ISSN- O: 2458 - 868X, ISSN–P: 2458 – 8687
Index Copernicus Value: 49. 23
PubMed - National Library of Medicine - ID: 101731606

International Journal of Medical Science and Innovative Research (IJMSIR)
IJMSIR : A Medical Publication Hub
Available Online at: www.ijmsir.com
Volume – 3, Issue – 6, December - 2018, Page No. : 231 – 242

Diagnosis of Genotoxicity by Comet Assay in Oral Cancer of Puducherry Population

Dr. Subash P, Ph.D., Associate Professor, Department of Biochemistry, KMCT Medical College, Kozhikode

Mrs. Jayanthi R, M.Sc., M.Phil., Ph.D., Lecturer, Department of Biochemistry, Mahatma Gandhi Medical College & Research Institute, Puducherry

Corresponding Author: Dr. Subash P, Ph.D., Designation: Associate Professor, Department of Biochemistry, Kerala University of Health Science, KMCT Medical College, Kozhikode

Type of Publication: Original Research Paper

Conflicts of Interest: Nil

Abstract

Background: Single cell gel electrophoresis (SCGE) or "comet assay" is a simple, sensitive and non-invasive fluorescent microscopic method for measuring and analyzing single stranded DNA breaks at individual cell level. Oxidative stress 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.

Aim and 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 90 subjects aged 30 – 70 were included for the study. Of these 30 were healthy controls, 25 patients with oral leukoplakia and 35 clinically and histologically diagnosed patients with OSCC. Blood samples were evaluated for malondialdehyde (MDA), comet assay and antioxidants. All study variables assessed through Kolmogorov-Smirnov one sample test (K-S test). Comparison among multiple groups was performed by one-way ANOVA with LSD post hoc test. Correlation of DNA damage with TAS was assessed by Pearson correlation coefficient.

Conclusions: 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 among three groups. 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, Oral cancer is the 6th most common cancer[1]. The most common type of oral cancer is squamous cell carcinoma, that develops from the stratified squamous epithelium which lines the mouth and pharynx, that accounts approximately 9 of every 10 oral malignancies in men and women[2]. Therefore the prevention of oral cancer with its early diagnosis and treatment is our desirable goal and thus there is a need for early diagnostic marker 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", also known as single cell gel electrophoresis (SCGE), is a simple, sensitive and reliable technique for quantifying and assessing DNA damage in an individual cells, originally developed by Ostling and Johansson in 1984[4]. 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[5]. Though several studies reported on cellular malignant changes in peripheral blood like T-cell leukemia, monocytes and polymorphonuclear leukocytes, the DNA SSB have not been studied in detail[6].

Similarly, quite a lot of evidence suggests that deleterious oral habits such as betel chewing, smoking, alcohol consumption have strong association with oral cancer[7], no study has revealed the association of DNA damage with decreased total antioxidant status in Puducherry population.

The purpose of this study was to emphasize comet assay as a promising tool for the detection of oxidative DNA damage in assessing oral cancer. Although increased oxidative stress and decreased antioxidant capacity was investigated before in previous studies, but their association between lymphocyte DNA damage and complete enzymatic and non-enzymatic antioxidant status in oral leukoplakia and OSCC has not been investigated in Puducherry population so far.

Materials and Methods

A total of 90 subjects aged 30-70 years were included for the study. Of these, controls (n=30), oral leukoplakia (n=25) and oral squamous cell carcinoma (n=35). Complete history was taken and oral habits were recorded for all the subjects. Detailed oral examination was carried out by a well trained clinical oral pathologist. The study was approved by both research and ethics committee of our institute. A fully informed consent was obtained from these subjects before participation in the study.

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

Blood sample collection

Peripheral venous blood samples of 5ml were drawn from all the subjects in the fasting state and placed in heparinised tubes. Of which 1ml blood was pipetted into another tube immediately for the analysis of lymphocyte DNA damage by comet assay. Remaining 4ml blood was centrifuged at 3000 rpm x 10 min for plasma separation and used for the analysis of TAS, protein thiols, vitamin C, vitamin E, Catalase (CAT), fasting blood glucose and lipoid profile. Glutathione peroxidise (GPX), superoxide dismutase (SOD), glutathione-s-transferase (GST) and Malondialdehyde (MDA) were analysed in hemolysate.

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 antioxidant

Vitamin C, vitamin E, protein thiols, GSH and 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[8-12].

Enzymatic antioxidant

CAT, SOD, GPX and GST were assayed by the method of Aebi (1984), Winterbourn et al (1975), Flohe and Gunzler (1984) and Coombes B & Stakelum GS (1961) respectively[13-16].

Measurement of Oxidative Markers

Lipid peroxidation product, MDA was estimated by Ohkawa et al (1979)[17]. Lymphocyte DNA damage by comet assay was determined by the method of Singh et al[18].

Measurement of Oxidative DNA damage by Comet Assay

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

Lymphocyte separation

Isolation of lymphocyte was carried out by using Histopaque 1077 (Sigma). 1mL of heparinized blood was carefully layered over 1mL 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.

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. It was allowed to stand for 2 minutes and sample placed on a slide and then with cover slip. 3. Hundred cells were scored and the number of dead cells (blue) and viable cells (shiny) were recorded.

An amount of 10µL of fresh lymphocyte cell suspension (around 20,000 cells) 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).

Scoring of slides in comet assay- Randomly, 100 chosen nuclei (50 cells from each of two replicate slides) were visually analyzed by manual scoring[19]. As shown in Fig.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), so that the total score of slides was between 0 to 400 Arbitrary Units (AU). The extent of DNA damage was detected by a single observer.

Total Antioxidant Status (TAS)

Plasma total antioxidant status was assessed by ferric reducing antioxidant power assay[20-22], 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 593 nm 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 χ^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 was assessed by Pearson correlation coefficient. Values of probability less than 0.05 was considered statistically significant. The statistical analysis was performed with SPSS 12 for windows.

Results

The base line characteristics like age, sex and habits and clinical details such as site of lesion, oral and histopathological diagnosis in OLP and OSCC patients were described and compared with control groups was summarized in Table 1. Overall 73% of the subjects in this study were women.

Table 1 Baseline characteristics and clinical details of study subjects.

Characteristics	**Control**	**OLP**	**OSCC**
Number of subjects (F/M) [a]	30 (5/25)	25 (8/17)	35 (11/24)
Age Mean ± S.D.	48.0±8.7	53.6±7.8	54.2±8.4
35 – 50	17	9	11
51 – 60	10	10	15
61 – 70	3	6	9
Habits			
Tobacco smokers (%)	2 (6)	4 (16)	8 (23)
Tobacco & Lime chewers (%)	N/A	6 (24)	7 (20)
Tobacco & Betel nut chewers(%)	4 (13)	8 (32)	14 (40)
Tobacco chewers & Smokers (%)	N/A	7 (28)	6 (17)
Clinicopathologic entity (Site of lesion)	N/A	Tongue (3) Buccal mucosa (9) Alveolus (6) Palate (2) Floor of the mouth (3) Lips (2)	Tongue (5) Buccal mucosa (11) Alveolus (9) Palate (4) Gingiva (3) Floor of the mouth (2) Lips (1)

Oral Clinical Diagnosis	N/A	Homogenous flat white leukoplakia (18) Speckled, nodular & Verrucous leukoplakia (7)	Stage II (10) Stage III (19) Stage IV (6)
Histopathoogical Diagnosis	N/A	Mild dysplasia (12) Moderate dysplasia (8)	Moderately differentiated OSCC (13)
		Severe dysplasia (5)	Well differentiated OSCC (22)

OLP, Oral Leukoplakia; **OSCC,** Oral squamous cell carcinoma.

Age was expressed as mean ± S.D. [a]χ^2-test

Table 2 Blood glucose and lipid profile of study subjects

Characteristics	**Control**	**OLP**	**OSCC**	***p*-Value**
Glucose (mg %)	97±7.0	93.0±6.7	100.6±11.4	NS
Total Cholesterol (mg %)	153.9±15.1	169.7±24.0*	166.6±24.7†	p<0.01
TAG (mg %)	95.9±15.3	135.7±20.9*	112±33.7†‡	p<0.01
HDL Cholesterol (mg %)	40.7±4.1	35.0±6.2	36.9±4.6	NS
LDL Cholesterol (mg %)	94±15.0	107.7±21.7*	106.2±23.5†	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*.

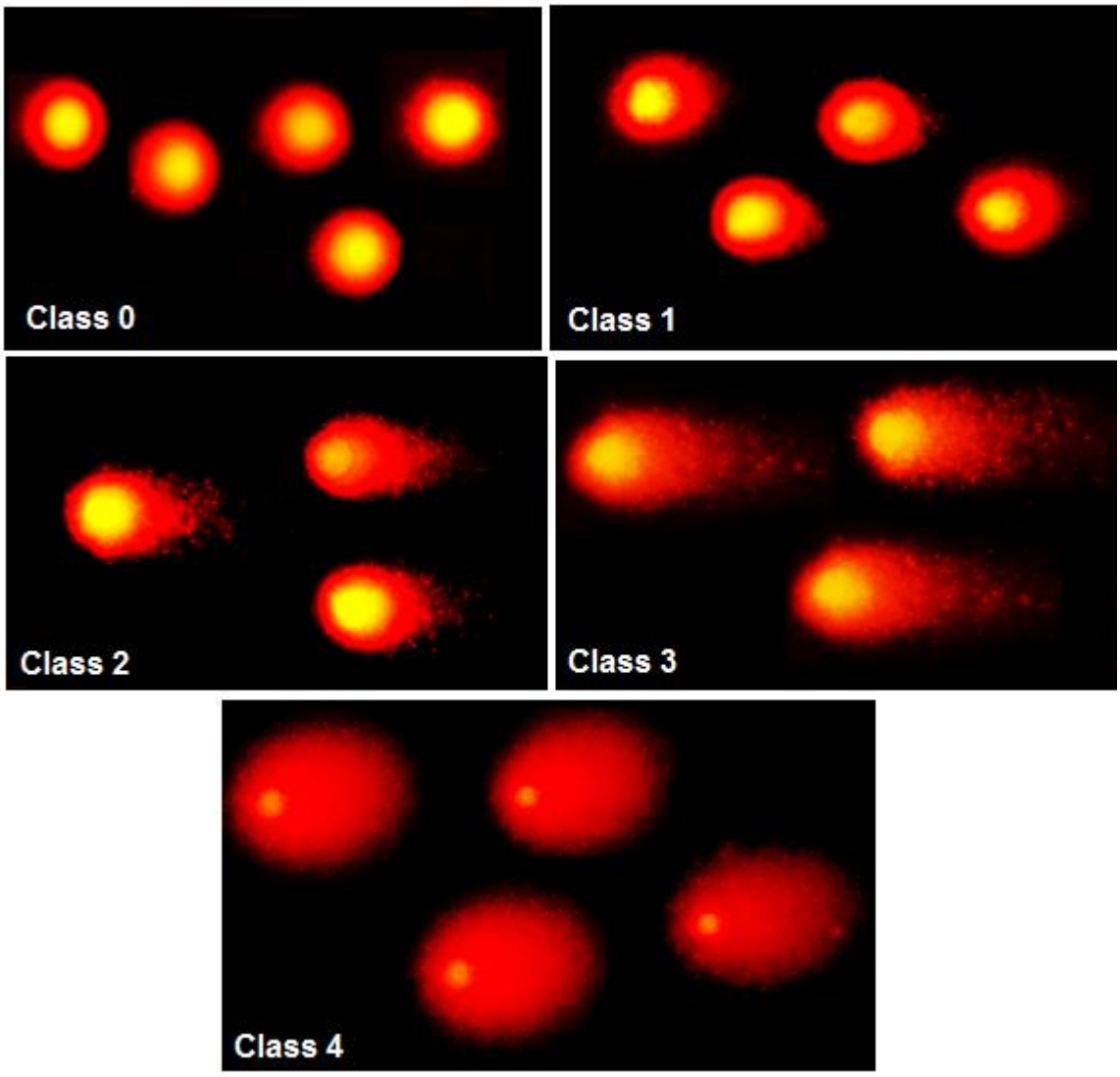
*P-value significant compared with control (P<0.05).

†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.

Fig. 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).

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

Parameters	**Control**	**OLP**	**OSCC**	***p*-Value**
Vitamin C (mg %)	1.3±0.2	0.5±0.1*	0.7±0.1†‡	p<0.001
Vitamin E (mg %)	1.4±0.2	1.0±0.1*	1.0±0.1†	p<0.001
Protein thiol (µM/L)	295.2±52.6	143.4±35.7*	160.3±22.0†‡	p<0.001
GSH (mg/g Hb)	3.7±1.0	2.4±0.4*	2.7±0.5†‡	p<0.05
TAS (µM/L)	911±169	691.2±105.8*	807.1±152.2†‡	*p*<0.001
Catalase (K/ml)	27.6±2.8	17.0±1.4*	20.0±3.9†‡	p<0.001

SOD (U/g Hb)	955.5±163.9	700.8±80.2*	849.1±102.9†‡	p<0.001
GPX (U/g Hb)	21.7±3.9	15.3±1.2*	21.1±2.7†‡	p<0.001
GST (mM/CDNB-GSH/min/mg protein)	4.3±1.0	1.4±0.2*	2.3±0.5†‡	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**	**OLP**	**OSCC**	***p*-Value**
MDA (µM/g Hb)	0.2±0.0	0.7±0.2*	0.5±0.1†‡	p<0.001
Lymphocyte DNA Damage (AU)	14.5±4.2	36.2±11.4*	23.2±9.7†‡	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*.

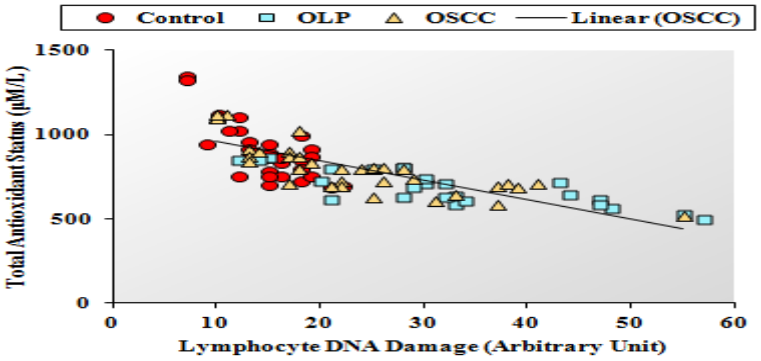
*****P-value significant compared with control (P<0.05).

‡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 2: The correlation of lymphocyte DNA damage with TAS in oral cancer



Group	**N**	**Correlation**	**p-Value**
Control	30	-0.772	p < 0.0001
OLP	25	-0.807	p < 0.0001
OSCC	35	-0.795	p < 0.0001

The lymphocyte DNA damage was negatively correlated with total antioxidant status and the *p*- value was highly significant ($p<0.0001$) in all the three groups, respectively (Control, **OLP**, Oral Leukoplakia and **OSCC**, Oral Squamous cell carcinoma).

Most of the patients in OLP and OSCC groups belongs to the age group 51-60. In OSCC group 37% patients with moderately differentiated OSCC and 63% patients diagnosed with well differentiated OSCC. Mean blood glucose and lipid profile of study subjects were summarized in Table 2.

In Table 3 the mean non-enzymatic and enzymatic antioxidants found to be decreased in OLP compared to OSCC patients, except for vitamin E and also decreased antioxidants were observed in OSCC patients compared to control group. Mean total antioxidant status (TAS) of OSCC group was significantly lower than control group ($p<0.05$) and mean total antioxidant status of OLP was found to be significantly lower when compared OSCC patients ($p<0.05$). In Table 4 the mean lymphocyte DNA damage and mean MDA of OLP patients was found at high level compared with OSCC and control groups ($p<0.05$) and mean lymphocyte DNA damage and MDA of OSCC patients was found significantly high compared with control group ($p<0.05$).

Lymphocyte deoxyribonucleic acid damage level in OLP, OSCC and normal individuals were assessed by using SCGE. The photomicrograph of the level of DNA damage shown 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: $AU = N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4$, 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 (-0.772), OLP (-0.807) and OSCC (-0.795) and it is highly significant ($p<0.0001$) in all the three groups.

Discussion

Oral leukopakia is a precancerous condition characterized by a white patch, frequently associated with oral habits. In this study, we assessed the high endogenous levels of oxidative markers in blood (MDA in RBC and oxidative DNA damage in lymphocytes) and in contrast diminished antioxidant status (both enzymatic and non-enzymatic) was observed in OLP and OSCC patients.

Our study is evidence for occurrence of oxidative DNA damage in oral cancer patients compared to controls in Puducherry population. 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.

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[23]. 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 oral leukoplakia and oral squamous cell carcinoma patients of puducherry population. This condition has high cancer turnover potentiality and if detected early it can be prevented and treated successfully[24]. 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[25].

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.

Oxidative DNA damage is a chief mechanism for cancer initiation. Radiation induced DNA damage has been assessed by comet assay.[18] Estimation of oxidative DNA damage in lymphocyte by comet assay technique is widely used in various studies to detect cervical cancer[26], lymphoblastic leukemia,[27], prostate cancer[28] and oral cancer[29].

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 OLP compared to OSCC. Negative correlation of TAS and comet assay further reveals that oxidative stress and DNA damage may be the root of cause for oral cancer. Therefore, comet assay could serve as a new biomarker for evaluating the oral precancerous stage and the potential risk of development of OSCC.

Conclusion

The present study emphasize that, oxidative DNA damage caused by ROS occurs more in OLP patients than in persons with OSCC 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 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 supplementation 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.

References

1. Parkin DM, Laara E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer 1988; 41(2):184-97.
2. Campo-Trapero J, Cano-Sanchez J, Palacios-Sanchez B, Sanchez-Gutierrez JJ, Gonzalez –Moles MA, Bascones-Martinez A. Update on molecular pathology in oral cancer and precancer. J Anticancer Res 2008; 28:1197-205.

3. Liu RH, Hotchriss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. Mutat Res 1995; 339: 73-89.
4. Ostling O, Johansson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 1984; 123: 291-8.
5. Rojas E, Lopez MC, Valverde M. Single cell gel electrophoresis: Methodology and applications (Review). J Chromatogr B Biomed Sci Appl 1999; 722: 225-54.
6. Korabiowska M, Voltmann J, Honig JF, Bortkiewicz P, Konig F, Cordon-Cardo C et al. Altered expression of DNA double-strand repair genes Ku 70 and Ku80 in carcinoma of the oral cavity. Anticancer Res 2006; 26: 2101-5.
7. Proia NK, Paszkiewicz GM, Nasca MA, Franke GE, Pauly JL. Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer: A review. Cancer Epidemiol Biomarkers Prev 2006; 15: 1061-77.
8. Omaye ST et al. Selected methods for determination of ascorbic acid in cells, tissues and fluids. Methods Enzymol 1979; 6: 3-11.
9. Desai ID. Vitamin E analysis method for animal tissues. Method Enzymol 1984; 105: 138-142.
10. Sedlak J, Lindsay RH. Estimation of total protein bound and non protein sulphydryl groups in tissue with Ellman's reagent. Anal Biochem 1968; 25: 192-205.
11. Beutler E., Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61(5):882-8.
12. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power. The FRAP assay. Analytical biochemistry 1996; 239: 70-6.
13. Aebi H. Catalase in vitro. Method enzyme. 1984; 105: 121-6.
14. Winterbourn CC, Hawkins RE, Brian M, Carrell RW. The estimation of red cell superoxide dismutase activity. J Lab Clin Med 1975; 85: 337- 41.
15. Flohe L and Gunzler WA. Assay of glutathione peroxidase. In methods in Enzymology. New York. Academic press. 1984.
16. Coombes B and Stakelum GS. A liver enzyme that conjugates sulfobromophtalein with glutathione. Journal of clinical Investigation 1961; 40: 981- 8.
17. Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxide in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351.
18. Singh PN, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175(1): 184–91.
19. Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR. et al. Fourth International Workshop on Genotoxicity testing: resulting of the in vivo Comet assay workgroup. Mutat Res 2007; 627: 31-5.
20. Benzie IF, Strain JJ. Ferric reducing antioxidant power assay. Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 1999: 299:15-27.
21. Erdogan C. The evaluation of oxidative stress in patients with CRF. CCA 2002; 322: 157 – 67.
22. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power.

The FRAP assay. Analytical biochemistry 1996; 239(1): 70-6.

23. Warnakulasuriya S. Significant oral cancer risk associated with low socioeconomic status. Evid Based Dent 2009; 10(1): 4-5.
24. Mukherjee S, Ray JG, chaudhuri K. Evaluation of DNA damage in oral precancerous and squamous cell carcinoma patients by single cell gel electrophoresis. Indian J Dent Res 2011; 22(5): 735-6.
25. Ribeiro DA. The single cell gel (comet) assay as a promising tool for the detection of DNA damage induced by compounds used in dental practice: the oral cancer risk assessment. Crit Rev Oncog 2008; 14(2):165.
26. Anuradha U, Malini J, Rajeswari N, Neena Desai, Jain S, Balakrishna N, et al. Risk assessment in cervical dysplasia patients by single cell gel electrophoresis assay: a study of DNA damage and repair. Mutation Research 1998; 412:195-205.
27. Patricia A. Escobar, Martyn T. Smith, Ananth Vasishta, Alan E. Hubbard and Luoping Zhang. Leukaemia-specific chromosome damage detected by comet with fluorescence in situ hybridization (comet-FISH). Mutagenesis 2007; 22(5): 321-7.
28. Kristin L.Lockett, M.Craig Hall, Peter E.Clark, Shu-Chun Chuang, Brittany Robinson, Hui-Yi Lin, et al. DNA damage levels in prostate cancer cases and control. Carcinogenesis 2006; 27(6): 1187-93.
29. Sajith vellappally, Mohammed A Binmgren, Sahar Bin Huraib, Mohamed I Hashem, Shankargouda Patil, Sukumaran Anil. Assessment of DNA damage in Leukoplakia Patients with different degrees of dysplasia. The Journal of Contemporary Dental Practice 2015; 16(12): 971-6.