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Galaxy Publication

Evaluation of 8-OHDG, SOD, and GPx Biomarkers among Individuals with OPMD and OSCC

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ABSTRACT

Oxidative stress arises when the generation of free radicals exceeds the capacity of the body's antioxidant defense and repair mechanisms, leading to unchecked oxidative damage. These reactive oxygen species (ROS) significantly contribute to molecular degradation, promote neoplastic alterations at the cellular level, and exert a profound impact on all phases of the cancer development process. In the Indian subcontinent, oral potentially malignant disorders such as leukoplakia and oral submucous fibrosis are widespread, and often associated with habits such as tobacco chewing and areca nut use. This investigation focused on quantifying salivary concentrations of 8-OHDG, SOD, and GPx in patients diagnosed with OPMD and OSCC, both with and without concurrent diabetes mellitus. The levels of biomarkers were determined using an ELISA assay protocol. Specifically, 40 µL of sample diluent was dispensed into each well along with 10 µL of the saliva sample. Following proper sealing, the plates were incubated for 45 minutes at 37 °C. Post incubation, the wells underwent aspiration and a series of five washing cycles, each lasting approximately 1–3 minutes. Subsequently, an HRP-linked diagnostic antibody was added to initiate signal detection. This study represents an early effort to analyze markers of oxidative stress—namely 8-OHDG, SOD, and GPx—in saliva samples from OPMD and OSCC patients, with and without diabetes mellitus, aiming to understand their diagnostic relevance.

Keywords: Well-being, Leukoplakia, Good health, Oral submucous fibrosis, Neoplastic transformation, Diabetes mellitus

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Introduction

Oxidative stress emerges when the pace of oxidative reactions in cells exceeds the body's intrinsic antioxidant defense capabilities, resulting in an imbalance that favors the formation of free radicals. These unstable molecules, produced under oxidative stress, have been identified as central contributors to degenerative pathologies such as oral cancer [1]. The reactive oxygen species (ROS), characterized by their instability and high reactivity, induce significant genomic alterations including mutations in DNA bases and breaks in single or double strands. These disruptions not only damage cellular structures but also trigger neoplastic transformations, influencing each phase of the cancer development process [2].

Oral potentially malignant disorders (OPMDs) are defined as a category of lesions and conditions that may not necessarily evolve into cancer but exhibit varying degrees of malignant potential [3, 4]. In essence, some of these lesions, though not all, may progress to malignancy [5]. According to the WHO's 1978 classification, several conditions once considered precancerous—including oral submucous fibrosis (OSMF), actinic keratosis, and discoid lupus erythematosus—are now placed under the umbrella of potentially malignant disorders, alongside other precancerous and smoking-related lesions such as leukoplakia and palatal changes [6, 7].

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Among these, OSMF stands out as the most widespread premalignant oral condition in India, with a strong association with areca nut and tobacco use [8, 9]. In recognition of its harmful effects, the International Agency for Research on Cancer (IARC) declared areca nut a confirmed human carcinogen in 2003 [10]. Additionally, tobacco has been recognized by the World Health Organization as one of the top ten global health hazards. It contains over 400 carcinogenic substances, including well-known agents like carbon monoxide, nicotine, and aromatic hydrocarbons [11, 12]. Furthermore, both areca nut and tobacco contain a range of reactive oxygen species and free radicals such as nitric oxide (NO), hydroxyl radicals (OH-), and singlet oxygen (O2) [13].
Although the relationship between OSMF and reactive oxygen species was previously considered limited, emerging evidence now suggests a stronger connection, particularly given the role of environmental carcinogens like tobacco [14]. Reactive oxygen species, a subclass of oxygen-derived radicals, include highly reactive entities such as hydrogen peroxide (H2O2), singlet oxygen (O2), hydroxyl radicals (OH), and superoxide anions (O2−). These molecules are implicated in a variety of physiological and pathological processes, including inflammation, aging, atherosclerosis, pre-cancerous changes, and tumor formation [6, 14-16]. Against this backdrop, the present investigation seeks to evaluate the concentrations of 8-OHDG, SOD, and GPx in saliva samples of individuals diagnosed with OPMD and OSCC, both with and without diabetes mellitus.

Materials and Methods

Study location

This investigation was conducted at the Department of General Pathology, Blue Lab, Saveetha Dental College, and Hospitals in Chennai. Saliva samples were collected aseptically in sterile containers from three subject groups: 10 individuals diagnosed with OSCC and OPMD along with diabetes mellitus, 10 patients with OSCC and OPMD but without diabetes mellitus, and a control group of 10 healthy individuals. Eligibility criteria included patients diagnosed with OSCC before surgical intervention, both with and without diabetes mellitus. Exclusion criteria encompassed patient demographics such as age and gender. Salivary concentrations of TIMP1 and MMP2 in OSCC patients were quantified using enzyme-linked immunosorbent assay (ELISA) kits. Data acquisition was performed using an automated micro ELISA reader.
All results were compiled into Microsoft Excel and statistical evaluation was performed using SPSS software version 23.0, reporting findings as mean ± standard deviation.

Assay principle – 8-OHDG, SOD, and GPx

The analytical procedure employed was based on a sandwich ELISA format. Polyclonal antibodies specific to 8-OHDG, SOD, and GPx were immobilized onto 96-well plates. Detection involved the application of biotinylated antibodies targeting the same analytes. After introducing standards, samples, and the biotin-tagged antibodies into the wells, the plates were washed with buffer to remove unbound elements. The next step involved adding the Avidin-Biotin-Peroxidase Complex, followed by another wash. A chromogenic reaction was initiated using TMB as the substrate. Upon catalysis by horseradish peroxidase (HRP), TMB turned blue, then yellow after the addition of a stop reagent. The intensity of this color change was directly proportional to the concentration of 8-OHDG captured in each well. Quantification was done by measuring absorbance at 450 nm.

Assay procedure

Before starting, the ABC working reagent and TMB solution were brought to room temperature (37 °C) for 30 minutes. Standards were used to generate a calibration curve for each assay. The layout of the pre-coated ELISA plate was carefully mapped, allocating wells for standards, samples, and controls. Each concentration and test sample was measured in duplicate. For the standards, 0.1 ml of a dilution series ranging from 10,000 pg/ml to 156 pg/ml was added into designated wells. Control wells received 0.1 ml of the standard/sample diluent (Kit Component 3). Test wells were filled with 0.1 ml of appropriately diluted sample types, which included human serum, plasma, cell culture supernatants, tissue lysates, and other biological fluids. Plates were incubated for 90 minutes at 37 °C with covers in place.
Following incubation, contents were discarded onto absorbent paper, and 0.1 ml of biotin-labeled anti-human 8-OHDG antibody was dispensed into each well. Care was taken to coat only the bottoms of the wells without touching the side walls. Plates were again incubated at 37 °C for one hour. Plate washing was performed three times using one of two methods:

Manual Wash: Discard well contents carefully without contacting the plate edges. Fill each well with Wash buffer (Kit Component 10), shake on an ELISA shaker for two minutes, and drain. Repeat the process twice more.
Automated Wash: Aspirate contents and refill each well three times with Wash buffer, ensuring overfill. Following the final wash, invert the plate onto absorbent paper. A one-minute soak is recommended during automated cycles.
Each well then received 0.1 ml of the ABC working solution and was incubated for 30 minutes at 37 °C. This was followed by five additional washes, with each wash allowing a 1–2 minute dwell time. Then, 90 µl of TMB substrate (Kit Component 8) was introduced to each well, and the plate was incubated in the dark at 37 °C for 25–30 minutes. A color gradient from light to deep blue was observed in the more concentrated standard wells. Upon the addition of 0.1 ml of the stop solution to each well, the blue transitioned to yellow. Optical density was then read at 450 nm using a microplate reader, within 30 minutes of stopping the reaction.
The relative optical density (O.D.450) was computed as follows: (O.D.450 of test or standard well) minus (O.D.450 of the zero control). A standard curve was created by plotting relative O.D.450 values (Y-axis) against their known concentrations (X-axis). Concentrations of Human MMP-2 in the unknown samples were calculated by interpolation from this curve.

Results and Discussion

This investigation focused on evaluating the levels of 8-OHDG, SOD, and GPx enzymes in patients diagnosed with OPMD and OSCC, both with and without the presence of type 2 diabetes mellitus. It was observed that individuals with OSCC and OPMD who also had diabetes exhibited heightened oxidative stress, which was accompanied by a reduction in the activity of antioxidant enzymes. Among all the biomarkers analyzed, 8-OHdG demonstrated the most statistically significant variation in mean salivary levels. Detailed values including the mean and standard deviation for SOD, GPx, and 8-OHDG across patient groups are presented in **Tables 1–3**. Corresponding visual representations of these findings are depicted in **Figures 1–3**.

Table 1. The mean and standard deviation of SOD value in OPMD and OSCC patients

Sample	Mean	Standard Deviation
control	1.53	0
OPMD + ND	0.695	0.04
OPMD + D	0.523	0.02
OSCC + ND	0.643	0.03
OSCC + D	0.489	0.02

Table 2. The mean and standard deviation of GPx value in OPMD and OSCC patients

Sample	Mean	Standard Deviation
control	1.782	0
OPMD + ND	0.767	0.02
OPMD + D	0.627	0.02
OSCC + ND	0.603	0.03
OSCC + D	0.411	0.02

Table 3. The mean and standard deviation of 8-OHDG value in OPMD and OSCC patients.

Sample	Mean	Standard deviation
control	0.376	0
OPMD + ND	0.486	0.04
OPMD + D	0.634	0.03
OSCC + ND	0.57	0.02
OSCC + D	1.35	0.01

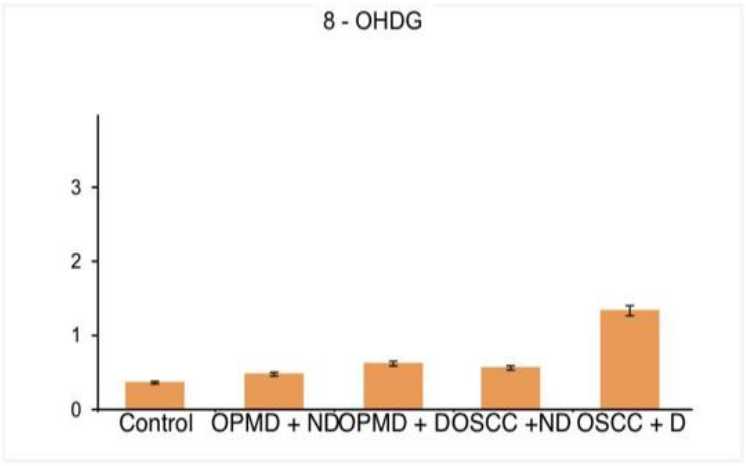

Figure 1. The 8-OHDG enzyme level in different patients such as control OPMD + ND, OPMD + D, OSCC + ND, OSCC + D; and it shows higher levels in OSCC patients who are diabetic.

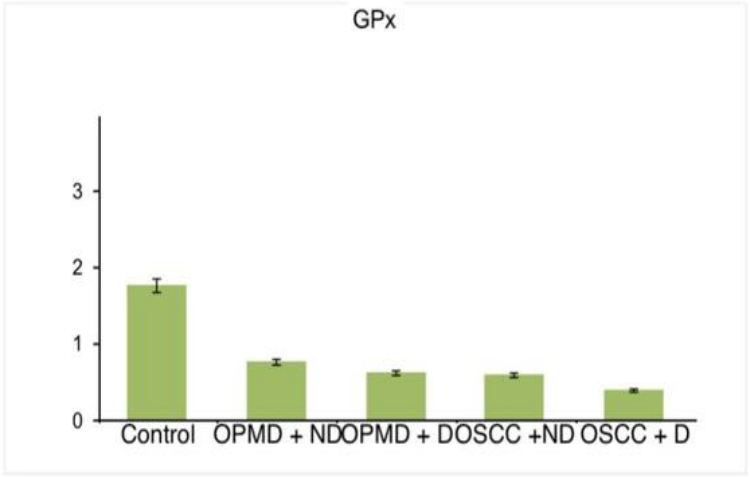

Figure 2. The GPx enzyme level in different patients such as control OPMD + ND, OPMD + D, OSCC + ND, OSCC + D; and it shows the lowest level in OSCC patients who are diabetic.

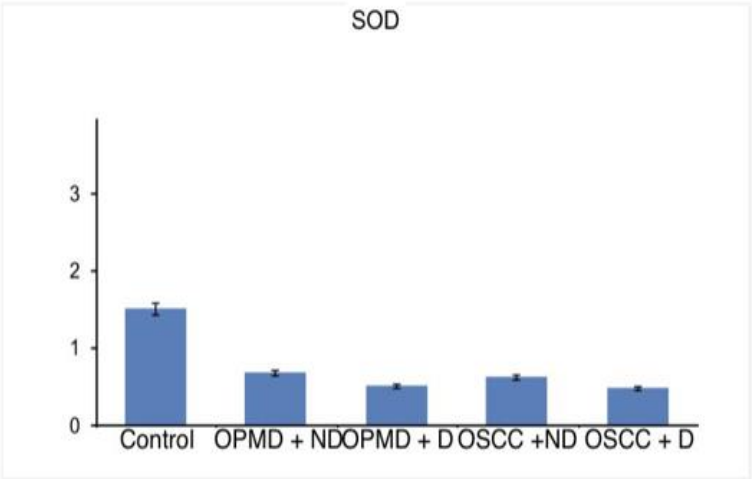

Figure 3. The SOD enzyme level in different patients such as control OPMD + ND, OPMD + D, OSCC + ND, OSCC + D; and it shows the lowest level in OPMD patients who are diabetic.

This research highlighted that individuals diagnosed with OSCC and OPMD in conjunction with type 2 diabetes mellitus exhibited a marked increase in oxidative stress, paralleled by a decline in the activity of key antioxidant enzymes. Oral potentially malignant disorders (OPMD) represent a spectrum of tissue alterations, not all of which inevitably evolve into malignancies. However, certain lesions within this group are believed to possess a higher probability of malignant transformation. The general prevalence of OPMD ranges between 0.2% and 0.5%, with a malignant progression rate estimated at 7.6% [5, 17].

Among the disorders categorized under OPMD is oral submucous fibrosis (OSMF), which was previously regarded as a precancerous condition. Areca nut consumption has been widely implicated as the primary etiological factor, with supporting evidence from Sinor *et al*. Furthermore, in 2003, the International Agency for Research on Cancer formally classified areca nut as a carcinogen for humans. The carcinogenic potential of areca nut is primarily linked to its ability to generate reactive oxygen and nitrogen species, both of which contribute to cellular alterations leading to malignancy. Our research group has a well-established background in this domain, demonstrated by a series of impactful publications [18–24].

Oxidative stress arises when the physiological antioxidant systems fail to neutralize excessive free radicals, leading to the accumulation of reactive species capable of inducing DNA damage. Such genotoxic stress may result in cell death, mutation, or cancer initiation. Free radical-induced deterioration of DNA, proteins, and lipids is considered a hallmark of oxidative damage, though quantifying these molecular changes poses challenges due to their fleeting stability [25–27].

In scenarios involving OPMD and OSCC, particularly when co-existing with type 2 diabetes mellitus, there is a pronounced escalation in oxidative stress accompanied by a reduction in antioxidant enzyme concentrations. Our analysis revealed a considerable suppression in the enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx), alongside an elevated presence of 8-Hydroxyguanosine, an oxidative stress biomarker. These findings imply that patients with both diabetes and oral pathologies may experience an additive or synergistic oxidative burden, contributing to antioxidant depletion [28].

When comparing OSMF and OSCC groups with healthy controls, there was a clear ascending trend in mean salivary 8-OHdG levels. This progressive increase points to heightened DNA oxidative damage and an increasingly oxidative microenvironment in affected individuals. Supporting literature includes findings by Totan *et al*. who reported significant disparities in 8-OHdG concentrations in both saliva and serum between OLP patients and healthy subjects. Similarly, Kumar *et al*. noted elevated 8-OHdG levels in OSCC patients relative to controls, reinforcing the biomarker's potential as a robust indicator of oxidative damage in oncogenic settings.

To the best of our knowledge, the present investigation marks the initial effort to assess salivary levels of 8-OHDG, SOD, and GPx among OPMD and OSCC patients with and without diabetes mellitus. Although statistical significance in antioxidant variations could not be firmly established due to sample size limitations, this foundational work sets the stage for more comprehensive future studies aimed at clarifying these biochemical alterations across patient subgroups.

Conclusion

The findings underscore a pronounced imbalance in redox homeostasis among individuals affected by OSCC and OPMD with coexisting type 2 diabetes mellitus, as evidenced by amplified oxidative stress and suppressed antioxidant enzyme activity. Notably, salivary 8-OHdG levels varied significantly between OSMF and OSCC cases, with the latter exhibiting the most pronounced elevation. This biochemical trend positions 8-OHdG as a promising salivary biomarker for evaluating oxidative DNA insult and tracking the pathological evolution from OSMF to OSCC.

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Conflict of Interest: None

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Ethics Statement: The research protocol was reviewed and authorized by the Institutional Ethical Committee, with all procedures conducted in full alignment with ethical research standards.

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