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THE MICRONUCLEUS ASSESSMENT OF BUCCAL MUCOSA: A NONINVASIVE METHOD IN SCREENING OF SMOKERS POTENTIALLY EXPOSED TO ORAL CANCER

JAHANFAR JAHANBANI[1], DONIA SADRI[2], SHILAGHASEMI MORIDANI[3], SAREH FARHADI[4*]

1, 2: Associate professor, Oral & Maxillofacial Pathology department, Dental branch of Tehran, Islamic Azad University, Tehran, Iran

3. Oral & Maxillofacial Pathologist, Toronto, Canada

4. Assistant professor, Oral & Maxillofacial Pathology department, Dental branch of Tehran, Islamic, Azad University, Tehran, Iran

*Corresponding author: Sareh Farhadi: No.:177, 5thGolestan st., Pasdaran st., Tehran, Iran; Email: dr.sfarhadi@gmail.com; Tell:+982122542238

Key Words: Micronucleus; smoking; cancer; oral; Buccal mucosa

ABSTRACT

Background: Micronucleus is a chromatin structure in cytoplasm which can be an indicator of chromosome damage and carcinogenesis. The present study assessed micronuclei in exfoli ated oral mucosal cells of heavy smokers.

Methods: This historical cohort study was performed on 48 individuals (24 smokers and 24 n on-smokers) whowere selected according to gender an age. The age of non-smokers equaled 34.42±16.38 and the age of smokers (case group) equaled 36.42± 16.17. Buccal mucosa sme ar was obtained with a moist wooden spatula and after placing the smear on glass slides and Papanicolaou staining, 1000 cells of each sample were inspected under light microscope and cells with micronucleus were counted. Data were analyzed by T-test and level of significance was set as lower than 0.05.

Results: The percentage of micronucleus was 21.21±2.18 and 3.5±1.17 respectively and this difference was determined to be statistically significant based on T-test and with 99% confide nce level. ($p<0.01$)

Conclusion: The average frequency of micronucleus was higher in smokers compared to non - smokers in this study, therefore this noninvasiveassessmentmay help in early diagnosis of or al cancer.

Key words: Micronucleus; smoking; cancer; oral; Buccal mucosa

INTRODUCTION

Oral cancer is one of the ten most common human cancers with 575000 new cases and 320000 mortalities per year worldwide. [1] The prevalence of oral cancer in the world i s often correlated with the pattern of tobacc o products consumption and a dose-respons e relationship existsbetween the level of co nsumption of tobacco products and prevale nce of oral cancer [2]. Oral cancer has a co mplex karyotype which is accompanied by many types of chromosomal deletion, transl ocation and chromosome abnormalities. Oft en, an error occurs in chromosome separati on so that chromosome or part of it is left b ehind from the nucleus and remains in the c ytoplasm in anaphase which is called micro nucleus. [3] Micronucleus assay in buccal mucosal cells has been used for evaluation of chromosome damages and can help in ea rly diagnosis of malignancies with simple b iopsy and minimum invasion without causi ng stress to individuals. The advantage of t his method is its simplicity because micron ucleus assay is quick and does not require a ny specific specialty. Kamboj et al.showed that evaluation of epithelial cells of buccal mucosa is a reliable marker in early diagno sis of premalignant and malignant lesions,[4] in a way that assessment of micronuclei i n exfoliated epithelial cells of mucosa has b een proposed as a method for specification of cancer risk. [3,5,6] in another study in 20 11, number of micronuclei in oral mucosal cells of malignant and potentially malignan t groups was significantly higher than that o f the control group and it has been proposed to use this method for prognosis screening and also as an instructive method in public centers. [7] This genomic damage can occu r due to exposure to genotoxic substances, medical interventions (radiation and chemic al substances), micronutrients deficiency (f olic acid), life style (alcohol, cigarettes, nar cotics and stress), air pollution, chronic con tact with arsenic and chromium and genetic factors such as defect in DNA metabolism or repair. [8] Because of the importance of micronucleus assay, Human Micronucleus Project (HUMN) was held in 1997 to stand ardize the assessment of this index in perip heral blood lymphocytes and the results of t his project have been published [9, 10, 11]. Based on our knowledge from available res ources, till now only few studies assessed t he diagnostic value of the mentioned metho d in smokers.[12] Therefore, considering th

e importance of early diagnosis of cancers, the present study was performed to evaluate the relation between cigarette smoking and number of micronuclei and to compare the results with that of non-smokers.

MATERIALS AND METHODS

This study was performed on 48 individuals including 24 subjects in the control group (non-smokers) and 24 subjects in the case gr oup (smokers) with a history of smoking fo r more than 5 years and more than 20 cigar ettes per day which are considered as heavy smokers. [13] Subjects in both groups were males residing in Tehran and were similar regarding socio-economic status and time o f assessment and were homogenized regard ing age. Subjects with recent viral disease, medicine consumption, alcohol consumptio n, addiction to narcotics, high risk occupati ons such as paint industry and radiotherapy were excluded i.e. selected smoking subject sonly smoked cigarettes and non-smokers were completely normal.

After receiving an informed consent, subjec ts were asked to rinse their mouth with wat er. Buccal mucosal cells were scrapped by a moist spatula under medium pressure and were placed on clean glass slides and were fixed with path fix spray. Standard Papanic olaou staining was used for micronucleus a ssay. 1000 Cells were inspected under light microscope (Nikon, Japan) with ×400 and ×1000 magnification. The criteria by Tolbe rt et al. were adapted for micronucleus coun t, [14, 15] which consists of the followings: 1- Round and smooth periphery which indi cates a membrane. 2- Less than 1/3 of the d iameter of the nucleus but big enough for di stinction of shape and color. 3- Color intens ity similar to the nucleus. 4- Consistency an d texture similar to the nucleus. 5- Focal le ngth similar to the nucleus. 6- No connectio n or overlapping with the nucleus. Cells wit h distinctive margins and nuclei were count ed. Micronuclei were not counted in areas with cell overlap. Also, dead and degenerat ed cells and nuclear bubbles were excluded from cell count. Finally, data were analyzed by T-test and level of significance was set as lower than 0.05.

RESULTS

The age of non-smokers equaled 34.42±16. 38 and the age of smokers (case group) equ aled 36.42± 16.17. Chi-square test showed t hat the two groups have no significant age difference. T-test was used for statistical an alysis and $p<0.01$ was considered as statisti cally significant. Mean, standard deviation and mean difference of data were calculate d. The frequency of micronucleus are prese nted in table 1 based on case and control gr oups and show that the minimum percentag e of micronucleus is in non-smokers (2.4) a nd this value equaled 9.9 in smokers with st atistical significant difference between the t wo groups. ($p<0.01$). Figure 1 shows cell w

it-hmicronucleus.

The average frequency of micronucleus in s tudy groups is presented in table 2.

The percentages of micronucleus per 1000 counted cells divided by case and control gr oups are presented in diagram 1.

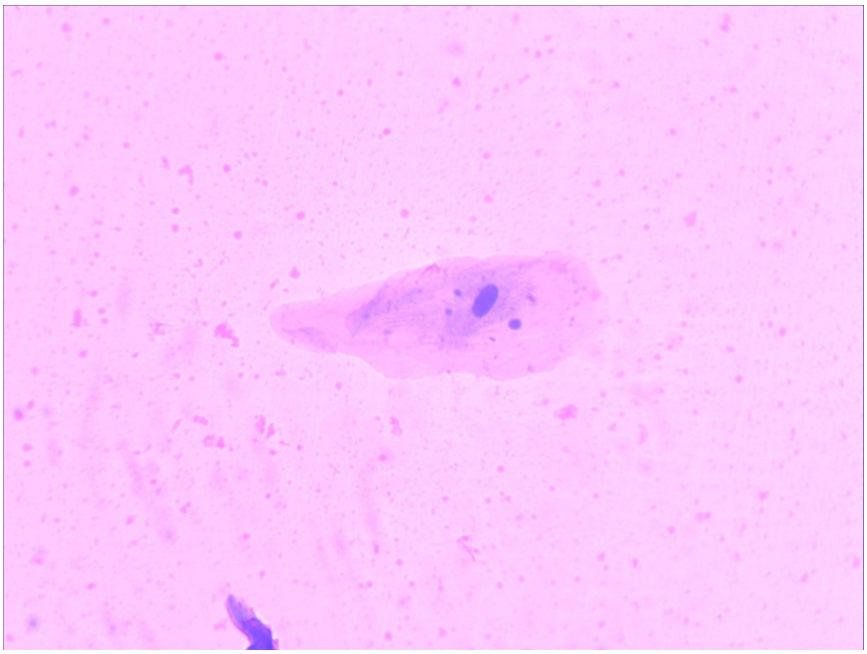

Figure 1 – Micronucleus in Papanicolaou staining (×1000 magnification)

Table 1 – Percentage of micronucleus based on case and control groups

Micronucleus / Cigarette smoking	Amount	Minimum-Maximum	Variation coefficient
Non-smoker (control) (N=24)	3.5±1.17	2.4 – 6.1	33.43
Smoker (case) (N=24)	12.12±2.18	9.9 – 18.5	17.99

Table 2 – Average number of micronucleus in study groups

Group	Sample size	Average number of micronucleus per 1000 counted cells in each sample	T-test result P< .01
Smoker	**24**	**121.25**	**Significant**
Control	**24**	**35**	

DISCUSSION

Considering the heavy expenses of therapeutic and rehabilitating procedures of malignant lesions, the path of recent studies has been directed towards prevention and early diagnosis of these lesions. In this regard, the present study assessed micronucleus frequency in buccal mucosa of heavy smokers and showed that the average percentage of micronucleus in smokers was significantly higher than that of non-smokers. This finding is in line with

previous studies which had shown that smoking elevates micronucleus frequency. [2, 3, 5, 12, 16, 18]. In other studies, smoking either had no effect on micronucleus frequency (20) or even decreased micronucleus in some cases. [21] These studies were performed on peripheral blood lymphocytes and it should be considered that the level of effective chemical substances in cigarettes may be lower in peripheral blood compared to other organs. In addition, smoking can damage lymphocytes in a way that they will be unable to survive or lose the ability to divide during stages of cell culture with cytokinesis-block micronucleus (CBMN) method. [11] In our study, smokers with a history of at least 5 years of smoking and at least 20 cigarettes per day which are considered as heavy smokers [1] were evaluated to better specify the effect of toxic substances of cigarette on production of micronucleus. In a comprehensive study by Bonassi et al. micronucleus frequency in peripheral blood lymphocytes of light or moderate smokers showed no significant increase. While in heavy smokers this increase was significant. [11]Bonnasi et al. mentioned that in 33 researches among 37 published articles, no correlation was found between cigarette smoking and micronucleus increase. But in the majority of these studies heavy smokers have not been considered which can justify the neutral effect of cigarette smoking in these studies. [11] But in our study, smokers had smoked at least 20 cigarettes per day for more than 10 years. Nevertheless, note that the level of effective chemical substances of cigarette in peripheral blood may be lower than other organs and this can justify the lack of increase in micronucleus frequency in light smokers when micronucleus assay is performed on peripheral blood cells [11]. JalayerNaderi et al. performed a study in 2012 entitled micronucleus assay of buccal mucosa cells in smokers of Iranian population with a history of smoking less and more than 10 years and compared the results with that of non-smokers and used Feulgen staining. They concluded that the percentage of micronucleus and also the average number of micronuclei in each cell in non-smokers are significantly lower than that of smokers. Although, the average number of micronuclei of oral mucosa cells was higher in smokers with more than 10 years of smoking compared to less than 10 years of smoking, but this difference was not significant. [12] Kamath et al. showed that individuals with a history of 5 to 10 years of smoking had more micronuclei than individuals who had smoked more than 10 years or less than 5 years. [2] But no such correlation was seen in our study. In the

present study, for example a 26-year-old individual who had smoked for 12 years had 185 micronucleus but a 73-year-old individual with a history of 40 years of smoking had 132 micronucleus. This difference can be due to the level of cell resistance against toxic elements in different individuals and also due to harmful factors other than cigarettes which of course this issue needs more evaluation. In addition, it is worth mentioning that the present study was performed on heavy smokers, while in other studies either heavy smoking criteria were not mentioned or subjects were not heavy smokers. Nevertheless, Wu et al. showed that a significant correlation exists between number of cigarettes per day and number of micronuclei and heavy smokers have more micronuclei in oral mucosa [22]. The applied staining methods for micronucleus specification in exfoliated oral mucosal cells have been different in various studies and include Fulgen-Fast green, fluorescent staining such as Diamidino-2-phenylinodole DAPI, Hoechst, Acridine orange, Propidium iodide, May-Grunwald Giemsa and Papanicolaou. [1] It seems that Feulgen-Fast green staining is of higher interest among researchers due to its specificity for DNA and clear staining of cytoplasm which eases micronucleus count. [1] Nevertheless, Papanicolaou staining has been used in many recent studies [2, 3, 23] including the present study with acceptable results. This method neither needs complex procedures such as cell culture or preparation of metaphase nor requires DNA-specific staining [8]. In other studies, mostly cigarettes and other forms of tobacco have been compared or study participants were using multiple substances while in the present study, only the effect of cigarette smoking on the average number of micro nucleated cells was assessed. Because based on the results of some researches when participants use multiple substances, for example cigarette and chewing tobacco, interaction between these substances causes nucleus degeneration and an appearance similar to micronucleus structure which affects the study results. [12] In the present study, confounding factors especially alcohol consumption were eliminated and the synergic effect of these two substances on the number of micronuclei was omitted and in this way, significant increase in number of micronuclei in smokers was more decisively correlated to cigarette smoking. It is clear that the synergic effect of cigarette smoking and alcohol consumption reaches to more than 5.5-fold [24]. Bansal et al. assessed micronuclei in tobacco consumers of Indian Punjabi population in 2011 with Papanicolaou staining method

and concluded that micronucleus in smokeless tobacco consumers are significantly more than that of smokers and the control group. Also, the number of micronuclei was higher in smokers compared to the control group. [3] Studies performed by Motgi et al. [23], Patel et al. [25] and Palaskar et al. [26] also revealed similar results and indicated the higher carcinogenicity of smokeless tobacco compared to cigarette. In another study, Caplash et al. assessed micronucleus in exfoliated oral mucosa cells of chewing tobacco users, smokers and the control group. They concluded that micronucleus frequency in tobacco consumers was significantly higher than the control group. Two interfering factors of age and exposure duration showed a significant correlation, while the third interfering factor (alcohol) showed no significant correlation. [19] On the other hand, in some studies controversies are seen among the results regarding the gender of patients in a way that some studies reported higher frequency of micronucleus in males and others reported it higher in females. [27-29]This controversy also exists regarding the age of individuals. [17, 28, 30, 31] In the present study, all subjects were males and were homogenized regarding their age to omit the effect of age and gender on the results. Although Sarto et al. reported that micronucleus frequency due to chromosome breakage is doubled in smokers compared to non-smokers [16] but in our study this amount was approximately 3.5-fold. This difference and other mentioned differences can be due to different staining methods, sample sizes and assessment of different smokeless and smoking substances. Note that the chemical composition of cigarettes of different manufacturers and that of smokeless substances are highly different. Therefore, more definite results require standardizing of applied methods. All mentioned studies and the present study show that unusual oral habits increase micronucleus (4) In other words, micronucleus assay can be applied as a quick and simple index and a valid biomarker for early diagnosis of premalignant and malignant oral lesions. Nevertheless, further researches with more precise techniques are required in this regard.

CONCLUSION

This study showed that the frequency of micronucleus in buccal mucosa of heavy smokers is significantly higher than that of non-smokers with 3.5-fold increase. ($p<0.01$) therefore, this noninvasive assessment may help in early diagnosis of oral cancer. Nevertheless, to reach justifiable and reliable results, larger groups should be assessed for longer time periods. Usually, a limited number

of patients are willing to reveal duration an d frequency of cigarette smoking and other habits. Also, buccal cells should be reassess ed after habit weans to see whether or not micronuclei have decreased in number. In a ddition, other genetically harmful factors in the living environment of each individual s hould also be considered.

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CONFLICT OF INTEREST:

The authors have no conflict of interest in t his study.

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