Role of Measurement of Antioxidant Enzymes in Evaluation of Antioxidant Therapy in Tobacco Abusers with Oral Leukoplakia

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Abstract Antioxidants are widely used in chemoprevention of malignancy. Numerous studies in medical literature have reported the evaluation of this treatment protocol by indirect methodology—epidemiology, invitro studies, pharmacology and animal models etc. However, there is a paucity of literature on the measurement of antioxidant enzymes as a parameter for assessing the outcome of antioxidant therapy. This study explores the efficacy and outcome of antioxidant enzyme assay in relation to antioxidant therapy in tobacco abusers, hitherto unreported in medical literature. A prospective cohort study with control in 50 patients carried out at a tertiary care teaching Institution (Institute of Medical Sciences, Banaras Hindu University, Varanasi, India). Out of these patients, 10 patients acted as control, rest 40 patients—all tobacco

users in some form, were divided into three groups on the basis of histopathological grading of dysplasia—no dysplasia, mild or moderate dysplasia. The levels of Lipid peroxidase (LPO), Superoxide dismutase (SOD) and Catalase (CAT) in mucosa and serum were assayed in each group, and re-evaluated at the end of 3 months after intervention with antioxidant treatment. To detect any alteration in degree of dysplasia a repeat biopsy was also done at the end of 3 months. The results were statistically analysed using paired *t* test. A statistically significant decrease in level of LPO and SOD, and an increase in CAT levels were recorded both in mucosa and serum. However, no change in dysplasia and no new case of dysplasia were observed. Further, antioxidant treatment was continued for a year and the final outcome of the lesion was assessed by “Carter’s criteria”. A final success rate of 74.19% was recorded in terms of partial or complete regression of the lesion. This study confirms the therapeutic efficacy of antioxidants in oral leukoplakia, and cites the importance of LPO, SOD and CAT in evaluating the efficacy of antioxidant treatment. However, the study failed to elucidate any relationship between enzyme measurement and the final outcome of the lesion.

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Introduction

Oral leukoplakia is the most common precancerous lesion in the oral cavity [1]. Association between tobacco and leukoplakia is well established in medical literature [2, 3]. The lesion has a malignant potential with a malignancy rate

reported in the range of 5–25% [4]. The transformation of a normal cell into a neoplastic cell proceeds through three phases: initiation, promotion and progression. The promotion phase is very slow in humans, thus it takes a latency period of 10–30 years for many cancers to develop [5]. This long time window not only aids in diagnosis of the lesion but also makes this phase an attractive target for intervention with cancer chemopreventive agents like beta-carotene and Vitamin E so that malignancy does not develop in the life time of the subject.

The efficacy of this treatment protocol is monitored by indirect methods like: epidemiology, invitro laboratory studies, animal models, pharmacology and reversal of lesion etc. [6]. Advances in laboratory medicine have led to enhanced understanding of pathophysiology of carcinogenesis. Enzymatic oxidants (glutathione peroxidase, superoxide dismutase and catalase etc.) and non enzymatic oxidants (vitamin E, reduced glutathione) act synergistically with one another to detoxify the effects of lipid peroxidation, which by cascade effect causes cell damage and thereby induces carcinogenesis. There measurement thus can also be used to evaluate the efficacy of antioxidant treatment. However, there is scant medical literature on this subject. Although a few earlier studies have demonstrated the status of lipid peroxidation and antioxidant defense mechanism in plasma and erythrocytes of oral cancer [7–10], there are no reports on lipid peroxidation and antioxidant therapy in patients with leukoplakia in a specific sub-group of tobacco abusers. Against this background, a prospective study with control was carried out in tobacco abusers:

1. To confirm the previously reported remission results for leukoplakia by antioxidant therapy.
2. To determine the efficacy of the said treatment protocol by evaluating the antioxidant enzyme status in serum and tissues of these patients.
3. To probe the predictive value of antioxidant enzyme assay in final outcome of leukoplakia with antioxidant treatment.

Materials and Method

A prospective (cohort) randomized study with control was carried out in 50 patients (10 patients acted as control—age matched healthy males) in the departments of ENT, Pathology and Pharmacology at Institute of Medical Sciences (a tertiary care “Central Government” university teaching hospital), Banaras Hindu University, Varanasi, India from 2002 to 2004. The protocol of the study was reviewed and approved by the institutional board of the university. The cases of leukoplakia were selected at

random from ENT out patient department (OPD) and recruited in the study design after an informed consent. Further, the following criteria were strictly adhered to:

Adult patients above 12 years of age with history of habitual (daily consumers) tobacco use in any form i.e. smoking or chewing for a period of 1 year or more.

The following patients were excluded from the study:

- Patients with any systemic diseases e.g. TB, Syphilis etc.
- Patients on any type of beta-carotene treatment

All the patients underwent a histopathological examination of the leukoplakic patch to detect any dysplasia. The patient profile along with the histopathology of the lesion was duly recorded in a “Performa” designed for this study. The patients were further divided into two groups: Group A (comprising of 10 normal people with no leukoplakia or history of tobacco abuse) and Group B (comprising of cases of leukoplakia). Group B was further split into three sub-groups on the basis of varying degree of dysplasia: Group BI (leukoplakia with no dysplasia), Group BII (leukoplakia with mild dysplasia) and Group BIII (leukoplakia with moderate dysplasia). No case of severe dysplasia was seen in this study.

The enzyme status in each group was evaluated both in the tissue and serum before any form of intervention (Tables 1, 2). Three enzymes were measured (Table 3): Lipid peroxidase (LPO), Superoxide dismutase (SOD) and Catalase (CAT). All the patients were put on an antioxidant capsule (Table 4) along with abstinence from hot spicy food, alcohol and tobacco in any form and a diet rich in salads, fruits and green leafy vegetables at least once a day.

The patients were kept on a regular monthly follow-up in the department of ENT. At the end of 3 months a repeat biopsy was done and the antioxidants enzymes were again measured and recorded in Table 1 and 2. A statistical analysis of the data was done by paired *t* test in order to draw an inference from the study.

Further an extended follow-up was kept up to a period of 1 year with patients still on the advised interventional plan. At the end of 1 year a subjective evaluation of the leukoplakic plaque was done using Carter’s criteria (Table 5).

Results

Of the 40 patients recruited in the study 9 were lost in follow-up. Thus in a group of 31 patients, 21 were males and 10 were females. In this group only 12 patients were asymptomatic; rest 19 had one or more of the following symptoms: pain, burning sensation, intolerance to hot & spicy food or alteration in taste.

Table 1 Status Of LPO, SOD and CAT in mucosa before and after treatment with antioxidants

Group	LPO (nmol/mg protein)			SOD (U/g wet tissue)			CAT (U/g wet tissue)		
	Before Rx	After Rx	<i>P</i> value	Before Rx	After Rx	<i>P</i> value	Before Rx	After Rx	<i>P</i> value
A(10)	0193 ± 0.444	0.170 ± 0.03		40.3 ± 6.1	39.0 ± 5.3		62.3 ± 7.2	64.8 ± 7.88	
BI(10)	0.219 ± 0.049	0.175 ± 0.043	<0.01	51.4 ± 6.4	34.9 ± 3.7	<0.01	37.7 ± 8.0*	53.5 ± 8.7	<0.001
BII(14)	0.316 ± 0.048	0.268 ± 0.046	<0.001	50.2 ± 4.0	34.3 ± 3.9	<0.001	48.8 ± 8.0	66.1 ± 8.7	<0.001
BIII(7)	0.289 ± 0.031	0.239 ± 0.024	<0.01	50.6 ± 4.7	33.8 ± 2.4	<0.01	43.4 ± 5.0	79.9 ± 6.5	<0.001

Note: Statistical analysis was done by paired *t* test

Results in Mean + SEM (standard error of mean)

Group A: control group i.e. having no leukoplakia—10 healthy individuals; Group BI: leukoplakia with no dysplasia—10 patients; Group BII: Leukoplakia with mild dysplasia—14 patients; Group BIII: Leukoplakia with moderate dysplasia—7 patients

LPO lipid peroxidase, SOD superoxide dismutase, CAT catalase, Rx Treatment

* *P* value < 0.05

Table 2 Status of LPO, SOD and CAT in serum before and after treatment with antioxidants

Group	LPO (nmol/mg protein)			SOD (U/g wet tissue)			CAT (U/g wet tissue)		
	Before Rx	After Rx	<i>P</i> value	Before Rx	After Rx	<i>P</i> value	Before Rx	After Rx	<i>P</i> value
A(10)	0.371 ± 0.079	0.034 ± 0.081		49.3 ± 1.99	48.8 ± 1.92		174.4 ± 16.3	183.2 ± 17.8	
BI(10)	0.532 ± 0.110	0.436 ± 0.102	<0.001	65.3 ± 2.78***	50.6 ± 2.10	<0.001	120.1 ± 13.6*	144.3 ± 17.7	<0.001
BII(14)	0.628 ± 0.070*	0.560 ± 0.072	<0.001	60.5 ± 2.48**	54.9 ± 1.40	<0.001	141.6 ± 21.1	180.9 ± 20.7	<0.001
BIII(7)	0.468 ± 0.024	0.396 ± 0.024	<0.05	71.7 ± 2.30***	52.2 ± 1.8	<0.001	130.2 ± 24.1	173.7 ± 26.4	<0.001

Note: Statistical analysis was done by paired *t* test

Results in Mean + SEM (standard error of mean)

Group A: control group i.e. having no leukoplakia—10 healthy individuals; Group BI: leukoplakia with no dysplasia—13 patients; Group BII: Leukoplakia with mild dysplasia—11 patients, Group BIII: Leukoplakia with moderate dysplasia—7 patients

LPO lipid peroxidase, SOD superoxide dismutase, CAT catalase, Rx Treatment

* *P* value < 0.05

** *P* value < 0.01

*** *P* value < 0.001

Table 3 Methodology of estimation of free radical generation in serum and tissue

The leukoplakic tissue sample and serum were homogenized (5%) in ice cold 0.9% saline with a potter–Elvehjem glass homogenizer for 30 s. The homogenate was centrifuged at 800×*g* for 10 min followed by centrifugation of the supernatant at 12000×*g* for 15 min and the obtained mitochondrial fraction was used for following estimation:

Superoxide dismutase (SOD)

The inhibition of reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazine metha sulphate and NADH was measured at 560 nm using *n*-butanol as blank. One unit of enzyme activity was defined as the amount of enzyme that inhibits rate of reaction by 50% in 1 min under the defined assay conditions and the results have been expressed as units (U) of SOD activity/g wet tissue [11].

Catalase activity (CAT)

Decomposition of H₂O₂ in presence of catalase was followed at 240 nm. One unit of (U) CAT was defined as the amount of enzyme required to decompose 1 μmol of H₂O₂ per min, at 25°C and pH 7.0. Results are expressed as units (U) of CAT activity/g wet tissue [12].

Lipid peroxidation (LPO)

LPO product malodialdehyde was estimated using 1,1,3,3 tetraethoxypropane as the standard and is expressed in nmol/mg protein [13].

21 leukoplakic lesions were detected in buccal mucosa, 5 in tongue, 3 in commissure, 1 each in labial mucosa and palate. Histologically 10 patients had leukoplakia with no

dysplasia (Group BI), 14 had leukoplakia with mild dysplasia (Group BII) and 7 patients had leukoplakia with moderate dysplasia (Group BIII).

Table 4 Contents of the anti-oxidant capsule

Beta-carotene—30% dispersion	10 mg (5000 IU)
Vitamin E Acetate I.P.	25 IU
Vitamin C Acetate I.P.	100 mg
Copper(from copper sulphate)	1 mg
Manganese	1.5 mg
Zinc	7.5 mg
Selenium	150 mcg

Table 5 Carter's criteria of assessment

Objective response
A. Complete remission
(I) Lesion completely regressed
(II) No new lesion developed
B. Partial remission
(I) More than 50% regression in greatest diameter
(II) No new lesion developed
C. No response
(I) No progression
(II) Decrease in lesion less than 50%
D. Progressive disease
(I) Progression in the lesion
(II) Appearance of fresh lesion
Subjective response
1. Subjective sense of well being
2. Relief from pain
3. Relief from intolerance to hot and spicy food
4. Improvement in taste sensation

Tables 1 and 2 shows the status of antioxidant enzymes in mucosa and serum respectively for all the patients including control Group A prior to initiation of any treatment. Clearly the levels of LPO and SOD were found to be increased in all the three sub-groups of Group B when compared to the control Group A. Also a decrease in levels for enzyme catalase was recorded in all the three sub-groups of Group B.

Table 1 shows the values of enzyme oxidants in mucosa before and after treatment with antioxidant capsule. The data showed a decrease in LPO and SOD after intervention. The result was also found to be statistically significant ($P < 0.01$ or $P < 0.001$). Similarly a statistically significant

increase in catalase levels was recorded in all the three sub-groups of Group B.

Table 2 presents the statistical interpretation of the anti-oxidant enzymes in serum before and after treatment. Here again statistically significant decrease was recorded for SOD in all the sub-groups of Group B. For LPO, except in Group BIII, both other sub groups Of Group B recorded a statistically significant decrease. However, a statistically significant increase in catalase was seen in all the three sub-groups of Group B.

It would be prudent to note that no histological improvement at the end of 3 months was recorded in this study i.e. patients dysplasia in each group remained constant. However, it is noteworthy that no deterioration in status of dysplasia in any case and no new cases of dysplasia were observed.

Lastly using carter's criteria at the end of 1 year in a total of 31 cases, 9 cases showed complete response, 14 showed partial response and 8 cases showed no response. Also of the symptomatic 19 patients, 18 cases showed subjective response (Table 6).

Discussion

Does Antioxidant Therapy Influence Lipid Peroxidase?

The answer to the above question keeping our study in view is yes. We found a decrease in LPO level in Group A as compared to Group B prior to induction of treatment. Moreover a statistically significant decrease in LPO levels was observed in all the three sub-groups of Group B both in mucosa and serum after anti-oxidant treatment.

It is known that increase in lipid peroxidation increases the reactive oxygen species (ROS). ROS plays an effective role in pathogenesis of different pathological diseases including cancer [14, 15]. Free radical induced lipid peroxidation causes a loss of cell homeostasis by modifying the structure and functions of cell membrane. The most important characteristic of lipid peroxidation is to cause a considerable DNA–MDA adducts by interacting with cellular DNA [16, 17]. The decrease in lipid peroxidation recorded as the final result in our study thus may be due to radical scavenging activity of anti oxidants.

Enhanced lipid peroxidation with decline in antioxidants has been reported in venous blood of oral cancer patients

Table 6 Final Result (by Carter's criteria)

Total cases	Complete response	Partial response	No response	Progressive disease	Subjective response
31	9 (29.03%)	14 (45.16%)	8 (25.80%)	0	18 ^a

^a Note: Of the symptomatic 19 patients (out of 31 total cases) 18 showed improvement

and patients with oral squamous cell carcinoma at different interoral sites [7, 8, 10, 18, 19]. Moreover Studies carried out by Krinsky and Deneke and Mobahan et al. have also shown the beneficial effect of beta carotene and carotenoid rich vegetables in inhibition of lipid peroxidation [20, 21].

Does Antioxidant Therapy Influence Superoxide Dismutase?

The answer to this question keeping our study in view is yes. The levels of SOD were high prior to induction of treatment in all the cases of Group B when compared to Group A (both in serum and mucosa, although a statistically significant level was seen only in serum).

After treatment with antioxidants the levels of SOD were markedly decreased and were found to be statistically significant, both in serum and mucosa. SOD scavenges the superoxide radical O_2^+ one of the ROS responsible for lipid peroxidation. Increase in SOD level is in response to increased tissue O_2 speeding up their dismutation and converting it immediately into H_2O_2 [22]. Low SOD has been reported to cause accumulation of free radicals leading to damage to DNA, RNA and proteins. SOD is thus an inducible enzyme for protection against free radical stress leading to increased levels in serum and tissue in response to carcinogenesis [23, 24].

Does Antioxidant Therapy Influence Catalase Activity?

From our statistical analysis of data, the answer to the above question is again yes. The level of catalase in Group B prior to intervention therapy was low when compared to control Group A. Catalase scavenges the H_2O_2 generated by SOD. It is shown that SOD plays a major role in metabolism of ROS by directly dismuting the superoxide anion radical to H_2O_2 which is scavenged by catalase and glutathione peroxidase. So catalase level diminishes due to increasing radical generation. In our study after antioxidant treatment the levels of catalase increased towards normal both in serum and mucosa ($P < 0.001$) thereby indicating that antioxidant treatment prevents excessive wash up in leukoplakic tissue.

The antioxidant enzymes SOD and catalase (along with glutathione peroxidase) serve as a backbone of cellular antioxidant defense mechanism [25]. Lowered activation of these enzymes has been reported in various pathological conditions including oral carcinogenesis [19, 26]. Our results support these observations.

Our results as defined here in thus confirm the ability of B-carotene to produce response in tobacco abusers with oral leukoplakia. This modest experience is thus consistent with and corroborates reports from other groups that cite

advantages of “Antioxidant Therapy” in treatment of leukoplakia [1, 27–31].

It is imperative to note that the only practical approach for coming to a conclusion to putative chemopreventive activity is to consider accumulating evidence from other indirect lines of evidence for or against the agent (like: epidemiology, in vitro laboratory studies, animal models, pharmacology, effect on intermediate biomarkers in laboratory models and reversal of oral leukoplakia etc.); for it is virtually impossible to carry out randomized control trials for any cancer (including oral cavity) due to logistic and practical reasons. Histopathological evaluation has its own limitations: even when a second biopsy is taken as close as possible to the initial site, the patchy nature of dysplasia and the histological effects of trauma from the antecedent biopsy make conclusions about histological response based on two biopsies meaningless. Biopsy at best can be used to confirm a complete clinical response i.e. to show whether normalization has occurred at a microscopic level [32]. In this era of “Animal Conservation Rights” animal models have their own set of ethical and political problems, apart from professional difficulties. Several putative biomarkers have also been proposed to evaluate the role of antioxidants: however, none has been proven to be fully validated [33]. Assessment by “Carter’s criteria” is a two dimensional measurement of the lesion which can be misleading and has a marked subjective observational bias. In addition oral photography is difficult to apply consistently [34].

Antioxidants are essential in reducing free radical reaction which may cause DNA mutations, change in enzymatic activity and lipid peroxidation of cellular membranes [35]; an imbalance between antioxidant defense mechanism and lipid peroxidation process results in cell and tissue damage. The measurement of antioxidant enzymes may thus be regarded as a highly objective laboratory test to evaluate the efficacy of antioxidant therapy in a specific target group. However it is interesting to note that in spite of getting statistically significant result for levels of antioxidant assay, not all patients recorded remissions at the end of 1 year (only 75% success rate was recorded). This raises a doubt on the efficacy of this method in predicting the final outcome of the lesion and indicates that, pathways other than antioxidant defense and lipid peroxidation imbalance are also involved in expression of premalignant and malignant lesions. This is our limited professional experience and is being presented to offer a debate on the cited subject and thereby provide directions for future research.

There are caveats to our study design. As data from a single tertiary health care centre was used, a selection bias may have crept in. Moreover tobacco intake was not quantified. In addition, critics may contend that our sample size was small and has a high dropout rate. The study also

does not take into consideration the effect of habit changes on response i.e. cessation of tobacco use may have influenced the antioxidant enzyme status and resulted in more rapid improvement of leukoplakia. In the end the study also does not address the lesion of nonresponders (to antioxidant treatment). These factors certainly influence the final outcome, thereby limiting conclusions that can be drawn from our data. Unfortunately due to the rural background, poor socio-economic status and illiteracy of our patient profile these shortcomings became inherent to the study design, something beyond our control in spite of our best efforts. Nevertheless, the major strength of this study lies in its prospective character which allowed for accurate assessment of the data without depending upon recalled information. The true value of this study in context of existing literature is the evaluation of antioxidant treatment by analysis of antioxidant enzymes in strong correlation to the histopathological profile in tobacco users exclusively, hitherto unreported in medical literature. This study marries the realities of clinical practice with rigors of scientific investigations and thus may invite hypothesis for future prospective randomized trials.

In conclusion, the numerous lines of evidence in this study suggest a definitive role for antioxidants in preventing malignancy in oral cavity in tobacco users. The study also highlights the importance of measurement of antioxidant enzyme in the evaluation of antioxidant therapy.

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