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# Comet Assay and Urinary 8-OHdG: A Marker of Oxidative Stress in Oral Cancer with Puducherry Population

Authors
**Dr Subash.P[1], Jayanthi.R[2]**
[1]Associate Professor, Department of Biochemistry, KMCT Medical College, Kozhikode, Kerala
[2]Lecturer, Department of Biochemistry, Mahatma Gandhi Medical College & Research Institute, Puducherry
Corresponding Author
**Dr. Subash. P**
Associate Professor, Department of Biochemistry, KMCT Medical College, Kerala University of Health Sciences, Manassery 673602, Kozhikode, Kerala India
Email: *subash15p@gmail.com*

**Abstract**
**Background:** *Establishment of early prediction of oral cancer is important. We evaluated both lymphocyte DNA damage by Single cell gel electrophoresis (SCGE) and non-invasive urinary 8-hydroxy-2-deoxyguanosine (8-OHdG) biomarker of oxidative stress (OS). OS and poorly maintained antioxidant defense system may leads to increased intracellular reactive oxygen species (ROS) that can control basic cellular functions, such as proliferation and apoptosis which leads to the development of cancer.*
**Objectives:** *To evaluate total antioxidant status (TAS) and the extent of oxidative stress (OS) by measuring DNA damage in oral leukoplakia and oral squamous cell carcinoma (OSCC) in comparison to normal healthy individuals.*
**Methods:** *A total of 95 subjects aged 30 – 70 were included for the study. Of these 30 were healthy controls, 30 patients with oral leukoplakia and 35 clinically and histologically diagnosed patients with OSCC. Blood samples were evaluated for malondialdehyde (MDA), comet assay and antioxidants. Random urine specimens were collected for 8-OHdG estimation.*
**Results:** *Significant increase in DNA damage with decreased antioxidants status was observed in oral leukoplakia compared to OSCC patients and controls. TAS and lymphocyte DNA damage showed a strong negative correlation and comet assay and urinary 8-OHdG showed a strong positive correlation among three groups.*
**Conclusion:** *Oral cancer associated with OS causes genotoxic susceptibility in cancer. Lack of DNA repair mechanisms causes extensive DNA damage in oral leukoplakia patients, suggesting that OS is important in the pathogenesis of oral cancer.*
**Keywords:** *Antioxidants, comet assay, DNA damage, lymphocyte, oral cancer and oxidative stress.*

## Introduction

Globally, cancer being the utmost social fear, as it is one of the leading cause of death. According to WHO, oral cancer (OC) is the 3rd most common cancer in males and 6th most common cancer in females[1]. More than 90% of oral and oropharyngeal cancers are squamous cell carcinoma. Oral squamous cell carcinoma (OSCC)

is one of the most common cancer and more than 400,000 of new cases of this type of cancer are diagnosed worldwide and one out of every three patients diagnosed with oral cancer would die within 5 years of detection[2]. Therefore the prevention of OC with its early diagnosis and treatment is our desirable goal and thus there is a need for early diagnostic oxidative markers as it can make the essential contribution to the prediction of oral cancer. DNA damage is one of the important hallmarks for cancer progression. In diseased condition, body can develop the endogenous defense mechanism. However, it has been observed that decreased cellular antioxidant system or abnormally increased reactive oxygen species (ROS), leads to oxidative stress (OS). ROS and free radicals able to damage cellular DNA and cause DNA base alterations, single-stand breaks (SSB), damage tumor suppressor genes and enhance expression of proto oncogenes [3]. If detectable and quantifiable, these may contribute for an early detection and prediction of oral cancer development and prognosis.

The comet assay or single cell gel electrophoresis (SCGE) developed by Ostling and Johansson in 1984 and later modified by Singh *et al* in 1998. The cell with DNA damage appears in the form of comet while undamaged cell appears as a halo. The head is composed of intact DNA, while the tail consists of damaged or broken pieces of DNA. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. Several studies have been reported on cellular changes in peripheral blood in a diverse range of malignancies in monocytes and in polymorphonuclear leukocytes, but a very few studies reported on DNA SSB. Similarly, quite a lot of evidence suggests that deleterious oral habits such as betel chewing, smoking, alcohol consumption have strong association with oral cancer [4], no study has correlated the association of oxidative DNA damage by using two different techniques both in blood and urine.

The Purpose of this study is to emphasize comet assay and urinary 8-OHdG as a promising tool for the detection of DNA damage in blood and urine respectively in assessing oral cancer. Though increased oxidative stress and decreased antioxidant capacity were investigated, their association between lymphocyte DNA damage and complete antioxidant status in oral leukoplakia and OSCC has not been investigated in Puducherry population so far.

## Materials and Methods

A total of 95 subjects aged 30-70 years were included for the study. Of these 30 were healthy controls, 30 patients with oral leukoplakia and 35 clinically and histologically diagnosed oral squamous cell carcinoma patients. Complete history was taken and oral habits were recorded and fully informed consent was obtained from them prior to participation in the study. Detailed oral examination was carried out by a well trained clinical oral pathologist. All method performed in this study were in accordance with the ethical standards of the institutional research committee.

**Exclusion criteria**-Subjects with past history of diabetes mellitus, hypertension, coronary heart disease, renal disease, liver disease and who were on supplementation of antioxidants were excluded from the study.

## Blood and Urine sample collection

After overnight fasting about 5ml of peripheral venous blood sample were collected in heparinised tubes from all the subjects. Of which 1ml blood was pipette into another tube immediately for the analysis of comet assay to evaluate lymphocyte DNA damage. 4ml of blood was centrifuged at 3000 rpm x 10 min for plasma separation and used for the analysis of vitamin C, vitamin E, TAS, protein thiols, catalase, fasting blood glucose and lipid profile. Glutathione peroxidase, superoxide dismutase, glutathione-s-transferase and malondialdehyde were analysed in hemolysate. 5mL urine was collected into a sterile container to measure 8-OHdG.

## Measurement of Biochemical markers

Plasma triglyceride, total cholesterol, HDL, LDL and glucose concentration were estimated in Randox Daytona analyzer by using commercial kits.

**Non-Enzymatic antioxidants**

Vitamin C, vitamin E, protein thiols, Glutathione (GSH) and Total antioxidant status (TAS) were estimated by the method of Omaye *et al*, (1979), Desai (1984), Sedlak and Lindsay (1968), Beutlar *et al* (1963), and Ferric reducing antioxidant power (FRAP) respectively.

**Enzymatic antioxidants**

Catalase (CAT), Superoxide dismutase (SOD), Glutathione Peroxidase (GPX) and Glutathione-s-transferase (GST) were assayed by the method of Aebi (1984), Winterbourn *et al* (1975), Flohe and Gunzler (1984) and Coombes B & Stakelum GS (1961) respectively.

**Measurement of Oxidative Markers**

Lipid peroxidation product, Malondialdehyde (MDA) was estimated by Ohkawa [5] *et al* (1979). Lymphocyte DNA damage by comet assay was determined by the method of Singh *et al*. Urinary 8-OHdG was measured by enzyme-linked immunosorbent assay (ELISA).

**Measurement of Oxidative DNA damage by Comet Assay**

Lymphocyte DNA damage was determined by alkaline comet assay by the method of Singh [6] *et al* with few minor modifications.

**Lymphocyte separation**

Isolation of lymphocyte was carried out by using Histopaque 1077 (Sigma). 1 mL of heparinized blood was carefully layered over 1 mL Histopaque and then centrifuged for 30 min at 500 x g at 25°C. The buffy coat formed at the interface containing lymphocyte was washed with phosphate buffer saline (PBS) and then collected by 15 min centrifugation at 400 x g. The obtained pellets were resuspended in PBS to obtain 20,000 cells in 10µL sample.

**Cell Viability Test**

Lymphocyte cells membrane integrity was assessed by using Trypan Blue exclusion method.

1. An amount of 5 µL of trypan blue dye and 10 µL of sample were taken in a micro centrifuge tube.
2. Allowed to stand for 2 minutes, placed the sample on a slide and then with cover slip.
3. 100 cells were scored and the number of dead cells (blue) and viable cells (shiny) were recorded.

10µL of fresh lymphocyte cell suspension was mixed with 80µL of 0.7% low melting point agarose (LMA) (Sigma) in PBS at 37°C. Later, 80µL of this mixture was layered onto slides that had previously been coated with 1.0 % hot normal melting agarose (NMA), covered with a cover slip at 4°C for at least 5 min to allow the agarose to solidify. After removing the cover-slips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100mM EDTA-2Na; 10mM Tris-HCl, pH 10-10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 hour. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH and 1mmol/L $Na_2EDTA$, pH>13) at 4°C for unwinding (30 min) and then electrophoresis was performed (400 mA/24 V, 25 min).

**Note:** All the above steps were performed under red light or dim light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide (2µg/mL in distilled water; 80µL/slide), covered with a cover slip and analyzed by using a fluorescence microscope (Olympus, Shinjuku Monolith, Tokyo, Japan).

**Scoring of slides in comet assay**

Randomly, 100 chosen nuclei (50 cells from each of two replicate slides) were visually analyzed by manual scoring[7]. As shown in Figure 1 each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4), hence the total score of slides will be between 0 to 400 Arbitrary

Units (AU). The extent of DNA damage was detected by a single observer.

**Measurement of Urinary 8-OHdG**

Urine specimens were centrifuged at 3000g for 10 minutes or filtered through 0.45mm filter, prior to use in the assay. The supernatant was used for the measurement of the 8-OHdG levels using a competitive in-vitro ELISA kit (Cell Biolabs, Inc, San Diego, CA 92126, USA). The detection range of the ELISA assay was 0 to 20 ng/mL.

**Measurement of Total Antioxidant Status (TAS)**

Plasma total antioxidant status was assessed by ferric reducing antioxidant power assay, whereby at low pH, reduction of a ferric tripyridyl triazine ($Fe^{3+}$-TPTZ) complex to a ferrous form, which had an intense blue color, that can be monitored by measuring the absorbance at 593nm using spectrophotometer. It was directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture. The results were expressed as μM/L.

**Statistical analysis**

All data were expressed in Mean ± S.D. or frequency expressed as a percent, categorical variables was compared by using $\chi^2$-test. We assessed normality of all study variables through Kolmogorov-Smirnov one sample test (K-S test). Comparison among multiple groups was performed by one-way analysis of variance (ANOVA) with LSD post hoc test for continuous variables. Correlation of lymphocyte DNA damage with total antioxidant status and correlation of lymphocyte DNA damage with urinary 8-OHdG was assessed by Pearson correlation coefficient.

Values of probability less than 0.05 was considered statistically significant. The statistical analysis was performed with SPSS 17 for windows.

**Results**

The base line characteristics like age, sex, habits and clinical details such as site of lesion, Oral and histopathological diagnosis in OLP and OSCC patients were elucidated and compared with control group in Table 1. Overall 72% of the subjects in this study were men and remaining 28% of subjects were women. Most of the patients in OLP and OSCC groups and in control group belonged to the age group 51-60. In OSCC group 31% with moderately differentiated OSCC and 69% patients diagnosed with well differentiated OSCC.

In Table 2 the mean fasting blood glucose and lipid profile (Total cholesterol, Triacylglycerol, HDL and LDL cholesterol) of study subjects were summarized. The mean Glucose and HDL cholesterol Table 3 describes the status of non-enzymatic antioxidants such as vitamin C, vitamin E, protein thiols, glutathione, TAS was found to be substantially suppressed in OLP and OSCC patients in comparison to controls. However, the difference was not significant in vitamin C when the mean values were compared between OLP and OSCC patients. Similarly, the status of enzymatic antioxidants such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase were significantly ($p<0.001$) decreased in OLP and OSCC patients compared to control group. Except vitamin C, both enzymatic and non-enzymatic antioxidants were drastically decreased in OSCC patients compared to OLP patients.

In Table 4, the results of oxidative markers are summarized. Mean value of malondialdehyde (MDA) levels in OSCC and OLP was greater than in controls ($p<0.001$). OSCC patient had the highest MDA levels 0.8±0.2 μM/g Hb ($p<0.001$) as compared with controls and OLP patients.

The mean lymphocyte DNA damage and urinary 8-OHdG of OSCC patients was found at high level compared with OLP and control groups ($p<0.05$) and level of lymphocyte DNA damage and oxidized DNA product, 8-OHdG was observed to rise significantly ($p<0.001$) in OSCC patients compared to OLP patients. The photomicrograph showing the various extend of DNA damage in Figure 1 provides varying intensities of the fluorescence in the comet tail.

DNA damage was expressed as arbitrary units (AU). The total amount of DNA strand breakage

was expressed in total arbitrary units (AU) defined as: AU = $N_0$ x 0 + $N_1$ x 1 + $N_2$ x 2 + $N_3$ x 3 + $N_4$ x 4, Where N is the number of nuclei scored in each category.

In Figure 2, Lymphocyte DNA damage was independently shown negative correlation with TAS in control (-0.722), OLP (-0.774) and OSCC (-0.94) and it is highly significant ($p$<0.0001) in all the three groups. In contrast In Figure 3, Lymphocyte DNA damage was independently shown positive correlation with urinary 8-OHdG in control (0.981), OLP (0.964) and OSCC (0.925) and it is highly significant ($p$<0.0001) in all the three groups.

**Table 1** Baseline characteristics and clinical details of study subjects

| Characteristics | Control [n=30] | OLP [n=30] | OSCC [n=35] |
|---|---|---|---|
| **Number of subjects (F/M)** [a] | 30 (8/22) | 30 (9/21) | 35 (10/25) |
| **Age** Mean ± S.D. | 47.4±8.6 | 53.2±8.3 | 53.9±8.1 |
| 35 – 50 | 9 | 8 | 9 |
| 51 – 60 | 16 | 15 | 20 |
| 61 – 70 | 5 | 7 | 6 |
| **Habits** | | | |
| Tobacco smokers (%) | 1 (3) | 5 (17) | 6 (17) |
| Tobacco & Lime chewers (%) | N/A | 4(13) | 7 (23) |
| Tobacco & Betel nut chewers(%) | 2 (7) | 9 (30) | 15 (43) |
| Tobacco chewers & Smokers (%) | 1 (3) | 12 (40) | 6 (17) |
| **Clinicopathologic entity** (Site of lesion) | N/A | Tongue (2)<br>Buccal mucosa (10)<br>Alveolus (7)<br>Palate (2)<br>Floor of the mouth (5)<br>Lips (4) | Tongue (4)<br>Buccal mucosa (12)<br>Alveolus (10)<br>Palate (3)<br>Gingiva (2)<br>Floor of the mouth (3)<br>Lips (1) |
| **Oral Clinical Diagnosis** | N/A | Homogenous flat white leukoplakia (22)<br>Speckled, nodular & Verrucous leukoplakia (8) | Stage II (8)<br>Stage III (20)<br>Stage IV (7) |
| **Histopathological Diagnosis** | N/A | Mild dysplasia (9)<br>Moderate dysplasia (16) | Moderately differentiated OSCC (11) |
| | | Severe dysplasia (5) | Well differentiated OSCC (24) |

OLP, Oral Leukoplakia; OSCC, Oral squamous cell carcinoma.
Age was expressed as mean ± S.D.
[a] $\chi^2$-test

**Table 2** Blood glucose and lipid profile of study subjects

| Characteristics | Control [n=30] | OLP [n=30] | OSCC [n=35] | *p*-Value |
|---|---|---|---|---|
| **Glucose (mg %)** | 97.2±7.2 | 99.7±11.0 | 96.4±5.2 | NS |
| **Total Cholesterol (mg %)** | 153.3±14.4 | 165.1±24.4* | 168±23.0† | p<0.01 |
| **TAG (mg %)** | 95.1±15.1 | 111±32.4* | 134.8±19.1†‡ | p<0.01 |
| **HDL Cholesterol (mg %)** | 40.9±3.7 | 35.7±3.7 | 34.3±7.2 | NS |
| **LDL Cholesterol (mg %)** | 93.4±16.0 | 107.2±20.1* | 107.6±20.2† | p<0.01 |

Results are expressed as mean ± SD for all the parameters. One-way ANOVA and LSD post hoc were used to derive the *p-value*.
*$P$-value significant compared with control ($P$<0.05).
†$P$-value significant compared with control ($P$<0.05).
‡$P$-value significant compared with Oral Leukoplakia ($P$<0.05).
TAG, Triacylglycerol; HDL, High density lipoprotein; LDL, Low density lipoprotein.

**Table 3** Comparison of antioxidant status between control and oral cancer patients

| Parameters | Control [n=30] | OLP [n=30] | OSCC [n=35] | *p*-Value |
|---|---|---|---|---|
| **Vitamin C (mg %)** | 1.5±0.2 | 1.0±0.1* | 1.0±0.1† | p<0.001 |
| **Vitamin E (mg %)** | 1.4±0.2 | 0.8±0.1* | 0.6±0.1†‡ | p<0.001 |
| **Protein thiols (µM/L)** | 298.5±49.2 | 161.6±21.0* | 145.1±34.5†‡ | p<0.001 |
| **GSH (mg/g Hb)** | 3.6±1.0 | 2.6±0.4* | 2.3±0.3†‡ | p<0.05 |
| **TAS (µM/L)** | 913.8±169.6 | 800.3±126.7* | 616.7±85.6†‡ | *p*<0.001 |
| **Catalase (K/ml)** | 28.1±2.7 | 19.9±2.8* | 16.2±2.1†‡ | p<0.001 |
| **SOD (U/g Hb)** | 958.8±159.9 | 848.2±101.6* | 710.2±78.2†‡ | p<0.001 |
| **GPX (U/g Hb)** | 21.8±3.7 | 20.8±2.9* | 14.8±1.7†‡ | p<0.001 |
| **GST (mM/CDNB-GSH/min/mg protein)** | 4.4±1.1 | 2.2±0.4* | 1.5±0.3†‡ | p<0.001 |

Results are expressed as mean ± SD for all the parameters. One-way ANOVA and LSD post hoc were used to derive the *p-value*.
**P*-value significant compared with control (*P*<0.05).
†*P*-value significant compared with control (*P*<0.05).
‡*P*-value significant compared with Oral Leukoplakia (*P*<0.05).
**GSH,** reduced glutathione**; TAS,** total antioxidant status**; SOD,** superoxide dismutase**; GPX,** glutathione peroxidase**; GST,** glutathione-s-transferase.

**Table 4** Comparison of MDA & Lymphocyte DNA damage between Control and Oral cancer patients

| Parameters | Control [n=30] | OLP [n=30] | OSCC [n=35] | *p*-Value |
|---|---|---|---|---|
| **MDA (µM/g Hb)** | 0.2±0.1 | 0.6±0.1* | 0.8±0.2†‡ | p<0.001 |
| **Lymphocyte DNA Damage (AU)** | 14.3±4.0 | 22.9±10.8* | 35.4±11.8†‡ | p<0.001 |
| **Urinary 8-OHdG (ng/mgCr)** | 5.4±2.2 | 11.1±2.9* | 16.1±5.9†‡ | p<0.001 |

Results are expressed as mean ± SD for all the parameters. One-way ANOVA and LSD post hoc were used to derive the *p-value*.
**P*-value significant compared with control (*P*<0.05).
‡*P*-value significant compared with control (*P*<0.05).
¥*P*-value significant compared with Oral Leukoplakia (*P*<0.05).
**MDA,** malondialdehyde; **AU,** arbitrary units;

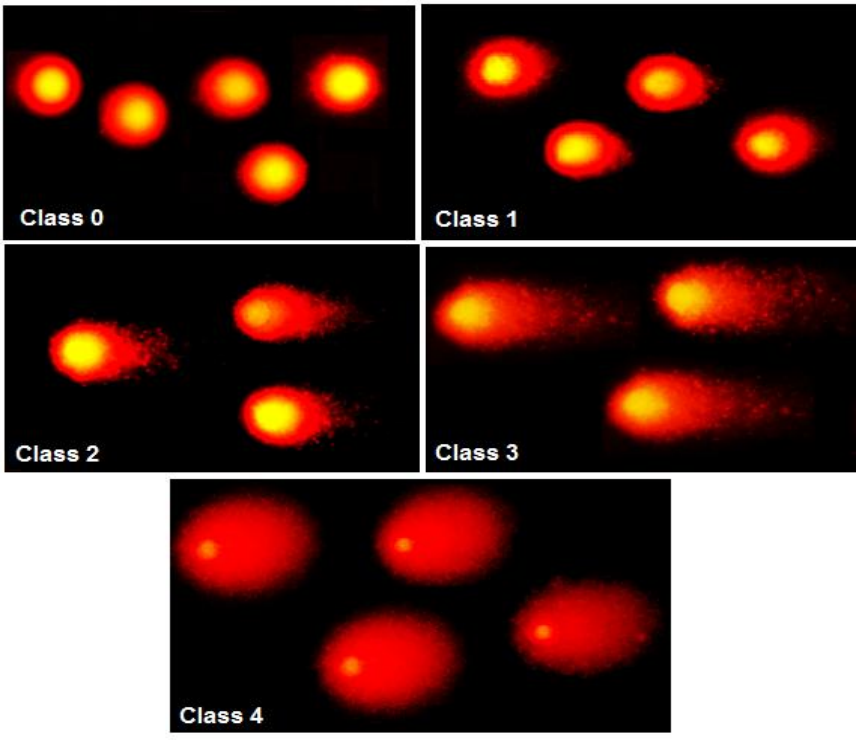


**Figure 1:** Lymphocyte DNA damage in Oral cancer
Photomicrographs showing varying intensities of the fluorescence in the comet tail
(Class 0, undamaged; Class 1, 2 & 3 increasingly damaged and Class 4, maximally damaged).

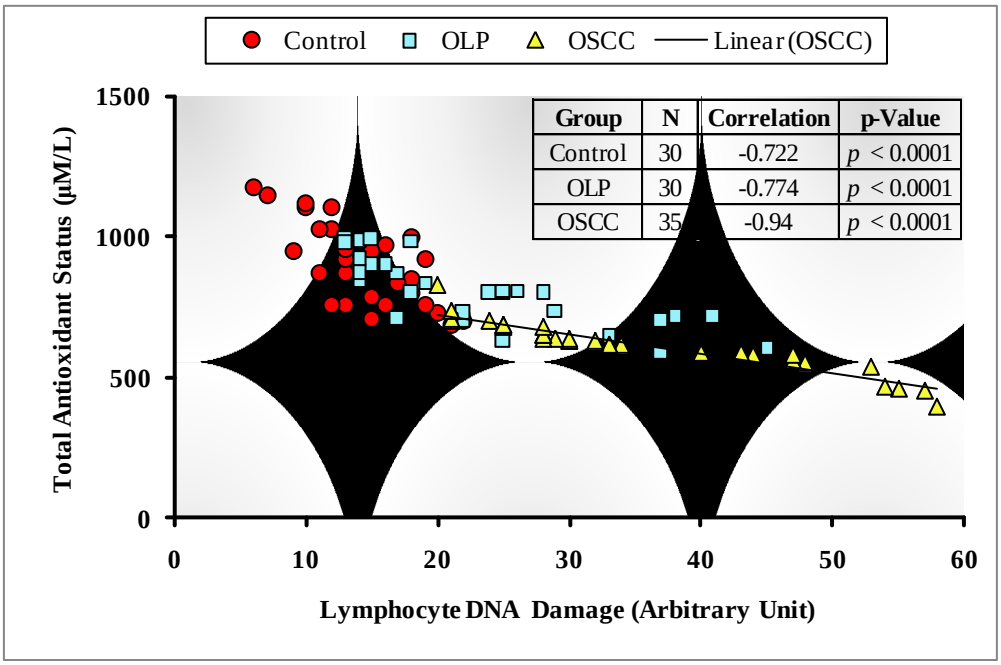


**Figure 2:** The correlation of lymphocyte DNA damage with TAS in study groups

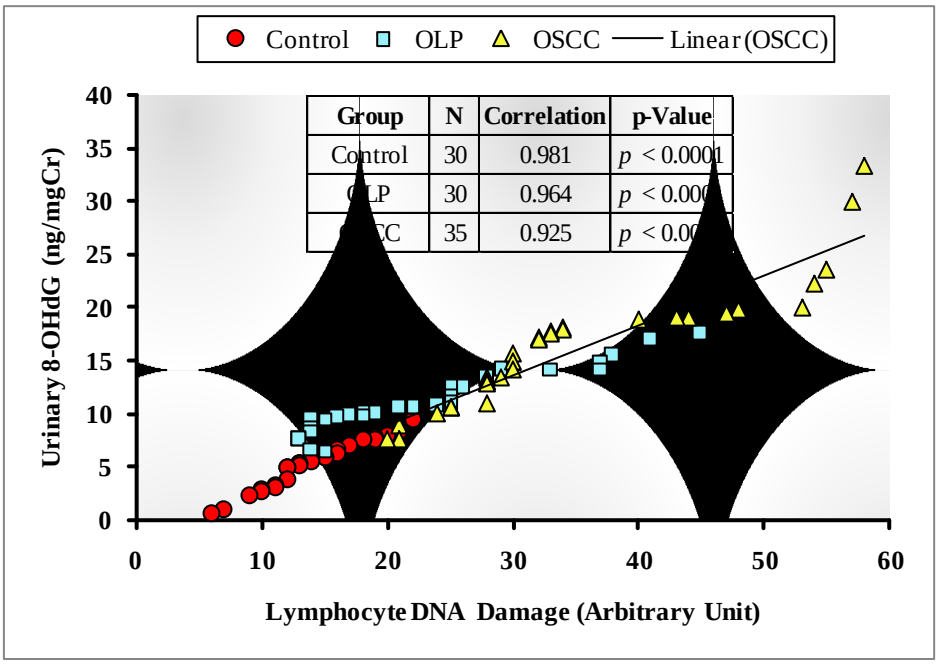


**Figure 3:** The correlation of lymphocyte DNA damage with Urinary 8-OHdG in study groups

**Discussion**

In this study, we assessed the high endogenous levels of oxidative markers in blood (MDA in RBC and oxidative DNA damage in lymphocytes) and increased excretion of DNA oxidized product, 8-OHdG in urine. In contrast diminished antioxidant status (both enzymatic and non-enzymatic) was observed in OLP and OSCC patients.

Our study is a strong evidence for occurrence of oxidative DNA damage in oral cancer patients compared to controls. Comet assay serves as sensitive technique for the assessment of DNA damage in an individual cell; it has been made known that oxidative stress in oral cancer influences the comet assay response in lymphocyte. Lymphocytes are readily available and widely used as a sentinel cell type to provide early warning signals for adverse effects.

ROS formation results in oxidative modification of macro molecules and subsequently, genomic

instability leads to the formation of 8-OHdG is regarded as a useful indicator of oxygen radical induced DNA damage. Intracellular accumulation of this compound has been demonstrated in animal models of oesophageal squamous cell carcinoma[8]. Hence excretion of 8-OHdG has been used as a sensitive oxidative biomarker in this study.

Several clinical studies have demonstrated clearly the association of harmful paranormal oral habits, such as betel quid chewing, tobacco chewing, paan chewing, smoking bidi or cigarette with occurrence of oral cancer[9]. But the genomic studies and its association with antioxidant status were very few, so an attempt was made to see the prevalence of oxidative DNA damage in blood and to support further, its excretory products 8-OHdG in urine was also measured in OLP and OSCC patients of Puducherry population. This condition has high cancer turnover potentiality and if detected early it can be prevented and treated successfully [10]. By means of rapid advance in human biomonitoring and genotoxic testing and with comet assay as a tool for the estimation of oxidative DNA damage, provide a base to determine the progression of cancer [11].

Oral cancer development is multifactorial, depend on the extent of oxidative DNA damage which in turn reflects the magnitude of oxidative stress and on the other hand efficiency of antioxidant defense, competence of cellular DNA repair mechanism. If this equilibrium is imbalanced either by the reduction in the antioxidant levels or by increased ROS levels, DNA is oxidized and thereby cancer emerges. This is precisely what we noticed in lymphocyte of OLP and OSCC patients in the present study.

Estimation of oxidative DNA damage in lymphocyte by comet assay technique is widely used in various studies to detect cervical cancer [12], lymphoblastic leukemia [13] and prostate cancer [14]. Similarly, in recent years 8-OHdG emerged as a non-invasive and technically less involved marker of oxidative stress to indicate the extent of oxidative DNA damage. ROS can produce DNA break, deletions and nucleotide modifications. Upon DNA repair, 8-OHdG is ecreted in the urine. The association of ROS and the use of 8-OHdG as a biomarker of OS have been investigated in many degenerative diseases including bladder and prostate cancer[15], cystic fibrosis [16], atopic dermatitis [17] and rheumatoid arthritis [18].

This study was conduct in a well controlled sample processing procedure (within 1 h after phlebotomy), viable lymphocyte obtained from blood often reflect DNA damage/repair of the target tissue. With the limited sample size, the present data suggest that increased oxidative DNA damage was observed in OSCC compared to OLP. Negative correlation of TAS and comet assay further reveals that oxidative stress and DNA damage may be the root of cause for oral cancer. Furthermore, the positive correlation between lymphocyte DNA damage and urinary 8-OHdG firmly support that ROS generation in the body by either internal or external sources which ultimately lead to DNA damage and carcinogenesis. Therefore, comet assay in blood and urinary 8-OHdG in urine could serve as new biomarkers for evaluating the oral precancerous stage and the potential risk of development of OSCC.

**Conclusion**

The present study reinforce that, oxidative DNA damage caused by ROS occurs more in OSCC patients than in persons with OLP compared to healthy controls. Hence, the study shows strong association with increased oxidative stress and reduced antioxidant status. Furthermore, genomic damage is one of the most important risk factors of cancer. Physicians do not know in their every day practice who might have more severe DNA damage, among oral cancer patients. For that reason, we need rapid and very sensitive and accurate method such as COMET assay in blood and 8-OHdG in urine for measuring DNA damage, so that we can we can go for more appropriate medications and treatment for oral cancer patients who have more DNA damage.

**Limitation of our Study**

Accordingly, it remains within the future scope to conduct large scale case-control and follow up studies are warranted to further test the potential application of the alkaline comet assay in oral cancer risk assessment and prevention.

However, further studies are required to document the effectiveness of antioxidant supplementations in the form of diet or as medications to improve the antioxidant levels in the body and ultimately to trim down the patient output.

**Compliance with Ethical Standards**

**Conflict of interest**

The author declares no conflict of interest.

**Ethical Approval All procedures**

All study procedures performed involving human participants were in accordance with the ethical standards of the institutional research committee. The study was approved by both research and ethics committee of our institute.

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