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# Comparison of Antioxidant Status in Saliva of Smokers, Leukoplakia and Oral Squamous Cell Carcinoma Patients

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**Type of Publication:** Original Research Article

**Conflicts of Interest:** Nil

## Abstract

**Introduction:** Oral cancer is the 6th most common cancer worldwide which is mainly due to use of tobacco, causes irreversible cellular changes and ROS mediated DNA alterations. Antioxidants help in eliminating those free radical formations. As diagnostic utility saliva can be more reliable because it is in direct contact with the lesion also is non-invasive and ease of collection, where analysis of antioxidants in saliva can be a useful early diagnostic biomarker. This study is aimed to compare the salivary antioxidant levels viz. Malondialdehyde (MDA), Glutathione (GSH) and Catalase (CAT) between different groups.

**Materials and Method:** The study and control group comprised of 5 patients each. The study group is further divided into 3 sub-groups based on age, sex, clinical presentation and cytological grading. Group 1: Normal patients (N), Group 2: Smokers (S), Group 3 Leukoplakia (L), Group 4 squamous cell carcinoma (C). Exfoliative cytology smears stained with PAP were evaluated and graded by double blinding method and saliva sample collected from the patients were evaluated for MDA, GSH and Catalase activities.

**Result:** The mean values of antioxidants like Catalase and GSH were significantly increased in normal patients whereas decreased in early smokers and cancer patients

compared to control and gradual increase in MDA levels were observed in smokers and Cancer patients as compared to controls. Cytology gradation revealed cancer patients at Grade 4.

**Conclusion:** Antioxidant constitutes a major role in development of malignancy and thereby is a valuable diagnostic marker in diagnosis of oral cancer and pre-cancer patients.

**Key words:** oral squamous cell carcinoma, antioxidants, exfoliative cytology, pre-malignant lesion, smoking, leukoplakia

## Introduction

Among the head and neck cancer, oral cancer is the 6$^{th}$ most common cancer worldwide which predominately develops as squamous cell type [1, 2]. The etiology for oral squamous cell carcinoma is multifactorial and the main predisposing factor includes usage of tobacco and associated products [3]. The chemicals present in tobacco causes oxidative stress in the cells and reactive oxygen species (ROS) induced DNA damages to the cell. These ROS are by-products of normal cellular activity and plays a role in cellular signaling pathway. Increase in the ROS production, results in altered cellular structure, function and homeostasis [4]. Oxidative stresses are formed when there is imbalance between the reactive oxygen species and the cells capacity to detoxify them, through enzymatic or non-enzymatic reactions, where these ROS are eliminated by anti-oxidants [5]. On Chronic usage of tobacco, causes redox imbalances which act as a main risk factor for development of cancers and other lesions [4]. It also, causes irreversible changes in oral mucosal surface and results in potentially malignant lesions, these lesions have a high risk for transformation to oral cancer. It is evident that, ROS production promotes tumour initiation and progression by genetic alteration and also produces cytotoxic effect on the cell by activating cellular pathways. The early diagnoses of these lesions are also important in management of disease progression [6]. Early detection and lack of reliable early biomarkers are the reasons for the poor prognosis and high mortality of the disease. Several studies have reported alterations in the serum anti-oxidants levels in malignant and pre-malignant lesions [7]. As saliva is non-invasive and easy to collect also it is in direct contact with the lesion, there can be disease related concentration alteration in the saliva, which can provide a better result than serum sample [1]. Since, tumour cell contains high ROS level as a result of oxidative phosphorylation; we aimed to study the levels of salivary lipid peroxidase, catalase and glutathione peroxidase in early smokers, oral leukoplakia and oral squamous cell carcinoma patients

## Material and Methods

Saliva collection and processing was approved by the Chettinad Academy of Research and Education - Institutional Human Ethics Committee (CARE – IHEC, ethical clearance reference number: 381/IHEC/10-17, dated 23-10-2017). Informed consent was obtained from all individual participants included in the study.

### Patients grouping

The study comprise of 20 subjects of selected age ranging from 20-50 years reporting to the department of oral medicine and radiology. Among the 20 subjects, 5 subjects of early smokers, 5 subjects of clinically and cytologically confirmed oral leukoplakia (OL) and 5 subjects of clinically, cytologically and histopathologically confirmed oral squamous cell carcinoma (OSCC). Buccal mucosal smear was taken and classified according to the cytology grading as class1, class2, class 3, class 4 and histopathology grading (Fig 1-7). Age and sex matched subjects were taken for the study. Patients with systemic illness, under medication and



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undergoing treatment for the pathological lesions were excluded from the study.

**Saliva sample collection**

The subjects were informed about the study and informed consent was obtained from each subjects participating in the study. Thorough intra oral examination was done using mouth mirror and explorer. Unstimulated whole saliva was collected. The subjects were instructed to not eat or chew any food or drink water 30 mints prior to the saliva sample collection procedure. Then the subjects were asked to spit in the 5 ml container with 5 minutes interval for 30 minutes. Immediately the saliva samples are stored at -20℃.

**Biochemical assay**

The fresh saliva samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for the assay. Total protein estimation was done in the sample for neutralization using Lowry's total protein estimation method.

Salivary estimation of Lipid peroxidase was determined by Buege and Aust 1984 method, Total MDA activity was estimated by TBA-TCA assay. Saliva sample and TCA-TBA HCL were mixed and heated for 15 minutes in the boiling water bath, after cooling the mixture is centrifuged at 1000rpm in cooling centrifugation machine for 10 minutes. Precipitate is removed and the absorbance of the Supernant was measured spectrophotometrically at 535nm.

Catalase by Chance et al 1955 method, Total catalase activity was estimated by adding 50mM phosphate buffer, 15mM $H_2O_2$ to the saliva sample, the reaction mixture was prepared and the absorbance was measured spectrophotometrically at 240nm.

Glutathione peroxidase by Sedlak 1968 method, Total thiol content was estimated by adding 4% Sulfosalicyclic acid and 0.1M sodium phosphate buffer to the saliva sample and reaction mixture was prepared to 3 ml. To the 1 ml of test solution 6µg DTMB was added. The solution was allowed to stand for 1 hour and 15 minutes and vortexes for 15 seconds. The absorbance was measured spectrophotometrically at 412nm.

The calculation was done using extinction coefficient and the units were expressed in µM /Min/µg protein.

**Statistical analysis**

Data obtained from at least three to five independent experiments performed in triplicate manner were presented as mean ± SEM. The unpaired *t* test (two-tailed) was used to determine significant differences between two groups of data (control and activator or inhibitor treated). *P*-values of <0.05, <0.01 and <0.001 were considered as statistically significant, and are indicated by asterisks (*, ** and *** respectively). All data's were analyzed by using Graph Pad V7.00 software

**Results**

The mean salivary MDA levels of group Normal was 0.13 µM /Min/µg protein, Smoker was 0.193 µM /Min/µg protein, Leukoplakia was 0.22 µM /Min/µg protein, OSCC was 0.26 µM /Min/µg protein (Fig 8, Table 1). The mean MDA levels of smokers were higher than normal control but lower than leukoplakia and OSCC, leukoplakia was higher than smokers but lower than OSCC and OSCC was higher than leukoplakia, smoker and normal control (P=<0.05) (Table 4).

The mean salivary CAT levels of group Normal was 3.081 µM /Min/µg protein, Smoker was 0.725 µM /Min/µg protein, Leukoplakia was 0.763 µM /Min/µg protein, OSCC was 1.078 µM /Min/µg protein(Fig 9, Table 1). The mean CAT levels of smokers were lower than normal control, leukoplakia was mildly higher than smokers but lower than normal control and OSCC was higher than smoker and leukoplakia but lower than normal control.



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The mean salivary GSH levels of group Normal was 0.66 µM /Min/µg protein, Smoker was 0.65 µM /Min/µg protein, Leukoplakia was 0.54 µM /Min/µg protein, OSCC was 0.08 µM /Min/µg protein (Fig 10, Table 1). The mean GSH levels of smokers were lower than normal control but higher than leukoplakia and OSCC, leukoplakia was lower than smokers and normal control but higher than OSCC and OSCC was lower than leukoplakia, smokers and normal control ($P=<0.001$) (Table 4).

Co-relation between cytology, histopathological grading and levels of anti-oxidants stated that OSCC has higher rate of ROS production corroborating with the cytology and histopathological results (Table 2, 3)

**Discussion**

Lipid peroxidase, catalase and glutathione peroxidase are considered to be the most important antioxidant enzymes that plays an important role in the regulation of cellular redox state in normal and tumorigenesis. In our current study, we observed that increased oxidative stress which was evaluated by levels of MDA in saliva the increasing levels of MDA indicates the oxidative stress which is enhanced in OSCC. These antioxidants play a vital role in preventing the DNA damage and cell death against cancer. The increased levels of MDA can be due to the reason that the cancer cell has a potential to produce more free radicals that leads to increase in lipid peroxidation, when there is high oxidative stress there is peroxidation of membrane lipids. This is accordance to the recent study, increased MDA level in Oral leukoplakia and Oral squamous cell carcinoma. Another study showed increased levels of MDA in colorectal cancers when compared with the normal tissue [8, 9]. Catalase is one of the enzymatic forms of antioxidant acts as a catalyst which converts the hydrogen peroxide ($H_2O_2$) to less reactive gaseous oxygen ($O_2$) and water ($H_2O$) molecule. It neutralizes the effect of hydrogen peroxide in the cell. In this study, there was reduced levels of catalase was found this is with accordance with the recent study, were there is reduced levels of catalase activity was seen in tissue samples of OSCC. Another study revealed the reduced Catalase activity in breast cancer. The possible reason for the decreased levels of catalase can be due to excessive production and accumulation of $H_2O_2$ that leads to DNA damage and progression of cancer [3, 10]. The glutathione (GSH) is an enzymatic form of antioxidant and also a major endogenous antioxidant that is present predominantly in mitochondria. This enzyme plays an important role in preventing the cell from high oxidative stress which directly involves in the neutralization of free radicals and reactive oxygen compounds. In this study, decreased levels of GSH were noted in the OSCC when compared to the normal sample. This is accordance to the study (FIASCHI et al) decrease in GSH enzyme levels in blood of OSCC patients may be due to increased scavenging of lipid peroxides as well as sequestration by tumor cells [11, 12]. Tumor cells have been found to sequester essential antioxidants such as GSH to meet the demands of a growing tumor. Deficiency of GSH in blood of oral cancer patients may be responsible for the reduction [13]. Another study states that decreased levels of this enzyme in cancer patients are due to increased turnover to counteract the oxidative damage [10]. Together compiling the datas from previous study, our current data suggests that anti-oxidants have important role in tumorigenesis and further studies on molecular mechanism and signaling pathway on ROS can provide a better insight in the development of anti-cancer drugs and novel cancer therapeutic agents for cancer progression and recurrence. It can be stated from our study that antioxidants plays an important role in preventing the cells from free radicals and reactive oxygen



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species and acts as a defence mechanism for the cell against carcinogenesis. And use of whole saliva for diagnosis can provide a new tool for screening and treatment strategies. A non-invasive estimation of salivary concentration of antioxidants can help in analysing the defence capacity of oral mucosa and prevent from pathology. Hence, the panel of salivary antioxidants can be used as a diagnostic biomarker in early diagnosis of cancer and pre-cancer [10, 14]. Further increasing the sample size and including variety of pathological conditions along with grading systems for oral cancer clinically and histopathologically can improve the specificity of the antioxidants in diagnostics and prognostic evaluation. And can be used in mass screening programs were saliva is the non-invasive and cost efficient source of sample.

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**Legends Figures and Tables**

| Study groups | n | Gender | Mean (µM /Min/µg protein) | | |
|---|---|---|---|---|---|
| | | | MDA | CAT | GSH |
| Normal control | 5 | Male | 0.13 | 3.081 | 0.66 |
| Smokers | 5 | Male | 0.193 | 0.725 | 0.65 |
| Leukoplakia | 5 | Male | 0.22 | 0.763 | 0.54 |
| OSCC | 5 | Male | 0.26 | 1.078 | 0.08 |

Table 1: Mean salivary MDA, CAT and GSH levels in study group

| Study groups | Cytology grading | Mean (µM /Min/µg protein) | | |
|---|---|---|---|---|
| | | MDA | CAT | GSH |
| Normal control | Class 1 | 0.13 | 3.081 | 0.032 |
| Smokers | Class 2 | 0.193 | 0.725 | 0.007 |
| Leukoplakia | Class 3 | 0.22 | 0.763 | 0.014 |
| OSCC | Class 4 | 0.26 | 1.078 | 0.012 |

Table 2: Mean salivary MDA, CAT and GSH based on cytology grading

| Parameter | Site | H/P | Anti-oxidant levels (µM/Min/µg protein) | | |
|---|---|---|---|---|---|
| | | | MDA | CAT | GSH |
| OSCC | Buccal mucosal ulcer | Well-differentiated | 0.163 | 1.38 | 0.16 |
| | Tongue ulcer | Well-differentiated | 0.422 | 1.324 | 0.15 |
| | Buccal mucosal ulcer | Well-differentiated | 0.292 | 1.05 | 0.11 |
| | Vestibular growth | Well-differentiated | 0.247 | 0.317 | 0.01 |
| | Tongue ulcer | Well-differentiated | 0.179 | 1.319 | 0.01 |

Table 3: Clinical co-relation with MDA, CAT and GSH levels

| Study groups | P value | | |
|---|---|---|---|
| | MDA | CAT | GSH |
| Normal control Smoker | Not significant | Not significant | <0.01* |
| Normal control Leukoplakia | Not significant | Not significant | Not significant |
| Normal control Oscc | <0.05* | Not significant | <0.001* |
| Smoker Leukoplakia | Not significant | Not significant | Not significant |
| Smoker Oscc | Not significant | Not significant | <0.01* |
| Leukoplakia Oscc | Not significant | Not significant | Not significant |

Table 4 statistical intergroup difference

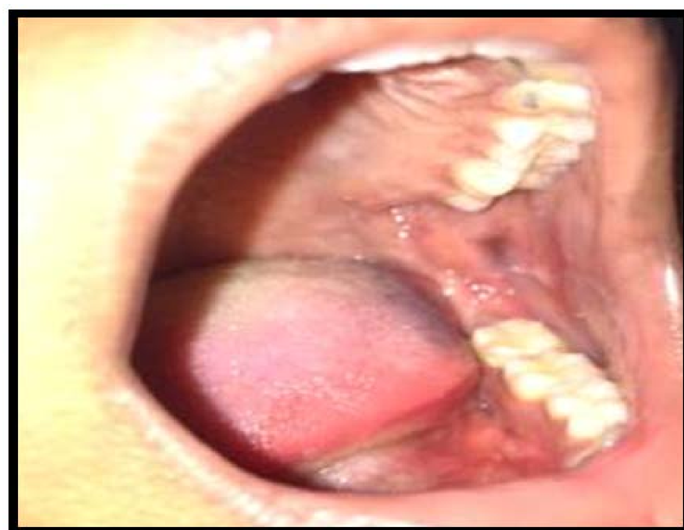

Fig 1: Normal oral mucosa – group 1

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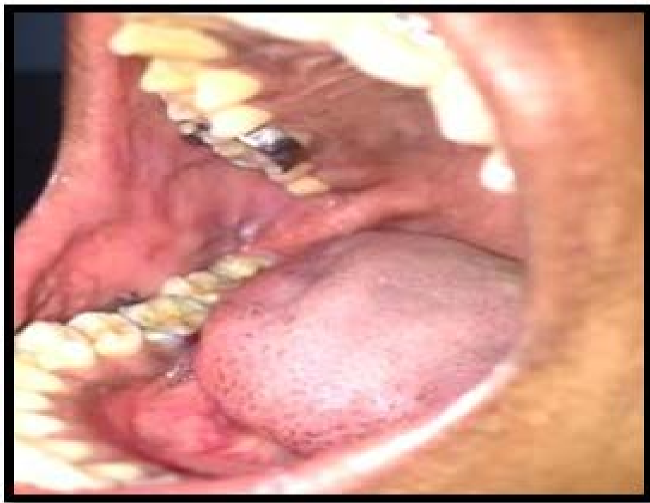

Fig 2: early smokers – group 2

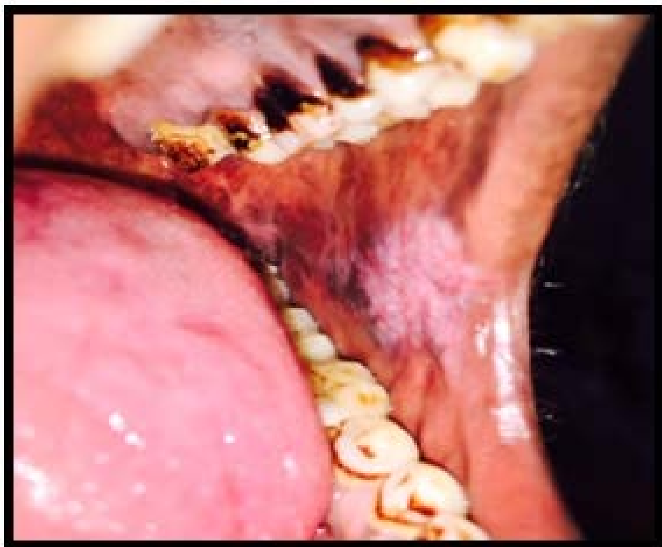

Fig 3: potentially malignant lesions – group 3

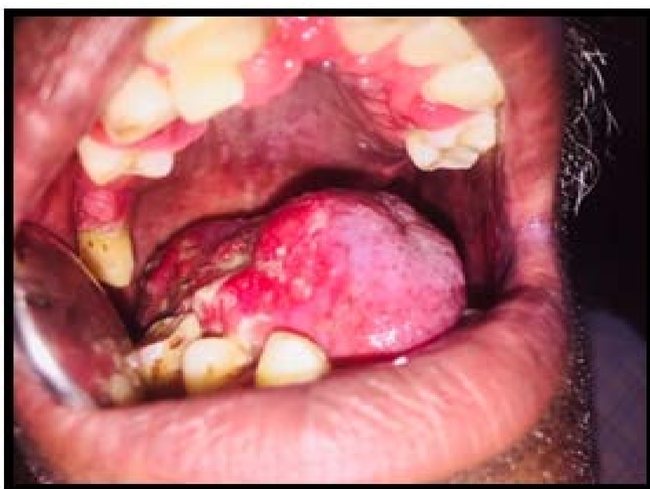

Fig 4: Oral squamous cell carcinoma – group 4

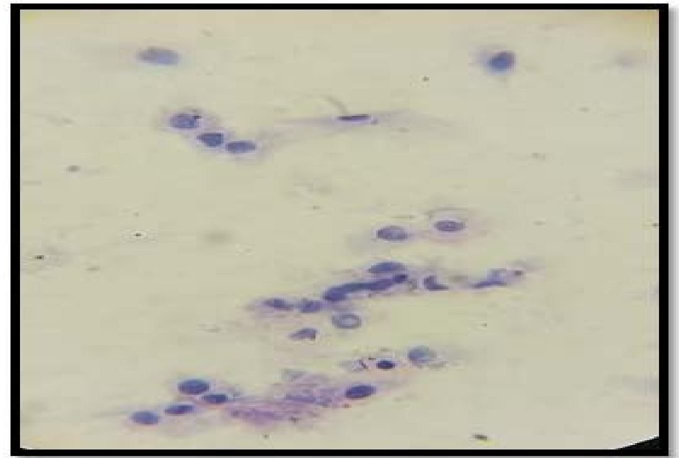

Fig 5: (10X) PAP smear showing altered N: C- grade 3

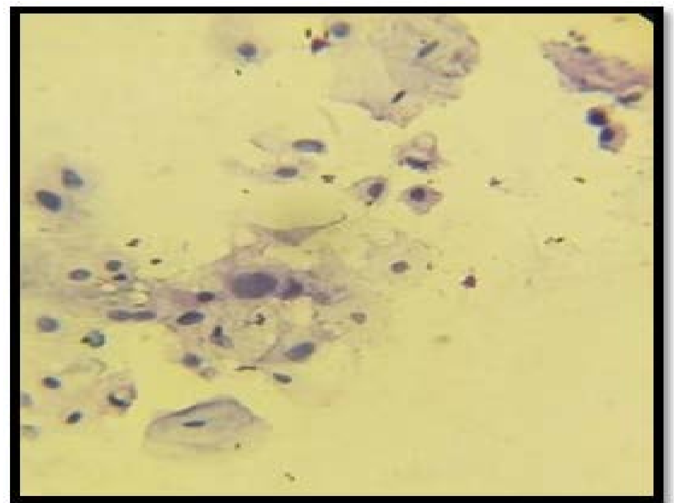

Fig 6: (10X) PAP smear showing nuclear hyperchromatism, altered N: C, nuclear pleomorphism-grade 4

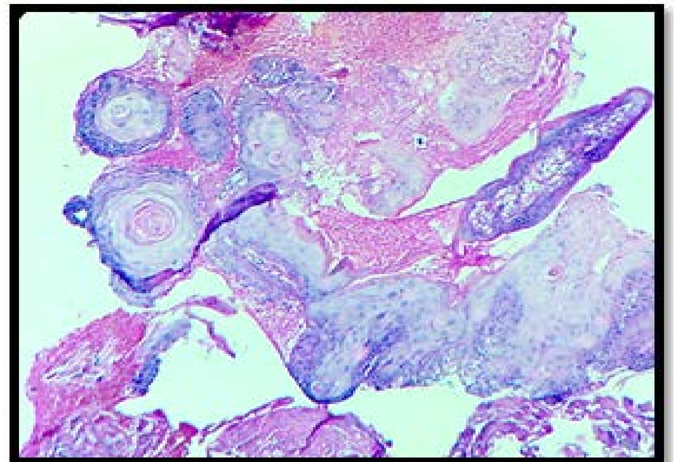

Fig 7: (40X) H and E section showing nuclear hyperchromatism, altered N: C, nuclear pleomorphism and



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keratin pearl formation with breach in the basement membrane – well-differentiated

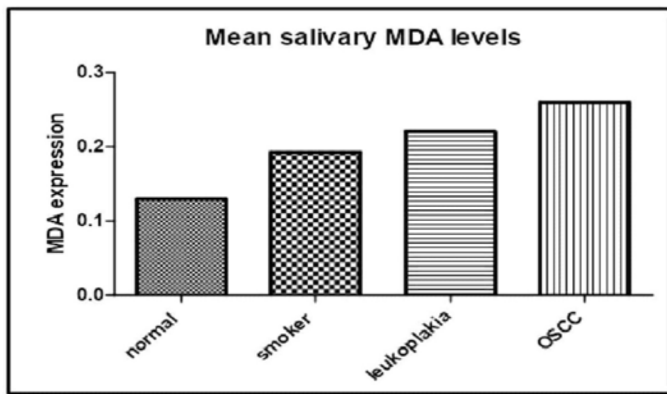


Fig 8: Mean salivary MDA levels in study group

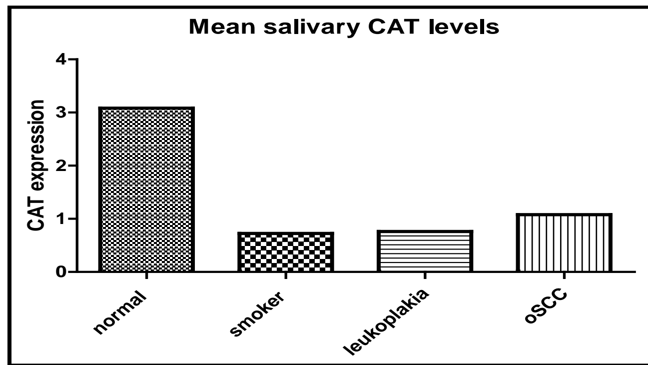


Fig 9: Mean salivary CAT levels in study group

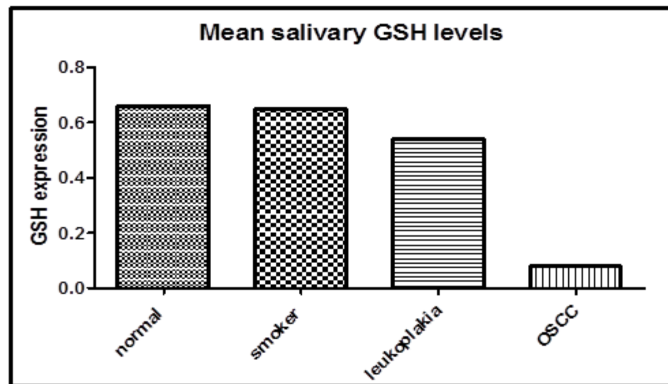


Fig 10: Mean salivary GSH levels in study group



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