and a co-carcinogenic agent.<sup>10</sup>

Under normal physiological conditions, cells are capable of counterbalancing the noxious effects of reactive oxygen species with antioxidant defense system which consists of free radical scavengers including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase and non enzymatic antioxidants such as thiols and various other substances taken along with food such as vitamin E, vitamin C,  $\beta$ -carotene and flavanoids. Antioxidant defense systems work cooperatively to alleviate the oxidative stress caused by enhanced production of the free radicals. Any change in one of these systems may break this equilibrium and cause cellular damage and ultimately malignant transformation.

Tobacco, an exogenous source of reactive oxygen species, is believed to play a significant role in mutagenesis and carcinogenesis. Tobacco has been recognized as a major etiological factor for the head and neck cancer.<sup>11</sup> Alkaline saliva generated by chewing betel quid also plays an important role in ROS mediated DNA damage in head and neck cancer patients.<sup>12</sup> The head and neck cancer refers to a group of biologically similar cancers originating from the upper aerodigestive tract including the lip, oral cavity,

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nasal cavity, para-nasal sinuses, pharynx and larynx. Most head and neck cancers are squamous cell carcinomas, originating from the mucosal lining of these regions.

The aim of this study has been to evaluate the extent of oxidative stress in head and neck squamous cell carcinoma patients before the start of any oncological treatment by analyzing the plasma levels of the free radical scavenging enzyme superoxide dismutase and antioxidants total thiols and ascorbic acid. Free radical mediated damage was also assessed simultaneously by measuring the plasma levels of MDA.

## Results

The inclusion criteria for patients comprised of the following parameters: the age group between 20–70 years and Karnofsky Performance Score (KPS) >70. Subjects (both control and the patients) with other co-morbidity and diseases were excluded from the study. The patient group comprised of 17 male patients (mean age  $52.71 \pm 14.40$ ). Tumor and node size was noted and patients were staged according to AJCC-2002, TNM classification. Personal profiles and clinical parameters of the patients are presented in Table 1. Ten out of the total 17 patients had their cancer in third stage while seven patients had their cancer in stage IV. Eleven patients had well differentiated cancers while five possessed moderately differentiated tumors. Only one patient had poorly differentiated cancer. Eight of the total patients had their primary tumor located in oral cavity while seven had in their pharyngeal region (oropharynx 3, hypopharynx 2 and nasopharynx 2). Two patients had laryngeal carcinoma. Ten patients were heavy smokers while five patients were betel chewers. Twelve age and sex matched healthy individuals from the same Indian socio-economic background as of the patients served as control. The controls were not habituated to tobacco chewing and smoking.

Results are expressed as mean  $\pm$  SD. Superoxide dismutase activity and levels of total thiols, ascorbic acid and MDA are presented in Table 2 and the individual observations are presented in Figures 1–4. The specific activity of superoxide dismutase in head and neck squamous cell carcinoma patients is decreased significantly when compared to the control ( $p < 0.05$ ). Also, the levels of non-enzymatic antioxidants, total thiols and ascorbic acid are significantly reduced in cancer patients ( $p < 0.005$ ) when compared to healthy persons. The extent of lipid peroxidation as evidenced by the levels of MDA is significantly increased in the patients ( $p < 0.00005$ ) compared to control.

## Discussion

Cancer is a heterogeneous disorder with multiple etiologies including somatic and germ line mutations, cellular homeostatic disturbances and environmental triggers. Certain etiologies are characteristic of head and neck cancer and include agents such as Epstein-Barr virus, the use of tobacco and consumption of alcohol. ROS may be critically involved in the causation of malignant diseases through their possible impact on proto-oncogenes and tumor suppressor genes. ROS may also affect the balance between apoptosis and cellular proliferation. If apoptotic mechanisms are overwhelmed, uncontrolled cellular formation may follow,

**Table 1 Personal profile and clinical parameters of the patients**

No. of Patients	17
<b>Stage:</b>	
II	0
III	10
IV	7
<b>Differentiation:</b>	
Poorly differentiated	1
Moderately differentiated	5
Well differentiated	11
<b>Location:</b>	
Oral cavity	8
Oropharynx	3
Hypopharynx	2
Nasopharynx	2
Larynx	2
Smokers/Nonsmokers	10/7
Betel chewers/non chewers	5/12

potentially leading to tumor formation.<sup>13</sup> ROS are also formed in response to tumor promoters and that cellular consequence of their actions may play a role in the process of tumor promotion.<sup>6</sup> ROS cause DNA damage, activate procarcinogens, initiate lipid peroxidation, inactivate enzyme systems and alter the cellular antioxidants defense system.<sup>14</sup>

High levels of oxidative stress results in peroxidation of membrane lipids with the generation of peroxides that can decompose in to multiple mutagenic carbonyl products such as lipid hydroperoxides and malondialdehyde. MDA can interact with cellular DNA and cause formation of DNA-MDA adducts.<sup>15</sup> These adducts appear to be promutagenic as they induce mutation in oncogenes and tumor suppressor genes in many human cancers and are also correlated with altered cell cycle control and gene expression in cultured cells.<sup>16</sup> The plasma MDA levels reflect the extent of lipid peroxidation hence serve as the marker for free radical mediated damage. In the present study, we observed increased plasma levels of MDA in head and neck squamous cell carcinoma patients, which could be attributed to increased formation or inadequate clearance of free radicals by cellular antioxidants.

Cellular free radical scavenging enzymes and antioxidants protect cells against toxic oxygen derived radicals. Free radical scavenging enzymes such as superoxide dismutase and catalase provide the first line of defense against free radicals. These enzymes react directly with oxygen free radicals to yield non-radical products. In our study, SOD activity was lowered in cancer patients when compared to healthy controls. The decreased activity of SOD has also been reported in other malignancies.<sup>17-19</sup> SOD metabolizes free radicals and dismutates superoxide anion to  $H_2O_2$  and protects the cell against superoxide mediated lipid peroxidation. Catalase acts on  $H_2O_2$  by decomposing it, thereby neutralizing its

**Table 2 Plasma levels of superoxide dismutase, total thiols, ascorbic acid and MDA in the patients and healthy persons**

	Superoxide dismutase (activity/mg protein)	T-thiols ( $\mu$ moles/ml)	Ascorbic acid (mg/dl)	Malondialdehyde (nmoles/ml)
Healthy (n = 12)	2.71 $\pm$ 0.71	231.65 $\pm$ 38.02	0.43 $\pm$ 0.10	1.16 $\pm$ 0.48
Patients (n = 17)	2.11 $\pm$ 0.47	163.22 $\pm$ 50.18	0.27 $\pm$ 0.08	2.74 $\pm$ 1.05
p-value	p < 0.05	p < 0.0005	p < 0.0005	p < 0.00005

The data are the mean  $\pm$  SD. p > 0.05 not significant; p < 0.05 significant; p < 0.005 highly significant.

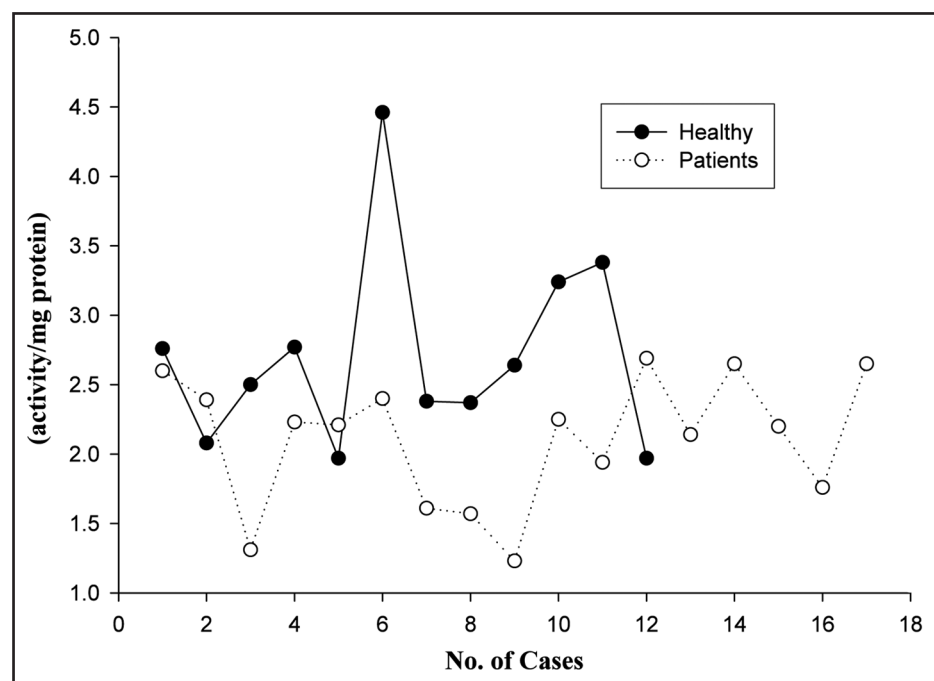


Figure 1. Activity of superoxide dismutase in plasma of healthy subjects and patients.

toxicity, but there is little or no catalase activity in plasma.<sup>20</sup>  $H_2O_2$  is, therefore, not detoxified and accumulates to high levels and causes inhibition of SOD activity.<sup>21,22</sup> Reduction of SOD activity may also be due to increased endogenous production of ROS as evidenced by elevated MDA levels.

Plasma thiol groups are contributed mainly by proteins and a smaller part from free-SH group containing substances, such as glutathione and certain other coenzymes which are critical endogenous antioxidants that act concurrently in scavenging and/or reducing free radicals. The plasma thiol groups break the peroxidative chain and allow the repair of oxidatively damaged molecules.<sup>23</sup> A significant depletion of plasma thiols observed in our study reflects enhanced pro-oxidant milieu of the cells and correlates with the increased lipid peroxides in the circulation of cancer patients.

Ascorbic acid is an important extracellular antioxidant that is capable of completely inhibiting oxidative modification of lipids by aqueous peroxy radicals.<sup>24</sup> It disappears faster than other

antioxidants when plasma is exposed to oxygen free radicals, thus sparing other antioxidants. Significantly lowered ascorbic acid levels in the patients compared to healthy individuals may be linked to increased oxidative stress. A strong synergism exists between thiols and ascorbic acid since regeneration of ascorbic acid requires thiols; diminished plasma thiol levels might be responsible for the low levels of ascorbic acid.

To summarize, our results suggest that there is increased oxidative stress as highlighted by the elevated plasma MDA levels and depletion in antioxidant defense system.

The low levels of antioxidants in head and neck squamous cell carcinoma patients could be a result of this increased oxidative stress, or it could be that low values aggravated the free radical damage and increase the chance of developing cancer, remains to be elucidated by further research.

## Materials and Methods

All the chemicals employed in the study were of analytical grade of Qualigens or equivalent. All the biochemicals used, were from Sigma Chemical Co., St. Louis, MO. The study was conducted in biopsy proven head and neck squamous cell carcinoma patients registered in the Department of Radiotherapy, CSM Medical University, Lucknow. The study was approved by the Departmental Ethical Committee. Informed consent was taken from all the subjects enrolled in the study.

Sample from all the subjects was collected in the morning between 10–11 A.M. Taking aseptic precautions, venous blood (3.5 ml) was withdrawn and transferred into the polypropylene tubes containing 0.5 ml 3.8% sodium citrate; pH 7.2. The tubes were gently rotated to mix the contents and centrifuged at 2,000 xg for 20 min at 4°C to separate plasma. All the analyses were carried out on the plasma.

**Assay of superoxide dismutase.** Superoxide dismutase was assayed by the method described by Misra and Fridovich.<sup>25</sup>

Three ml reaction mixture consisted of 1.5 ml of 0.1 M sodium carbonate buffer, pH 10.2; 0.01 ml of 0.03 M EDTA, suitable aliquot of enzyme preparation and water to make up the volume to 2.94 ml. The reaction was started by addition of 0.06 ml of 0.015 M epinephrine. Change in absorbance was recorded for one minute at 15 sec interval. Control lacking the enzyme, was run simultaneously.

One unit of the enzyme activity has been defined to cause 50% inhibition of epinephrine auto-oxidation by 1 ml enzyme.

**Protein analysis.** Protein was estimated by the method of Lowry et al.<sup>26</sup> using Folin phenol reagent with bovine serum albumin as standard.

The specific activity of the enzyme has been expressed as activity/mg protein.

**Total thiols.** Total thiols in plasma were measured by the method of Hu.<sup>27</sup> In final volume of 4.0 ml. was added 0.05 ml plasma; 0.6 ml of 0.25 M tris buffer containing 0.02 M EDTA, pH 8.2 followed by addition of 0.04 ml of 0.01 M 2,2-dithiobisnitrobenzoic acid (DTNB) in absolute methanol and 3.31 ml of absolute methanol. The tubes were capped and color was developed for 15 min at room temperature. The tubes were then centrifuged at 3,000 xg for 20 min. Supernatant was collected and absorbance measured at 412 nm. Total thiol groups were calculated using molar extinction coefficient of 13,600 at 412 nm.

**Lipid peroxidation.** Lipid peroxidation was monitored in terms of malondialdehyde (MDA) by the method of Ohkawa, Ohishi and Yagi<sup>28</sup> using thiobarbituric acid (TBA) reagent. To 0.2 ml plasma was added 0.2 ml of 8.1% [w/v] sodium dodecyl sulphate; 1.5 ml 20% [v/v] acetic acid, pH 3.5; 1.5 ml of 0.8% TBA and water to make up the volume to 4.0 ml. The tubes were heated in a water bath at 95°C for one hour and cooled immediately under running tap water. To each tube, 1.0 ml chilled water and 5.0 ml of butanol and pyridine [15:1 v/v] were added and the tubes vortexed and centrifuged at 800 xg for 20 min. The upper layer was aspirated out and color intensity measured at 532 nm. 1,1,3,3-tetra ethoxy propane (TEP) was used as the reference.

**Ascorbic acid.** Ascorbic acid was measured by the method of Omaye, Turnbull and Sauberlich.<sup>29</sup> This method is based on the

oxidation of ascorbic acid to dehydroascorbic acid, which reacts with 2,4 dinitro phenyl hydrazine to form a colored complex with absorption maximum at 520 nm. L-ascorbic acid was used as reference.

Statistical analyses were carried out by student's t-test using Sigma plot version 8.0. A value of  $p < 0.05$  was considered as statistically significant.

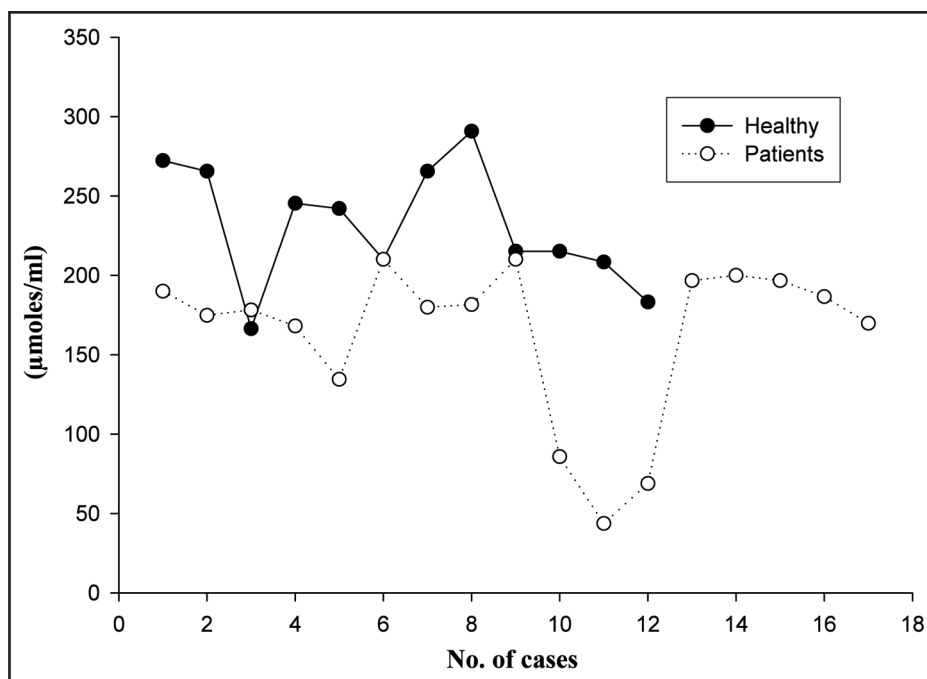


Figure 2. Plasma levels of total thiols in healthy subjects and patients.

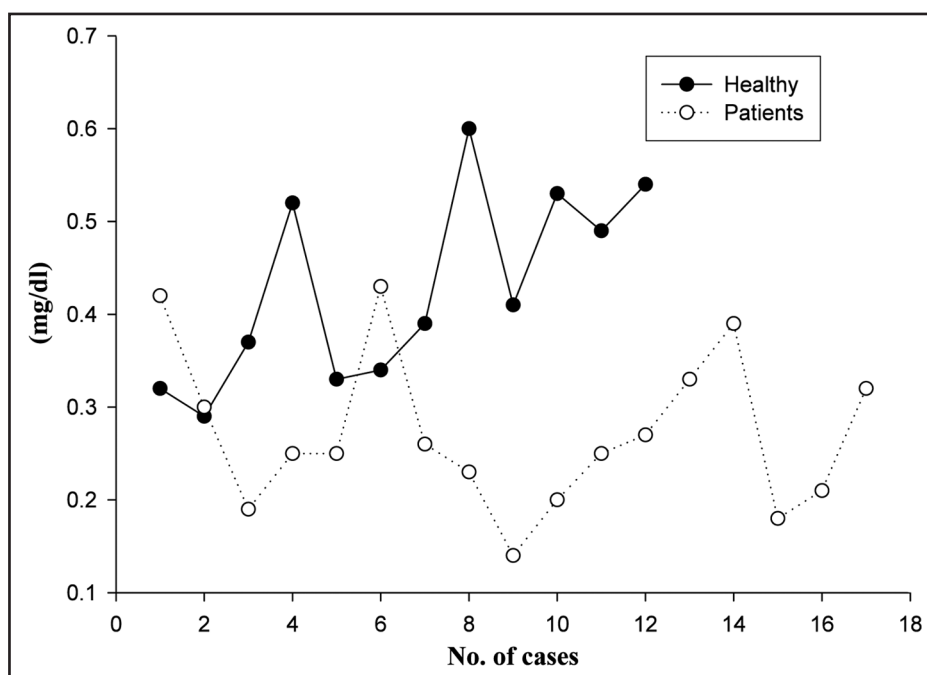


Figure 3. Ascorbic Acid levels in plasma of healthy subjects and patients.

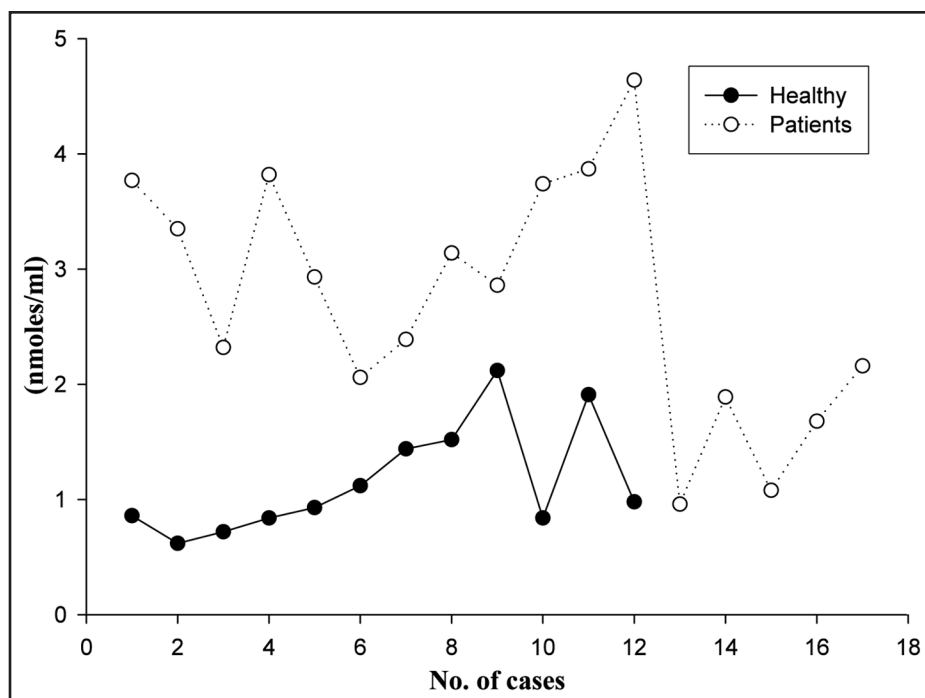


Figure 4. Plasma MDA levels in patients and healthy subjects.

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