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STUDIES ON THE OXIDATIVE STRESS PARAMETERS IN ORAL CANCER PATIENTS

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ABSTRACT
Oral cancer in India ranks number one among all cancers in male patients and number three among cancers in female patients in India. There are a number of substances which seem to stimulate the production of free radicals. Enzymatic and non-enzymatic antioxidant defense systems helps to protect cells against those free radicals produced during normal metabolism and after an oxidative insult. Alteration in any one of these systems results in break in this equilibrium and cause cellular damages which ultimately transform the cell to malignant. Our study in patients with oral cavity cancer reveals elevated levels of lipid peroxidation products and nitric oxide products and effective depletion shown in the oral cancer patient. However, the degree of effectiveness with which the antioxidant system can be restored or maintained with dietary and nutritional supplements, so that oral cancer atients can actually be benefited remains to be elucidated. Further investigation can be continued with the same approach in oral cancer patient of various age groups, among sex, among children. This will enlighten about the enzymatic and non enzymatic parameters under study in above various groups and may guide us to prevent the oral cancer.

KEYWORDS: Oral cancer, ROS, peroxidation, glutathione.

INTRODUCTION
Oral cancer in India, ranks number one among all the cancers that are seen in male patients and number three in female patients, because of chewing of betel quids containing tobacco leaves or stem and other tobacco habits has been extensively studied.[1]

Cancer is caused from the damage free radicals react with DNA or the cell membrane. The DNA is, in essence, the reproductive map of the cell slightly changes; the cell may duplicate the change when it reproduces. These mutations also start a chain reaction and leads to the development of mutated, cancerous tissue such as tumors and lesions.

Oral cavity cancer is an important tumor globally and is one of the ten most frequent cancers worldwide. The Reactive Oxygen Species (ROS) such as hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2) and super oxide radicals (O2•) play an important role in human cancer development. They have been shown to possess several characteristics of carcinogens. ROS can cause DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of proto-oncogenes.[2]

Even with their potential for damage, the presence of a limited number of free radicals is not dangerous as free radicals are actually part of the normal metabolism of a healthy body. Many times the immune system of cells creates free radicals deliberately in order to neutralize viruses and bacteria. The excess of free radicals present than the normal level can be dangerous. There are a number of substances which may stimulate the production of free radicals such as radiation, pollution, tobacco and pesticides etc.

But the real cause of cancer is not the substances but excessive of free radicals present in the system. Apart from cancer, free radicals are also reported to have link in heart disease, aging, cataracts and impairment of the immune system. Researchers have also noted that free radical damage accumulates with age. Since there is no direct method to measure free radicals in the body, scientists have reached the conclusion by measuring the by-products released as the product from free radical reactions.[3]

The burst of ROS has been reported in the development of oral cavity cancer in tobacco chewers and continuous smokers.[4] Tobacco consumption in any form have carcinogenic and genotoxic effects and has significant

correlation with accumulation of DNA damage. Hence tobacco is believed to have direct influence on cellular DNA damages in the human oral cavity.[5] Oxidative modification of nucleic acids by reactive oxygen species could result in the transformation of normal cells into malignant cells.[6]

Lipid peroxidation induced by ROS has been found in malignant transformation. The major targets of peroxidation by ROS in the membrane lipids are the polyunsaturated fatty acids (PUFA). Furthermore, these peroxidized lipids decomposition releases a variety of compounds including malondialdehyde (MDA) and lipid hydroperoxides (LHP) as an end products. The levels of these lipid peroxides serve as markers of cellular damages and indicate the extent of lipid peroxidation.[7]

Enzymatic and antioxidant defense systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamins E, C and A and ß carotene. They protect cells against free radicals especially ROS, synthesized during metabolism and after an oxidative insult. Antioxidant defense systems work cooperatively to regulate and maintain the oxidative stress caused by enhanced free radical production in control. Any minor changes of these systems may break this equilibrium and results in cellular a damage which ultimately leads to malignant transformation.[8]

Nitric oxide (NO•) an uncharged molecule with an unpaired electron is a free radical and plays variety of roles in cellular signaling mechanisms.[9] This highly reactive molecule is produced by the isoenzyme nitric oxide synthase (NOS) using L-arginine.[10] Reaction of NO• with oxygen or other free radicals results in reactive nitrogen species (RNS), which can cause multiple biological effects.[11] NO• is either cytostatic or cytotoxic. Different tissues display various responses to NO•, that may relate to the presence of cellular antioxidants such as GSH, CAT, SOD, etc.[12]

In chronic inflammation over expression of NOS can lead to genotoxicity. NO• may mediate DNA damage through the formation of nitrosamines, generation of RNS and inhibition of DNA damage repair mechanism. It can thus be considered as a tumor initiating agent.[13] However, NO• may also have an impact on other stages of cancer development. These effects of NO• ranging from cellular transformation and formation of neoplastic lesions to the regulation of various other aspects of tumor biology.[12]

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

The present study has the following objectives.

1. To correlate the tobacco consumption and oral cancer.
2. To study the marker enzymes of oral cancer in selected risk group of patients.
3. To study the extent of oxidative stress and the nitric oxide levels in oral cavity cancer patients by analyzing the levels of lipid peroxidation products (MDA), antioxidants products (GSH, Vitamin E and Vitamin C).

Oral cancer

Oral cancer occurs in a region of the body that is easily accessible for physical examination. The 90% of all squamous cell cancers arise in the following: the floor of the mouth, the ventro lateral aspect of the tongue, and the soft-palate complex. Leukoplakia and erythroplastic lesions are the earliest and most serious signs of squamous cell carcinoma.

Epidemiology

There are many diseases that cause threats to public health in the developed and developing world. One among them is cancer which stands second in the developed countries. According to the World Health Report cancer accounted for 7.1 million deaths and it is estimated the overall number of new cases will rise by 70% in the next 10 years .The prevalence of oral cancer is high among men. Incidence rates for oral cancer vary in men from 1 to 10 cases per 100 000 population in many countries.

In south-central Asia, oral cavity cancer ranks three and the most common types of cancer. In India, the age standardized incidence rate of oral cancer is 15.1 per 100 000 population. It is important to note that there is an sharp increases in the incidence rates of oral/pharyngeal cancers have been reported for several countries and regions such as Denmark, France, Germany, Scotland, central and Eastern Europe and to a lesser extent Australia, Japan, New Zealand and the USA.

The cancer epidemic in developed countries, and increasingly in developing countries, is due to the combined effect of the ageing and the high levels of prevalence of cancer risk factors. It has been estimated that 45% of cancer deaths worldwide are due to tobacco, unhealthy diet habits, physical inactivity and infections. Tobacco use and high alcohol consumption have been reported for about 90% of cancers in the oral cavity; the oral cancer risk increases when tobacco is used in combination with alcohol or areca nut.[1]

Etiology

Oral cancer is the excess growth of a tissue located in the mouth. It may arise as a primary lesion or by metastasis with a distant site of origin, or by extension from a neighboring anatomic structure especially from the nasal cavity, the maxillary sinus etc. Oral cancers originate in any of the tissues of the mouth, and may be of many

histologic types such as teratoma, adenocarcinoma or lymphoma or other lymphoid tissue, or melanoma. The most common oral cancer is squamous cell carcinoma, originating in the tissues that line the mouth and lips.[14]

Oral or mouth cancer commonly involves the tissue of the lips or the tongue. It may also occur on the floor of the mouth, cheek lining, gums or palate. Most oral cancers look very similar under the microscope. These are malignant and tend to spread rapidly.[15] Smoking and other tobacco use are reported with about 75 percent of oral cancer cases, which are caused by irritation of the mouth mucous membranes from smoke and heat of cigarettes. Tobacco contains over 19 known carcinogens, and the combustion of it as well as it's by products from this process, is the primary mode of involvement.[16] Chewing betel, paan and Areca is known to be a strong risk factor for developing oral cancer. In India such practices are common, oral cancer represents up to 60% of all cancers. There is known to be a strong correlation reported on oral cancer risk for those subjects who are heavy smoker and drinker. Some oral cancers begin as leukoplakia a white patch (lesion), red patches, (erythroplakia) or non healing sores that have existed for more than 14 days. Human Papilloma Virus, (HPV) particularly versions 16 and 18 (there are over 100 varieties) is a known risk factor and independent causative factor for oral cancer. Oral cancer in this group tends to favor the tonsil and tonsillar pillars, base of the tongue, and the oropharnyx.

Tumor markers

Tumor markers are the molecules that occurr in blood or tissue that are associated with oral cancer and whose measurement or identification is very important in diagnosis or clinical management. Tumor markers include many substances such as superoxide, hydrogen peroxide, hydroxyl radical, Malondialdehyde (the end-product of lipid peroxidation), antioxidants such as reduced Glutathione (G-S-SH), vitamin C, and vitamin E as well as enzymatic antioxidant such as catalase, superoxide dismutase and various peroxidases that are readily systematically organized and they are either produced by the tumor cell or by the body response to the tumor cells. They are typically substances that are released in to the circulation and thus measured in the tumor condition. Lactate dehydrogenase is a tetrameric enzyme and is recognized as a potential tumor marker in assessing the progression of the proliferating malignant cells. In oral cancer patients the levels of aspartate amino transferase and alanine amino transferase can be used as indicators as if any cellular damage or the loss of functional integrity of the cell membrane will lead to the release of these enzymes into the blood circulation. Similarly alkaline phosphatase and another enzyme have to be known to be over expressed at the surface of various solid tumors. The rise/decrease in their activities is shown to be good correlation with the number of transformed cells in cancer conditions.[17]

METHODOLOGY

Subjects and Sample Collection

This prospective study was conducted on 30 male patients with biopsy proven squamous cell carcinoma of the oral cavity with clinical stage II/III. All the patients included in the investigation were either smokers or tobacco chewers. The control groups consisted of 10 non-smoking healthy volunteers from similar socioeconomic backgrounds. The age range was 30 to 50 years for both patients and controls.

All the carcinomas were graded as well-differentiated squamous cell carcinoma. Samples are collected from the patients and control subjects who didn't have concomitant diseases such as diabetes mellitus, rheumatoid arthritis and liver disease. All the patients and the control subjects were not on any medical treatment or any supplementation of antioxidants. A thorough dental checkup was done for all the subjects. The case history of the patient was recorded after examination of the subjects, the blood samples were collected for various assays.

Grouping of Patients

Group –1 Healthy male.
Group –2 Male in early stage of cancer.

The results of Group 1 were taken as control and it is compared with Group 2. Number of samples taken for each group is 30.

Analysis in Blood

After overnight fasting, the blood samples (approximately 10 ml) from the subjects were collected in appropriate sterile vials by venous arm puncture. 5ml of blood was collected with EDTA as an anti coagulant for plasma and another without anticoagulant for the separation of serum. Plasma and sera were separated by centrifugation at 1000 g for 15 min. Biochemical estimations were carried out immediately.

1. Estimation of Malondialdehyde (MDA)

The level of MDA was estimated by the method of Draper and Hadley. To 0.5 ml of sample add 0.5 ml of distilled water and o.5 ml, 1.0 ml of TCA, TBA respectively. Mix and heat the contents in boiling water bath for 10 minutes. After heating cool the contents in ice cold water bath for 5 minutes. Finally add 1.0 ml of distilled water. The intensity of the colour developed is read at 530 nm. The units are expressed in m moles / 100ml plasma.[18]

2. Estimation of Reduced Glutathione (GSH)

The Reduced Glutathione content in the blood was estimated by the method of Thomas and Skrinska. 0.2 ml of blood was made upto 1.0 ml by addition of 5% TCA and the protein in the sample was precipitated by centrifugation. 0.2 ml of the protein free supernatant was used for the assay. 2.0 ml of DTNB was mixed with 0.2 ml of the supernatant and the final volume was made

up to 3.0 ml with phosphate buffer and the optical density was measured at 412 nm in a spectrophotometer within 60 seconds, against the blank. The blank contained 0.2 ml of TCA and 2.0 ml of DTNB, which was made upto 3.0 ml with phosphate buffer. The standard glutathione was prepared in separate tubes at a concentration range of 5 to 20 µg were treated with 2.0 ml of DTNB and the volume was made upto 3.0 ml with phosphate buffer. The blank and the standard were also measured at 412 nm. The amount of reduced Glutathione in the plasma was expressed in mg / dl.[19]

3. Estimation of Vitamin E (Vit.E)

The level of Vitamin E was estimated by the method of Baker and Frank.

Extraction of Vitamin E

Add 3 ml of petroleum ether and 1ml of ethanol to 0.5 ml of plasma or serum sample taken in a centrifuge tube. Mix well for 3-5 minutes using a cyclomixer. Centrifuge for 10 min at 2500 rpm. Two layers will be separated out after centrifugation. Pipette out 2 ml of the ether layer (top layer) in to a fresh tube and leave the tube at room temperature (incubator switched off) overnight for evaporation. Next day make it up with 3 ml ethanol and mix well.

Procedure

To 3.0 ml of extracted Vitamin E add 0.2 ml of Bathophenanthroline and $FeCl_3$. The tubes were mixed well in a cyclomixer for 1 minute. Add 0.2 ml ortho phosphoric acid. The tubes were mixed well for 1 minute. The colour developed was read at 540 nm in photochem colorimeter. The units are expressed as mg / 100ml.[20]

4. Estimation of Vitamin C (Vit.C)

The level of Vitamin C was estimated by the method of Jacob. To 0.5 ml of sample add 3.0 ml of distilled water. To this add 0.1 ml of 4% thiourea solutions and 1.0 ml of 2, 4 – Dinitrophenyl hydrazine was added. The tubes were kept for boiling in a water bath for 10 minutes and cooled to room temperature. Add 6.0 ml of 85% Sulphuric acid was added with constant stirring. The absorbance was read at 540 nm (Green filter).The units are expressed as mg / 100ml.[21]

5. Estimation of Lactate Dehydrogenase (LDH) (E.C.1.1.1.27)

Lactate Dehydrogenase was assayed by the method of King. 1.0 ml of buffered substrate and 0.2 ml NAD solution was taken and incubated at 37^{o}C for 15 minutes. 0.2 ml of enzymes was added. The tubes were incubated at 37^{o}C for 30 minutes. To the control tubes, enzymes were added after arresting the reaction with 1.0 ml DNPH reagent. The tubes were kept at room temperature for 20 minutes. Then 0.5 ml 0.4N Sodium Hydroxide was added and the colour developed was read at 540 nm in photochem colorimeter. The activities of the enzyme are expressed as IU/L serum. One unit is the amount of enzyme that liberates 1 µmole of pyruvate per minute under incubation condition.[22]

6. Estimation of Aspartate aminotransferase (AST) (E.C; 2.6.1.2) and Alanine aminotransferase (ALT) (E.C; 2.6.1.10)

Aspartate aminotransferase and Alanine amino transferase was assayed by the method of King.

1.0 ml of substrate was taken and incubated at 37^{o}C for 10 minutes then 0.2 ml of enzymes was added. The tubes were incubated at 37^{o}C for 30 minutes. To the control tubes, enzymes were added after arresting the reaction with 1.0 ml DNPH reagent. The tubes were kept at room temperature for 20 minutes. Then 0.5 ml 0.4N Sodium Hydroxide was added and the colour developed was read at 540 nm in photochem colorimeter.

The activities of the enzyme are expressed as IU/L serum. One unit is the amount of enzyme that liberates 1 µmole of pyruvate per minute under incubation condition.[17]

7. Estimation of Alkaline phosphatase (ALP) (E.C.3.1.3.1)

Alkaline phosphatase was assayed by the method of King. To the tube containing 0.5 ml of acetate buffer (pH 8.0), 0.5 ml of substrate (p – nitrophenyl phosphate) and 0.1 ml of enzyme source was added. The reaction mixture was incubated at 37^o C for one hour. The enzyme reaction was arrested after one hour by adding 5.9 ml 0.1 N Sodium hydroxide. A blank solution is prepared without the substrate and was replaced by 1.0 ml of distilled water to which 0.1N Sodium hydroxide was added. The intensity of the colour developed was estimated at 420 nm in photometer. The activities of the enzyme are expressed as IU/L serum. One unit is the amount of p – Nitrophenol liberated due to the enzyme activity with p – nitrophenyl phosphate.[23]

8. Estimation of Erythrocyte Sedimentation Rate (ESR)

The level of ESR was assayed by Westergren's method. Westergren's tube is 300m long and opened on both ends. 1.6ml of blood is mixed with 0.4ml of 3.8 percent sodium citrate anticoagulant and loaded in the Westergren's tube. The tube is fitted to the stand vertically and left undisturbed. The reading is taken at the end of 1 hour.

RESULTS

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.

Table – 1: Levels of MDA and GSH in plasma of oral cancer patients and control subjects (mean ± SD)

Tests	MDA (nmol/ml)	GSH (mg/dl)
Control group (n = 30)	2.12 ± 0.23	10.50 ± 0.55
Patient group (n = 30)	5.58 ± 0.98	2.89 ± 0.65

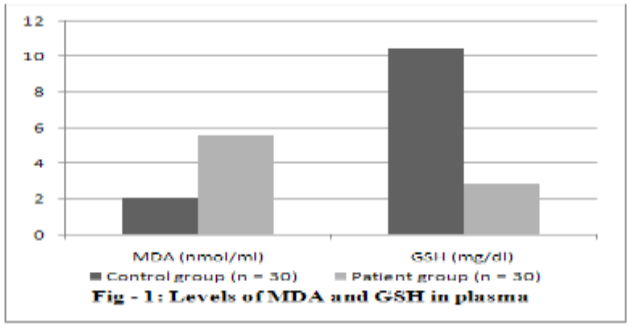

Fig - 1: Levels of MDA and GSH in plasma

The Table – 1 and Fig. 1 shows the levels of MDA in plasma were significantly increased in the oral cancer patients (p< 0.001).

Table – 2: Levels of GSH, Vitamin C and Vitamin E in plasma of oral cancer patients and control subjects (mean ± SD)

Tests	GSH (mg/l)	Vitamin C (mg/l)	Vitamin E (mg/l)
Control group (n =30)	10.02 ± 0.55	1.92 ± 0.35	8.50 ±0.75
Patient group (n = 30)	3.09 ± 0.53	0.58 ± 0.09	1.89 ± 0.35

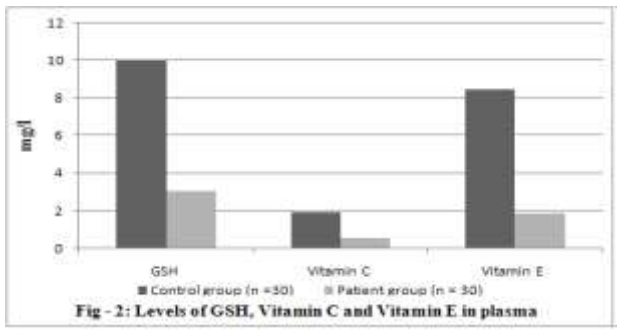

Fig - 2: Levels of GSH, Vitamin C and Vitamin E in plasma

The Table – 2 and Fig. 2 shows the levels of non enzymatic antioxidants such as GSH, Vitamin C and Vitamin E levels in plasma of the oral cancer patients were significantly lowered. (P< 0.001)

Table – 3: Levels of LDH, AST and ALP in plasma of oral cancer patients and control subjects (mean ± SD)

Tests	LDH (mu/ml)	AST (mu/ml)	ALP (mu/ml)
Control group (n =30)	48.36±6.03	11.92± 2.35	38.50±6.69
Patient group (n =30)	88.80±13.93	18.26±6.29	65.89±19.35

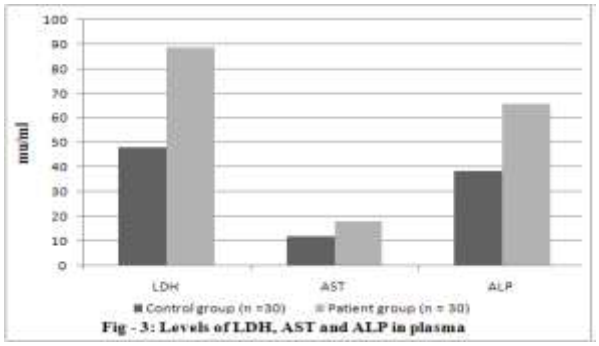

Fig - 3: Levels of LDH, AST and ALP in plasma

The Table – 3 and Fig. 3 shows the levels of various enzyme markers such as LDH, AST and ALP levels in plasma of the oral cancer patients were significantly increased. (P< 0.001)

Table – 4: Levels of Erythrocyte Sedimentation Rate of oral cancer patients subjects.

Tests	ESR
Control group (n =30)	9.23
Patient group (n = 30)	19.5

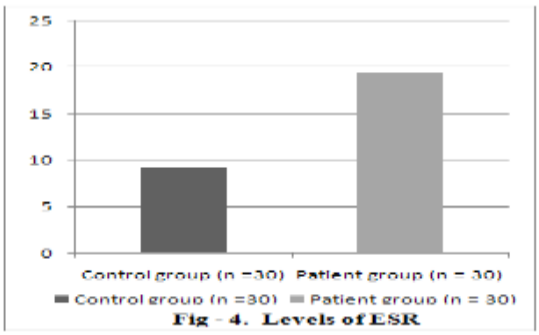

Fig - 4. Levels of ESR

Table – 4 and Fig. 4 shows the levels of ESR in the oral cancer patients were significantly increased.

DISCUSSION

Cancer is an event occurring at the gene level and the ultimate step resulting in carcinogenesis is DNA damage. Many factors such as chemicals, irradiation, the genetic makeup of the individual etc. play a very important role in carcinogenesis. The agents that cause DNA damage are ROS and RNS which is believed that occurs naturally as well. The magnitude of oxidative stress or oxidative damage depends not only on ROS/RNS levels but also on the levels of various cellular antioxidants. Oxidant / antioxidant imbalance in the body seems to play a causative role in carcinogenesis. ROS/RNS are reported to be effectively involved in initiation and also in promotion of multistep carcinogenesis by first causing DNA damage, then activate procarcinogens, lipid peroxidation, followed by inactivating the enzyme that are important in the cellular antioxidant defense system.[24]

High levels of oxidative stress causes peroxidation of membrane lipids which generates peroxides which can decompose to many mutagenic carbonyl products that results in the formation of LHP and MDA as lipid peroxidation end products. They are considered to be

highly mutagenic and carcinogenic.[25] They can also promote tumors by modulating the expression of genes related to tumor.

The levels of LHP and MDA reflect the extent of lipid peroxidation. Our study reveals increased levels of MDA in patients with oral cavity cancer because of increased formation or inadequate clearance of free radicals by the cellular antioxidants. Increased levels of the products of lipid peroxidation support the hypothesis that the increased number of free radicals directly cause cancer cells[2] and that there exists a relationship between free radical activity and malignancy.[26] Observations similar to our findings have been reported in studies on various human cancers.[27]

Cellular antioxidants inhibit initiation as well as promotion in carcinogenesis and counteract cell immortalization and transformation.[10] Different antioxidant enzymes show different function during neoplastic transformation and tumor cells, when compared to their appropriate normal cell.[28]

Cellular antioxidant enzymes and free radical scavengers protect a cell against toxic oxygen radicals. Reduced Glutathione (GSH), an important non-protein thiol, conjugates with glutathione transferase (GST) and GPx, plays a vital role in scavenging ROS and protects the cell from damage.[34] A significant depletion of plasma GSH observed in our study shows enhanced pro-oxidant milieu of the cells and correlates with the increased lipid peroxides circulation of oral cavity cancer patients.

Antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase protect the cell against toxic free radicals. These enzymes reduce the oxygen free radicals to yield non-toxic products. Selenium dependent GPx removes both H_2O_2 and LHP using GSH. This prevents H_2O_2 mediated intracellular DNA damage that can lead to cause carcinogenesis.[8] Oxidative damage to the cell membrane has been reported to inactivate GPx. SOD metabolizes free radicals and dismutates superoxide anions ($O_2^{\bullet}$) to H_2O_2 and protects the cells against $O_2^{\bullet}$ mediated lipid peroxidation. CAT decompose H_2O_2 thereby neutralizing its toxicity.

GSH, vitamin E and vitamin C acts synergistically to prevent cellular damage. Vitamin E is an important antioxidant present in both erythrocyte membrane and plasma in their lipid domain. It can be readily exchanged between erythrocytes and plasma, in favor of plasma to balance the reduced and oxidized state. Vitamin C, extracellular antioxidant disappears faster than other antioxidants when plasma is exposed to oxygen free radicals. Vitamin C utilizes GSH and together with vitamin E prevents the oxidation of GSH. To reduce vitamin E and vitamin C again, our cellular system requires GSH, and hence level of plasma GSH showed lowered in our study in turn might be responsible for the low levels of other antioxidants.[29]

CONCLUSION

Our study in patients with oral cavity cancer reveals Oxidative stress is increased because of elevated levels of lipid peroxidation products and nitric oxide products. As the oxidative stress increases, to compromise enzymatic and non-enzymatic antioxidants are utilised as the first line defence and hence there are efective depletion shown in the oral cancer patient. As the levels of available antioxidants decreased, the defense system present in the mucosal cells becomes more vulnerable to the toxic effect of ROS that alters intracellular environment which can mutate DNA and hence there will be more favorable for DNA damage and disease progression.

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