Chapter - 2
MICRONUCLEUS INVESTIGATION IN
HUMAN BUCCAL EPITHELIAL
CELLS OF GUTKHA USERS
MICRONUCLEUS INVESTIGATION IN HUMAN BUCCAL EPITHELIAL CELLS OF GUTKHA USERS

2.1. Introduction

It is well known that betel quid chewing with or without tobacco is carcinogenic to humans. (IARC, 1985; 2004). Gutkha is a mixture of areca nut, catechu, lime, cardamom, unspecified flavouring agents and tobacco. Gutkha is responsible for a number of oral diseases due to the presence of areca nut and tobacco (Kumar, 2008). Areca nut is a main component of gutkha, that is responsible for oral submucous fibrosis (OSMF) (Tilakaratne et al., 2006). OSMF is an incurable disease that may finally lead to oral cancer (Murti et al., 1985). After long time of smoking the adverse effects are seen but in case of gutkha users, OSMF develops within a very short span of time (Babu et al., 1996). The intake of gutkha and OSMF is very common among young persons (Gupta et al., 1998). Areca nut increases the chances of formation of pre-cancerous lesions and OSMF. Micronuclei are small chromatin bodies that appear in the cytoplasm by the condensation of acrocentric chromosomal fragments or by whole chromosomes, lagging behind during cell division. Thus, it is the only biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells, that are easily detected in interphase cells (Norppa et al., 2003). MN assay has been used as a biomarker of genetic damage in buccal mucosa cells (Stick and Rosin, 1983; Speit and Schmid, 2006). An elevated micronucleated cell frequency was reported in the buccal mucosal epithelium of areca nut chewers (Stich et al., 1982). The aqueous extract of N-Nitroso compounds related to areca nut, that is, 3-(Methylnitrosamino) propionitrile is highly cytotoxic and genotoxic in cultured human buccal epithelial cells and enhances the induction of tumors in betel quid chewers (Sundqvist et al., 1989). The MN assay in buccal cells can be used to detect cancerous or pre-cancerous lesions and also to monitor the effects of a number of chemopreventive agents (Stich and Rosin, 1984; Stich and Dunn, 1987). In the present study the effect of gutkha was studied on the micronucleus frequency in buccal epithelial cells.
2.2. Materials and Methods

Survey

The study comprised of sixty male individuals out of which 30 individuals were having the habit of chewing gutkha (cases), these were compared with remaining 30 individuals who were non users (control: Those who did not involve in any addiction). A written consent was taken from each individual, and the samples were taken from the Department of Ziauddin Ahmed Dental College and Hospital, A.M.U. Aligarh. U.P. The period of the study was almost 6 months.

Chemicals

Trizma hydrochloride (Tris-HCl) and ethylene di amine tetra acetic acid (EDTA) was purchased from SRL, India. Giemsa stain, sodium chloride, methanol and sodium hydroxide pellets was purchased from Merck (India). The buffer solution was prepared by dissolving 0.1M EDTA, 0.001M Tris-HCl and 0.02M NaCl in a sterile 1L distilled water. The pH of the buffer was adjusted to 7.0 with NaOH.

Oral Mucosa Cell Collection and Processing

Oral mucosa cells were collected from each subject using a soft tooth brush gently from the oral mucosa of cheeks (Surrelles et al., 1997). The brush was then swirled into a centrifuge tube containing a buffer solution of pH 7.0 there by creating a cell suspension. The cells were washed three times by centrifugation at 1500 rpm for 10 minutes in the buffer solution. (Surrelles et al., 1997). About 15 mL of buffer in a 30 mL conical tube was used in every washing step. About 50-100 µL of the cell suspension was laid and spread on clean, pre-heated (37°C) glass slide and allowed to air dry for 5-10 minutes. The slides were fixed in methanol, stained with 5% Giemsa and observed under microscope (Rajeswari et al., 2000). A total of 2000 oral mucosal cells were scored per individual.

Statistical Analysis

Statistical analysis was carried out by Student’s t-test using commercial software Statistica Soft Inc 12.
2.3. Results

MN frequency among individuals having chewing habit was found to be four times higher (21.3 ± 1.788) as compared with the control (4.56 ± 0.331) (Table 2.1). The number of micronucleated cells in the controls and cases were 4.53 ± 0.331 and 17.4 ± 0.944, respectively (Table 2.1). The distribution of micronucleated cells is given in (Table 2.2) and (Figure 2.1). The age distribution of cases and controls is given in (Table 2.3). Among users, the youngest was of 12 years and the oldest one was of 65 years of age. (Figures 2.2-2.4) shows the MN in buccal epithelial cells of the users.

Table 2.1: Total micronucleus frequency per 2000 cells per individual in the buccal region.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of individuals</th>
<th>Age range</th>
<th>Age (mean ± SE)</th>
<th>MC (mean ± SE)</th>
<th>TM (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>16-85</td>
<td>31.4 ± 2.51</td>
<td>4.53 ± 0.331</td>
<td>4.56 ± 0.331</td>
</tr>
<tr>
<td>Cases</td>
<td>30</td>
<td>12-65</td>
<td>29.7 ± 2.46</td>
<td>17.4 ± 0.944a</td>
<td>21.3 ± 1.788a</td>
</tr>
</tbody>
</table>

MC: micronucleated cell; TM: total number of micronuclei. *significant at p<0.05 compared with control.

Table 2.2: Micronucleus distribution from combined data.

<table>
<thead>
<tr>
<th>Number of micronucleus</th>
<th>Number of controls</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>3.</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>4.</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total number of individuals</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
Figure 2.1: Total micronucleus frequency in 2000 cells.

Figure 2.2: Buccal epithelial cell with (a) one micronuclei (b) two micronuclei (c) three micronuclei.
Table 2.3: Age distribution of cases and controls.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Control</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 24</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>25-40</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>&gt;40</td>
<td>05</td>
<td>06</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

2.4. Discussion

MN has been used since 1937 as an indicator of genotoxicity (Heddle et al., 1983). Studies on MN frequencies support that MN as a product of early events in human carcinogenic processes, particularly in oral regions. (Stich and Dunn, 1987; Stich et al., 1982; Roberts, 1997). MN test is especially used for the identification of pre-clinical steps of the cancer (Ramirez et al., 1999). Various studies from 1985 till date have shown significant increase in micronucleated frequency in betel quid chewers as compared with healthy individuals (Nair et al., 1991). The present study shows the higher frequency of MN among the gutkha users. This has also been proved in other previous studies (Gandhi and Kaur, 2000; Siddique et al., 2008). The main carcinogens in gutkha are derived from their ingredients (arecanut, catechu and tobacco). Tobacco specific nitrosamines are formed due to chewing of gutkha (Nair et al., 1999). A high level of nitrite and nitrate reductase activity has been reported in the saliva of gutkha chewers (Murdia et al., 1982). Swallowing of the quid leads to the nitrosation of secondary and tertiary amines due to the acidic pH of stomach. Urinary levels of N- nitrosopropylene were 4 to 6.5 fold higher in gutkha chewers (Nair et al., 1986; Chakradeo et al., 1994). Aqueous extracts of arecanut and catechu generates reactive oxygen species that leads to the genotoxic damage in buccal epithelial cells (Nair et al., 1987). Variations in the number of micronucleated cells depends on the ingredients in the quid, the number of quids per day and different life styles, gender, age and food habits (Holland et al., 2008). The differences observed in the frequencies of micronucleated cells in the control group, is due to the different food habits of the population groups. Individuals ingest various types of chemicals in their daily diet, which was the reason for the variable levels of micronucleated cells (Kamboj and Mahajan, 2007). The duration of addiction of the chewing habit in the present study of 30 individuals was in average of 1-20 years and their frequency was 2-18
pouches/day. A majority of degenerative and developmental diseases are caused by genomic damage, which is produced by the exposure of radiation, chemicals, micronutrient deficiency and lifestyle factors (alcohol, smoking, drugs, gutkha, pan masala and stress. The MN assay in buccal cells serves as an excellent biomarker (Tolbert et al., 1992).

2.5. Conclusion

This study reveals that gutkha is highly genotoxic and may be responsible for oral cancer in near future, so it is important to increase the awareness programs to inform and educate the public regarding the adverse health consequences and possible cancer risk associated with gutkha.
Micronucleus investigation in human buccal epithelial cells of gutkha users

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Abstract
Background: Gutkha is a cheap and convenient betel quid substitute, which is popular among all age groups. Various studies reveal its carcinogenic nature that leads to oral submucous fibrosis and increases the chances of oral cancer. The micronucleus (MN) assay in exfoliated mucosal cells is a useful method for observing genetic damage in humans.

Aim: To observe the genotoxic effect of gutkha on human buccal epithelial cells.

Materials and Methods: The MN assay was performed to assess the frequency of MN in human buccal epithelial cells. The study comprises 60 individuals of which 30 individuals were gutkha chewers and another 30 were nonusers (control). The MN frequency was scored to estimate the genotoxic damage.

Results: In gutkha users, the frequency of MN was highly significant (17.4 ± 0.944) as compared with nonusers (control) groups (4.53 ± 0.331). (P < 0.001).

Conclusions: The MN assay in human buccal epithelial cells is a useful and minimally invasive method for monitoring genetic damage in humans. A significantly higher frequency of micronucleated cells are found among gutkha users.

Key Words: Buccal, epithelial cells, gutkha, micronuclei

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INTRODUCTION

Gutkha and pan masala are in more demand among all age groups. It is revealed that betel quid chewing with or without tobacco are carcinogenic in humans. Gutkha is the mixture of areca nut, catechu, lime, cardamom, spices, unspecified flavouring agents, and tobacco. Gutkha is supposed to be responsible for a number of oral diseases and has addictive effects that leads to the addiction due to the presence of areca nut and tobacco. Areca nut is a main component of gutkha, which is responsible for oral submucous fibrosis (OSMF). OSMF is incurable disease, and finally leads to oral cancer. After long time of smoking, adverse effects are seen but in case of gutkha users, OSMF develops within a very short span of time. The intake of gutkha and OSMF is very common in young persons. Areca nut increases the chances of formation of precancerous lesion and OSMF. Micronuclei are small chromatin bodies that appear

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the cytoplasm by the condensation of acrocentric chromosome fragments or by whole chromosomes, giving behind during cell division. Thus, it is the only marker that allows the simultaneous evaluation of clastogenic and aneugenic effects in a wide range cells, that are easily detected in interphase cells. An MN assay has been used as a biomarker of genetic damage in mucosa cells. An elevated micronucleated frequency is found in the buccal mucosal epithelium area of areca nut chewers. The aqueous extract of nitroso compounds related to areca nut, that is, nitrates, nitrites, and nitrofurans in cultured human buccal epithelial cells, and enhance the induction of tumors by betel quid were. The MN assay in buccal cells can be used to detect cancerous or precancerous lesions and also monitor the effects of a number of chemopreventive agents. In the present study, the effect of gutka was tested on the micronucleus (MN) frequency in buccal epithelial cells.

III and Objectives

The present study showed the frequency variation of MN in chewers and nonchewers of gutka by performing an assay.

MATERIALS AND METHODS

The study comprised 60 male individuals out of whom individuals were having the habit of chewing gutka, these were compared with the remaining individuals who were nonusers (control: Those who did not involve in any addiction). A written consent was taken from each individual, and the samples were taken at the Department of Ziauddin Ahmed Dental College Hospital, A.M.U. Aligarh, U.P. The period of the study was almost 8 months.

Biochemicals

Tris hydrochloride (Tris–HCl), ethylene diamine tetraacetic acid (EDTA) from SRL, India. Griesa stain, uranyl acetate, methanol, and sodium hydroxide pellets from Merck (India). The buffer solution was prepared by adding 0.1 M EDTA, 0.001 M Tris–HCl and 0.02 M NaOH in a sterile 1 L distilled water. The pH of the buffer was 7.0 with NaOH.

I. Mucosa Cell Collection and Processing

Mucosa cells were collected from each subject using a toothbrush gently from the oral mucosa of cheeks. The brush was then swirled into a centrifuge tube containing a buffer solution of pH 7.0, thereby creating a suspension. The cells were washed three times centrifugation at 1500 rpm for 10 min in the buffer solution. About 15 mL of buffer in a 50 mL conical tube used in every washing step. About 50–100 mL of the cell suspension was laid and spread on clean, preheated (37°C) glass slide and allowed to air dry for 5–10 min. The slides were fixed in methanol, stained with 5% Giemsa and observed under microscope. A total of 2000 oral mucosal cells were scored per individual.

Statistical Analysis

Statistical analysis was carried out by Student's t test using commercial software Statistica Soft Inc.

RESULTS

MN frequency among individuals having chewing habit was found to be 4 times higher (21.3 ± 1.788) as compared with the control (4.56 ± 0.331) (Table 1). The number of micronucleated cells in the controls and cases were 4.53 ± 0.331 and 17.4 ± 0.944, respectively (Table 1). The distribution of micronucleated cells is given in (Table 2) and Figure 1. The age distribution of cases and controls is given in Table 3. Among users, the youngest was of 12 years and the oldest one was of 65 years of age. Figures 2–5 shows the MN in buccal epithelial cells of the users.

DISCUSSION

MN has been used since 1937 as an indicator of genotoxicity. Studies on MN frequencies support that MN as a product of early events in human carcinogenic processes, particularly in oral regions. MN test is especially used for the identification of preclinical steps of the cancer. Various studies from 1985 till date have shown significant increase in micronucleated frequency in betel quid chewers as compared with healthy individuals.

The present study shows the higher frequency of MN

<p>| Table 1: Total micronucleus frequency per 2000 cells per individual in the buccal region of 30 cases and 30 controls |
|---------------------------------|-------|--------|-------|-------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Individuals</th>
<th>Age range (Mean±SE)</th>
<th>Age MC (Mean±SE)</th>
<th>Age TM (Mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>31.4 ± 2.518</td>
<td>4.53 ± 0.331</td>
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<tr>
<td>Cases</td>
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<td>29.7 ± 2.46</td>
<td>17.4 ± 0.944</td>
</tr>
<tr>
<td>MC</td>
<td>micronucleated cell; TM, total number of micronuclei. Significant at P&lt;0.001 compared with control.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| Table 2: Micronucleus distribution from combined data |
|---------------------------------------------------|-------|--------|-------|</p>
<table>
<thead>
<tr>
<th>Number of micronuclei</th>
<th>Number of controls</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total number of Individuals</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

<p>| Table 3: Age distribution of cases and controls |
|---------------------------------|-------|-------|</p>
<table>
<thead>
<tr>
<th>Age group</th>
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</tr>
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<tbody>
<tr>
<td>≤ 24</td>
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<td>13</td>
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<tr>
<td>&gt;40</td>
<td>05</td>
<td>06</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
among the users of gutkha, this was proved by previous studies.\textsuperscript{28,29} The main carcinogen in gutkha is derived from their ingredients areca nut, catechu, and tobacco. Tobacco-specific nitrosamines are formed due to chewing of gutkha.\textsuperscript{29} That leads to exposure of buccal cells to volatile nitrosamines derived from areca nut alkaloids.\textsuperscript{28} A high level of nitrite and nitrate reductase activity have been reported in the saliva of gutkha chewers.

\textsuperscript{28} Swallowing of the quid leads to the nitrosation of secondary and tertiary amines due to the acidic pH of stomach. Urinary levels of N-nitrosopropylene were 4- to 6.5-fold higher in gutkha chewers.\textsuperscript{27,28} Aqueous extracts of areca nut and catechu responsible for the generation of reactive oxygen species that cause the genotoxic damage in buccal epithelial cells.\textsuperscript{29} Variations in the number of micronucleated cells may be affected by the ingredients in the quid, the number of quids per day and different lifestyles, gender, age, and food habits.\textsuperscript{28} We observed the difference in the frequencies of micronucleated cells in the control group, which may be due to the different food habits of the population groups. Individuals ingest various types of chemicals in their daily diet, which was the reason for the variable levels of micronucleated cells.\textsuperscript{21} The duration of addiction of the chewing habit in the present study of 30 individuals was in average of 1-20 years and their frequency was 2-18 pouches/day. A majority of degenerative and developmental diseases are caused by genomic damage, which is produced by environmental exposure of radiation, chemicals, micronutrient deficiency, and
lifestyle factors, for example, alcohol, smoking, drugs, gutkha, pan masala and stress. So it is important to biomonitor, identifying, and treatment of diseases caused by, or associated with genetic damage. The MN assay in buccal cells serves as an excellent biomarker. Supplement of vitamins and beta-carotene found to be an effective measure used for reduction in the number of micronucleated cell frequency in healthy chewers as well as precancerous lesions.

CONCLUSIONS

This study reveals that gutkha is highly genotoxic and responsible for oral cancer in near future, so it is important to increase the awareness programs to inform and educate the public regarding the adverse health consequences and possible cancer risk associated with gutkha.

ACKNOWLEDGMENTS

The authors are thankful to the Council of Science and Technology (CSST/33908), Lucknow, UP, for awarding the project titled “Genotoxicity assessment in exfoliated Mucosal cells of Pan masala and Gutkha Chewers.” We are also thankful to the Chairman, Department of Zoology, for providing laboratory facilities and to the Chairman, Department of Periodontics and Community Dentistry, for the support in providing the samples.

REFERENCES


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