COMPARISON OF FREQUENCY OF MICRONUCLEI ON LESIONAL AND NORMAL SIDE IN PATIENTS WITH TOBACCO INDUCE KERATOSIS & LEUKOPLAKIA: THE PILOT STUDY

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ABSTRACT

The assessment of micronuclei in exfoliated cells is a promising tool for the study of the effect of carcinogens on oral epithelium. The technique involves examination of epithelial smears to determine the prevalence of cells containing micronuclei, extra-nuclear bodies composed of chromosomes or chromosomal fragments that failed to be incorporated into daughter nuclei at mitosis. Usually the neoplastic changes take place at the site of chronic stimulus. It passes through various phases of dysplastic changes. Clinically, shows varied spectrum of red and white lesions. Thus it can be hypothesized that micronuclei frequency is higher in that particular lesional tissue compare to normal contra lateral side. Aim of the present study was to compare frequency of micronuclei in Leukoplakia & tobaccos induce keratosis to that of normal contra lateral side. The study consist of 10 patients with clinical diagnosis of Leukoplakia, tobacco induce keratosis. Oral exfoliative cytology from the lesions as well as normal mucosa stained with papanicolaou stain used for counting micronuclei. Result shows that statistically significant difference in
frequency of micronuclei on lesional side and non lesional side was found. Perhaps the
difference between the frequency of micronuclei on lesional side and normal side almost
found to be constant. Thus it can be concluded that, higher frequency of micronuclei suggest
the localized effect of carcinogens on the oral mucosa. Perhaps the difference between both
sides that is lesional and normal almost remains constant which may be because of the
generalized effect of carcinogen on the oral mucosa.

**KEYWORDS:** Biomarker, Leukoplakia, Micronuclei, Tobacco induced keratosis.

**INTRODUCTION**

Squamous cell carcinoma is by far the most common oral mucosal malignant tumor. The
basic alteration for development of squamous cell carcinoma taking place in the proliferation
of cell that is related to nucleus. Hence it is the nucleus that expresses the phenotypic
alterations caused by genotoxic damage in the process of malignancy. On the other hand
exfoliative cytology is a method that gives better insight of the nuclear changes in individual
cells.\(^1\)

Micronuclei are extra nuclear cytoplasmic bodies. They are induced in cells by numerous
genotoxic agents that damage the chromosomes. The damaged chromosomes, in the form of
acentric chromatids or chromosome fragments, lag behind in anaphase when centric elements
move towards the spindle poles. After telophase, the undamaged chromosomes, as well as the
centric fragments, give rise to regular daughter nuclei. The lagging elements are included in
the daughter cells, too, but a considerable proportion is transformed into one or several
secondary nuclei, which are, as a rule, much smaller than the principal nucleus and are
therefore called micronuclei.\(^2\)

Micronuclei are induced in oral exfoliated cells by a variety of substances, including
genotoxic agents and carcinogenic compound in tobacco, betel nut, and alcohol. Tobacco-
specific nitrosamines have been reported to be potent clastogenic and mutagenic agents
which are thought to be responsible for the induction of chromatids/chromosomal aberrations
resulting in production of micronuclei. The genotoxic and carcinogetic chemicals released
from betel nut and tobacco and also the calcium hydroxide content of lime present in the betel
quid are thought to be responsible for promotion of reactive oxygen species from areca nut
extracts. These reactive oxygen species can in turn cause damage to the DNA.\(^3\)
Usually the neoplastic changes take place at the site of chronic stimulus. It passes through various phases of dysplastic changes. Clinically, it manifests as white lesion usually non-specific lesion known as Leukoplakia. Also because of local action of caustic agents & heat produced changes manifestate as tobacco induced keratosis. The local alterations and genotoxic effect result in production of micronuclei. Thus it can be hypothesized that micronuclei frequency is higher in that particular lesional tissue where continuous exposure tobacco takes place, compared to the normal contra lateral side.

Thus, the aim of present study is to compare frequency of micronuclei in Leukoplakia & tobacco induced keratosis to that of normal contra lateral side. The micronuclei count is examined by exfoliative cytology.

MATERIALS & METHODS

1. Patient selection

The samples include unilateral cases of Leukoplakia, tobacco induced keratosis. Who have not received any therapy prior. For the purpose of study the criteria for tobacco induced keratosis must be defined. Habitually chewing tobacco leaves or dipping snuff results in the development of a well-recognized white mucosal lesion in the area of tobacco contact, called smokeless tobacco keratosis, snuff dipper’s keratosis, or tobacco pouch keratosis. They are significantly different from true Leukoplakia and have a much lower risk of malignant transformation. Smokeless tobacco contains several known carcinogens, including N-nitrosonornicotine (NNN), and these have been proven to cause mucosal alterations.

Definition of Axéll 1976, Snuff dipper's lesion is a lesion of the oral mucosa at the exact site of the regular placing of snuff. For the purpose of study criteria are defined for diagnosis of tobacco induce keratosis the criteria included are

1. That shows Changes associated with the use of smokeless tobacco are seen in the area contacting the tobacco, rather than normal mucosa.
2. The surface of the mucosa appears white and is granular or wrinkled with a folded character.
3. Commonly noted is a characteristic area of gingival recession with periodontal-tissue destruction in the immediate area of contact
4. This recession involves the facial aspect of the tooth or teeth and is related to the amount and duration of tobacco use.
5. The usually stretched mucosa appears fissured or rippled, and a “pouch” is usually present.
6. This white tobacco pouch may be soft, leathery or nodular in consistency.

2. COLLECTION OF EXFOLIATED CELLS
Subjects were asked to rinse their mouth gently with water. Oral mucosal cells were scraped from lesional tissue of Leukoplakia & tobacco induced keratosis with slightly moistened wooden spatula. Using same technique oral mucosal cells were scraped from normal buccal mucosa of same patient. The cells were immediately smeared on pre cleaned microscopic slides. Just prior to drying, the smears were fixed with commercially available spray fixative for 15 min. The slides were coded & immediately taken for staining by papanicolaou technique (PAP) staining.

3. CYTOLOGICAL STAINING AND EVALUATION
All the cytological smears were stained by papanicolaou technique using a commercially available staining kit RAPIDPAP™ (Biolab Diagnostics, Tarapur, Maharashtra). The slides were mounted with cover glass using DPX mountant. All the slides were observed under light microscope using low magnification (×100) for screening and high magnification (×400) for counting of micronuclei.

4. SCORING CRITERIA
The most commonly used method, i.e., the zigzag method, was followed for screening of slides. One thousand cells with intact nuclei with cell boundaries were counted on each slide. The criteria for designating an extra nuclear body as 'micronucleus' were as follows:
1. Diameter less than one third of the main nucleus.
2. Staining intensity similar to, or slightly weaker than, that of the nucleus. Round-to-oval shape.
3. Texture same as that of the main nucleus.
4. Close proximity but no actual contact with the nucleus.
5. Plane of focus same as that of the main nucleus.

Only those structures fulfilling the above-mentioned criteria were recorded as micronuclei. Micro nucleated cells were counted out of 1000 intact epithelial cells, and they were expressed as percentages.
RESULTS
Table 1: Showing comparison of number of micronuclei frequency on lesional area to that of micronuclei on normal mucosa.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Number of Micronuclei in normal mucosa</th>
<th>Number of Micronuclei in lesional area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>5</td>
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<tr>
<td>5</td>
<td>6</td>
<td>8</td>
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<td>9</td>
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<tr>
<td>10</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>80</td>
</tr>
</tbody>
</table>

After applying paired test (table 2) shows the result showing results are statistically significant results.

Table 2: Paired Samples Test

<table>
<thead>
<tr>
<th>Paired Differences</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
<th>95% Confidence Interval of the Difference</th>
<th>t</th>
<th>Df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micronuclei in normal - micronuclei in lesion</td>
<td>3.000</td>
<td>1.054</td>
<td>.333</td>
<td>-3.754</td>
<td>-2.246</td>
<td>-9.000</td>
<td>9</td>
</tr>
</tbody>
</table>

Thus, statistically significant difference in frequency of micronuclei on lesional side and non lesional side was found. Perhaps the difference between the frequency of micronuclei on lesional side and normal side almost found to be constant.

DISCUSSION

Squamous cell carcinoma of the oral mucosa accounts for 90% to 95% of all oral malignancies. Oral exfoliative cytology has been used extensively for screening cellular alteration in oral squamous cell carcinoma cases. Oral exfoliative cytology can reveal various cellular alterations in squamous cell carcinoma. It includes karyorrhexis, karyolysis, micronucleus formation, pyknosis, binucleation, broken-egg nucleus, anucleation, etc.
On the other hand early detection of carcinogen related interaction is important so that the preventive strategies can be applied. Hence in many researches are directed towards the development of screening strategies indicating individual cancers with certain biomarkers. Biomarkers are instruments of individual tumor prevention and help to detect high-risk patients. They allow statements concerning environmental and occupational exposition and further give information on the status of susceptibility. Biomarkers are divided in three groups: the first to define the exposure to carcinogenic agents, the second to show biological effects on the target tissue and the third to give information about the individual susceptibility.[8] Micronuclei (MN) are one of such biomarkers that are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from acentric chromosome fragments.[8]

In the present study, all cases were observed having habit of keeping tobacco in buccal vestibule only on one side and never placed on other side vestibule. All patients having white lesion in the buccal vestibular area. Thus the comparison between the lesional side and normal side can be made. Thus whatever the difference is there is because of tobacco. We have not considered the gender difference all patients were male. Age range is from 21 yr to 78 yr.

On the normal side the frequency of micronuclei count is found to be 0.03 to 0.07%. In the previous study by Palve D & J.V. Tupkari the frequency of micronuclei in normal is 0 to 0.5%.[1] The present study also showing almost the same result as that of previous study. But the frequency found to be somewhat high in present study is mainly because of the presence of tobacco habit. While previous study was excluded the patient with tobacco habit.

In previous study of Koneru et al comparison of micronuclei frequency on chewing & non-chewing side, slight difference has been observed. This difference was not statistically significant.[9] because in previous study patients with oral submucous fibrosis were included. As already been established that oral submucous fibrosis is generalized state. But in present study the difference was statistically significant. One of the reasons for that patient having tobacco induce keratosis & Leukoplakia has been considered and that only related to the localized effect of tobacco.

Though the frequency of micronuclei is statistically significant the difference in each patient is relatively constant suggesting the generalized effect of carcinogens. In present study the
patients who chew & keep tobacco has been considered. The only patient having chewing habit or keeping habit has not been differentiated. That can be one of the factors for generalized effect of carcinogens. Previous theory of “field cancerization” (that is generalized effect of carcinogen) is acceptable up to certain extent by the present study.

In the present study the frequency of micronuclei on lesional side ranging from 0.05 to 0.10. results are in correlation with that of Haldar et al study. They suggested the micronuclei in normal patient range from 0 to 0.35, for precancer 0.5 to 0.75, for cancer cases 1.16 to 1.66. Thus, the micronuclei frequency can be used as the useful biomarker to evaluate the effect of carcinogen on the tissue. Still limitation of biomarker should be considered that include Genotoxic effect by carcinogen may manifestate by various ways like apoptosis, nuclear degeneration and only counting micronuclei cannot consider as marker for Genotoxicity, The micronuclei formation takes place after cell division only. High turnover rate only allow the visualization of micronuclei, If slow turnover then micronuclei may get degenerate.

Also this study having some limitations like histopathological evaluation has not been considered. The genotoxic mitotic interferences take place in basal layer of epithelium and here only exfoliative cytology is considered.

Thus it can be concluded that 1. The comparison of frequency of micronuclei shows statistically significant difference in between lesional and normal side. This is due to localized effect of carcinogens on the oral mucosa. 2. Perhaps the difference between both sides that is lesional and normal almost remains constant suggesting the generalized effect of carcinogen on the oral mucosa.

REFERENCES