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Oxidative Stress & Antioxidants Status in Patients With Different Stages of Oral Squamous Cell Carcinoma – in-Vitro Treatment With Neem Leaf Extract(Nle)

KEYWORDS: Oxidative- stress, Antioxidant

Abhishek Sharma
Department of Biochemistry, N.S.C.B Medical College, Jabalpur, M.P

Nidhi Sharma
Department of Microbiology, Mimer Medical College, Maharashtra

Najmul Islam
Department of Biochemistry , J .N medical college, AMU Aligarh

ABSTRACT *Objective: to study level of oxidative stress and antioxidant status in oral cancer patients with various clinical stages before and after in-vitro treatment with Neam Leaf Extract(NLE) in different concentrations.*

Methods: Blood samples of 60 oral cancer patients with various clinical stages of oral cancer and 20 age and sex matched healthy subjects were collected. Plasma and erythrocytes levels of TBARS, GSH, and activities of SOD, CAT and GPx and ferric reducing ability of plasma ,were assayed using specific colorimetric methods. The statistical comparisons were performed by ANOVA followed by Student's t-test.

Results: The TBARS levels were gradually increased whereas antioxidants were gradually reduced from stage II to stage IV of oral cancer patients and in comparative study done after treating the whole blood with different concentrations of NLE, it was found that oxidative stress was ameliorated and antioxidant status was increased .

conclusion: The altered lipid peroxidation in plasma and erythrocytes of oral cancer patients may be related to their compensatory changes in the antioxidants defense system.

Introduction-

Oral cancer is a major form of cancer worldwide and is one of the most common malignancy in India accounting for 30-40 per cent of all cancers. Squamous cell carcinoma of the oral cavity is responsible for considerable morbidity and mortality in India where 60,000 new cases of oral cancer are reported to occur every year[1]. Tobacco chewing with betel quid, tobacco smoking and alcohol consumption are the most important aetiological factors associated with the incidence of oral cancer in India[2]. Reactive oxygen species plays an effective role in the pathogenesis of different pathological diseases including cancer[3]. Free radical induced lipid peroxidation causes a loss of cell homeostasis by modifying the structure and functions of cell membrane[4]. The most important characteristic of lipid peroxidation is to cause a considerable DNA-MDA adducts by interacting with cellular DNA . However, mammalian cells possess elaborate antioxidant defense mechanisms to neutralize the deleterious effects of free radical induced lipid peroxidation[5] . Enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] and non-enzymatic antioxidants [Reduced glutathione (GSH)] act synergistically with one another to detoxify the effects of lipid peroxidation[6]. earlier the status of lipid peroxidation and antioxidant defense mechanism in plasma and erythrocytes of oral cancer patients has been demonstrated. . However, there are no reports on lipid peroxidation and antioxidant status in patients with various clinical stages of oral squamous cell carcinoma. The present study was therefore, undertaken to evaluate the status of lipid peroxidation and antioxidants in plasma and erythrocytes of oral cancer patients with various clinical stages.

Material & Methods

Blood samples were obtained consecutively from 60 oral cancer patients admitted during 2009-2010, in radiotherapy department of J.N medical college hospital, AMU , Aligarh,U.P india.The patients were categorized into three different groups of 20 each (stages II, III and IV) according to TNM (Tumour, Node, Metastasis) system of cancer classification. 20 age matched healthy males were also investigated as control. The subjects who were diagnosed as cancer free were categorized as control subjects. The controls were healthy subjects not habituated to tobacco chewing and smoking and were of the same age, sex and socio-economic strata as the oral cancer patients. The subjects were ranging in age from 40-60 yr (Control: 51.3 ± 4.6; Oral cancer patients: 53.2 ± 5.1). As only 20 patients with stage IV carcinoma could be admitted and included during the study period the number in other subgroups with stages II and III and control group was also restricted to 20 in each group. So the first 20 patients with stage II or III were included. Study protocol was approved by the ethics committee of JNMC&H. Oral tumour tissues obtained at the time of OPD visit from buccal mucosa of patients were immediately fixed in 10 per cent formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The tumours were histopathologically confirmed as well, moderately and poorly differentiated squamous cell carcinoma.

Blood samples (6 ml) were obtained from patients and controls by venous arm puncture and the plasma was separated by centrifugation at 1000 *g* for 15 min. After plasma separation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of erythrocytes was lysed with hypotonic phosphate buffer, pH 7.4. The haemolysate was separated by centrifugation at 3500 *g* for 15 min at 20^0C. TBARS , antioxidants and FRAP estimations were carried out in plasma of healthy subjects and oral cancer patients.

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Thiobarbituric acid reactive substances (TBARS) released from the endogenous lipoperoxides, reflecting the lipid peroxidation processes, were assayed in plasma.

The reduced glutathione was determined by the method of Beutler and Kelly[7]. The technique involves protein precipitation by meta-phosphoric acid and spectrophotometric assay at 412 nm of the yellow derivative obtained by the reaction of the supernatant with 5-5'dithiobis-2-nitrobenzoic acid.

Cytoplasmic Cu/Zn superoxide dismutase activity was assayed by the method of McCord and Fridovich *et al* [8]. One unit of enzyme is taken as the amount of enzyme required to give 50 per cent inhibition of nitro blue tetrazolium (NBT) reduction. The activity of catalase was assayed by the method of Sinha [9], based on the utilization of H_2O_2 by the enzyme. The colour developed was read at 620 nm. One unit of the enzyme is expressed as μ moles of H_2O_2 utilized per minute. Glutathione peroxidase activity was assayed by the method of paglia and valentine *et al* [10] based on the utilization of reduced glutathione by the enzyme. One unit of enzyme is expressed as μ moles of GSH utilized per minute.

The method of Benzie and Strain[11] (1996) was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimate the "total antioxidant power", with minor modification. Ferric to ferrous ion reduction at low pH results in the formation of a coloured ferrous-tripyridyl triazine complex. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of plasma in 0.1 ml and 900 µl of freshly prepared FRAP reagent, prepared by mixing 10.0 ml of 22.78 mM Sodium acetate buffer, pH 3.6, 1.0 ml of 20 mM ferric chloride and 1.0 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine solution prepared in 40 mM HCl. Before starting the reaction, both FRAP reagent and plasma samples were pre incubated for 5 min at 30 C. Incubation was done for 5 min at 30 C and absorbance was recorded at 593 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Ferrous sulphate was used as a standard for calculating the "total antioxidant power"

As depicted in Table 1 in control group the mean value of GPx was 49.94±4.89 , and mean values of Catalase, SOD and MDA and FRAS were 34.94±4.89, 27.58±3.68, 9.71±2.15, 714.13±2.15 respectively.

TABLE .1

Parameter	GPx(U/ml)	Catalase(U/ml)	SOD(U/ml)	MDA(Nm/ml)	FRAS(nM/min/ml)
Mean± SD	49.94±4.89	34.94±4.89	27.58±3.68	9.71±2.15	714.13±2.15

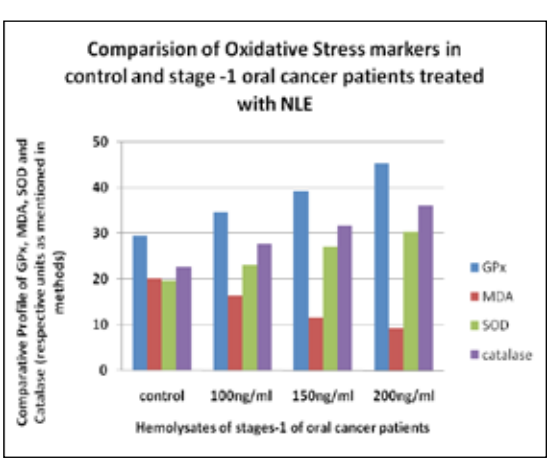
TABLE .2 Oxidative stress-related parameters and antioxidant enzymes in controls and different stages (Stage-II, III and IV) of oral cancer patients.

Parameter	Control (n=20)	O.C Stage-II (n=20)	O.C Stage-III (n=20)	O.C Stage-IV (n=20)
GPx (U/ml)	49.93+4.89	22.71+4.24a	16.52+4.14 b	10.74+3.93c
SOD(U/ml)	27.58+3.68	13.44+4.17	9.97+4.17	5.88+4.17
CAT(U/ml)	34.93+4.89	16.42+4.17	12.85+4.17	8.65+4.17
MDA(nM/ml)	9.7+2.14	14.12+4.19	18.30+4.19	26.19+4.19
FRAS(nM/min/ml)	714.12+12.95	667.98+23.09	584.49+21.93	570.42+23.58

Values are indicated by mean±SD. Comparisons were carried out by unpaired **t-test.** MDA: erythrocyte malondialdehyde; GPx:erythrocyte glutathione peroxidase; SOD: erythrocyte superoxide dismutase; CAT: erythrocyte catalase;FRAS:ferric reducing activity of serum.O.C=oral cancer
a as compared with controls P <0.05
b as compared with control P<0.05
c as compared with control P<0.05

Dose response effect of Neem leaf extract (NLE) on oxidative stress markers

Samples (n=20) of fresh heparinised human blood of stage -1 of oral cancer were exposed to varying concentrations of neem leaf extract (100ng/ml, 150ng/ml and 200ng/ml) for 90 minuts and plasma was separated from blood by centrifugation at 2,500 rpm for 30 min, aliquoted and stored at 4°C for further study. Serum was separated from clotted blood sample .



Statistical analysis: The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Student's t-test.

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Results & Discussion

Both normal subjects and oral cancer patients were males and females ranging in age from 40-60 yr (control: 51.3 ± 4.6; oral cancer patients: 53.2 ± 5.1).

The level of TBARS was significantly increased in plasma, and haemolysate of oral cancer patients as compared to healthy subjects, and were gradually increased from stage II to stage IV of oral cancer patients (Table 2). FRAP and reduced glutathione were significantly decreased in oral cancer patients as compared to healthy subjects, and were gradually decreased from stage II to stage IV of oral cancer patients (Table 2). The activities of superoxide dismutase, catalase and glutathione peroxidase were also significantly decreased in oral cancer patients as compared to healthy subjects, and were gradually decreased from stage II to stage IV of oral cancer patients .

In The present study lipid peroxidation products like malondialdehyde (MDA) were significantly augmented, whereas enzymatic antioxidants (GPx, CAT and CuZn-SOD) were significantly lowered in the circulation of oral and lung cancer patients when compared to control subjects. Our findings were also in agreement with the earlier reports of Joshua *et al*, and Nisha *et al*,[12,13] on the elevated lipid peroxidation with concomitant antioxidant depletion as evident by FRAS assay in the oral cancer patients.

The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase serve as the backbone of cellular antioxidant defense mechanism. Lowered activities of these enzymes have been reported in various pathological conditions including oral carcinogenesis. Our results support these observations.

In addition, in the present study (table-2), we also found decreased antioxidant enzyme (GPX, SOD and CAT) levels in the oral and lung cancer groups versus controls. SOD, GPX and CAT are considered primary antioxidant enzymes, as they are involved in direct elimination of ROS. 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. Selenium dependent GPX removes both H_2O_2 and lipid hydroperoxides using glutathione [5]. SOD metabolizes and protects the cells against O_2^- mediated lipid peroxidation, while CAT acts on H_2O_2 by decomposing it, thereby neutralizing its toxicity. Any changes in one of these systems may break the equilibrium and cause cellular damages and ultimately lead to a malignant transformation [3].The activities of these enzymes were reported to be decreased in oral and lung cancers [1,12].

Our findings were in agreement with the reports of Izabela *et al*, [14] who demonstrated that the reduction in several antioxidant defense mechanisms correlates with the emergence of the malignant phenotype.

In present study, Neem leaf extract (100-200 ng/ml) pretreated erythrocytes showed an appreciable ameriolation in levels of GPx, CAT and SOD activities in erythrocytes of oral and lung cancer patients. These results are in accordance with previous reports by Coates *et al*., 1989; Wu *et al*., 2004. Thus, we have shown that Neem leaf extract play protective role against oxidative stress in oral cancer patients.

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