Original Article

Comparison of Repair Index in Cigarette and Waterpipe Smokers: A Bio-Monitoring Assessment Using Human Exfoliated Buccal Mucosa Cells

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Abstract

Background: Repair index (RI) using DNA changes reveals the activity of carcinogenesis. Cigarette and waterpipe smoking are important contributors to oral cavity malignancy. The RI in cigarette and waterpipe smokers has not been compared so far. The aim of this study was to compare the RI in cigarette and waterpipe smokers using the human exfoliated buccal mucosa cells. **Methods:** The exfoliated buccal mucosa cells of 60 cigarette and waterpipe smokers and 20 nonsmokers were evaluated in a case-control study. The number of micronuclei (MN), broken egg (BE), karyorrhexis (KR), and karyolysis (KL) were counted in 1000 cells from randomly selected fields. The RI = (KL + KR)/(MN + BE) was calculated and compared between subjects and controls. Data were analyzed by one-way analysis of variance (ANOVA), Tukey's Honest Significant Difference (HSD), and Spearman's correlation coefficient test at $P < 0.05$ probability level. **Results:** The difference of MN ($P < 0.0001$), BE ($P < 0.0001$), KR ($P < 0.0001$), and KL ($P < 0.0001$) count was significant between cigarette smokers, waterpipe smokers, and nonsmokers. The RI was significantly different between groups ($P = 0.007$). The RI was significantly higher in cigarette smokers compared to waterpipe smokers ($P = 0.04$) and nonsmokers ($P = 0.009$). **Conclusions:** The RI was significantly higher in cigarette smokers compared to waterpipe smokers. The finding suggests that due to higher interrupted cellular hemostasis, the risk of carcinoma in waterpipe smokers can be greater than that in cigarette smokers.

Keyword: *Micronucleus assays, mouth mucosa, smoking*

Introduction

Biological markers display the biological effects of genotoxic and cytotoxic agents on cells and simply demonstrate the susceptibility to the development of cancers. DNA alterations that show with micronucleus (MN) count and cellular death features are reliable biomarkers to evaluate the cellular/nuclear changes.[1]

The exfoliated buccal mucosa is a good source to biomonitor the impact of genotoxic agents. For the first time, Stich *et al*., outlined the effect of genotoxic exposure on exfoliated buccal cells using MN count.[2] MN are formed from chromosome fragments or aberrant chromosomes. The generation of MN corresponds to aneuploidy and activation of oncogenes, which leads to malignancy.[3] Cellular death stimulates by mutagenic agents and damaged cells are removed.[4] A degenerated nucleus passes from clastogenic, mutagenic, and carcinogenic steps. During a genotoxic event, MN improves from genomic instability. Developing a broken egg (BE) is a former step of MN formation. Throughout apoptosis, the nucleus follows karyorrhexis (KR) and karyolysis (KL) stages, which are consistent with the disintegration and dissolution of nuclear material. The repair index (RI) = (KL + KR)/(MN + BE) reveals the epithelial cells homeostasis. Disruption of the steady-state of the cell leads to carcinoma.[5]

Cigarette and waterpipe smoking are important contributors to develop proliferative lesions and oral cavity malignancy. Studies showed a higher count of MN and cellular death features in exfoliated buccal mucosa of cigarette and waterpipe smokers than nonsmokers.[6-8] However, the knowledge on the cellular

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dynamics of carcinogenesis in smokers is very scant.[9] RI using MN count and cellular death frequency provides a simple assessment of cellular dynamics. Considering the higher cellular changes in both cigarette and waterpipe smokers, the RI may be different from nonsmokers. RI in cigarette and waterpipe smokers has not been compared so far. The aim of this study was to investigate and compare the RI in cigarette and waterpipe smokers using the human exfoliated buccal mucosa cells in a biomonitoring assessment.

Methods

This was a case–control study and was completed in the faculty of Dentistry, Shahed University during 2018–2019. Eighty subjects comprising 30 cigarette smokers, 30 waterpipe smokers, and 20 nonsmokers who never smoked waterpipe and cigarette were enrolled in the study. Male subjects aged 20-50 years were enrolled in the study. Smokers who left the smoking in past three years, and less than three years smokers,[10] subjects who suffered from oral and systemic disease, drugs and/or alcohol consumers, subjects who exposed to dental radiography beam in recent 6 months, laborers' who work with industrial materials and pesticides were excluded.

The number of smoked cigarettes per year and the number of smoked waterpipe per year (Pack × years = PY) were registered. The protocol of the study was approved by the ethics committee of Shahed University and take the number IR.SHAHED.REC.1397.099. Participants gave signed inform consent and, their names and families were kept secret.

Sampling

Exfoliated mucosal cells from left buccal mucosa were scrapped using a sterile, disposable plastic spatula and spread onto the glass slides. Samples were fixed with a fixative (methanol and glacial acetic acid in a ratio of 3:1) for 30 minutes and then dried at room temperature. Before sampling, the mouth was rinsed twice with water. Samples were stained with the Feulgen method based on the modified method of Thomas *et al*.[11]

The number of MN, BE, KR, and KL were counted in 1000 cells from randomly selected fields.[9] Cellular count was completed using an optic microscope (OLYMPUS BX40) equipped with a digital camera (Sony ExWaveHAD, Model No. SSC-DC58AP; Tokyo, Japan).The fields with higher clusters of cells with distinctive cellular margins were selected. The overlapped cells were not evaluated. The features were counted blind at 400× (10× ocular and 40 × objective lenses) magnification. The RI = (KL + KR)/(MN + BE) was calculated and compared in subjects and controls.[5]

Based on the study by Tolbert *et al*.[4], disintegrated nucleus and dissolved nucleus were considered as KR and KL features, respectively. The cytoplasmic structure with 1/3 to 1/5 size of the nucleus and nuclear stain was considered as MN.[12] A structure smaller than a nucleus that was connected to it by a filament was considered as broken egg (BE) [Figure 1].[13]

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), Tukey's Honest Significant Difference (HSD), and Spearman's correlation coefficient test at $P < 0.05$ probability level. The Statistical Package for the Social Sciences (SPSS) statistical software package (Version 25; IBM Company, Chicago, IL, USA) was used.

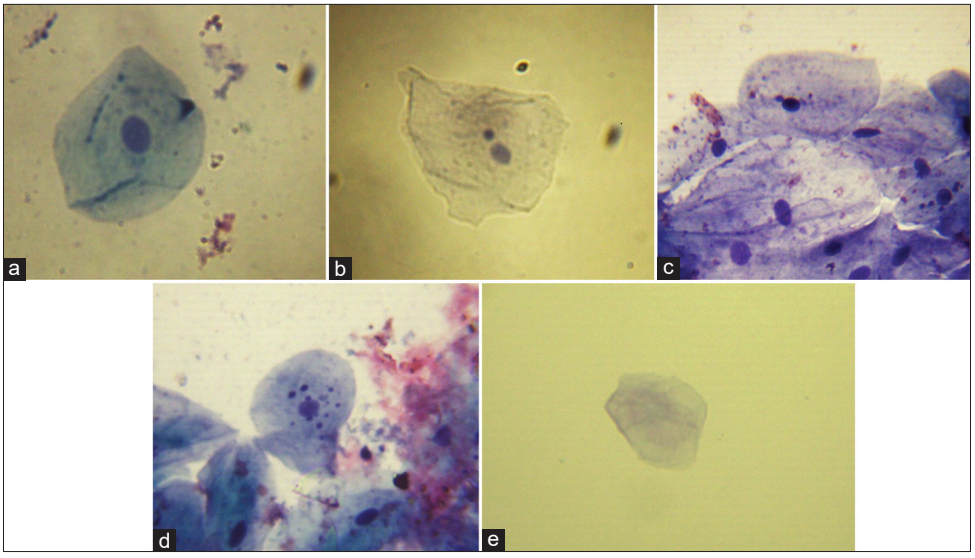

Figure 1: DNA alterations (Micronucleous and Broken egg) and cellular death features (Karyorrhexis and Karyolysis) of exfoliated human buccal cells (Feulgen staining, ×400), (a) Normal cell, (b) Micronucleous, (c) Broken egg, (d) Karyorrhexis, (e) Karyolysis

Table 1: The frequency (Mean±SD) of repair index and nuclear features in cigarette smokers, waterpipe smokers and nonsmokers

	Micronucleus	Broken egg	Karyorrhexis	Karyolysis	Repair index
Cigarette smokers	4.28±3.58	0.25±0.43	0.37±0.75	5.78±4.35	2.46±3.05
Waterpipe smokers	9.16±2.80	5±0.62	3.96±1.77	6.16±4.75	1.18±1.26
Nonsmokers	3.05±2.79	0.20±0.52	0.15±0.67	1.60±1.14	0.64±0.80

Table 2: Difference between nuclear features within smokers and nonsmokers using Tukey's Honest Significance Difference

Nuclear features	Cigarette smokers vs. nonsmokers	Waterpipe smokers vs. nonsmokers	Cigarette smokers vs. Waterpipe smokers
Micronucleus	0.35	< 0.0001*	< 0.0001*
Broken egg	0.21	< 0.0001*	< 0.0001*
Karyorrhexis	0.79	< 0.0001*	< 0.0001*
Karyolysis	0.001*	< 0.0001*	0.924
Repair index	0.009*	0.64	0.04*

*The mean difference is significant at the 0.05 level

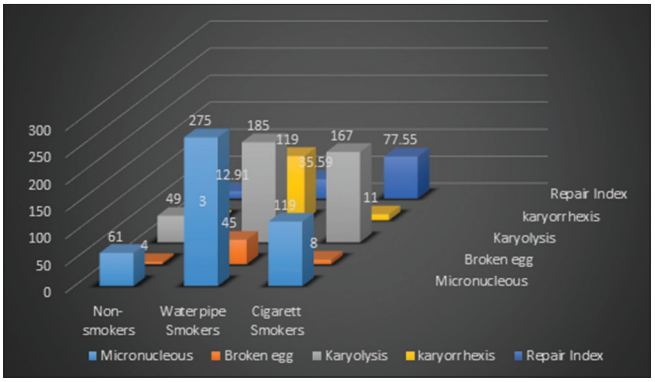

Figure 2: Distribution the total number of nuclear features per 1000 cells

Results

The mean ages of cigarette smokers, waterpipe smokers, and nonsmokers were 30.34 ± 9.97, 26.8 ± 3.7, and 26.2 ± 8.16 years, retrospectively. Table 1 shows the frequency of nuclear features and RI in cigarette smokers, waterpipe smokers, and nonsmokers. Figure 2 demonstrates the distribution of nuclear features per 1000 cells.

The one-way ANOVA revealed a significant difference between cigarette smokers, waterpipe smokers, and nonsmokers in terms of MN ($P < 0.0001$), BE ($P < 0.0001$), KR ($P < 0.0001$), and KL ($P < 0.0001$) count. The RI was significantly different between groups ($P = 0.007$).

The Tukey HSD revealed that the count of MN, BE, and KR were significantly higher in waterpipe smokers compared to cigarette smokers ($P < 0.0001$, $P < 0.0001$ and $P < 0.0001$, respectively) and nonsmokers ($P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively). KL was significantly higher in cigarette smokers and waterpipe smokers compared to non-smokers ($P < 0.0001$). The RI was significantly higher in cigarette smokers compared to waterpipe smokers ($P = 0.04$) and nonsmokers ($P = 0.009$) [Table 2].

Using Spearman's correlation coefficient test, in cigarette and waterpipe smokers, a significant correlation was found between RI and MN count ($P = 0.018$ and $P < 0.0001$, respectively), and KL ($P < 0.0001$ and $P < 0.0001$, respectively). In waterpipe smokers, the correlation between RI and KR was significant ($P = 0.002$). In nonsmokers, significant correlation was indicated between RI and KL ($P = 0.001$) and KR ($P = 0.001$) [Table 3].

Spearman's correlation coefficient test showed that the correlation between the RI and exposure time to smoke (P × Y) was significant in cigarette smokers ($P = 0.03$). The difference was not significant in waterpipe smokers ($P = 0.55$).

Discussion

The findings showed that the RI in cigarette smokers was significantly higher compared to waterpipe smokers. The RI in those who were not smokers was significantly lower than that in both cigarette and waterpipe smokers.

MN is a chromosomal fragment isolated from the nucleus during mitosis. The presence of MN has been suggested as an indicator for displaying the genotoxic and carcinogenic effects. Various studies have used lymphocytes and exfoliated oral mucosa cells to investigate the damage of human genome due to various environmental and chemical factors.[14,15] The findings of the study showed that MN count was significantly higher in waterpipe smokers compared to cigarette smokers and controls. This finding is consistent with that of El-Setouhy *et al*.[6] where MN counts are used in human buccal mucosa and that of Khabour *et al*.[16], which is based on the evaluation of sister chromatid exchanges on lymphocytes of waterpipe smokers.

BE,[4] also referred to nuclear blebbings[17] and nuclear buds[18] is a nuclear structure resemble a MN attached to the nucleus with a narrow stalk. Since BEs are formed before MN at the time of cell division, researchers believe that in addition to MN, a quantitative count of BE is also necessary.[5] Findings showed that the number of BEs was higher in waterpipe smokers compare to cigarette smokers and controls. The higher number of MN and BE in waterpipe smokers suggests a higher risk of genomic variation in waterpipe smokers compare to cigarette smokers.

Table 3: Correlation between nuclear features within smokers and nonsmokers (Spearman's correlation coefficient test)

Nuclear features	Micronucleus	Broken egg	Karyorrhexis	Karyolysis	Repair index
Micronucleus	1.000	0.504**	0.615**	0.113	-0.199
Broken egg	0.504**	1.000	0.720**	0.248*	0.081
Karyorrhexis	0.615**	0.720**	1.000	0.421**	0.248*
Karyolysis	0.113	0.248*	0.421**	1.000	0.796**
Repair index	-0.199	0.081	0.248*	0.796**	1.000

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed)

Waterpipe smoking increases free radicals in the body. Free radicals can cause oxidative damage to DNA and cause chromosomal alterations.[19] The amount of toxic material that enters the oral cavity in every time of waterpipe smoking is equivalent to 10 cigarettes.[20] Since the average time of waterpipe smoking is longer than cigarette smoking; it can be expected that the waterpipe will accompany higher genotoxic effects.

Cytotoxic agents of chemicals cause cellular death. These changes are observed in cytologic studies in the forms of a decomposed nucleus (KR) and a non-nucleated cell with a vanished margin (KL).The findings of the study showed that KR and KL were higher in waterpipe and cigarette smokers compared to controls. This is consistent with the findings of previous studies that have shown the higher rate of KR and KL in cigarette smokers than that in nonsmokers.[7,9,21]

Passing a mutagenic event to a carcinogenic process is associated with quantifying changes in the MN count and an increase in cell death. The RI = (KL + KR)/(MN + BE), reflects the dynamics of the squamous epithelial cells. The higher rate of KL and KR, reflects the effort of the cell to repair genomic damage.[5] A higher count of KL, which is the last stage of cell death, has been recognized as an adaptive process in response to cytotoxic damage.[22] Ramirez and Saldanha believed that the continued impact of genotoxic substances increases cell death.[5] The findings showed that KL was significantly higher in smokers compared to controls, but there was no significant difference between waterpipe and cigarette smokers. The MN count was higher in waterpipe smokers than that in cigarette smokers, but KL did not differ significantly. This finding indicates that both cigarettes and waterpipe cause apoptotic changes in human buccal mucosa but the disruption of the dynamic balance of cells, which can increase the risk of cancer, was higher in waterpipe smokers.

The results showed that the RI was significantly correlated with the $P \times Y$ of cigarette smoking, while the RI was not related to the duration of waterpipe smoking. This finding, in line with previous studies,[7,9] indicates that the genotoxic effects of waterpipe are higher than cigarettes and that the likelihood of apoptosis increased by waterpipe smoking. The cumulative effect of this event may be associated with carcinogenic results. Waterpipe smoke contains toxic substances such as carbon monoxide and heavy metals. Some of these substances, such as carboxyhemoglobin, are three times higher than cigarettes.[23,24] Therefore, waterpipe smoke has greater cytotoxicity than cigarette smoking.

Studies examining the MN count have demonstrated that exposing to various chemical agents such as tobacco,[6,7,9,25] asphalt smoke,[1] materials used for carpet production,[13] construction-industrial paints,[26] arsenic in the glass industry[27] and gasoline[28] can disrupt dynamic of cells. Based on studies, MN count can be a simple bio-monitoring assessment of various chemical agents. The RI by showing the dynamic relationship between the MN count and apoptosis can be a useful indicator in determining the effect of genotoxic factors and even disease course of patients with oral carcinoma during the disease and after treatment.[5,29] This study was completed on male subjects, it is suggested that future studies assess the effect of tobacco smoking in females and younger age of subjects.

Conclusions

MN count was higher in waterpipe smokers compared to cigarette smokers, but KL was not significantly different from cigarette smokers. The findings showed that the RI in cigarette smokers was significantly higher compared to waterpipe smokers. The finding suggests that waterpipe smoking can disrupt the dynamic balance of the squamous cell. Due to higher interrupted cellular hemostasis, the risk of carcinoma in waterpipe smokers can be greater than that in cigarette smokers.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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