

Comparative Evaluation of Mitochondrial Antioxidants in Oral Potentially Malignant Disorders

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Summary: Various endogenous mitochondria-associated antioxidants protect mitochondria from oxidative stresses such as Superoxide Dismutase 2 (SOD2), Catalase, Glutaredoxin 2 (GLRX2), reduced Glutathione (GSH), Glutathione Peroxidase (GPx), and Thioredoxin 2 (TXN2). They protect mitochondria from Reactive Oxygen Species (ROS). Excess ROS causes mitochondrial DNA damage and respiratory chain dysfunction leading to carcinogenesis. In an earlier study we found complex alterations of mitochondrial antioxidants in different stages of oral squamous cell carcinoma. Here, we profiled mitochondrial antioxidants in different oral potentially malignant disorders such as oral leukoplakia (OL), oral lichen planus (OLP), and oral submucous fibrosis (OSMF). Mitochondria was prepared from oral tissues from OL, OLP, and OSMF patients. Biochemical assays and immunoblotting were performed to investigate expression of various mitochondrial antioxidants. Catalase was expressed as Control > OL > OSMF > OLP. GLRX 2 was expressed as OLP > OL > Control > OSMF. GPX1 and GPX4 were expressed as Control > OSMF > OLP > OL. GSH increased in OL and OSMF, but decreased in OLP. SOD2 was expressed as Control > OSMF > OLP > OL. PRX3 was expressed as OL > OLP > OSMF > Control. TXN2 expression was nearly the same in all groups except OL, which showed elevated expression. We conclude that endogenous mitochondria-associated antioxidants show different levels of expressions in various oral potentially malignant disorders.

Key words mitochondrial antioxidants, oral precancerous disorders, oral leukoplakia, oral lichen planus, oral submucous fibrosis

INTRODUCTION

Reactive oxygen species (ROS), when produced in excess by oxidative phosphorylation, overwhelms the antioxidant capacity of the target cell, resulting in oxidative stress [1]. ROS accumulation can be directly associated with reversible or irreversible cell

injury and initiate formation of a microenvironment favorable for carcinogenesis [2]. ROS-activated microenvironment may contribute to the formation of newly activated pre-neoplastic cells or may enable the clonal proliferation of latent pre-neoplastic cells leading to carcinogenesis [1]. Several metabolic alterations have been observed in cancerous cells such as

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Abbreviations: ANOVA, Analysis of variance; df, Degrees of freedom; DTNB, Dithiol(2-nitrobenzoic acid) (DTNB); GLRX2, Glutaredoxin2; GPx, Glutathione Peroxidase; GSH, Reduced Glutathione; GSSG, Oxidized glutathione; MnSOD, Manganese superoxide dismutase; NADPH, Nicotinamide adenine dinucleotide phosphate; OL, Oral Leukoplakia; OLP, Oral Lichen Planus; OSCC, Oral Squamous Cell Carcinoma; p value, Probability Value; OSMF, Oral Submucous Fibrosis; ROS, Reactive Oxygen Species; SD, Standard Deviation; SOD2, Superoxide Dismutase2; SPSS, Statistical Package for Social Sciences; TNB, Titrobenzoic acid; TXN2, Thioredoxin2; VDAC1, Voltage Dependent Anion Channel1.

mitochondrial dysfunction [3]. Oxidative stress causes severe mitochondrial DNA damage with subsequent progressive respiratory chain dysfunction thereby promoting carcinogenesis [4]. Oral cancer is one of the most common cancers that can occur de-novo or may be preceded by precancerous lesions or conditions like oral leukoplakia (OL), oral submucous fibrosis (OSMF), and oral lichen planus (OLP) [5]. Significant alterations of cytosolic antioxidant levels have already been investigated in different oral precancerous pathologies [5,6], but profiling of endogenous mitochondria-associated antioxidants in those oral potentially malignant disorders have not yet been reported. The various antioxidants that regulate ROS and redox-sensitive pathways in the mitochondria are manganese superoxide dismutase (MnSOD or SOD2), mitochondrial glutaredoxin 2 (GLRX2), reduced glutathione (GSH), glutathione peroxidase (Gpx), catalase, and thioredoxin 2 (TXN2) [7]. Earlier, we reported alterations in the levels of various mitochondrial antioxidants in the mitochondrial redox landscape as a result of stress response programs within oral squamous cell carcinoma tissue. We obtained our earlier results by means of immunoblotting and biochemical assays [8]. We found significant alterations of various mitochondrial antioxidants in oral tissues at different stages of oral cancer. This led us to investigate whether mitochondrial antioxidants also differ in oral precancerous lesions. The pathogenesis differs in various oral potentially malignant disorders like OL, OLP, and OSMF. Therefore, we decided to find out if altered mitochondrial antioxidant levels play any significant role in the pathogenesis of those oral potentially malignant disorders. In our current study, we investigated the levels of these antioxidants in tissue samples of OL, OLP, and OSMF patients of different age groups and compared them with control patients to get an insight into the pathogenesis of the diseases in relation to mitochondrial antioxidants.

MATERIAL AND METHODS

Patient Selection

The study was conducted in the outpatient department of Burdwan Dental College. The study subjects were 12 newly diagnosed patients with oral leukoplakia (OL), 14 cases of oral lichen planus (OLP) and 12 cases of oral submucous fibrosis (OSMF) who had not received any previous treatment for the same. The provisional diagnosis of OL was made with the clinical appearance of a whitish patch or plaque which could not be characterized clinically or pathologically

as any other disease, and was not associated with any physical or chemical agent except the use of tobacco [9]. Confirmatory diagnosis of the lesion was done histopathologically, thereby excluding other specific disorders. All OL patients had positive tobacco (smoking and/or chewing) or alcoholic history. A provisional diagnosis of OLP was made based on its clinical appearance, and the final diagnosis was confirmed histopathologically. There was no positive association of a specific habit among the OLP patients, but psychological stress had a common association. Clinical diagnosis of OSMF was made on the basis of definite features like blanching of mucosa, palpable fibrous band on the buccal mucosa, shrunken uvula, restricted mouth opening, and reduced tongue protrusion. The confirmatory diagnosis was made by biopsy. OSMF patients had the habit of chewing areca nut with or without tobacco. Twenty age- and sex-matched control subjects were selected and tissue specimens were obtained by vestibuloplasty. Patients suffering from a systemic disease such as diabetes, hypertension, gout (serum uric acid levels >than 7.2 mg/dl), hepatic diseases (serum GGT levels >45 U/L), cardiovascular diseases, and adrenal dysfunctions were excluded from the study. All subjects were interviewed before being clinically examined in the outpatient department. A questionnaire was prepared to obtain data on demographic factors, types of habits, frequency, and duration of habits (Table 1). The study was independently reviewed and approved by the Ethical Committee, Burdwan Dental College & Hospital in the year 2013, and written consent was obtained from all the subjects after detailed explanation of the study objectives and methodologies. The patients gave voluntary consents to the study and the experiments were conducted in full accordance with standard ethical principles.

Tissue Sample Collection and Preparation of Mitochondria

Surgically resected tissues were collected from each oral precancerous patient category and kept immersed in an isotonic homogenization buffer. Purification of mitochondria from the tissue samples was carried out as described earlier by differential centrifugation (Beckman Coulter, Indianapolis, IN, USA) [9,10]. To check the purity of the mitochondrial fractions as obtained from various tissues, the crude homogenate and the mitochondrial fractions were immunoblotted against various protein markers like PCNA (nuclear marker), α -tubulin (cytosolic marker), Calreticulin & Calnexin (Endoplasmic reticulum)

to rule out contamination of cytosol, nucleus, and endoplasmic reticulum. The fractions were also immunoblotted with anti VDAC1, anti TOMM20, anti TIM44, and anti COX IV to confirm successful isolation of mitochondria. Oral tissue mitochondria from control group was collected by vestibuloplasty.

Measurement of mitochondrial Reduced Glutathione (GSH) content

The chemicals were purchased from Sigma-Aldrich (Bangalore, India). The contents of mitochondrial oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured by Ellman's method. Briefly, GSH reduces 5, 5'-dithiol (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) and gets oxidized to GSSG. GSSG then gets reduced to glutathione (GSH) by glutathione reductase in presence of NADPH in a coupled reaction. Measurement of TNB was done by a UV-VIS Spectrophotometer at 412 nm.

Catalase activity assay

Catalase activity in the mitochondrial extracts of oral tissues of OL, OLP, and OSMF patients was determined at 25°C [11]. Catalase oxidizes methanol to formaldehyde (HCHO) in the presence of hydrogen peroxide. HCHO then oxidizes 4-amino-3-hydrazine-5 mercapto-1,2,4 triazole (Purpald) resulting in a change of color to purple, which was read in an UV-VIS Spectrophotometer at 540 nm.

Measurement of mitochondrial lipid peroxide content

Mitochondrial lipid peroxide content was measured according to the method described by Ogura et al [12]. Briefly, thiobarbituric acid reacted with the breakdown products of mitochondrial lipid resulting in formation of a chromogen that was detected spectrophotometrically at 532.5 nm.

Measurement of Superoxide Dismutase activity

Superoxide Dismutase activity was measured according to standard methodology [13]. Briefly, a red fomazan dye is produced from 2-(4-indophenol)-3-(4-nitrophenol)-5phenyltetrazolium chloride after it gets reduced by superoxide radicals generated by xanthine oxidase. The reduction rate of superoxide anion has a linear relation to Xanthine Oxidase activity and is inhibited by superoxide dismutase. Superoxide dismutase activity is measured by the degree of inhibition of this reaction. [13]

Measurement of Glutathione Peroxidase activity

Glutathione peroxidase activity is measured according to standard methodology [14]. Briefly, Glutathione peroxidase catalyzes oxidation of glutathione. As oxidized glutathione gets reduced, NADPH is oxidized and gets converted into NADP. This oxidation-reduction is observed at 340 nm and activity of glutathione peroxidase is measured.

Western Blotting

Antibodies were purchased either from Sigma-Aldrich (Bangalore, India) or Abcam (Abcam, Cambridge, MA, USA). Total tissue homogenate (50 µg protein) and total mitochondrial extract (100 µg protein) were 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 then probed with polyclonal rabbit anti PCNA, anti α -tubulin, anti-Calreticulin, anti-Calnexin, anti TOMM20, anti TIM44, anti Cytc, anti SOD2, anti-Catalase, anti GLRX2, anti PRX3, anti TXN2, anti GPX1, and anti GPX4 antibodies at 1:2000 dilutions for 2 hours at 4°C in parallel sets. Incubation with secondary antibody was done using mouse anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP), at 1:10,000 dilutions for 1 hour at room temperature. Immunoblotted protein bands were detected by Chemiluminescence method using ECL kit (Amersham Pharmacia Biotech/ GE Healthcare, Kolkata, India). VDAC1 was taken as the loading control and incubation was done with polyclonal antiVDAC1 (Voltage Dependent Anion Channel1) antibody. Relative quantifications of the various protein bands were done with respect to the loading controls using Image J software on the western blot films.

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) Version 15.0 Statistical Analysis Software (Chicago, IL, USA). The different biochemical parameters were compared between case and control groups by using independent student t- test. The values were expressed as mean \pm SD. One way ANOVA (Analysis of variance) test was used to compare the parameters in different precancerous lesions. $P < 0.05$ was considered to be statistically significant. Tukey HSD test was performed to obtain statistical difference in mean values between different precancerous groups. $P < 0.05$ was taken to be signifi-

cant.

RESULTS

The mitochondrial fractions prepared by differential centrifugation from various tissues were found to be pure without any cytosolic, nuclear, and other organelle contamination as checked by immunoblotting with various organellar marker specific antibodies such as anti PCNA, anti α Tubulin, anti Calnexin, anti Calreticulin, anti VDAC1, anti TOMM20, anti TIM44, and anti COX IV (Figure 1). The expression levels of mitochondrial antioxidants and mitochondrial lipid peroxide content were investigated in control and precancerous tissue samples either/or by biochemical assays and western blotting of mitochondrial extracts. The concentration/activities of reduced glutathione, lipid peroxide, and catalase were measured by a biochemical approach (Figure 2). The concentration of reduced glutathione increased in OL (12.4 ± 0.432 mM) and in OSMF (12.5 ± 0.638 mM) and decreased in OLP (10.2 ± 0.374 mM) when compared to control mitochondria (11.3 ± 0.716 mM). Mitochondrial lipid peroxide content increased in all precancerous tissues viz OL (2.02 ± 0.165 nmol/mg of protein), OLP (2.25 ± 0.198 nmol/mg of protein) and OSMF (1.87 ± 0.172 nmol/mg of protein) when compared to control (1.57 ± 0.286 nmol/mg) group. The activity of catalase decreased significantly in all three precancerous lesions compared to the control group. The catalase activity was found to get reduced in OL (3.1 ± 0.156 U/mg/min), OLP (1.39 ± 0.118 U/mg/min) and OSMF (3.91 ± 0.142 U/mg/min) when compared to control (6.4 ± 0.268 U/mg/min). ANOVA analysis found that expression levels of reduced glutathione, mitochondrial lipid peroxide content, and catalase varied significantly across OL, OLP, and OSMF patients (Supplementary Table 1). For correlation analysis among OL, OLP and OSMF groups, post hoc Tukey's HSD test was applied on reduced glutathione, lipid peroxide, and catalase using the values obtained from the SPSS software. A p value < 0.05 indicates statistical significance. Levels of glutathione showed significant changes between control and OL, control and OLP, and control and OSMF groups. Within intragroup comparisons, statistically significant changes in glutathione levels were observed between OL and OLP, OLP, and OSMF but non-significant changes were observed between OL and OSMF (Supplementary Table 2). Mitochondrial lipid peroxide content showed statistically significant changes when control group was

compared separately with OL, OLP, and OSMF. Intragroup comparisons of mitochondrial lipid peroxide content showed no statistically significant changes between OL and OSMF, but these changes were significant in the other groups (Supplementary Table 3).

We did post hoc Tukey HSD test on catalase activity across these various groups. When the control group was compared individually to all precancerous groups, no significant changes were observed. However, intra precancerous group comparison of catalase activity showed statistically significant changes between OL and OLP, OL and OSMF, and OLP and OSMF (Supplementary Table 4).

We also tested the expression levels of various antioxidant proteins in the control group and different precancerous groups by immunoblotting. Immunoblotting with anti-catalase antibody showed a decrease in catalase expression levels in OL, OLP, and OSMF

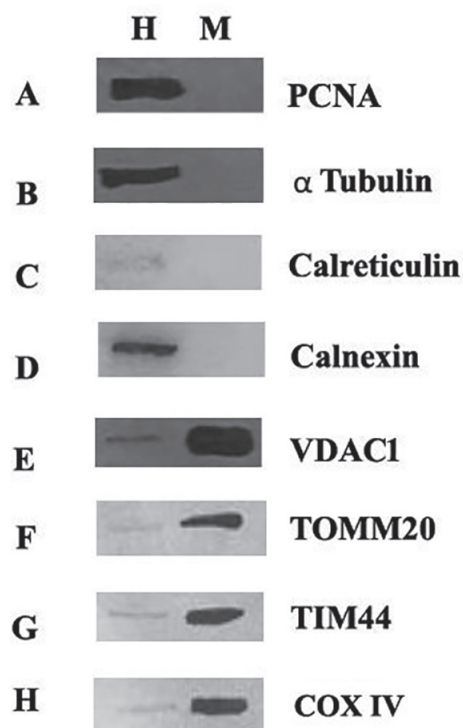


Fig. 1. Purity of oral tissue mitochondria as tested by Immunoblotting against different mitochondrial protein markers in the entire blot membrane. Total tissue homogenate is indicated by "H" and Mitochondrial fraction obtained after differential centrifugation is indicated as "M" (A) PCNA, Nuclear Marker (B) α Tubulin, Cytosolic Marker (C) Calreticulin, Endoplasmic Reticulum Marker (D) Calnexin, Endoplasmic Reticulum Marker (E) VDAC1, Mitochondrial Marker (F) TOMM20, Mitochondrial marker (G) TIM44, Mitochondrial marker (H) COX IV, Mitochondrial marker.

when compared with controls. Quantification of immunoblot signals of catalase was done using Image J software (Table 2). Among the groups, mean expression levels of catalase was in the order of Control > OSMF > OL > OLP (Table 2)

The expression levels of SOD2 decreased across precancerous groups in comparison to the control group and the mean expression level was in the order of Control > OSMF > OLP > OL (Figure 3). Similar findings were also observed for SOD2 activity in biochemical assay (Supplementary Figure 1A). SOD2 activity decreased in the order Control > OSMF > OLP > OL.

For GLRX2, there was an increase in immunoblot signal in OL and OLP, but a decrease in expression in OSMF when compared to control. The expression levels were in the order of OLP > OL > Control > OSMF.

The immunoblot patterns of GPX1 and GPX4 were similar. The expression levels of both GPX1 and GPX4 were less in OL, OLP, and OSMF when compared with controls. Comparison with Image J software revealed the order of expression as Control > OSMF > OLP > OL. Similarly, activity of mitochondrial glutathione peroxidase decreased in the following order Control > OSMF > OLP > OL as shown by biochemical assay (Supplementary Figure 1B).

PRX3 and TXN2 showed different patterns in immunoblots. For PRX3, the mean expression levels

were in the order of OL > OLP > OSMF > Control. For TXN2, OL group showed the highest expression, whereas Control, OLP, and OSMF showed similar levels of expression.

For catalase, the control group showed the highest expression in immunoblots, and there was a decrease in OL, OLP, and OSMF in the order of Control > OL > OSMF > OLP. VDAC1 was used as a loading control in mitochondrial immunoblot analysis, and its levels were unchanged in control and other precancerous groups.

DISCUSSION

Precancerous lesions have broadly been used in literature to describe a group of lesions that have the potential to develop into cancer [15]. Initially, these group of lesions were termed as precancerous lesions and conditions, but in the 2005 WHO meeting held in London a common terminology was adopted, i.e., "Potentially malignant disorders." This new terminology indicates that not all disorders described under this group may convert into malignancy [16]. In a clinicopathological study conducted by Phookan et al, oral leukoplakia (OL) was reported as the most common potentially malignant disorder involving 20.65% of patients. On the other hand, oral submucous fibrosis (OSMF) and oral lichen planus (OLP) had very simi-

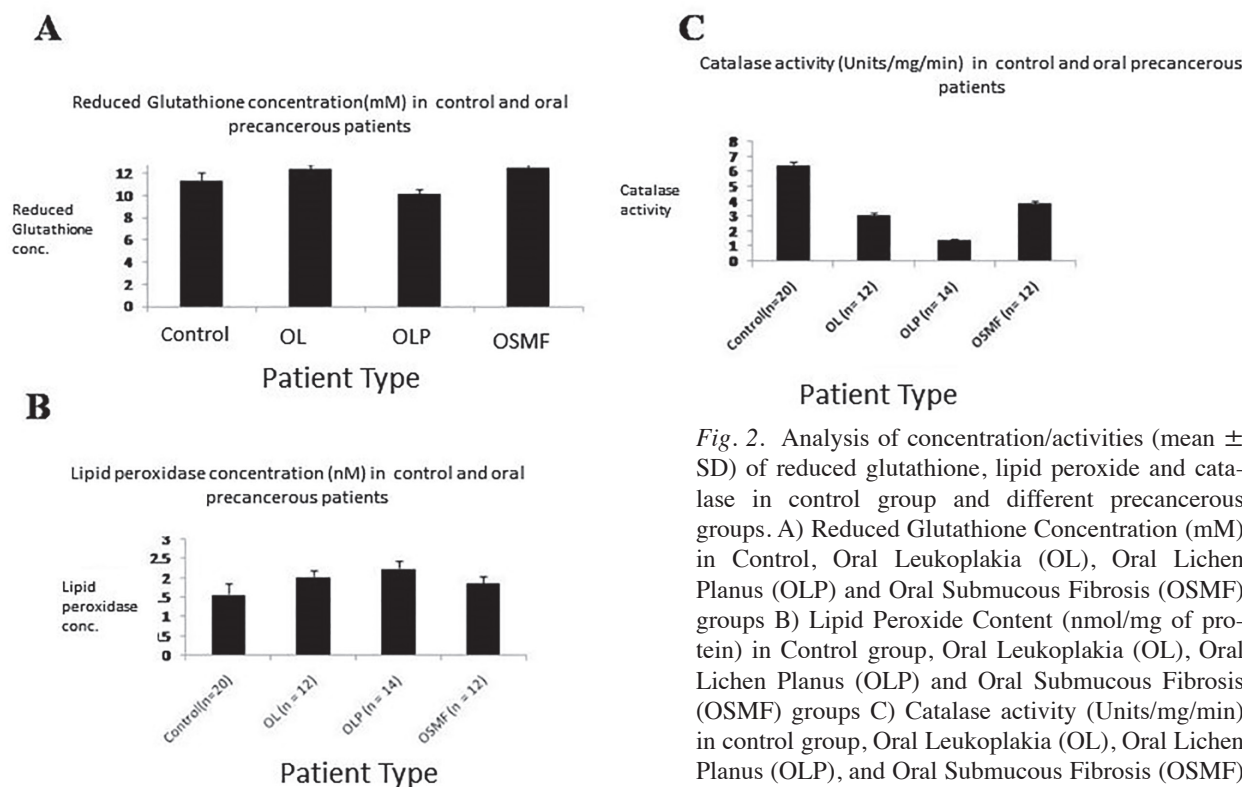


Fig. 2. Analysis of concentration/activities (mean \pm SD) of reduced glutathione, lipid peroxide and catalase in control group and different precancerous groups. A) Reduced Glutathione Concentration (mM) in Control, Oral Leukoplakia (OL), Oral Lichen Planus (OLP) and Oral Submucous Fibrosis (OSMF) groups B) Lipid Peroxide Content (nmol/mg of protein) in Control group, Oral Leukoplakia (OL), Oral Lichen Planus (OLP) and Oral Submucous Fibrosis (OSMF) groups C) Catalase activity (Units/mg/min) in control group, Oral Leukoplakia (OL), Oral Lichen Planus (OLP), and Oral Submucous Fibrosis (OSMF) groups. n= No. of Individuals.

lar distribution (0.62% of patients) [17]. The etiology of potentially malignant disorders of the oral cavity is multifactorial and not fully understood [18]. Tobacco

chewing, smoking and alcoholism can be directly associated with oral leukoplakia although several other factors are also contributory [19]. OSMF can mainly

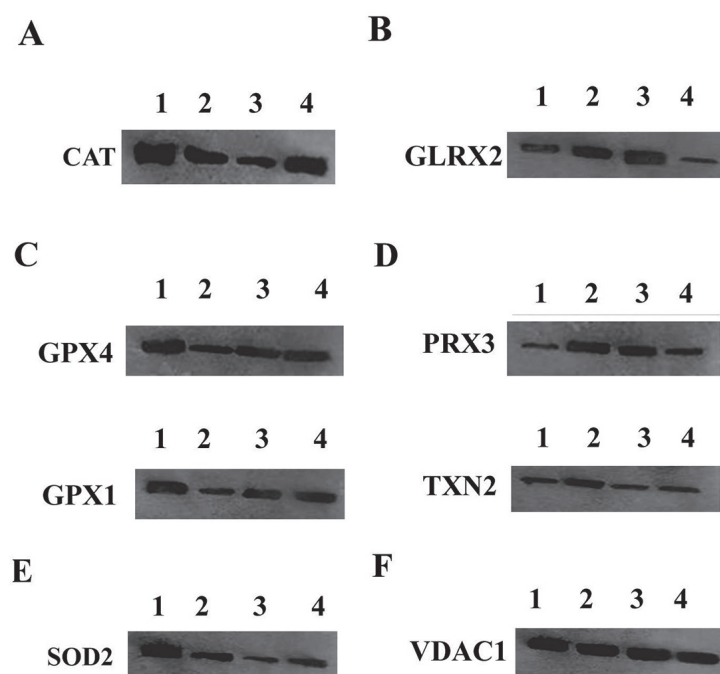
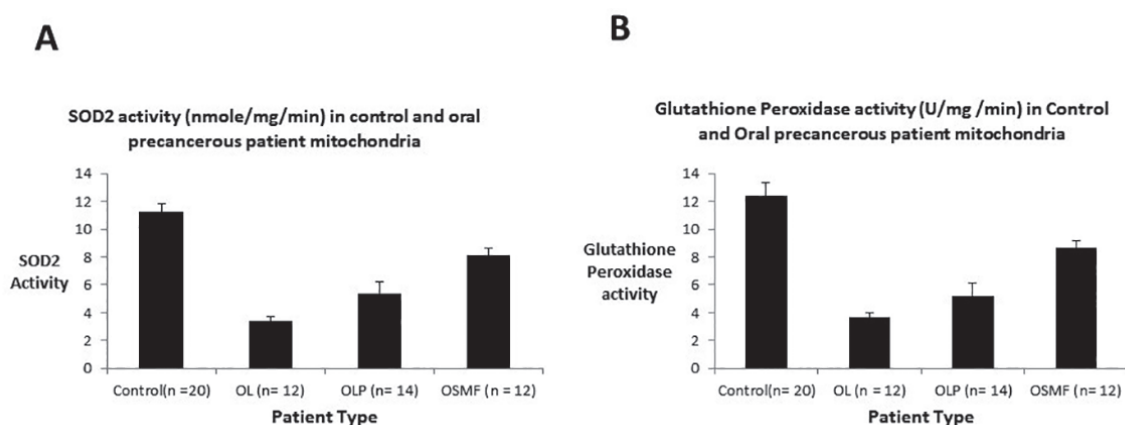
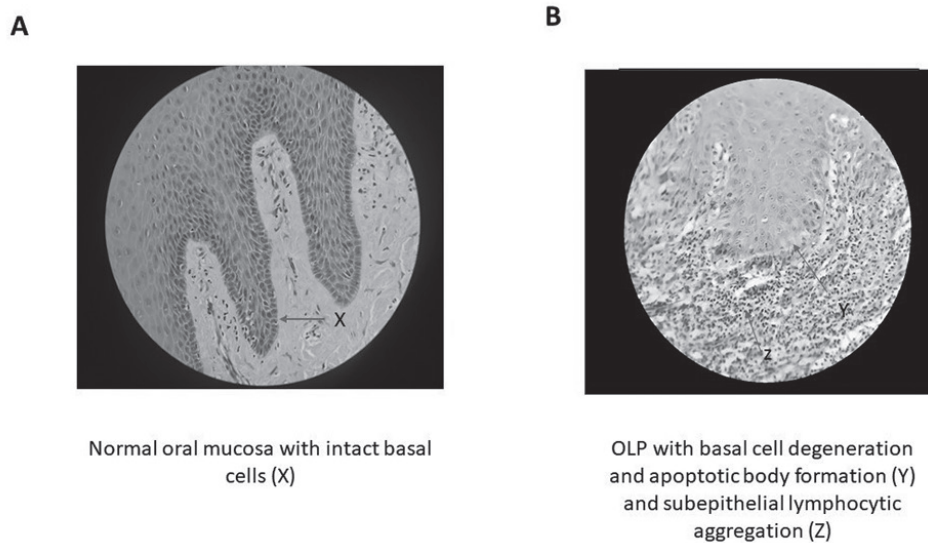


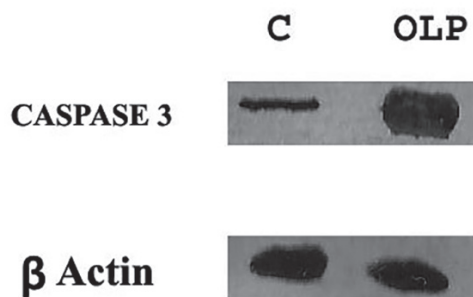
Fig. 3. Expression levels of mitochondrial antioxidants in various stages of Oral Leukoplakia, Oral Submucous Fibrosis, and Oral Lichen Planus. (A) Catalase, CAT (B) Glutaredoxin 2, GLRX2 (C) Glutathione Peroxidase (GPX4) & Glutathione Peroxidase 1 (GPX1) (D) Peroxiredoxin3 (PRX3), Thioredoxin2 (TXN2) (E) Superoxide Dismutase 2, (SOD2) (F) Voltage-Dependent Anion Channel 1 (VDAC1) as a housekeeping protein in mitochondrial immunoblot analysis. Lane 1: Control Group. Lane 2: Oral Leukoplakia group. Lane 3: Oral Lichen Planus group. Lane 4: Oral Submucous Fibrosis Group.



Supplementary Fig. 1. Analysis of SOD2 activity (Units/min/mg of protein) and Glutathione Peroxidase activity (Units/min/mg of protein) in control and oral precancerous groups. (A) Activity of SOD2 in control group and Oral Leukoplakia (OL), Oral Lichen Planus (OLP), and Oral Submucous Fibrosis (OSMF) groups. n= No. of Individuals (B) Activity of Glutathione Peroxidase in control group and Oral Leukoplakia (OL), Oral Lichen Planus (OLP), and Oral Submucous Fibrosis (OSMF) groups. n= No. of Individuals.



Supplementary Fig. 2. Histological analysis of oral mucosa at 40x magnification. (A) Control group, X indicates intact basal cells. (B) Oral Lichen Planus (OLP) patients, Y indicates apoptotic body formation and X indicates subepithelial lymphocytic aggregation.



Supplementary Fig. 3. Immunoblotting of apoptotic marker Caspase 3 in Control (C) group and Oral Lichen Planus (OLP) patients. β -actin is the loading control.

be associated with chewing of areca nut, the main component of betel quid. Other than that, ingestion of chilies, alterations of immunity, genetic contribution, and nutritional deficiency are also major contributory factors [15]. OLP is considered an immune-mediated T cell disorder [20]. OLP can be associated with genetic background, response to the dental materials, several drugs, infectious agents, autoimmunity, immunodeficiency, food allergies, stress, habits, trauma, diabetes, hypertension and much more [21,22,23]. On the other hand, histologically, the tissues are found to react quite differently in response to threatening agents. Histopathologically, the hallmark of OL is epithelial hyperplasia and hyperkeratosis of ortho- or parakeratotic type of the epithelium, with various degrees of chronic inflammatory infiltrates in lamina propria. It also can be associated with various degrees of epithelial dysplasia [24]. OSMF shows a variable

degree of fibrosis of the sub-epithelial connective tissue, associated with inflammatory changes. The epithelial thickness in OSMF is variable [25]. Typical histopathological examination of the OLP biopsy reveals hyper orthokeratosis or hyperparakeratosis, with acanthosis, which is thickening of the granular layer with intercellular edema. Subepithelially, there is mononuclear infiltration of the T-cells and histiocytes forming a typical band like appearance. The intraepithelial T-cells and degenerating keratinocytes form eosinophilic homogeneous globules, also known as civette bodies. Ultrastructurally, the civette bodies are apoptotic keratinocytes revealing DNA fragmentation in these cells [26]. Histological specimens from OLP patients in our study also revealed basal cell degeneration (Supplementary Figure 2). We also found a significant increase of apoptotic marker Caspase 3 in OLP compared to control group (Supplemental Figure 3). Whatever differences there are in the basic pathology of these different premalignant lesions, one common association has been noted i.e. oxidative stress leading to reactive oxygen species (ROS) and free radical generation [27]. The generation of ROS is associated with damage to protein, carbohydrate, lipid, and nucleotides, finally contributing to malignant transformation. On the other hand, there is a strong association between ROS production and mitochondrial dysfunction [28]. The mitochondrial DNA (mtDNA) is more sensitive to ROS-induced damage than nuclear DNA. Mutated mtDNA contributes to malfunction

of the subunits of respiratory complexes that in turn increase ROS production creating a vicious cycle [29,30,31,32]. A direct association between oxidative stress and OLP has been established [33] and it is postulated that oxidative stress may constitute a significant pathogenic mechanism in OLP and its complications. The deleterious effects of oxidative stress influence the basic mechanisms of

signal transfer and transduction, resulting in the dysfunction of keratinocytes and their impaired apoptosis [34]. Oxidative stress has been found directly contributing to the apoptosis of the basal keratinocytes by altering mitochondrial regulation and the regulation is dependent on CD8+ lymphocytes, which in turn occurs through their influence on nuclear factor kappa B activity (NF- κ B) [35,36].

TABLE 1.
Clinical and demographic details of the precancerous subjects

Characteristics	Oral Leukoplakia patients (n=12)	Oral Lichen Planus patients (n=14)	Oral Submucous Fibrosis patients (n=12)
Sex	Male :7 Female : 5	Male: 6 Female:8	Male: 9 Female: 3
Age Mean	52.69 \pm 5.35	34.73 \pm 3.82	43.13 \pm 7.39
Habits	Smoker and chewers (n=5) Only tobacco chewers (n=3) Active smokers (n=2) Smoking and alcohol (n=1) Tobacco chewers and alcohol (n=1)	No habit associated (n=9) Smoking (n=3) Tobacco chewers (n=2)	Betel leaf+arecanut+tobacco (n=8) Areca nut +lime (n=2) Areca nut only (n=2)
Duration of Habits Mean	10.34 \pm 3.12 years	6.23 \pm 8.16 years	8.03 \pm 2.48 years
Sites affected	Buccal mucosa (n=6) Lower labial mucosa including attached gingiva in relation to lower anteriors. (n=4) Floor of the mouth (n=2)	Buccal mucosa (n=7) Labial mucosa (n=4) Generalized attached gingiva (n=3)	Buccal mucosa (n=8) Tongue, limited tongue protrusion (n=2) Soft palate and uvula (n=2)
Clinical diagnosis	Homogenous leukoplakia (n=10) Speckled leukoplakia (n=2)	Reticular (8) Ulcerative(6)	stage I (n=8) Stage II (2) Stage III (3)
Histopathological diagnosis	No dysplasia (n=3) Mild dysplasia (n=6) Moderate dysplasia (n=2) Severe dysplasia (n=1)	Oral lichen planus (no dysplasia) (n=14)	Oral submucous fibrosis (n=12)

Supplementary TABLE 1.

ANOVA test on the concentration/activity levels of reduced glutathione, lipid peroxide, and catalase as derived in Table 2

	Sum of squares	Degrees of freedom (df)	Mean of square	F	p	Significance
<i>Reduced Glutathione</i>	47.41	3	15.80	47.23	0.000	S
<i>Lipid Peroxide</i>	4.033	3	1.344	27.04	0.000	S
<i>Catalase expression</i>	222.1	3	74.03	1964	0.000	S

S= statistically significant (P<0.05), NS = statistically non significant (P>0.05)

TABLE 2.

Mean Relative Expression Levels of various mitochondrial oxidants in control and Oral Leukoplakia, Oral Lichen Planus and Oral Submucous Fibrosis patient samples as quantified by Image J after normalizing to the loading control marker, mitochondrial marker, VDAC1.

Experimental Groups	Loading Control protein VDAC1	CAT	GLRX2	GPX4	GPX1	PRX3	TXN2	SOD2
Control Patient	100	98±0.32	90±0.57	95±0.43	85±0.32	150±0.65	102±0.70	85±0.2
OL Patient	100	75.35 ±0.56	146.17 ±0.43	48.58 ±0.46	25.28 4±0.55	432.15 ±0.67	146.11 ±0.87	40.8 ±0.44
OLP Patient	100	45.76 ±0.32	225.29 ±0.65	76.75 ±0.65	61.32 ±0.21	354.74 ±0.89	75.72 ±0.43	24.41 ±0.98
OSMF Patient	100	84.56 ±0.45	40.96 ±0.32	80.84 ±0.34	69.45 ±0.33	212.70 ±0.54	106.0 ±0.86	38.20 ±0.73

Supplementary TABLE 2.

Analysis of significant/non-significant changes of reduced glutathione in Control group and different precancerous lesions by post-hoc Tukey's HSD test

SN	Comparison	Mean Difference	q	95%CI	"p"	Significance
1.	Control vs OL	1.1000	-0.60	0.5398 to 1.6602	0.0000	S
2.	Control vs OLP	1.1000	-2.49	1.6346 to -0.5654	0.0000	S
3.	Control vs OSMF	1.2000	0.56	0.6398 to 1.7602	0.0000	S
4.	OL vs OLP	-2.2000	0.52	-2.8036 to -1.5964	0.0000	S
5.	OL vs OSMF	0.1000	0.78	-0.5264 to 0.7264	0.9743	NS
6.	OLP vs OSMF	2.3000	0.39	1.6964 to 2.9036	0.0000	S

S= statistically significant (P<0.05), NS = statistically non-significant (P>0.05)

Supplementary TABLE 3.

Analysis of significant/non-significant changes of lipid peroxide in Control group and different precancerous lesions by post-hoc Tukey's HSD test

SN	Comparison	Mean Difference	q	95%CI	"p"	Significance
1.	Control vs OL	-0.45	1.114	-2.97 to 2.07	0.0000	S
2.	Control vs OLP	-0.68	1.683	-3.2 to 1.48	0.0000	S
3.	Control vs OSMF	-0.30	0.70	-2.94 to 1.34	0.0000	S
4.	OL vs OLP	-0.23	0.504	-3.07 to 2.16	0.0000	S
5.	OL vs OSMF	0.15	0.317	-2.80 to 3.1	0.8213	NS
6.	OLP vs OSMF	0.38	0.833	-2.46 to 3.22	0.0000	S

S= statistically significant (P<0.05), NS = statistically non-significant (P>0.05)

Supplementary TABLE 4.

Analysis of significant/non-significant changes of catalase activity in Control group and different precancerous lesions by post-hoc Tukey's HSD test

SN	Comparison	Mean Difference	q	95%CI	"p"	Significance
1.	Control vs OL	-3.3000	-0.36	-3.4879 to -3.1121	0.9375	NS
2.	Control vs OLP	-5.0100	-2.82	-5.1893 to -4.8307	0.9375	NS
3.	Control vs OSMF	-2.4900	0.39	-2.6779 to -2.3021	0.9206	NS
4.	OL vs OLP	-1.7100	0.88	-1.9125 to -1.5075	0.0032	S
5.	OL vs OSMF	0.8100	0.56	0.5999 to 1.0201	0.0000	S
6.	OLP vs OSMF	2.5200	0.67	2.3175 to 2.7225	0.8691	NS

S= statistically significant ($P < 0.05$), NS = statistically non-significant ($P > 0.05$)

Altered expression of salivary antioxidants has already been reported in both OL and OSMF [5,6,37], but all the enzymatic analysis has been done either in saliva or serum samples. In this study, an attempt has been made to profile the endogenous mitochondrial antioxidants including manganese superoxide dismutase (MnSOD or SOD2), mitochondrial glutaredoxin (GLRX2), reduced glutathione (GSH), glutathione peroxidase (Gpx), catalase, and thioredoxin 2 (TXN2) systems in different potentially precancerous disorders and to find any possible correlation in their pathogenesis which could indicate a possible treatment modality in future. In mitochondria, GSH is found mainly in reduced form and has a multidirectional role in protecting mitochondrial environment against oxidative stress. The mitochondrial oxidative stress is mainly regulated by GSH, with participation of either GSH peroxidase or peroxiredoxin, and by the later conversion of oxidized glutathione (GSSG) back into GSH by the NADPH-dependent GSSG reductase (GR) [38]. Among the different GSH peroxides (Gpxs) that detoxify hydrogen peroxide is Gpx1, which is present in mitochondrial matrix. Mitochondrial GSH also stabilizes the mitochondrial membranes by reducing the oxidative stress on phospholipids and other lipid peroxides through the actions of mitochondrial GSH-S transferases (GSTs) that exhibit modest Se-independent Gpx activity [39,40]. Gpx4, a membrane-associated enzyme, that is partly localized to the intermembrane space, acts as a critical defense enzyme in protecting membranes against oxidative stress because of its capacity to reduce hydroperoxide groups on phospholipids,

cholesterylesters, and lipoproteins. Moreover, GSH can act nonenzymatically by reacting with electrophiles that are generated as a consequence of metabolic processes involving both endogenous compounds and xenobiotics, and this reaction is greatly enhanced in presence of GSTs [41]. The GSH system also collaborates with the glutaredoxin (Grx) system and acts as electron donor in electron transport chain and reduces the protein mixed disulphide concentration preventing the malfunctioning of these proteins [42,43]. The higher amounts of GSH noted in OSMF and OL could be a protective mechanism adopted to prevent oxidative stress-induced DNA damage that aids malignant transformation process [37], but in OLP, GSH was found to be exhausted due to apoptotic pathway activation and proves the involvement of mitochondrial apoptotic pathway in the pathogenesis [35].

SOD2 is a potent mitochondrial endogenous antioxidant associated with regulation of cell proliferation and has significant anti-apoptotic function [44,45,46]. In our experiments, SOD2 level was found to be reduced in all the lesional groups in comparison to the control group as shown by both biochemical assay and immunoblotting. It is expressed almost in similar levels in OL and OSMF, but was found to be significantly reduced in OLP indicating the probable loss of regulation of anti-apoptotic pathway.

GLRX2 promotes cell proliferation [47] and also prevents apoptosis by inhibition of cytochrome c release and caspase activation [48]. We found an increase in expression of GLRX2 immunoblot signal in OL and OLP, but a decrease in expression in OSMF when compared to control. The expression of GPX1

and GPX4 were similar in immunoblots showing reduced expression of both the enzymes in the lesional groups in comparison to the control group. The maximum reduction was found in OL. Both GPX1 and GPX4 have anti tumorigenic property and decreased level of GPX1 can be correlated with increased cancer risk [49].

PRX3 have a protective role in hypoxic condition and it prevents hypoxia- induced apoptosis [50]. In OSMF, due to progressive fibrosis, there is constriction of the blood vessels in advanced stages leading to the creation of a hypoxic microenvironment [51]. The increased level of PRX3 in OSMF compared to control group might contribute to the atrophic nature of the epithelium. Similar increased levels of PRX3 are also observed in OL and OLP, which may correlate with the proliferative nature of the epithelium in OL and OLP.

The proliferative cells have a constant requirement for DNA synthesis and this phenomenon correlates with the increased level of TXN2 in OL [52].

Oxidative stress can affect the biological membrane by lipid peroxidation and the breakdown products of lipid peroxides may serve as “oxidative stress second messengers,” due to their prolonged half-life and their ability to diffuse from their site of formation, compared to free radicals. The lipid peroxidation and the breakdown products can lead to changes in the functional integrity of the membrane lipid bilayer and can dramatically alter cell function [53]. Altered levels of lipid peroxidation have been reported in many precancerous and cancerous lesions [54,55,56]. In our study, we found an increased level of mitochondrial lipid peroxide content in OL, OLP, and OSMF patients in comparison to the control group.

Catalase has a protective role in cancer progression and invasion [57]. p53, the tumor suppressor gene and its downstream targets, p53-inducible ribonucleotide reductase (p53R2) and p53-inducible gene 3 (PIG3), are reported to interact with catalase for efficient regulation of intracellular ROS, depending on stress intensity [58]. The antioxidant functions of p53 are mediated by p53R2, which maintains increased catalase activity and thereby protects against endogenous ROS. After genotoxic stress, high levels of p53 and PIG3 cooperate to inhibit catalase activity, leading to a shift in the oxidant/antioxidant balance toward anti oxidative status. In our study, we found reduction of catalase activity in OLP, which can play an important role in oxidative stress generation. Similarly reduction in catalase activity in OL and OSMF in comparison to control group as shown in our study indicate

loss of catalase mediated protective mechanism under oxidative stress.

CONCLUSION

Our data shows mitochondrial antioxidants differ in expression in various oral potentially malignant disorders. Detailed investigation of these molecular players in the context of the mitochondrial redox landscape can help us in understanding the pathogenesis of oral leukoplakia, oral lichen planus, and oral submucous fibrosis.

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