EVALUATION OF DNA DAMAGE BY COMET ASSAY IN PATIENTS OF ORAL SUBMUCOUS FIBROSIS (OSMF)

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ABSTRACT
In OSMF Oral mucosa become pale and ulcers appears in the mouth and fibrotic bands appear in the mucosa, this leads to hardening of the tissue along with difficulty in opening of mouth and tissue hardening. Chewing of tobacco and its related products are mainly responsible for pre malignant conditions such as OSMF. The DNA damage can be assessed by comet assay. The study was done on 30 control and 30 OSMF patients. In OSMF patients DNA damage were higher as compared to control group. The study conclude that the comet assay can be used in evaluation of DNA damage.
KEYWORDS: *OSMF, Comet assay, Tail length, Tail moment*

1. INTRODUCTION

Schwartz (1952) was the first person who reported the OSMF and he used the term 'atrophica idiopathica (tropica) mucosae oris' for it. Later on in 1953, Joshi termed it as OSMF on the basis of its histological nature (1, 2). In OSMF Oral mucosa become pale and ulcers appears in the mouth and fibrotic bands appear in the mucosa, this leads to hardening of the tissue along with difficulty in opening of mouth and tissue hardening (3). Gupta et al. in their cohort study for a 10 year duration reported that lesions of OSMF are solely associated with betel quid chewing habit. The risk factor further increases if this chewing habit is associated with cigarette smoking and alcohol drinking habit (4). In the cases of OSMF inflammation occurs at sub-epithelial sites, then the fibro-elastic changes occur in the lamina propria along with atrophy of the epithelium. In a few cases, even vesicles appears at the onset of OSMF (5). OSMF is a benign but potentially malignant condition. Upon continuation of chewing habit in the beginning, preneoplastic lesions occur in the oral cavity that subsequently lead to malignancy (6).

Comet assay or the single cell gel electrophoresis (SCGE) is a very popular and widely used method for quantification and evaluation of the DNA damage. The reason for its popularity is ease of use, flexibility and its sensitivity. It is named comet assay because of appearance of DNA like a comet after electrophoresis. The damage to the DNA can be calculated through various parameters like tail length and tail moment.

2. OBJECTIVES

The evaluation of DNA damage in patients of oral sub mucous fibrosis is important to identify as

OSMF develops within a very short span of time and can lead to oral carcinoma. Chewing of tobacco damages the DNA and thus the patients which are at high-risk for developing cancer can be identified by comet assay.

3. METHODOLOGY

A total of 30 patients of OSMF (See figures) and 30 normal controls was chosen for the present study. The controls did not have the history of tobacco consumption in any form. The OSMF patients chosen for the present study were the patients visiting to OPD of National Institute of Medical Sciences & Research and Hospital (Jaipur). The study was approved by the Institutional Ethical Committee. The subjects were informed about the study and a written consent was obtained from them.

The methodology used was as follows:

1. Collection of blood sample

The help of a medical practitioner was taken for the collection of blood samples. About three milliliters of whole blood were withdrawn from the median cubital vein of each individual under aseptic conditions in sterile tubes containing EDTA anticoagulant. Blood samples were stored at −80°C till used.

2. Slides preparation

A. Pre-coating: Microscopic slide were cleaned by detergent and allowed to dry. Then the slides were made grease free by rubbing with acetone soaked cotton followed by flamed drying. Cleaned slides were placed on a level platform and coded prior to the experiment. Now 1.0 ml of 1% normal melting agarose (1gm in 100ml water) solution was poured on the slide by a 5.0 ml glass pipette. With the help of pipette layer was made evenly on the slide. Slides were kept as such at room temperature till it dried completely. Usually it takes 12 hours and these dried slides can be stored in a slide box for future use. The precoating procedure prevents separation of gel from the slide during the experiment.

B. First layer: 75 µL of 1% normal melting agarose (1gm in 100ml PBS) was dropped on the precoated slide and then it was covered with 18mm x 18mm cover slip and was kept on ice for 10 minutes. The cover slip was removed gently, so that the underline gel was not injured.

C. Second layer: EDTA blood was mixed with 1% Low melting agarose (1gm in 100ml PBS) at the ratio of 1:10 (Blood: Agarose). 75 µL of this blood agarose mixture was used to make a second layer. Then the slides were covered with cover slip to flatten out the molten agarose layer. The slides were kept on ice again for 10 minutes and then cover slip was removed gently. Care was taken while putting or removing the cover slips to avoid the entry of air bubbles and wear and tear to gel layer.

D. Third layer: 75 µL of 1% Low melting agarose (1gm in 100ml PBS) was added as a third layer in the same way on the slide and covered with cover slip. It was kept on ice for 1 hour. The purpose of the third layer is to fill in any residual holes in the second layer, to increase the distance between the cell and gel surface, and to protect the sample containing layer from wear and tear during experiment.

3. Lysing

The slides were placed in a freshly prepared ice cold lysis solution consisting of high concentration of salt and detergents at 4°C for overnight. The chilled lysis solution was used to maintain the stability of the agarose gel. Most of the proteins and membranes (organelle's and cellular) are lysed by the lysis solution to expose the nucleoids. Most of the histones are removed, and nucleosomes are disrupted, but the DNA remains super coiled.

4. Unwinding of DNA

Electrophoresis tank was carefully poured with a freshly prepared cold electrophoresis solution to submerge the slides completely without formation of air bubbles over the agarose gel. The slides were taken out and put into the horizontal electrophoresis tank side by side. The level of the electrophoresis solution was kept approximately 2-3 mm above the slides with no bubbles on the slides. The slides were then left for about 20 minutes in the electrophoresis buffer prior to the electrophoresis at room temperature. This allows the DNA to unwind for the expression of alkali labile sites as single strand breaks before the electrophoresis. Cold electrophoresis is used to avoid DNA damage due to heat generated during flow of current.

5. Electrophoresis

After unwinding, the electrophoresis was carried out at 300 mA and 19 V for 15min at room temperature.

6. Neutralization

After electrophoresis, the slides were lifted gently from the electrophoresis tank and placed on a staining tray. The slides were washed with neutralization buffer 3 times, for about 10 minutes. This was done to remove any traces of detergent and alkali, which would otherwise interfere with staining. The slides were then allowed to dry gently.

7. Staining

The slides were stained with 100µL of Ethidium bromide (10µg/ml) stain. Ethidium bromide is a known DNA intercalating agent, so precautions were taken to avoid any contact with the stain. Slides stained with

ethidium bromide cannot be stored hence should be analyzed immediately.

8. Observation

The slides were observed under fluorescent microscope. DNA (ethidium bromide) in lymphocyte cells emits reddish color. 100 cells for each subject were chosen randomly. The photographs were taken and analyzed by comet score software. The following comet parameters were used in the study

> ➤ % DNA in head
> ➤ Tail length
> ➤ % DNA in tail
> ➤ Tail moment
> ➤ Olive tail moment

4. STATISTICAL DESIGN

Data was entered into Microsoft Excel spreadsheet. It was analysed using Develve software for statistical analysis. The data between OSMF patients and control groups was analysed by One way Annova Test. A p-value <0.05 was considered as statistically significant.

5. RESULTS

The control group has 19 males and 11 females, and in OSMF patient group has 24 males and 6

7. FIGURES

These pictures are showing different sites of OSMF.

females. The control had an average age of 49.36 years (age range: 27-74 years), while OSMF group had an average age of 37.76 years (age range: 23-50 years). The % head DNA in controls was 90.36± 6.48,while in OSMF patients it was 50.86± 19.83. The tail length (TL) in controls was 1.80± 1.65, while in OSMF patients it was 34.70± 9.17. The % tail DNA in controls was 9.64± 6.48,while in OSMF patients it was 49.15± 19.83. The tail moment (TM) in controls was 0.23± 0.29,while in OSMF patients it was 16.57± 6.31. The olive tail moment (OTM) in controls was 1.27± 1.27,while in OSMF patients it was 15.01± 5.94. On comparison between controls and OSMF patients by One Way ANOVA the difference was highly significant [P value <0.001 (HS)] (Graph 1).

6. CONCLUSION

The increased tail length, % tail DNA, tail moment and olive tail moment was more in OSMM patients as compared to controls. This shows that the DNA damage was more in OSMF patients , so the comet assay is an effective tool to assess the DNA damage. Such a method is cost effective and can be done even in the rural hospitals as a screening test for early prediction of oral malignancy.

8. GRAPHS
Graph-1 showing DNA damage among controls and OSMF patients.
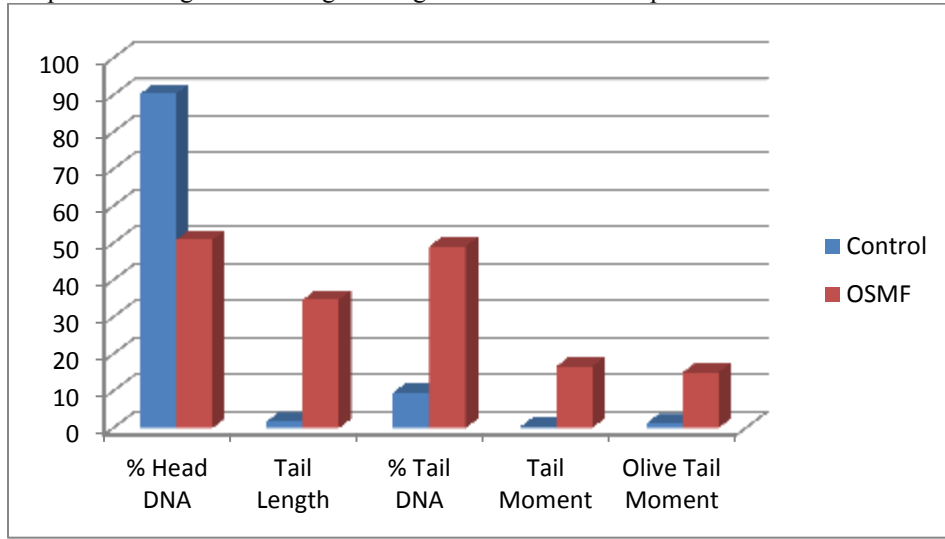
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