A Case control study to evaluate oxidative stress in plasma samples of oral malignancy

KUMAR CHANDAN SRIVASTAVA, RAVI DAVID AUSTIN, DEEPTI SHRIVASTAVA1, S. SETHUPATHY2, S. RAJESH3

Abstract

Background: Imbalances between the oxidant –antioxidant status have been implicated in the pathogenesis of several diseases, including cancer. Aim: The aim of this study was to evaluate the extent of lipid peroxidation and antioxidants in the venous blood samples of oral squamous cell carcinoma patients of different Clinicopathologic stages in comparison with the healthy controls.

Setting and Design: A Case control study was designed in a hospital (Rajah Muthiah Dental College and Hospital, Annamalai University) based setting. Materials and Methods: Twenty new histopathologically proven oral carcinoma patients, and equal number of age, sex and habit matched healthy subjects were recruited for this study. Their blood samples were subjected to evaluation of Thiobarbituric Acid Reactive Substances (TBARS) and antioxidant enzymes, namely, superoxide dismutase (SOD), Catalase (CAT) reduced glutathione (GSH) and glutathione peroxidase (GPx) using spectrophotometric methods. Statistical Analysis: The data are expressed as mean±SD. The statistical comparisons were performed by independent Student’s t-test and One Way ANOVA. P value <0.05 was considered statistically significant. Karl Pearson correlation was performed for the biochemical parameters within the group and between the groups. For statistically significant correlations, linear regression was performed. Results: Significant enhanced lipid peroxidation (P<0.001) with decrease in antioxidants (P<0.001) was observed in the venous blood of oral squamous cell carcinoma patients as compared with the healthy controls. Accordingly, significant (P<0.001) pattern of progression in TBARS levels was observed at various clinical stages of patients. (GSH) showed significant (P<0.01) negative correlation with TBARS and positive correlation (P<0.001) with SOD. On linear regression analysis, GSH showed significance for SOD (P<0.001), GPx, CAT and TBARS (P<0.01). It was also found that, 70% of variance in SOD can be attributed to the influence of GSH alone. Conclusion: Enhanced lipid peroxidation and compromised antioxidant defense in plasma indicate development of oxidative stress. Amongst the antioxidant enzymes, (GSH) appears to have a profound role in carcinogenesis.

Keywords: Antioxidants, lipid peroxidation, oral cancer, oxidative stress

Introduction

Cells under aerobic environment are always threatened by highly reactive oxygen species (ROS) and Reactive nitrogen species (RNS). However, they are efficiently neutralized by highly powerful Cellular antioxidant enzymes and the free-radical scavengers. When the balance between ROS production and antioxidant defense is lost, it results in oxidative stress, leading to oxidative damage of the cellular macromolecules. Lipid peroxidation, oxidation of proteins, and DNA damage are its well known outcomes leading towards cellular pathology and ultimately cell death.[1] Conditions, like gastrointestinal ulcerogenesis, rheumatoid arthritis, cardiovascular diseases, metabolic disorders, neurodegenerative disease, and cancer have been reported as ROS-mediated disorders.[2] Oral conditions, like, lichen planus, recurrent aphthous ulcer and periodontitis have reported involvement of oxidative stress during their clinical course. Recent studies have also demonstrated the association between oxidative stress with precancerous conditions like oral submucous fibrosis (OSMF) and Leukoplakia.[3]

Great concern for oral cancer has been generated worldwide as it accounts for the sixth most common malignancy in the world. Annually, across the globe around 275,000 patients are reported with oral cancer. Its incidence exhibits a marked geographical variation, with preponderance seen in developing countries like India (south central Asia).[4] Though it remained the most explored area of research, the overall mortality rate remained high, at approximately 50%. In this scenario, significance of prognostic markers associated with...
advanced stage of disease can make a crucial contribution to the prediction of survival. Various investigators have studied the role of oxidative and nitrosative damage in oral cancer by estimating various antioxidant enzymes assay and lipid peroxidation in saliva. Plasma and tumor tissue. Previous studies clearly stated the implication of oxidative stress in oral squamous cell carcinoma (OSCC), but emphasis was not laid on the individual interactions and associations between the enzymes and TBARS and also, among the antioxidant enzymes. Efforts made in these lines may give us a more crucial independent variable/risk factor, which may be looked upon as the prognostic marker. Only a few studies have been reported in the south Indian rural population and the usage of statistical tools also appeared to be limited.

The purpose of the study is to address the specific role and association of enzymes and TBARS in the progression of the disease. The study hypothesizes that the burden caused by oxidative damage and the compromised antioxidant enzymes keeps on increasing as the disease progresses from initial stages to advanced stages of cancer.

This study aims to evaluate and compare the status of oxidative stress and antioxidant enzymes in plasma of patients with various clinicopathologic stages of oral malignancy.

Materials and Methods

Study design

The institutional ethical committee of Rajah Muthiah institute of health sciences, Annamalai Nagar, India had approved this case control study.

Study sample and its characteristics

The study subjects (sample size, n=40) were derived from the population of patients who presented to the department of oral medicine and radiology at the Rajah Muthiah Dental College and Hospital, Annamalai University, Annamalai Nagar, India, for evaluation and management of oral diseases. These subjects were divided into two groups. Group I/OSCC consisted of 20 random selected, newly diagnosed Subjects with histopathologically confirmed diagnosis of oral cancer. The exclusion criteria for enrollment of Subjects were previously treated cases of Oral cancer, subjects with the history of diabetes, hypertension, anemia, jaundice and liver or kidney disorders and had other systemic diseases and patients with reported carcinoma elsewhere in the body. Group II/control group consisted of Age, sex and habit matched 20 healthy subjects belonging to the similar socioeconomic group, as of group I.

Data collection management

Written informed consent was obtained from all the participants of the study after being explained the purpose of the study. A detailed case history of the patients with emphasis on their habits (chewing betel nut and/or tobacco, smoking and alcohol) was taken and recorded on a standard Performa along with thorough clinical examination. Histopathologically, group I (OSCC) patients were divided into well, moderate or poorly differentiated carcinoma. OSCC patients’ were also categorized clinically into Stage II/III/IV on the basis of TNM staging system.

Blood sample collection

Blood samples (5 ml) were collected from both study and control group patients by venous arm puncture under aseptic precautions and transferred into a presterilized EDTA vials. The collected samples were then subjected to centrifugation at 3000 rpm for 10 min to segregate plasma and erythrocytes. After plasma separation, theuffy coat was removed and the packed cells were washed three times with normal physiological saline. A known volume of erythrocytes were lysed with hypotonic phosphate buffer, (ph 7.4). The hemolysate was separated by centrifugation at 3500 rpm for 15 min at 2°C. (TBARS) and antioxidant enzymes estimation were carried in plasma and erythrocyte lysate.

Procedure for estimation

Lipid peroxidation was estimated as evidenced by the formation of TBARS. It was analyzed in plasma by the method of Yagi1978. In this method, Malonaldehyde and TBARS react with 2-thiobarbituric acid in an acidic condition to generate a pink color chromogen which was read at 532 nm.

Reduced glutathione (GSH) was estimated by the method of Ellman. This method was based on the development of yellow color, read at 412 nm spectrophotometrically, when 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulfhydryl groups. Superoxide dismutase (SOD) was assayed by the method of Kakkar et al., (1984) based on the 50% inhibition of the formation of NADH-phenazinemethosulphathentritolbuetetrazolium (NBT) formazan at 520 nm. The activity of Catalase was assayed by the method of Sinha (1972), based on the utilization of H₂O₂ by the enzyme. The color developed was read at 620 nm. Glutathione peroxidase activity was estimated by the method of Rotruck et al., (1973) with modification. A known amount of enzyme preparation was incubated with H₂O₂ in the presence of GSH for a specified time period. The amount of H₂O₂ utilized was determined by the method of Ellman.

Statistical analysis

All quantitative data were expressed as mean±SD, whereas qualitative data in numbers and percentiles. Tabulation and graphical presentation of the results was carried out for oral cancer and control group. All the variables from the study were statistically analyzed for the mean values, SD and P value. The statistical comparison of biochemical parameters between case and control group was performed by independent Student’s t-test. Analysis of variance (ANOVA) was used to compare parameters in various TNM staging. Means of TBARS with
that of individual antioxidant enzymes and within enzymes themselves were correlated by using Pearson’s correlation. For statistically significant correlations, linear regression was performed. The data were analyzed using SPSS13.0 package. In all the above tests, $P$ value $<0.05$ was considered statistically significant; $P$ value $>0.05$ was taken to be statistically not significant; $P$ value $<0.01$ was taken to be statistically highly significant and $P$ value $<0.001$ as very highly significant.

### Table 1: Clinicopathological characteristics of oral cancer patients (group I) and control group (group II) participated in the study

| Characteristic                          | No. of patients (%) | $P$ value | Inference                  | Comparison between groups          |
|-----------------------------------------|---------------------|-----------|---------------------------|-----------------------------------|
| Sample size ($n$)                       |                     |           |                           |                                   |
| Group I (OSCC)                          | 20                  |           |                           |                                   |
| Group II (control)                      | 20                  |           |                           |                                   |
| Age                                     |                     |           |                           |                                   |
| Group I                                 |                     |           |                           |                                   |
| Range (in years)                        | 38-85               |           | Very highly significant   | 0.000***Very highly significant   |
| Mean                                    | 56.35±12.58         | 0.000*    |                           |                                   |
| SEM                                     | 2.81                |           |                           |                                   |
| Group II                                |                     |           |                           |                                   |
| Range (in years)                        | 25-60               |           | Very highly significant   |                                   |
| Mean                                    | 39.55±9.22          | 0.000*    |                           |                                   |
| SEM                                     | 2.06                |           |                           |                                   |
| Gender                                  |                     |           |                           |                                   |
| Group I                                 |                     |           |                           |                                   |
| Male                                    | 12 (60)             | 0.371**   | Not significant           | 0.038 *Significant                |
| Female                                  | 8 (40)              |           |                           |                                   |
| Group II                                |                     |           |                           |                                   |
| Male                                    | 15 (75)             |           | Significant               |                                   |
| Female                                  | 5 (25)              | 0.025**   |                           |                                   |
| Duration of habit (in years) - for Group I|                     |           |                           |                                   |
| 0-10                                    | 0 (0)               | 0.000*    | Very highly significant   | -                                 |
| 11-20                                   | 2 (10)              |           |                           | -                                 |
| >20                                     | 18 (90)             |           |                           | -                                 |
| Mean                                    | 31±13.13            |           |                           | -                                 |
| SEM                                     | 2.93                |           |                           | -                                 |
| Frequency of habit(Times/day) - for Group I|                     |           |                           |                                   |
| 0-5                                     | 9                   | 0.000*    | Very highly significant   | -                                 |
| 6-10                                    | 7                   |           |                           | -                                 |
| 11-15                                   | 4                   |           |                           | -                                 |
| Mean                                    | 8.25±3.38           |           |                           | -                                 |
| SEM                                     | 0.75                |           |                           | -                                 |
| Site-for Group I                         |                     |           |                           |                                   |
| Buccal Mucosa                           | 11 (55)             | 0.086**   | Not significant           | -                                 |
| Alveolus                                | 6 (30)              |           |                           | -                                 |
| Tongue                                  | 3 (15)              |           |                           | -                                 |
| Clinical stage (TNM)-for Group I        |                     |           |                           |                                   |
| II                                      | 5 (25)              | 0.705**   | Not significant           | -                                 |
| III                                     | 7 (35)              |           |                           | -                                 |
| IV                                      | 8 (40)              |           |                           | -                                 |

*One sample t-test; $P<0.001$, **Chi square test; $P<0.05$, *** Independent t-test; $P<0.001$, *binomial test; $P<0.05$
**Results**

Table 1 shows the Clinicopathological Characteristics of oral cancer patients and Control subjects Participated in the study.

Table 2 shows comparison of lipid peroxidation end products (TBARS) and various antioxidant enzyme profiles, between oral cancer patients and control subjects. Lipid peroxidation was found to be significantly ($P<0.001$) increased, whereas all antioxidant enzymes were significantly ($P<0.001$) decreased, when compared with the normal subjects. Levels of TBARS showed a significant ($P<0.05$) increase from stage II to stage IV of oral cancer patients as well, but significant decrease along the stages were not seen for antioxidant enzymes [Table 3].

On analyzing the various biochemical parameters by Pearson’s correlation, it was observed that SOD ($P<0.01$) and Catalase (0.05) showed a significant negative correlation with TBARS [Table 4].

In an attempt to further probe the relationship among the antioxidants, it was found that GSH had a significant positive correlation with SOD ($P<0.001$), GPx ($P<0.01$) and Catalase ($P<0.01$) [Table 5], thus emphasizing the crucial role of GSH enzyme. Further exploration with regard to GSH was performed with linear regression model [Table 6]. It was found that if GSH is presumed to be an independent variable, then it can reliably predict the dependent variables like GPx, Catalase, TBARS ($P<0.01$), and most importantly SOD ($P<0.000$). A significant proportion of variance ($R^2=0.70; 70\%$) in the SOD thought to be brought by GSH. Regression equation ($SOD=0.37+0.034GSH$) will be able to predict the value of SOD, for a unit change in GSH.

**Discussion**

Despite the existence of endogenous defense mechanisms against ROS, it has been observed that whenever either the level of the cellular antioxidant systems goes down or when the ROS reach abnormally high levels, oxidative damage to the cells occurs, finally leading to several pathological conditions, including Cancer. Oral cancer is essentially an event occurring at the gene level, with DNA damage being the final step. Carcinogenesis is a multistage process definable by at least three steps or stages: Initiation, promotion, and progression, and ROS are found to be involved in all these stages. It participates in the above said stages, by way of causing DNA damage, activating procarcinogens, initiating lipid peroxidation, inactivating enzyme systems and altering the cellular antioxidant defense system. Peroxidation of membrane lipids generates peroxides that decompose to form multiple mutagenic carbonyl products. Lipid hydroperoxides (LHP) and Malondialdehyde (MDA) are well-characterized lipid peroxidation end products. They interact with cellular DNA and cause the formation of DNA-MDA adducts, causing DNA damage and interference with its repair. Measurement of lipid peroxidation and antioxidants is therefore valuable in assessing tumor burden at various stages of oral cancer. In addition, it is also important to identify the most influencing parameter, which may take us to a more specific therapeutic intervention.

Patients of oral cancer included in the present study had an average age of 56.35±12.58 ($P<0.000$) with maximum percentage (40\%) of patients falling in the range of >65 years. The study sample showed male predominance (60\%) with a male to female ratio of 3:2. These findings are consistent with age and gender distribution reported in the literature. The distribution of lesions seen, were minimum at the lateral border of tongue with 15\%, to maximum at the buccal mucosa (55\%). Alcohol contributed to 30\% of the cases. This is comparable to the distribution of oral cancer lesions obtained by Sankarnarayanan et al. [11] It is widely accepted that smoking, alcohol consumption, and betel nut chewing are leading risk factors for the development of oral cancer. We found that all our patients had habit of chewing tobacco with or without additives with an average duration of habit, 31±13.13 years ($P<0.000$) with average frequency of 8.25±3.38 times/day ($P<0.000$). This confirms the gravity of these risk factors.

In our study, we observed significantly increased ($P<0.001$) plasma levels of TBARS in patients with oral cancer as compared to control subjects. Similar significant ($P<0.001$) increasing trend was reported, while comparing clinical stages (TNM) of oral cancer. Poly unsaturated fatty acid (PUFA), a major component in the Cell membranes of erythrocytes and other cells, is considered highly susceptible to oxidative attack. Thus in plasma, erythrocytes cell membrane becomes the major substrate for ROS mediated damage. Alterations in respect to membrane’s fluidity and permeability are seen as a result of such damage. Thus, large volumes of TBARS in plasma could be attributed to its increased formation in erythrocytes with consequent leakage into the plasma or inadequate clearance of free-radicals by the cellular antioxidants. Hence the hypothesis made in the beginning, that the cancer cells produce large amount of free-radicals is proved with our results, as shown by the previous researchers too.

In the present study, the levels of Superoxide Dismutase (SOD), Reduced Glutathione (GSH), Glutathione Peroxidase (GPx) and Catalase (CAT) were significantly decreased ($P<0.001$) in oral cancer patients as compared to healthy subjects. Unlike TBARS, non-significant decrease for all antioxidant enzymes was noted from stage II to stage IV oral cancer patients. Such result can be attributed to small sample size in each category of stage II/III/IV (5, 8, and 7, respectively), considering the number of case groups. The antioxidant enzymes serve as the backbone of cellular antioxidant defense mechanism and their lowered activities have been reported in various pathological conditions including oral cancer. Our results support these observations. The premise that the host tumor cells supposedly sequester essential nutrients from the circulation to meet the demand of growing tumor, explains...
Table 2: Comparison of blood levels of TBARS, SOD, GSH, GPx and catalase between the normal controls and OSCC groups (all values are expressed in mean±SD)

| Groups          | TBARS (nM/mL) | SOD (U/g Hb) | GSH (mg/dl) | GPx (U/g Hb) | Catalase (U/g Hb) |
|-----------------|---------------|--------------|-------------|--------------|-------------------|
| Group I: OSCC   | 5.500±1.70    | 1.45±0.112   | 32.43±2.80  | 13.12±0.618  | 1.300±0.024       |
| Group II: Control | 2.050±0.944  | 2.280±0.301  | 48.93±0.863 | 21.68±1.18   | 1.95±0.489        |
| P-value         | 0.000         | 0.000        | 0.000       | 0.000        | 0.000             |
| Inference       | VHS           | VHS          | VHS         | VHS          | VHS               |

*Independent student t-test; VHS: P<0.001

Table 3: Comparison of TBARS, SOD, GSH, GPx and catalase among clinical stages of OSCC group. (all values are expressed in mean±SD)

| Parameters          | Group II: Control (n=20) | Stage II | Stage III | Stage IV |
|---------------------|--------------------------|----------|-----------|----------|
| TBARS (nM/mL)       | 2.050±0.944              | 3.20±1.09** | 5.42±0.53* | 7.12±0.35**|
| SOD (U/g Hb)        | 2.28±0.301               | 1.52±0.08* | 1.44±0.13* | 1.43±0.10*   |
| GSH (mg/dl)         | 48.93±0.863              | 34.58±2.01* | 32.80±0.50* | 30.76±2.64* |
| GPx (U/g Hb)        | 21.68±1.18               | 13.30±0.41* | 13.47±0.58* | 12.71±0.55*  |
| Catalase (U/g Hb)   | 1.95±0.489               | 1.32±0.01** | 1.29±0.01** | 1.29±0.02*   |

One way ANOVA; †compared to healthy subjects; *P<0.01; ¶compared to stage II; ‡compared to stage III

Table 4: Correlation between TBARS and antioxidant enzymes in OSCC group

| Parameter | Mean     | r        | P     | Inference |
|-----------|----------|----------|-------|-----------|
| SOD       | 1.45±0.112 | -0.413   | 0.070 | NC; NS    |
| GSH       | 32.43±2.80 | -0.588** | 0.006 | NC; HS    |
| GPx       | 13.12±0.618 | -0.389   | 0.090 | NC; NS    |
| Catalase  | 1.30±0.024 | -0.548*  | 0.012 | NC; SS    |

NC: Negative correlation; NS: Not statistically significant (P>0.05); **HS: Statistically highly significant (P<0.01); *SS: Statistically significant (P<0.05)

Table 5: Correlation among antioxidant enzymes in OSCC group

| Parameter | SOD        | GSH         | GPx         | Catalase |
|-----------|------------|-------------|-------------|----------|
| SOD       | -          | r=0.837***  | r=0.439     | r=0.557* |
| GSH       | r=0.383**  | -           | r=0.681**   | r=0.659**|
| GPx       | r=0.439    | r=0.681**   | -           | r=0.106  |
| Catalase  | r=0.557*   | r=0.659**   | r=0.557*    | -        |

PC: Positive correlation; NS: Not statistically significant (P>0.05); ***VHS: Statistically very highly significant (P<0.001); **HS: Statistically highly significant (P<0.01); *SS: Statistically significant (P<0.05)

Table 6: Linear regression analysis of GSH with TBARS, SOD, GPx and catalase

| Parameter | P value (sig) | R2 | Regression line |
|-----------|---------------|----|-----------------|
| TBARS     | 0.006*        | 0.346 | TBARS=17.13-0.35GSH |
| SOD       | 0.000*        | 0.70  | SOD=0.37+0.034GSH |
| GPx       | 0.001*        | 0.463 | GPx=8.246+0.15GSH |
| Catalase  | 0.002*        | 0.435 | CAT=1.1+0.006GSH |

*HS: Statistically highly significant (P<0.01); **VHS: Statistically very highly significant (P<0.001)

the observed decrease in antioxidants in plasma of oral cancer patients.[6]

As one of the objective of the current study, is to explore the individual interactions among the various biochemical parameters under consideration, correlation and regression statistical tools were used. Though the negative association of TBARS with antioxidant enzymes and vice versa has been widely documented in the literature, only GSH (P < 0.01) and Catalase (P < 0.05) showed a significant negative correlation with TBARS in the present study. On performing correlation analysis among the enzymes, only GSH showed significant (for SOD = P < 0.01; for GPx and CAT = P < 0.01) positive correlation with the rest of the enzymes. Since GSH showed promising results in correlation analysis, it was thought to further probe its role via linear regression analysis, taking it an independent variable against rest of the parameters. It showed very high significance (P < 0.001) for SOD and high significance (P < 0.01) for CAT and GPx. Also Considering the R² values, the contribution of GSH in the variance seen in SOD, GPx, CAT and TBARS was found to 70, 46, 43, and 34%, respectively. Derived Regression equations will help us...
to predict the levels of other parameters, provided GSH levels are known. Hence according to our results, GSH emerged as the most influencing independent parameter. Literature explains in great detail about multiple roles played by Glutathione against oxidative stress. It scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of GPX. GSH serves as a cofactor of several detoxifying enzymes against oxidative stress, e.g. GPx, glutathione transferase (GST). It also bears the capability to regenerate the most important antioxidants, Vitamins C and E, back to their active forms. The capacity of glutathione to regenerate the most important antioxidants is linked with the Redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) and therefore the intracellular “Redox homeostasis” or “Redox buffering” capacity is substantiated primarily by GSH. Our results contribute in strengthening the facts given above. Thus, in the present study, the antagonistic relationship between TBARS and antioxidant enzymes is restated with an emphasis on sole influencing role displayed by Reduced Glutathione (GSH).

Despite the small sample size, an interesting observation regarding the independent role of GSH and its highly significant association with SOD is made. Further studies of the molecular mechanisms of ROS-mediated carcinogenesis with an attempt to unfold the role of GSH may be beneficial in understanding the pathogenesis and thus evolving strategies for effective treatment for oral cancer.

**Conclusion**

Determination of lipid peroxidation and antioxidants in blood may be useful in evaluating tumor burden in patients of various stages of OSCC. Thus, normalization of the levels of these antioxidants especially GSH, might be used to improve the accuracy of prognosis of the patients.

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