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Original Article

Nuclear Changes Features in Buccal Mucosa Smear of Adult Male Smokers Using Pappaniculou Staining

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Abstract

Background: Tobacco smoke contains a combination of chemicals that could be harmful to the buccal mucosa as the first part of the body that has been exposed. Damage to the buccal epithelial cells has the potential to become a malignant lesion. This study aimed to describe changes in the nuclear epithelial of the buccal mucosa using Pappaniculou staining as an indicator of mucosal damage in smokers.

Methods: This was a descriptive analytical study, involving adult male participants from Bale Endah District, Bandung Regency, Indonesia aged >35 years, who had smoked for ≥10 years. A buccal mucosa smear was taken, and the features of nuclear epithelial changes were observed per 500 cells, each at 400x magnification with Pappaniculou stain to evaluate the features of micronucleus, broken egg, karyorrhexis, karyolysis. Those who did not smoke were recruited as a control group.

Results: Smokers were mostly light active smokers or kretek cigarettes, with a smoking duration of ≥15 years. The frequency of micronucleus ($p<0.001$), broken eggs ($p<0.001$), karyorrhexis ($p=0.001$), and karyolysis ($p=0.003$) in the buccal mucosal epithelial was significantly different between the smoker and non-smoker groups.

Conclusion: All epithelial nuclear changes have shown significant differences between smoker and non-smoker groups. Nuclear epithelial features in smokers may be associated with future malignancies, therefore, smoking cessation programs are necessary to substantially reduce tobacco use, thus fostering a healthy lifestyle for everyone.

Keywords: Buccal smear, nuclear changes, smokers

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Introduction

Cigarettes are products made entirely or partly from tobacco leaves, which are then burned, smoked, inhaled, or chewed. Cigars, white cigarettes, and clove cigarettes are typical tobacco products.[1] In Indonesia, the majority of adult smokers are men (33.5%).[2] West Java is one of the provinces with the highest number of smokers. In 2021, 23.52% of Bandung residents were active smokers.[3,4] Smoking is the world's most common cause of cancer and increases the risk of developing the disease in men and women aged 30 to 60 years.[5,6]

There are 70 of the 5,300 compounds contained in cigarette smoke that are carcinogenic.[7–9] The combination of chemicals produced from the combustion of cigarette smoke can harm the body. Reactive oxygen species (ROS), cytokines, chemokines, and prostaglandins as a result of deoxyribonucleic

acid (DNA) damage, inflammation, and oxidative stress.[7,10] induced the process of cell invasion, epithelial-mesenchymal transition (EMT), and proliferation tumor development.[10,11] Oral cancers, including cancers of the gums, tongue, palate, buccal, and tonsils, have been related to smoking. Patients with cancer usually have problems with the tongue and buccal mucosa.[12] The mucosa supports the defense system that surrounds the oral cavity.[13]

Nuclear cells appear altered when buccal mucosa changes are observed under the microscope. Oral exfoliative cytology is one of the basic, non-aggressive, and generally painless procedures that may be used to diagnose potentially malignant and malignant oral illnesses in tobacco chewers. The collection of exfoliated buccal cells is a simple, painless, and least invasive method that can be performed for mass screening and regular follow-up of potentially malignant disorders in humans caused by environmental exposures, such as tobacco and alcohol. Buccal mucosa smear provides the least invasive method for detecting pre-cancerous to malignant lesions of the oral cavity by evaluating changes in buccal cell nuclei, such as micronuclei, broken eggs, karyorrhexis, and karyolysis.[14]

Micronuclei and other nuclear abnormalities are examples of early microscopic alterations in the buccal mucosa. Micronucleus is a cell nucleus that surrounds the main cell nucleus with a one-third size of the main cell nucleus. It is oval, sharply outlined, and the same color as the main cell nucleus. Extensive chromatin aggregation characterizes a type of apoptosis called karyorrhexis, which fragments and disintegrates the cell nucleus.[14] The breakdown of cell chromatin defines the presence of karyolysis and represents the rate of cell death. Broken egg refers to changes in the nucleus of the cell leading to the phenomenon of cell nucleus rupture.[15,16] This study was conducted to observe the nuclear changes of buccal smears as an impact of smoking habits on the buccal mucosa cells of adult male smokers.

Methods

This study was a descriptive-analytical study comparing nuclear changes between 20 male smokers and 20 non-smokers at the Universitas Islam Bandung Laboratory, Indonesia conducted in July–August 2023. Secondary data on patient characteristics were obtained through questionnaires that met the inclusion criteria, namely male aged 35 years and over who had smoked for at least 10 years. The exclusion criteria were that participants did not have a history of systemic diseases such as diabetes mellitus, hypertension, and heart disease, no history of alcohol consumption and the habits of eating and drinking hot drinks. The inclusion criteria for non-smoker participants were male, aged 35 years and over, and had never smoked actively before.

Before filling out the questionnaire, participants were asked to read and provide informed consent. The questionnaire given to participants contained several questions about participant characteristics (name, date of birth, address) and smoking habits (smoking status, number of cigarettes per day, type of cigarette, duration of smoking). Researchers assisted participants in filling out the questionnaire completely. The questionnaire was previously validated using a security guard group in the Tamansari area, Bandung City, Indonesia.

The participants were asked to have breakfast and brush their teeth in the morning at least 3 hours before specimen collection (no later than 6 a.m.). Participants came to the research laboratory for specimen collection to be taken by researchers. Before collecting samples, all the participant rinsed their mouths with clean water to remove dirt from the oral cavity in front of the researchers. Sampling was carried out at 09.00 a.m by taking epithelial cell samples on both sides of the buccal mucosa. A circular motion was used to take the buccal mucosa smear samples with a toothbrush. The toothbrush was then revolved on the end of the object glass that had been dripped with a saline solution. With a little pressure, the toothbrush was spread across the object glass and then fixed by dripping a 96% absolute alcohol solution on it. Then, the object glass was stained with the Papanicolaou stain.

Papanicolaou staining began with rinsing the object glasses in tap water, staining with Harris hematoxylin for one to three minutes, and then rinsing it with running water. Next, the slides were dipped in 95% ethanol, then stained with eosin azure for 2–2.5 minutes, two 10-second dips in 95% ethanol, one minute in 100% ethanol, and two 10-second dips in xylene to remove the stain, and finally mounted with dibutyl phthalate xylene.

The slides were examined microscopically on 500 cells at a magnification of 400x to observe nuclear alterations (micronuclei, karyorrhexis, karyolysis, and broken egg cells) by two blinded pathologists.

The data was initially subjected to the Shapiro-Wilk test to ascertain its normality. To assess the differences between the two groups, an independent t-test was employed for variables with normal distribution, with the mean and standard deviation serving as the primary parameters. Conversely, for variables exhibiting non-normal distribution, the Mann-Whitney test was utilized, with the mean value and minimum and maximum values as the primary parameters. The study was approved by the Medical Ethics Committee of the Medical Faculty of Universitas Islam Bandung (ethics approval code: 196/KEPK-UNISBA/VII/2023).

Results

This study involved 40 participants in Bale Endah District, Bandung Regency, Indonesia consisting of 20 adult male smokers and 20 adult male non-smokers. All participants met the inclusion criteria.

This study comprised participants ranging in age from 35 to over 50 years. The participants' average age was 38.48 years in the smoker group and 46.34 years in the non-smoker group. Most smokers were light active smokers (40.0%) and used kretek cigarettes (60.0%) with a smoking duration of ≥15 years.

In microscopic examination of buccal mucosa smears using a light microscope with 400 x magnification, variations in cell nucleus changes were found, namely micronuclei, broken egg cells, karyorrhexis, and karyolysis. Results based on the proportion of respondents in the non-smoker and smoker groups exhibited nuclear changes; namely, in the smoker group, 59.4% experienced nuclear changes in the form of karyorrhexis, while in the non-smoker group, 87.5% experienced it (Table 1). Fisher's exact test revealed a statistically significant difference in the percentage of karyorrhexis changes based on smoking status. A total of 73.9% of smoker respondents and 82.4% of non-smoker respondents experienced nuclear changes in the form of karyorrhexis. The Chi-square test on karyorrhexis changes also demonstrated a significant difference.

Identification and counting of the nuclear changes frequency were performed by a pathologist per 500 cells using Papanicolaou staining at 400x magnification. The micronucleus was a nucleus with a size of one-third of the main cell nucleus that surrounds the main cell nucleus. It was oval, sharply outlined, and the same color as the main cell nucleus (Figure 1D).

Broken eggs (Figure 1A) referred to changes in the nuclear cells leading to the phenomenon of cell nuclear rupture. The average frequency of micronuclei was highest in smokers, followed by the frequency of broken eggs, which was the second highest.

Table 1 Number of Respondents with Nuclear Epithelial Changes in Buccal Mucosa Smears

Nuclear Epithelial changes	Number of Respondents (n=40)		Total (n%)	P-value
	Non-smoker (n=20)	Smoker (n=20)		
	n (%)	n(%)		
Micronucleus				
No	0	0		
Yes	20 (100)	20 (100)		
Broken egg				
No	0	0		
Yes	20 (100)	20 (100)		
Karyorrhexis				
No	7 (87.5)	1 (12.5)	8 (100)	0.04[a]**
Yes	13 (40.6)	19 (59.4)	32 (100)	
Karyolisis				
No	14 (82.4)	3 (17.6)	17 (100)	<0.01[b]**
Yes	6 (26.1)	17 (73.9)	23 (100)	

Note: [a]Fisher Exact Test, [b]Chi Square Test *no significant difference, **no significant difference

Table 2 Epithelial Nuclear Changes Features Based on Smoking Status

Epithelial Nuclear Changes Features	Non-Smoker	Smoker	Total	p-value
Micronucleus				
Mean±SD	8.65±4.47	24.85±9.74	16.75±11.10	<0.001*[a]
Median (Min–max)	9 (2–16)	25 (4–52)	15 (2–52)	
Normality test	0.300+	0.188+	0.104+	
Broken egg				
Mean±SD	7.35±2.79	16.7±4.55	12.05±6.04	<0.001*[a]
Median (Min–max)	7.5 (2–11)	15.5 (10–30)	11 (2–30)	
Normality test	0.255+	0.090+	0.161+	
Karyorrhexis				
Mean±SD	1.65±1.69	6.75±2.73	4.20±3.42	0.001*[b]
Median (Min–max)	1 (0–5)	7 (0–11)	4 (0–11)	
Normality test	0.004++	0.072+	0.008+	
Karryolisis				
Mean±SD	0.50±0.82	2.3±3.01	1.40±2.36	0.003*[b]
Median (Min–max)	0 (0–2)	1 (0–11)	1 (0–11)	
Normality test	<0.001++	<0.001++	<0.001++	

Note: [a]Independent t-test, [b]Mann-Whitney test *statistically significant, +normally distributed data, ++ non-normally distributed data

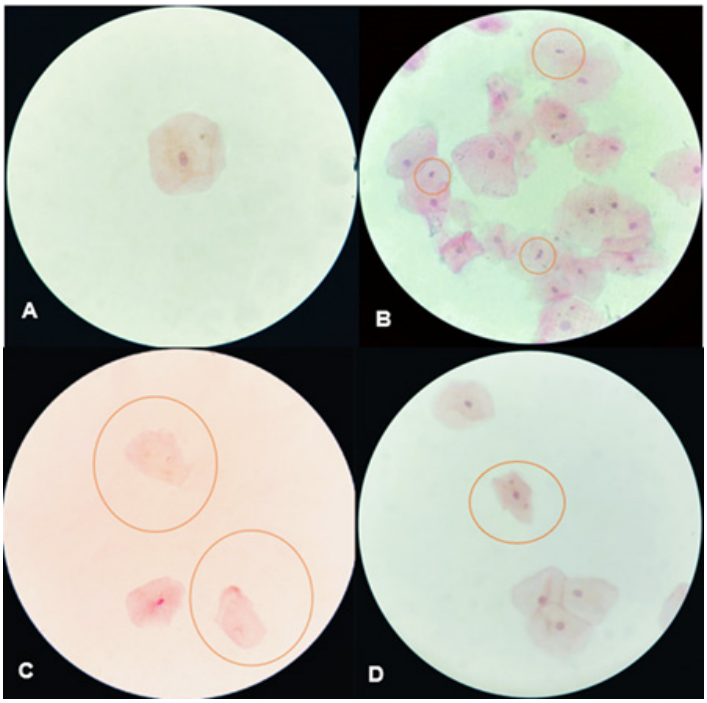

Figure 1 Nuclear Changes Features of Adult Male Smokers on Papaniculou Stain with 400x Magnification. A= Broken egg, B= Karyorrhexis, C= Karyolisis, D= Micronucleus

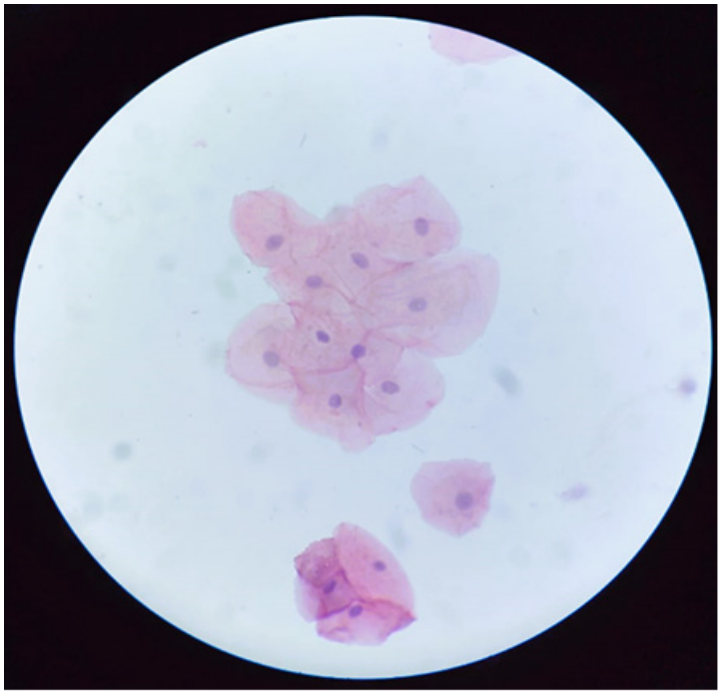

Figure 2 Normal Buccal Smear of Non-Smokers Group on Pappaniculou Stain with 400x Magnification

Similar findings were also found in the non-smoker group, including the dominance of nuclear alterations, such as micronuclei and broken eggs, feature in a considerably lesser proportion than the smoker group. Extensive chromatin aggregation characterized karyorrhexis nuclear change (Figure 1B), which fragmented and disintegrated the cell nucleus.

The presence of karyolysis (Figure 1C), defined by cellular chromatin impairment and representing the rate of cell death, was also evaluated. Several participants in both groups did not find any nuclear changes in the form of karyorrhexis and karyolysis. Most buccal mucosa smears in non-smoker were normal, containing epithelial cells with abundant amorphous to eosinophilic cytoplasm and normal nuclear size and shape (Figure 2).

Furthermore, it could be seen that in the buccal mucosa smear of smokers, the most nuclear epithelial changes were found in the form of micronuclei. This was also similar to the non-smoker group, with the quantity being less than in the smoker group (Table 2).

Moreover, the variations of nuclear changes based on smoking status indicated that all variations of nuclear changes showed significant differences between the non-smoker and smoker groups based on the mean values of changes in micronuclei and broken eggs, as well as based on the median of karyorrhexis and karyolysis. In all groups, nuclear changes showed significant differences, with the number of changes in the smoker group being significantly greater than in the non-smoker group. In terms of micronuclear changes, the average of the smoker group reached almost three times that of the non-smoker group. On the other hand, the number of changes in ruptured egg cells increased twofold compared to the non-smoker group. The range of karyorrhexis between the two groups showed a wider range (0–11) in the smoker group compared to smokers, with a median value of seven compared to one in the non-smoker group. Finally, regarding nucleus karyolysis changes, although the median value between the non-smoker and smoker groups was only one difference, the range was wider in the smoker group (0–11) than in the non-smoker group (0–2) (Table 2).

Discussion

Tobacco mostly contains nicotine, tar, and polycyclic hydrocarbons, one of the principal reasons for cigarette poisoning. Tobacco

toxicity testing with various types of tobacco reveals that tar and nicotine concentration have genotoxic effects on buccal cells, manifesting as diverse nuclear abnormalities that can be employed as biomarkers for potentially malignant illnesses.[17] Buccal cells are frequently in correspondence with the environment, emphasizing that the oral epithelium is an important target site for inhaled toxicants; hence, it is reasonable to expect evidence of genotoxicity.[18]

In this study, micronuclei were found to be dominant as one of the nuclear changes found in smokers. The results of this study are similar to previous studies that used the Papanicolaou staining to compare the presence or formation of micronuclei in buccal smears of tobacco users. Oral and maxillofacial pathology studies in India found a direct association between higher micronuclei in smokers and a correlation between the occurrence of micronuclei with age, duration, and frequency of smoking. Interestingly, maximum micronucleus detection was demonstrated among individuals aged three decades and above, while the consequences of smoking would be the most crucial consideration. A strong association was also found between micronucleus expression and increasing smoking frequency (>10 cigarettes per day). It proves that micronucleus would be a better indicator of such changes, and high-risk patients could be counseled with appropriate tobacco-related harmful effects.[19]

A previous study in India used acridine orange fluorescence labeling to examine the frequency of micronuclei in exfoliated buccal epithelial cells of oral submucous fibrosis (OSF) patients and found a significant increase in the frequency of micronuclei in OSF patients when compared to gutkha chewers and controls.[20] They determined that gutkha, in addition to smoking, is more hazardous to human health. Although using different staining, our study yielded similar results to the research. Besides micronuclei, various types of nuclear changes have been found, such as broken egg nuclei, karyorrhexis, and karyolysis.[20]

In this study, the frequency of broken eggs was quite high in the smoker group. This is in line with previous studies, which state that the percentage of cells experiencing micronuclei, karyorrhexis, karyolysis, and broken egg cells in the buccal mucosa of hookah users was significantly higher than in the control group.[15]

In this study, nuclear changes were also found in the form of karyorrhexis and karyolysis using Papanicolaou staining. These evaluations did not have as many frequencies as the number of micronuclei and broken eggs in both groups. Karyorrhexis is a chromatin that condenses, as part of the apoptosis process, further to rupture in cells with intact membranes. It is a catastrophic disintegration of a dying cell's nucleus, causing the chromatin to be distributed unevenly throughout the cytoplasm. It is frequently preceded by pyknosis, followed by karyolysis. Karyolysis is the total disintegration of a dying cell's chromatin matter caused by DNAase activity.[21]

A previous study has shown that karyorrhexis is significantly more common in non-smokers compared to smokers. Nuclear abnormalities such as karyolysis and ruptured egg nuclei are insignificant in smokers and non-smokers.[17] This may be due to differences in the use of staining in this study. Our study used Papanicolaou staining, while a previous study has revealed that the use of Fuelgen staining is considered better in identifying nuclear changes when compared to the use of Papanicolaou staining in buccal mucosa smears.

This study found no karyolysis in several participants, both in the smoker and non-smoker groups. This may be due to the use of the Papanicolaou staining could not clearly identify karyolysis. A previous study has suggested the use of Periodic Acid Schiff staining and Fast Green staining to evaluate for the presence of karyolysis.[22]

In this study, there are several limitations, such as the difficulty of finding male participants over 35 years old who do not smoke, so the number of research samples is not large enough. Further research should use a larger number of respondents with a wider age range and research area coverage.

In conclusions, all variations in epithelial nuclear changes (micronucleus, broken egg, karyorrhexis, karyolysis) show significant differences between non-smoker and smoker groups. Nuclear epithelial changes may reflect genetic changes and be associated with pre-malignant and or malignant lesions. Therefore, it is essential to educate smokers about the potential health implications of smoking.

References

1. Krishnan-Sarin S, O'Malley SS, Green BG, Jordt SE. The science of flavour in tobacco products. World Health Organ Tech Rep Ser. 2019;1015:125–42.
2. Ministry of Helath Republic of Indonesia, World Health Organization Indonesia,

Centers for Disease Control and Prevention. GATS:global adult tobacco survey fact sheet Indonesia 2021 [Internet]. 2022 [cited 2022 Nov 4]. Available from: https://cdn.who.int/media/docs/default-source/ncds/ncd-surveillance/data-reporting/indonesia/indonesia-national-2021-factsheet.pdf?sfvrsn=53eac4fd.
3. Kadar JT, Respati T, Irasanti SN. Hubungan tingkat pengetahuan bahaya rokok dengan perilaku merokok mahasiswa laki-laki di Fakultas Kedokteran. Proceeding of Bandung Meeting on Global Medicine & Health (BaMGMH); 2017 Sep 6; Bandung: FOKI; 2017.
4. Badan Pusat Statistik Kota Bandung. Persentase penduduk kota Bandung berusia 5 tahun ke atas yang merokok tembakau selama sebulan terakhir menurut jenis kelamin dan kebiasan merokok, 2020 [Internet]. [Cited 2023 January 20]. Available from: https://bandungkota.bps.go.id/id/statistics-table/1/MTUwNSMx/persentase-penduduk-kota-bandung-berusia-5-tahun-ke-atas-yang-merokok-tembakau-selama-sebulan-terakhir-menurut-jenis-kelamin-dan-kebiasan-merokok--2020.html.
5. O'Keeffe LM, Taylor G, Huxley RR, Mitchell P, Woodward M, Peters SAE. Smoking as a risk factor for lung cancer in women and men: a systematic review and meta-analysis. BMJ Open. 2018;8(10):e021611.
6. Schottenfeld D, Beebe-Dimmer JL, Buffler PA, Omenn GS. Current perspective on the global and United States cancer burden attributable to lifestyle and environmental risk factors. Annu Rev Public Health. 2013;34:97–117.
7. Khariwala SS, Ma B, Ruszczak C, Carmella SG, Lindgren B, Hatsukami DK, et al. High level of tobacco carcinogen-derived DNA damage in oral cells is an independent predictor of oral/head and neck cancer risk in smokers. Cancer Prev Res (Phila). 2017;10(9):507–13.
8. Khariwala SS, Hatsukami D, Hecht SS. Tobacco carcinogen metabolites and DNA adducts as biomarkers in head and neck cancer: potential screening tools and prognostic indicators. Head Neck. 2012;34(3):441–7.
9. Caliri AW, Tommasi S, Besaratinia A. Relationships among smoking, oxidative stress, inflammation, macromolecular damage, and cancer. Mutat Res Rev Mutat Res. 2021;787:108365.
10. Niklander SE. Inflammatory mediators in oral cancer: pathogenic mechanisms and diagnostic potential. Front Oral Health. 2021;2:642238.
11. Lee CH, Chang JSM, Syu SH, Wong TS, Chan JYW, Tang YC, et al. IL-1β promotes malignant transformation and tumor aggressiveness in oral cancer. J Cell Physiol. 2015;230(4):875–84.
12. Gowhar O. Comparison of exfoliative cytology of tongue and buccal mucosa among smokers and non-smokers using PAP Stain and AgNOR counts. International Journal of Contemporary Medical Research. 2017;4(7):1587–90.
13. Mescher AL. Digestive tracts. In: Mescher AL. Junqueira's basic histology text and atlas. 16th ed. New York: McGraw Hill; 2021.
14. Kabiraj A, Khaitan T, Bhowmick D, Ginjupally U, Bir A, Chatterjee K. Screening of oral potentially malignant disorders using exfoliative cytology: a diagnostic modality. J Cancer Epidemiol. 2016;2016:8134832.
15. Taghibakhsh M, Farhadi S, Babaee A, Sheikhi M. The effect of hookah use on buccal mucosa: evaluation of repair index. Asian Pac J Cancer Prev. 2019;20(4):1109–12.
16. Farhadi S, Mohamadi M, Mohamadi M. Repair index in examination of nuclear changes in the buccal mucosa of smokers: A useful method for screening of oral cancer. Asian Pac J Cancer Prev. 2017;18(11):3087–90.
17. Devadoss S, Raveendranath MC, Kathiresan TS, Ganesan K. Genotoxic effect of various forms of tobacco on oral buccal mucosa and nuclear changes as a biomarker. J Pharm Bioallied Sci. 2021;13(Suppl 2):S1141–8.
18. Hartwig A, Arand M, Epe B, Guth S, Jahnke G, Lampen A, et al. Mode of action-based risk assessment of genotoxic carcinogens Arch Toxicol. 2020;94(6):1787–877.
19. Kamath V, Anigol P, Setlur K. Meicronucleus as prognostic indicators in oral cytological smears: a comparison between smokers and non-smokers. Clin Cancer Invest J. 2014;3:49–54.
20. Jyoti S, Khan S, Afzal M, Naz F, Siddique Y H. Evaluation of micronucleus frequency by acridine orange fluorescent staining in bucccal epithelial cells of oral submucosus fibrosis (OSMF) patients. Egypt J Med Human Genet. 2013;14(2):189–93.
21. Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. Biomed Res

Int. 2014;2014:150845.
22. Prihastuti CC, Widodo AHB, Bagasworo NP, Widikusumo A, Novrial D, Wahyono DJ, et al. Morphological changes and apoptosis of buccal mucosa basal Epithelium in heads and necks during cancer radiotherapy. Indonesian Journal of Cancer. 2023;17(1):23–8.