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Evaluation of DNA damage levels in Oral leukoplakia by Single Cell Gel Electrophoresis Assay

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Abstract: *Comet assay also known as Single cell gel electrophoresis (SCGE) assay is a rapid and highly sensitive fluorescent molecular technique for detecting various forms of deoxyribonucleic acid (DNA) damage at individual cellular level. The present study was done to detect the extent of DNA damage in oral leukoplakia (OL) and compare with normal individuals. The sample population was obtained from Department of Dental Surgery, Sri Padmavathi Medical College for women, and Sri Venkateswara Institute of Medical Sciences (SVIMS) Tirupathi. Andhra Pradesh. In the period of 24months,from January 2017 to January 2019. A total of 180 consecutive patients with leukoplakia and 20 healthy normal volunteers were recruited for the study and assessed for the extent of DNA damage using SCGE following clinical diagnosis and histological grading. Peripheral blood was obtained by venipuncture and SCGE assay was performed. Mean comet tail length was recorded and analyzed statistically to compare the extent of damage in study subjects. The mean comet tail length seen in leukoplakia patients 1.65 ± 0.132 µm while for the control subjects, it was 0.42 ± 0.14 µm. The difference was statistically significant. On comparing within the leukoplakia and Control, a progressive trend of increasing tail length was observed with increasing in study subjects. Deoxyribonucleic acid damage as measured by SCGE was seen in leukoplakia. A stepwise increase in DNA damage levels from healthy controls, through patients have been observed indicating the extent of DNA damage in this study group.*

Keywords: *SCGE, Comet assay, DNA damage, Leukoplakia, Single cell gel electrophoresis assay.*

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I. Introduction

Oral leukoplakia, as traditionally defined by the World Health Organization (WHO), is a predominantly white lesion of the oral mucosa that cannot be characterised as any other definable lesion (1). Leukoplakia is often associated with tobacco smoking. Tobacco has been considered a risk factor and OL is six times more common in smokers than in non-smokers (2). Alcohol intake and the possible etiologic role of the human papilloma virus (HPV) in OL is not yet clear. The estimated prevalence of OL worldwide is 2 to 4.9%. Oral leukoplakia usually occurs after the age of 30 to 40 years with no gender predilection (3). An international working group has amended the earlier WHO definition as follows: "The term leukoplakia should be used to recognise white plaques of questionable risk having excluded (other) known diseases or disorders that carry no risk for cancer". Leukoplakia is commonly homogeneous and most are benign. Non-homogeneous leukoplakia - a predominantly white or white and red lesion (erythroleukoplakia) with an irregular texture that may be flat, nodular, exophytic, or papillary/verrucous - is more likely to be potentially malignant (4-6).

II. Materials and Methods

All patients of Oral leukoplakia attending the Dental OPD Department ACSR Govt. General hospital, Nellore, Andhra Pradesh, India were included in this study. The present study was conducted from January 2017 to January 2019 over a period of 24 months. The sample population was obtained from Department of Dental Surgery, Sri Padmavathi Medical College for women, and Sri Venkateswara Institute of Medical Sciences (SVIMS) Tirupathi. Andhra Pradesh. A total of 180 consecutive patients with leukoplakia and 20 healthy normal volunteers were recruited for the study and assessed for the extent of DNA damage using SCGE following clinical diagnosis. A detailed medical and dental history of the patients was taken and clinical details were recorded. The patients who were not willing to participate and those on medications or suspected of suffering from any systemic illness were excluded from the study. An informed consent was obtained from all patients. Subjects with histologically proven cases of leukoplakia were included. A control group (20 controls) consisting of healthy subjects were included. The healthy group consisted of individuals without any systemic illness and those free from habits, such as smoking or chewing tobacco.

II.1. Collection of Samples

After histopathological assessment and confirmation, intravenous blood samples were collected from the patients in sterile Sodium citrate vaccutainers. Single cell electrophoresis was carried out for both these groups and the DNA damage was quantified using a fluorescence microscope following the method outlined by Rao et al (8) and Singh et al (9).

II. 2. Preparation of the Reagents

The following reagents were prepared as per the protocol for using the single cell electrophoresis. All the necessary materials for preparation of the reagents were obtained from Sigma Chemical Company

***Dulbecco's phosphate buffered saline (Ca and Mg free)*:** Sodium chloride (8 gm), potassium chloride (0.2 gm), disodium hydrogen orthophosphate (1.15 gm) and potassium dihydrogen phosphate (0.2 gm) were dissolved in 500 ml of distilled water at 7.4 pH and stored at 4°C.

***Lysing solution*:** Sodium chloride (146.4 gm), Ethylene Diamine Tetra Acetic acid (EDTA; 37.2 gm) and Tris buffer (1.2 gm) were each dissolved in 1000 ml of distilled water to formulate a 2.5 H sodium chloride solution, 100 mm EDTA and 10 mm Tris solution respectively. Sodium hydroxide was then added and pH was set at 10. The final lysing solution was prepared with 36 ml of the above prepared solution with 1% Triton X-100 (0.4 ml) and dimethyl sulfoxide (4 ml), which was stored in amber coloured bottles at 4°C.

***Electrophoresis buffer*:** The stock solutions of 10 N sodium hydroxide and 200 mm EDTA was prepared by dissolving 50 gm of sodium hydroxide in 500 ml of distilled water and EDTA (7.4 gm) along with 1.2 gm sodium hydroxide in 100 ml distilled water respectively. Before each run, fresh buffer was prepared with 30 ml sodium hydroxide and 5 ml EDTA taken from the above stock solutions and made up to 1000 ml at a concentration of 300 mm sodium hydroxide and 1 mm EDTA respectively.

***Neutralization buffer*:** Tris (48.5 gm) was dissolved in 1000 ml of distilled water to prepare 0.4 M tris buffer solution which was then set at a pH of 7.5 with concentrated hydrochloric acid and stored at room temperature until further use.

***Staining solution*:** A stock staining solution of ethidium bromide was prepared by dissolving 10 mg of it in 50 ml distilled water. For the working solution, 1 ml of the above prepared solution was mixed with 9 ml of distilled water.

II.3. Preparation of the Slides

Slide preparation was performed under dim yellow light to prevent additional DNA damage. A total of 0.5% of low melting and regular agarose was prepared with agar (125 mg) and phosphate-buffered saline (PBS) (25 ml). Approximately 5 ml of the cell suspension was mixed with low melting agarose (70 ml) and placed over the frozen regular agarose on the slide with coverslips. After adequate solidification of the agarose, the coverslip was removed and the slide was slowly lowered into cold lysing solution and placed in a refrigerator overnight.

II.4. Electrophoresis of the Slides

The slides were removed from the lysing solution and placed in a horizontal gel electrophoresis system near the anode end, sliding them as closely together as possible. The buffer reservoir was filled with freshly made electrophoresis buffer solution until the liquid level completely covered the slides without any bubbles over the agarose. The slides were immersed in the alkaline buffer for 20 minutes to allow unwinding of the DNA. The power supply was adjusted to 25 V and 600 mA by slowly raising and lowering the buffer level.

II.5. Staining Procedure

The slides were removed from the buffer and placed on a staining tray. The slides were coated drop wise with neutralization buffer and left for 10 minutes. The procedure was repeated twice. Following which, the slides were drained and 50 ml of the prepared working solution of ethidium bromide was added and then covered with a fresh coverslip. The excess liquid was blotted with a tissue paper and the coverslips were sealed with vaseline.

II.6. Evaluation of DNA Damage

The ethidium bromide stained slides were examined for DNA damage using a fluorescent microscope (Olympus

B × 53, Tokyo, Japan) at 250× magnification. The images were captured and the measurement of the tail length is done using Image J software. A randomly selected 25 cells per slide were analyzed. The significance between the groups were assessed by ANOVA with post hoc test. A p-value ≤ 0.05 was taken as statistically significant.

III. Results

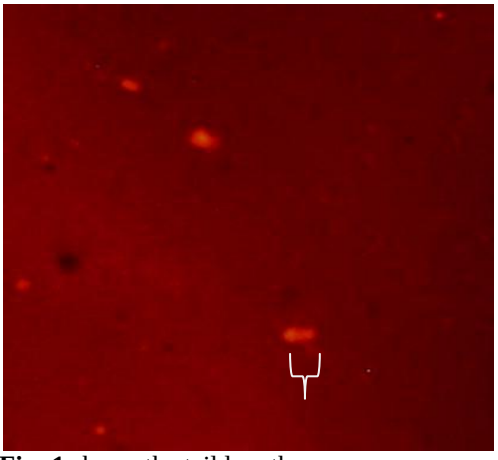

Fig -1 shows the tail length

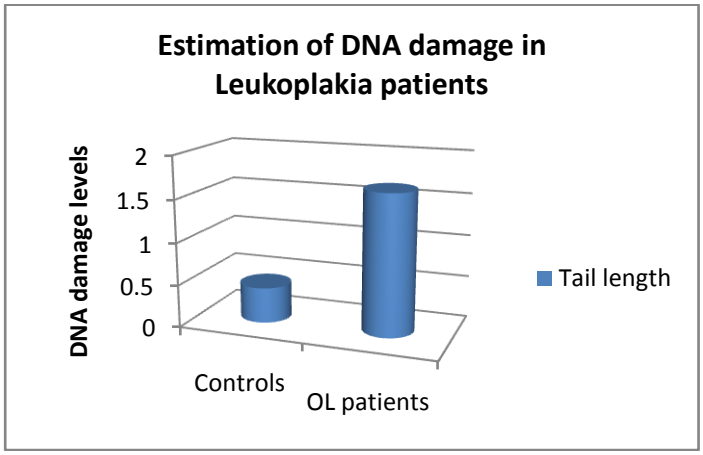

Fig -2 shows the graphical image of DNA damage in OL and controls

Deoxyribonucleic acid damage levels in OL (n = 180) patients and normal (n = 20) individuals were assessed using SCGE assay. The level of damage was expressed as the length of tail measured in µm. The mean tail length of leukoplakia patients was 1.65 ± 0.132 µm while for control it was 0.42 ± 0.14 µm. The difference was statistically significant. Results showed a significant increase in DNA damage in OL patients was statistical significance of $p < 0.001$ was found when compared with normal subjects.

IV. Discussion

Damage to DNA is suggested to be a common mechanism for cancer initiation. Deoxyribonucleic acid damage especially that which is radiation induced has been assessed by comet assay, (8, 9) but the use of this technique in cancer has been limited. Comet assay provides a generalized picture of a particular population that reflects whether it is an associated environmental carcinogenic stress, and may in turn become an epidemiologic asset. Number of epidemiological and clinical studies have demonstrated an independent, clear cut association of deleterious paranormal oral habits, such as smoking tobacco, betel quid chewing and chewing tobacco with occurrence of oral cancer (10). The precancerous condition of leukoplakia is characterized by a white patch, mainly associated with tobacco smokers, the prevalence of which is considered high in a developing country like India. This condition has high cancer turnover potentiality and if detected early can be prevented and treated successfully. With rapid advances in genotoxicologic studies and better assessment of the extent of DNA damage, the knowledge gained from these resources may provide a platform to determine the cancer progression (11). The association of the deleterious oral habits with DNA damage has been clearly established in a previous study. The results of the present study indicate that DNA damage in the study population is associated with leukoplakia. Tobacco smoking and pan chewing or presence of any deleterious oral habit may lead to DNA with greater tail length. The results are consistent with earlier reports in literature (12). Oral squamous cell carcinoma (OSCC) is often preceded by several changes in the overlying epithelium, which are observed histopathologically as dysplastic changes. It is important to quantify these dysplastic changes in oral epithelium as the degree of dysplasia is limited to the degree of probability to the development of malignancy (11-13).

The limitation of the study is that small sample size as well as the history of smoking among subjects. However all subjects in the study groups were smokers. Despite the above limitations, present study has been able to demonstrate that DNA damage in blood cells measured by SCGE is greater in leukoplakia than controls and establishes that deleterious oral habits are associated with such greater DNA damage (13).

V. Conclusion

The study also identifies potential situations where demonstrating DNA damage using SCGE may lead to further advancement in our understanding of the biology of oral leukoplakia and pre-cancer for developing possible worthy early detection tests for oral cancer.

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