Levels of salivary thiocyanate and its relation with occurrence of micronuclei using exfoliative cytology in smokers and nonsmokers

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ABSTRACT

Aims: To evaluate the levels of salivary thiocyanate and its relation with the occurrence of micronuclei (MN) using exfoliative cytology in smokers and nonsmokers.

Materials and Methods: One hundred and twenty patients were divided into 3 groups: nonsmoker group 1 (control), smokers group 2, and smokers group 3. Their saliva was collected and analyzed for thiocyanate levels, and exfoliative cytology was evaluated for the presence of MN.

Statistical Analysis Used: Fisher's exact test and ANOVA test were used.

Results: It was seen that as the grade of smoking increased, the levels of salivary thiocyanate and occurrence of MN increased.

Conclusions: Detection and quantification of “biomarkers” such as salivary thiocyanate and MN in noninvasive and painless procedures such as oral exfoliative cytology can be an upcoming research domain in the field of cancer prevention and therapeutics.

Key words: Exfoliative cytology, micronuclei, salivary thiocyanate

Oral cancers have been one of the leading causes of deaths, particularly in the developing countries. There is an increasing effort worldwide to determine the impact of environmental, genetic, and lifestyle factors on genomic stability in human populations. As a result of rapid globalization and changing social attitudes, tobacco smoking has been increasingly giving rise to this dreaded malignancy which seems a high price to pay for an innocuous-looking habit. Drastic phenotypic and biochemical changes occur during the metamorphosis of a normal cell into an invasive cancer cell, and such biochemical changes can be studied using certain biomarkers. Tobacco consumption is positively correlated with the accumulation of DNA damage, and exposure to tobacco-related chemical carcinogens is proved to have direct damaging effects on the cellular DNA in the oral cavity. Tobacco smoke consists of a mixture of combustion gases and particulate matter. Inhaled cigarette smoke contains hydrogen cyanide which is metabolized to thiocyanate. The direct contact between saliva and smoke makes the measurement of salivary thiocyanate, an attractive alternative to serum and tissue testing.

These chemical carcinogens such as thiocyanate present in tobacco can cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities. These effects of chromosomal damage can be observed as changes in chromosomal structure, chromosomal number, sister chromatid exchange, and micronuclei (MN).

MN are extranuclear cytoplasmic bodies induced in the oral exfoliated cells by a variety of substances including...
genotoxic agents in tobacco smoke.[7] The cytological changes can be an easily available tool to determine early dysplastic changes in smokers to avoid cancer by early detection and treatment. In addition, establishment of a correlation between the salivary thiocyanate levels and cytological changes in the form of MN may pave the path for its future use as a biomarker in prevalence studies and screening procedures and thus may improve the treatment plan, management, and prognosis of the patient to a greater extent.

With this view in mind, the present study was carried out to evaluate the salivary thiocyanate levels in smokers and nonsmokers and to compare and correlate their levels with the occurrence of MN visible in cytology of oral mucosal epithelium.

The present study was carried out by the Department of Oral Pathology and Microbiology on the patients who reported to the outpatient department (Department of Oral Medicine and Department of Periodontology).

MATERIALS AND METHODS

Source and method of collection of data
We chose the sample by convenient sampling technique, and the sample size was determined by sample size formulae depending on the prevalence of tobacco users in the area of research.

One hundred and twenty cases consisted of eighty adult smokers and the control group consisted of forty nonsmoker volunteers. Before collection of saliva sample, informed consent was obtained. The participants were classified into three groups as follows:

1. Nonsmoker (control) group 1 consisted of individuals between the ages 25 and 45 years without habit of tobacco in smoked or smokeless form ($n = 40$)
2. Smoker group 2 consisted of individuals between the ages 25 and 45 years with a habit of smoking 4–10 filtered cigarettes per day ($n = 40$)
3. Smoker group 3 consisted of individuals between the ages 25–45 years with a habit of smoking 11–20 filtered cigarettes per day ($n = 40$).

The subjects rejected for the study were as per the following criteria.

Exclusion criteria
- Patients with potentially malignant conditions
- Any other form of tobacco consumption
- Patients exposed to radiation
- Patient exposed to any other irritant, for example, sharp tooth.

Method of collection of saliva
Participants were asked to rinse their mouth gently with water, and 2 ml of unstimulated saliva was collected by spitting method in clean-coded sterile container. Saliva was then transferred into test tubes which were then frozen and stored at 4°C until analyzed for thiocyanate.

Procedure for determining the levels of thiocyanate in saliva
Saliva was treated with trichloroacetic acid (20%) and ferric nitric acid reagent by the method of Denson.[9] Twenty percent trichloroacetic acid solution was added to 1 ml of saliva in a test tube. The contents of the tube were mixed and allowed to stand for 10 min. Then, 3 ml of the filtrate was added to 2 ml of water, followed by 5 ml of ferric nitrate reagent. The color change produced was measured colorimetrically at 470 nm, and the results compared with a standard curve obtained by adding ferric nitrate solution to standard thiocyanate solutions. Using known standards of sodium thiocyanate, concentration was related to absorption.

Method of obtaining exfoliated cells
Participants were asked to rinse their mouth gently with water, and oral mucosal surface scrapings were obtained using a wooden spatula. The cells were immediately smeared on precleaned microscopic slides, and the smears were fixed with absolute alcohol.

Procedure for staining exfoliative cells
All the cytological smears were stained by Feulgen reaction. All the slides were observed under light microscope using low magnification ($\times$100) for screening and high magnification ($\times$400) for counting the MN.

Heddle initially described the well-established basic criteria for MN.[10] However, the criteria for identifying cells for inclusion into the MN frequency count were not provided. Later, Tolbert et al. developed the criteria for choosing the cells, and this is being most widely applied.[11,12] It consists of the following parameters:
- Cytoplasm intact and lying relatively flat
- Little or no overlap with adjacent cells
- Little or no debris
- Nucleus normal and intact, nuclear perimeter smooth and distinct.

In order for a cell to be considered MN, the cell must satisfy the above criteria regarding inclusion in total cell count and the suggested criteria for identifying MN are as follows:
- Rounded, smooth perimeter suggestive of membrane
- Less than one-third of the diameter of the associated nucleus but large enough to discern shape and color
- Feulgen positive (i.e., pink in bright field illumination)
- Staining intensity is similar to that of the nucleus
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• Texture similar to that of the nucleus
• Same focal plane as nucleus
• Absence of overlap or bridge to nucleus.

Tolbert et al. also recommended the scoring of at least 1000 cells, with an increase to 2000–3000 if fewer than 5 MN cells were observed after counting 1000 cells. The majority of the published studies have scored between 1000 and 3000 cells although it has been suggested that 10,000 cells may be needed to observe a statistically significant, 50% increase, in the MN frequency.[12,13]

Only those structures fulfilling the above-mentioned criteria were regarded as MN [Figures 1 and 2].

RESULTS

The following criteria were used:
• The occurrence of MN was studied in nonsmokers and smokers
• The levels of salivary thiocyanate were studied in nonsmokers and smokers
• Correlation of salivary thiocyanate and MN was studied in nonsmokers and smokers.

Using the above criteria, the following results were obtained.
• Out of forty patients of Group 1, 31 patients had MN in the range of 1–3 and nine patients had 0 MN
• Out of forty patients of Group 2, 11 patients had MN in the range of 1–3 and 29 patients had MN in the range of 4–6
• Out of forty patients of Group 3, 19 patients had MN in the range of 4–6 and 21 patients had more than 6 MN [Table 1 and Graph 1].

Using Fisher’s exact test, $p < 0.05$ was obtained; therefore, there is association between MN and Group 1, Group 2, and Group 3. Thus, it shows as the grade of smoking increased, the occurrence of MN increased.
• The mean micronucleus frequency (%) in Group 1 is found to be $0.13 \pm 0.97\%$ and the range being $0–0.3\%$
• The mean micronucleus frequency (%) in Group 2 is found to be $0.405 \pm 1.04\%$ and the range being $0.2%–0.6\%$
• The mean micronucleus frequency (%) in Group 3 is found to be $0.668 \pm 1.27\%$ and the range being $0.4%–0.9\%$ [Table 2 and Graph 2].

Using ANOVA test, $p < 0.05$ was obtained; therefore, there is significant association between mean MN frequency (%) in Group 1, Group 2, and Group 3.

Thus, as the grade of smoking increased, the mean micronucleus frequency (%) increased:
• Group 1 shows a mean thiocyanate value of 1.17 with standard deviation [SD] ± 0.51
• Group 2 shows a mean thiocyanate value of 3.51 with SD ± 0.91
• Group 3 shows a mean thiocyanate value of 5.31 with SD ± 0.51 [Table 3 and Graph 3].

Using ANOVA test, \( p < 0.05 \) was obtained; therefore, there is significant association between mean thiocyanate in Group 1, Group 2, and Group 3.

Thus, it shows as the grade of smoking increased, the mean value of salivary thiocyanate increased.

Out of 120 patients, nine patients had 0 MN and mean thiocyanate count of 0.61 with SD ± 0.18, 42 patients had MN in the range of 1–3 and mean thiocyanate value of 1.66 with SD ± 0.80, 48 patients had MN in the range of 4–6 and mean thiocyanate value of 4.30 with SD ± 0.83, and 21 patients had MN more than 6 and thiocyanate value of 5.61 with SD ± 0.19 [Table 4 and Graph 4].

Using ANOVA test, \( p < 0.05 \) was obtained; therefore, there is significant association between mean thiocyanate with respect to MN count. Thus, it shows that as the levels of salivary thiocyanate increased, there was an increase in the occurrence of MN.

Table 1: Distribution of cases in Group 1, Group 2 and Group 3 with respect to micronuclei group

| Micronuclei | Group 1 | Group 2 | Group 3 | Total | \( p \) |
|-------------|---------|---------|---------|-------|--------|
| 0           | 9       | 0       | 0       | 9     | <0.001 |
| 1-3         | 31      | 11      | 0       | 42    |        |
| 4-6         | 0       | 29      | 19      | 48    |        |
| >6          | 0       | 0       | 21      | 21    |        |
| Total       | 40      | 40      | 40      | 120   |        |

Table 2: Comparison of mean micronuclei frequency % between Group 1, Group 2 and Group 3

| Group     | Number of cases | Mean MN%±SD | Range%   | \( p \)   |
|-----------|----------------|-------------|----------|-----------|
| Group 1   | 40             | 0.13±0.97   | 0-0.3    | <0.001    |
| Group 2   | 40             | 0.405±1.04  | 0.2-0.6  |           |
| Group 3   | 40             | 0.668±1.27  | 0.4-0.9  |           |

Table 3: Comparison of mean thiocyanate in Group 1, Group 2 and Group 3

| Group     | Number of cases | Thiocyanate | \( p \) |
|-----------|----------------|-------------|--------|
|           |                | Mean SD     |        |
| Group 1   | 40             | 1.17 0.51   | <0.001 |
| Group 2   | 40             | 3.51 0.91   |        |
| Group 3   | 40             | 5.31 0.51   |        |

Table 4: Comparison of mean thiocyanate and micronuclei group

| Micronuclei | Number of cases | Thiocyanate | \( p \) |
|-------------|----------------|-------------|--------|
|             |                | Mean SD     |        |
| 0           | 9              | 0.61 0.18   | <0.001 |
| 1-3         | 42             | 1.66 0.80   |        |
| 4-6         | 48             | 4.30 0.83   |        |
| >6          | 21             | 5.61 0.19   |        |

**DISCUSSION**

Cancer is one of the most life-threatening diseases afflicting humankind. The word “cancer,” in itself, generates fear
among all human beings, to whichever strata of society they may belong.[14] It is often diagnosed at an advanced stage because of the lack of early diagnostic markers, and therefore, the survival rate is markedly reduced despite the best available treatment options.

The majority of human cancers are caused by tobacco and synthetic and natural chemicals of occupational, environmental, medical, and dietary origin.[15] Tobacco smoke consists of a mixture of combustion gases and particulate matter. Inhaled cigarette smoke contains hydrogen cyanide, which is metabolized to thiocyanate. Thus, thiocyanate is an end-product of detoxification of hydrogen cyanide present in cigarette smoke. Thiocyanate has a property to induce cancerous changes in epithelium as it is secreted in saliva, has a long half-life, and has continuous contact with epithelium through the saliva.[16]

These chemical carcinogens such as thiocyanate present in tobacco can cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities.[17] The MN are extranuclear cytoplasmic bodies associated with chromosomal aberrations. These are induced in oral exfoliated cells by a variety of substances, including genotoxic agents and carcinogenic compounds in tobacco, betel nut, and alcohol. The induction of MN cells by carcinogens and mutagens is a sign of the genotoxic effect of such substances.[18] The damaged chromosomes, in the form ofacentric chromatids or chromosome fragments, lag behind in anaphase when centric elements move toward 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 principle nucleus and are therefore called MN.[19] Bigger MN result from exclusion of whole chromosome following damage to the spindle apparatus of the cell (aneugenic effect), whereas smaller MN result from structural aberrations causing chromosomal fragments (clastogenic effect).[20] Thus, the present study was conducted to evaluate the association of occurrence of MN and the levels of salivary thiocyanate in smokers and nonsmokers. Wahi pointed out that heat application on the mucosa has a definite effect in enhancing the action of tobacco smoke on the mucosal cells.[21] The quantum of consumption of cigarettes and the quality and volume of tobacco also act as factors responsible for the increased genotoxicity in the affected mucosa, thereby causing an increase in the MN cell counts of an individual.[22]

Thus, the present study evaluated the levels of salivary thiocyanate and occurrence of MN in smokers and nonsmokers and found a positive association between salivary thiocyanate and occurrence of MN.

CONCLUSIONS

The present study demonstrated that among smokers, a significant increase in the levels of salivary thiocyanate was observed with increase in the count of MN, suggesting a strong association between the two. Thus, these new biomarkers are noninvasive, painless and prove to be an efficient tool in screening a large population as well as in aiding motivation of individuals for withdrawal of tobacco smoking. They can be extremely promising and should hopefully change the paradigm of oral cancer diagnostics.

Further studies are required to determine the value of these biomarkers in monitoring various treatment modalities and can also be used in finding out the risk of field cancerization.[22]

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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