Abstract: Reactive oxygen species, if produced in excess by oxidative phosphorylation, contributes to mitochondrial DNA damage and progressive respiratory chain dysfunction, leading to various diseases including carcinogenesis. Mitochondria are susceptible to oxidative stress (OS) owing to lack of introns, protective histones, and DNA repair enzymes. However, mitochondria are protected from OS by numerous antioxidants such as superoxide dismutase 2 (SOD2), catalase, glutaredoxin 2 (GLRX2), reduced glutathione (GSH), glutathione peroxidase (GPX), and thioredoxin 2 (TXN2). To obtain insights regarding expression of these mitochondrial antioxidants in oral squamous cell carcinoma (OSCC), we performed qualitative and quantitative estimations of key molecular players of mitochondrial antioxidants during various stages of OSCC by immunoblotting with specific antibodies against antioxidant enzymes and/or biochemical assays. Different mitochondrial antioxidants varied in their expression levels as OSCC progressed. The levels of GPX1, GPX4, and catalase reduced with progression of OSCC. However, GLRX2, PXR3, TXN2, and reduced GSH gradually increased. Expression of SOD2 decreased initially in Stages II and III of OSCC but increased in Stage IV. In conclusion, our findings indicate a complex interplay of various mitochondrial antioxidants in different stages of OSCC, and further insights regarding these molecular players can help us better understand the pathogenesis of OSCC in context of mitochondrial redox status.

Keywords: antioxidants; catalase; glutathione; glutathione peroxidase; mitochondrial superoxide dismutase 2; oral squamous cell carcinoma.

Introduction
Cancer is a multifactorial disorder, and reactive oxygen species (ROS) plays a vital role in its pathogenesis (1). Mitochondria are a significant source of ROS generation, which includes free radicals, and peroxides are produced as by-products of oxidative phosphorylation (2,3). In addition, mitochondrial DNA is ten times more susceptible to oxidative stress (OS) than nuclear DNA owing to lack of protective histones, introns, and DNA repair systems (4,5) and mitochondrial DNA mutations gradually lead to carcinogenesis (6-8). Moreover, certain physiodynamic alterations of mitochondria occur under OS, which enable tumor cells to react abnormally, showing the Warburg effect, and finally escape the apoptotic process, leading to uncontrolled cellular growth (9). Both exogenous and endogenous antioxidant systems maintain a well-coordinated balance in redox homeostasis within the mitochondria.

Various antioxidants function to regulate ROS and the redox-sensitive pathway in the mitochondria such as manganese superoxide dismutase (MnSOD or SOD2),
mitochondrial glutaredoxin (GLRX2), reduced glutathione (GSH), glutathione peroxidase (GPX), catalase, and thioredoxin 2 (TXN2) systems (10). Several studies have analyzed alterations in the cytosolic level of various cellular antioxidants in the progression of several cancers including oral cancer (2,11,12). However, estimation of these antioxidants in mitochondria at different stages of oral squamous cell carcinoma (OSCC) has not been explored yet. Here, we compared the levels of MnSOD or SOD2, mitochondrial GLRX2, GPX1 and GPX4, reduced GSH, catalase, and the TXN2 system comprising peroxiredoxin 3 (PRX3) and TXN2 in mitochondria of OSCC patients at different TNM stages. Moreover, we compared differences in mitochondrial lipid peroxidation between oral cancer and control groups by estimating the levels of thiobarbituric acid reactive substrates generated during peroxidation of phospholipids.

**Materials and Methods**

**Patient selection**

In total, 30 cases (25 men and 5 women) of newly diagnosed surgical biopsy specimens of OSCC were obtained from the outpatient department and included in the study (Group I). The patients’ age ranged 25-50 years. Histologically, 10 cases were well differentiated, 14 were moderately differentiated, and 6 were poorly differentiated. Clinically, the OSCC cases were categorized into Stage II/III/IV using the tumor, node, and metastasis (TNM) staging system (Table 1). Overall, 20 age- and sex-matched volunteers with tobacco chewing habits but without any present antioxidant medication, any local or systemic infection, and any visible oral lesions were included in the control group (Group II).

The study was approved by the Institutional Ethical Committee, Burdwan Dental College (Memo No: BDCH/164, ID No AC/11/EC/BDCH/2012, Protocol No: 0029/13/P version 9) in the year 2013, and written informed consent forms were obtained from all participants.

The exclusion criteria included patients with prior history, diagnosis, or treatment of cancer with other organ/system, presence of neoplasms at other anatomical site/system at the time of diagnosis of the primary OSCC, and past history or treatment of OSCC. Demographic details, type of habit, frequency, and duration of the habit of the study and control groups were collected in a detailed questionnaire as represented in Table 1.
Tissue sample collection and preparation of mitochondria

Surgically resected tumor tissues were immersed in an isotonic homogenization buffer. Mitochondria were isolated by differential centrifugation (Beckman Coulter, Indianapolis, IN, USA), as described (13). The purity of mitochondrial preparation was checked by immunoblotting the crude homogenate and the mitochondrial fraction with various protein markers specific for cytosol (α-tubulin), nucleus (PCNA), endoplasmic reticulum (calreticulin and calnexin), and mitochondria (VDAC1). On the other hand, control group samples were collected using vestibuloplasty.

Measurement of reduced glutathione concentration in mitochondria

All chemicals were purchased from Sigma-Aldrich (Bangalore, India) unless otherwise mentioned. The levels of oxidized glutathione (GSSG) and reduced GSH were measured using Ellman’s method (14). GSH reduces 5,5′-dithiol (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) and gets converted into GSSG. In a coupled reaction, GSSG is reduced by glutathione reductase and NADPH to reduced GSH. TNB was spectrophotometrically measured at 412 nm.

Catalase activity assay

The catalase activity of the mitochondrial extract was determined at 25°C (15). The enzyme reacts with methanol in the presence of hydrogen peroxide forming formaldehyde that reacts with 4-aminobenzene-3-hydrazine-5-mercapto-1,2,4-triazole (Purpald). Upon oxidation, it changes to purple in color, which was read in UltraViolet-Visible (UV-VIS) spectrophotometer (Eppendorf India Ltd., Kolkata, India) at 540 nm.

Measurement of mitochondrial lipid peroxide content

The mitochondrial lipid peroxide content was measured as described by Ogura et al (16). The breakdown products of mitochondrial lipid peroxide react with thiobarbituric acid (Sigma-Aldrich), and the resultant chromogen was spectrophotometrically detected at 532.5 nm.

Western blotting

All chemicals were obtained from Sigma-Aldrich unless mentioned otherwise. Antibodies were purchased either from Sigma-Aldrich or Abcam (Abcam, Cambridge, MA, USA). In brief, 100 μg of mitochondrial total protein was separated on 10% Tris-Glycine SDS-PAGE using Mini-Protean polyacrylamide gel electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA, USA), blotted onto nitrocellulose membrane (Amersham Pharmacia Biotech/GE Healthcare, Kolkata, India), and probed with polyclonal rabbit anti-α-tubulin, anti-PCNA, anti-calreticulin, anti-calnexin, anti-SOD2, anti-catalase, anti-GLRH2, anti-PRX3, anti-TXN2, anti-GPX1, and anti-GPX4 antibodies at 1:2,000 dilutions for 2 h at 4°C in parallel sets and then with mouse anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) at 1:10,000 dilutions for 1 h at room temperature. Chemiluminescence detection of protein bands was conducted using an ECL kit (Amersham Pharmacia Biotech/GE HealthCare). For loading control, rabbit polyclonal anti voltage-dependent anion channel 1 (VDAC1) antibody was used. Relative quantifications of SOD2 protein bands with respect to loading controls were performed using Image J software on the western blot films (Table 2).

Statistical analysis

Statistical analysis was conducted using the statistical package for social sciences (SPSS version 15.0; SPSS, Chicago, IL, USA) statistical analysis software. Comparisons of biochemical parameters between case and control groups were made using independent student t-test, and the values were expressed as mean ± SD. One-way analysis of variance (ANOVA) test was used to compare the parameters in different TNM staging. P values < 0.05 were considered statistically significant. Tukey’s honest significant difference (HSD) test was used to reveal statistical differences in mean values between different groups, and P < 0.05 was considered significant.

Table 2 Relative expression levels of SOD2 in various patient samples as quantified by Image J after normalizing to the loading control mitochondrial marker VDAC1

<table>
<thead>
<tr>
<th>Patient type</th>
<th>No. of patients</th>
<th>Mean of relative expression level</th>
<th>Standard deviation of relative expression level</th>
<th>Variance of relative expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Group 2)</td>
<td>20</td>
<td>98.5</td>
<td>0.87</td>
<td>0.765</td>
</tr>
<tr>
<td>Stage II</td>
<td>12</td>
<td>46.16</td>
<td>0.88</td>
<td>0.778</td>
</tr>
<tr>
<td>Stage III</td>
<td>7</td>
<td>16.55</td>
<td>0.48</td>
<td>0.229</td>
</tr>
<tr>
<td>Stage IV</td>
<td>11</td>
<td>72.70</td>
<td>1.29</td>
<td>1.687</td>
</tr>
</tbody>
</table>
Results
Mitochondria obtained after differential centrifugation were identified as pure without any cytosolic or other organellar contamination (Fig. 1). The extent of lipid peroxidation and the concentration/activity levels of various mitochondrial antioxidants were compared during various clinical stages of OSCC (Stages I, II, III, and IV) and control groups by either biochemical activity assays or western blotting of mitochondrial extracts of tissue samples. Table 3 enlists the concentration/activity levels of reduced GSH, catalase, and lipid peroxide, as measured using the biochemical approach. The concentrations of reduced GSH increased during different stages of OSCC, as determined in OSCC patients from Stage I (15.2 ± 3.87 mM), Stage II (13.2 ± 2.36 mM), Stage III (15.3 ± 2.63 mM), and Stage IV (17.2 ± 3.25 mM) in comparison with control or Group II participants (11.1 ± 2.38 mM). Moreover, the expression of lipid peroxide reduced in OSCC patients from Stage I (0.792 ± 7.491 nmole/mg of protein), Stage II (0.841 ± 8.25 nmole/mg of protein), Stage III (0.743 ± 3.30 nmole/mg of protein), and Stage IV (0.659 ± 5.33 nmole/mg of protein) in comparison with control or Group II participants (1.49 ± 0.189 nmole/mg of protein). Similarly, catalase activity reduced in Stage I (2.66 ± 0.314 mg/min), Stage II (3.13 ± 0.521 mg/min), Stage III (2.2 ± 0.321 mg/min), and Stage IV (2.0 ± 0.209 mg/min) in comparison with Group II participants (6.410 ± 0.285 mg/min). In order to understand the differences between groups, means, and the alterations during different stages of cancer, ANOVA was applied on the expression levels of reduced GSH, lipid peroxide, and catalase (Table 3) by putting the variables in an ANOVA chart using SPSS (version 15). The results of ANOVA are summarized in Table 4, detailing the statistical values of various parameters such as sum of squares, degrees of freedom, mean of squares, F, and P values, with P values being the most important parameter in ANOVA; P < 0.05 is considered statistically significant (denoted by “S” in Table 4), whereas P > 0.05 is considered statistically nonsignificant (denoted by “NS” in Table 4). According to the ANOVA analysis,
the concentration/activity of reduced GSH and catalase induced significant changes during OSCC (P = 0.000). On the other hand, lipid peroxide showed nonsignificant changes in OSCC (P = 0.986). To analyze the correlation between different groups, post-hoc Tukey’s HSD test was applied; the test was applied only for reduced GSH and catalase because the concentration/activity levels of these two enzymes alone were found statistically significant in ANOVA. All statistical parameters analyzed in Table 5 (representing analysis for reduced GSH) and Table 6 (representing analysis for catalase) was outcomes of post-hoc Tukey’s HSD test. Tables 5 and 6 present individual comparisons between all of the different study groups to determine if any study group showed any significant changes in reduced GSH and catalase expression in comparison with each other. In addition, Tables 5 and 6 also enlist the outcomes of post-hoc Tukey’s HSD for various statistical parameters such as mean differences, q, 95% CI, and P values, with P values being the most important; P values < 0.05 indicate statistically significant (denoted by “S”), whereas P value > 0.05 were considered statistically insignificant (denoted by “NS”). For reduced GSH, significant changes were observed between the control group and the OSCC Stage I, between the control group participants and Stage III patients, and between the control group participants and Stage IV patients. Intra group comparisons of OSCC patients revealed statistically significant changes between Stage II and Stage III patients and between the Stage II and Stage IV patients. However, Stage III and Stage IV patients revealed no statistically significant changes in reduced GSH levels. There is a significant change in reduced GSH level in Stage III in comparison to other cancer stages which indicate the vital role of this enzyme in cancer progression; however, additional studies are warranted to investigate this further.

On performing similar comparisons for catalase expression among different groups, statistically significant changes were noted among the control group participants and Stage I OSCC patients, controls and Stage III patients, and controls and Stage IV patients. Moreover, intra group comparisons for various stages for OSCC revealed statistically significant changes between Stage II and Stage IV patients as well as between Stage III and Stage IV patients. However, Stage II and Stage III patients revealed no significant differences. These findings indicate that alterations in catalase expression from Stage III to Stage IV might have a pivotal role in

<table>
<thead>
<tr>
<th>SN</th>
<th>Comparison</th>
<th>Mean difference</th>
<th>q</th>
<th>95% CI</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control vs Stage I</td>
<td>4.1000</td>
<td>−3.86</td>
<td>1.5477 to 6.6523</td>
<td>0.0002</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>Control vs Stage II</td>
<td>2.1000</td>
<td>−1.58</td>
<td>1.1284 to 5.3284</td>
<td>0.3709</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>Control vs Stage III</td>
<td>4.2000</td>
<td>−2.9</td>
<td>0.3173 to 8.0827</td>
<td>0.0274</td>
<td>S</td>
</tr>
<tr>
<td>4</td>
<td>Control vs Stage IV</td>
<td>6.1000</td>
<td>−4.52</td>
<td>2.7811 to 9.4189</td>
<td>0.0000</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>Group I vs Stage II</td>
<td>2.0000</td>
<td>−1.73</td>
<td>5.0199 to 1.0199</td>
<td>0.3526</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>Group I vs Stage III</td>
<td>0.1000</td>
<td>−0.08</td>
<td>3.6112 to 3.8112</td>
<td>1.0020</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>Group I vs Stage IV</td>
<td>2.0000</td>
<td>−1.71</td>
<td>1.1164 to 5.1164</td>
<td>0.3848</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>Stage II vs Stage III</td>
<td>2.1000</td>
<td>−1.22</td>
<td>1.0490 to 1.0390</td>
<td>0.0362</td>
<td>S</td>
</tr>
<tr>
<td>9</td>
<td>Stage II vs stage IV</td>
<td>4.0000</td>
<td>−2.26</td>
<td>0.3094 to 7.6906</td>
<td>0.0270</td>
<td>S</td>
</tr>
<tr>
<td>10</td>
<td>Stage III vs Stage IV</td>
<td>1.9000</td>
<td>−1.04</td>
<td>2.3748 to 6.1748</td>
<td>0.7267</td>
<td>NS</td>
</tr>
</tbody>
</table>

S = statistically significant (P < 0.05), NS = statistically nonsignificant (P > 0.05).
the progression of OSCC, which must be investigated in future studies.

Besides biochemical tests, immunoblotting of patient tissue samples was performed with antibodies against various antioxidants. Significant decrease in catalase expression was noted in immunoblotting (Fig. 2A). Moreover, GPX1 and GPX4 levels decreased with progression of OSCC (Fig. 2C). On the other hand, GLRX2, PRX3, and TXN2 showed increased expression in OSCC (Fig. 2B, 2D). Furthermore, mitochondrial SOD2 showed reduced expression as OSCC progressed (Stages II and III); however, at advanced stages (Stage IV), SOD2 expression increased (Fig. 2E). Quantification of SOD2 protein bands using Image J showed a relative decrease of SOD2 in Stage II and Stage III patients but an increase in Stage IV patients in comparison with the control group (Table 5). Although the control group (Group 2) showed a mean relative SOD2 expression level of 98.5 when western blot films were scanned using Image J, Stage II and Stage III patients showed a gradual decrease, with mean relative SOD2 expression levels of 46.16 and 16.15, respectively. However, Stage IV patients showed a significant increase in the mean relative SOD2 expression level (72.70). VDAC1 was taken as a loading control in mitochondrial immunoblot analysis, and its levels remained unaffected in both the control and OSCC groups (Fig. 2F).

**Discussion**

Our study presents an interesting profiling of various mitochondrial antioxidants in the mitochondrial redox landscape and its alteration during oral carcinogenesis. SOD2 is known to be a key controller of cell proliferation (17), but its role in carcinogenesis remains controversial. Depending on its concentration and specific contexts, it modulates the growth of cancer cells (18). In this study, SOD2 levels decreased in the early stages of OSCC (Stages II and III) but increased significantly in Stage IV. However, Liu et al. reported a constant increase of SOD2 levels with ascending grades of tongue squamous cell carcinoma (19). Most previous studies related to SOD2 levels in OSCC have been conducted in cell lines, and only few in vivo studies have been conducted. In this study, the altered levels of SOD2 may be attributed to the self-defense mechanism of the immune system and the biphasic control of SOD2 on cancer cells (18). On the other hand, the sudden increase of SOD2 in Stage IV OSCC compared to Stage III (Fig. 2E) supports the findings of Liu et al. (19) who also reported increased levels of SOD2 in metastatic tumors.

GRX2, another major antioxidant of mitochondria, blocks cytochrome c release and caspase activation, preventing apoptotic cell death under OS (20). It facilitates tumor angiogenesis, thus promoting additional cell proliferation (21). A gradual increase in GLRX2 levels with progressive stages of OSCC in comparison with the control group, as observed in the present study, support its antiapoptotic function. Both GPX1 and GPX4 have antitumorigenic properties (10). In this study, both of these enzymes significantly decreased with progressive stages of OSCC, which is similar to pancreatic and breast cancer (22,23). We also assessed the components of the TXN2 system, a prime target for anticancer therapy nowadays (10), and found an overall increase in these components (i.e., TXN2 and PRX3) with progression of OSCC. Tumor cells are more dependent on the TXN2 system owing to their constant demand of DNA; thus, suppression of the TXN2 system can be a promising strategy for oral cancer therapy. Such an example of targeting TXN2 was done in treatment of multiple myelomas (24). Reduced GSH plays a major role in maintaining intracellular redox balance. In various cancers, GSH levels are increased, making them more resistant to ROS (25,26). Similar findings were noted for mitochondrial GSH in the present study as well. For lipid peroxide levels, a decrease in...
mean values were noted in both controls and OSCC patients, which, however, was not statistically significant (Table 2). Phospholipids are potent substrates for peroxidation in the mitochondrial membrane of OSCC tissues, and a decline in lipid peroxide levels indicates loss of phospholipids. Membranes of cancer cells have more cholesterol than phospholipids as cholesterol decreases the susceptibility of cancer cells to ROS attack (27). The decrease in mean values of lipid peroxides in our cancer samples can indirectly signify the loss of phospholipids in the membrane, even if the decrease may not be statistically significant owing to small sample size. Catalase has a protective role in cancer progression and invasion (28). The decrease in catalase levels with progression of cancer stages signifies the loss of the catalase-mediated protective mechanism in cancer microenvironment. Our preliminary findings establish that mitochondrial antioxidants might be correlated with progression in OSCC. It would be worth studying if there is any molecular interplay of mitochondrial and cytosolic antioxidants in the context of OSCC.

Acknowledgments
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Conflict of interest
The authors have no conflict of interest to declare.

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