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Buccal Cell Micronuclei among Betel Quid Chewers and Non-Betel Quid Chewers from Selected Barangays in Zamboanga City

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ABSTRACT

Background. Betel quid chewing has been reported to have carcinogenic properties due to the presence of harmful compounds present in its ingredients. The oral mucosa is directly exposed to these carcinogenic compounds which could cause pathological changes and lead to malignancies. Micronucleus is a biomarker that indicates genetic alteration could form due to exposure from carcinogenic substances that can be attributed from betel quid chewing. Thus, a person's oral health status can be gauged through the detection of micronucleus in buccal cells.

Objective. A cross-sectional study was done to compare the presence of micronuclei in buccal epithelial cells between betel quid chewers and non-betel quid chewers in Zamboanga City.

Methodology. Purposive sampling was used to enroll the 104 participants (52 betel quid chewers and 52 non-betel quid chewers). The demographic profiles and betel quid chewing habits of the participants were obtained using a questionnaire. Buccal cells samples were collected using clean and dry tongue depressors and were smeared directly onto pre-cleaned glass slides. Slides were processed for Papanicolaou staining by a medical technologist. For each slide, 1000 buccal cells were examined using a light microscope with an attached camera. Photomicrographs of buccal cells with micronuclei were taken. Two pathologists separately validated the results through the photomicrographs. Intraclass correlation coefficient for inter-rater reliability gave a value of 1 which indicates high reliability among observers.

Results. The median of the frequency of micronuclei among betel quid chewers and non-betel quid chewers were 56.5 and 36, respectively. Mann-Whitney U test revealed a significant difference (p=0.031) at α=0.05 in the Micronuclei frequency between the 2 groups. There were 36.5% of betel quid chewers who have Micronuclei frequency above the cut-off value and on the other hand, 15.4% among the non-betel quid chewers. Pearson's correlation coefficient revealed that there was a very weak negative relationship (r=-0.072) between total Micronuclei frequency and length of time of betel quid exposure among the exposed group.

Conclusion. Betel-quid chewers have significantly higher frequency of micronuclei compared to non-betel quid chewers which puts them at higher risk for developing oral malignancies.

Key words: micronucleus, betel, quid, Areca, Papanicolaou

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INTRODUCTION

Betel quid chewing is one of the habits practiced by some Filipinos. Accordingly, as early as 1915, betel quid chewing was already associated with oral cancer in 70% of cases and this was rampant among the elderly people around the Philippines.[1] One of the noted ethnic groups to practice betel quid chewing in the country are the Ifugaos and it was found out that those who had this habit had 3.7% higher proportion of micronucleated cells compared to those who did not.[2] A betel quid usually comprises of an *Areca* nut cut into sections, betel piper vine leaf, a lime made from ground and burnt sea shell, and tobacco leaves (Figure 1).

Its addictive potential is attributed to its parasympathetic agonist properties brought about by alkaloids *arecoline* and *arecaidine* which are independent of synergistic

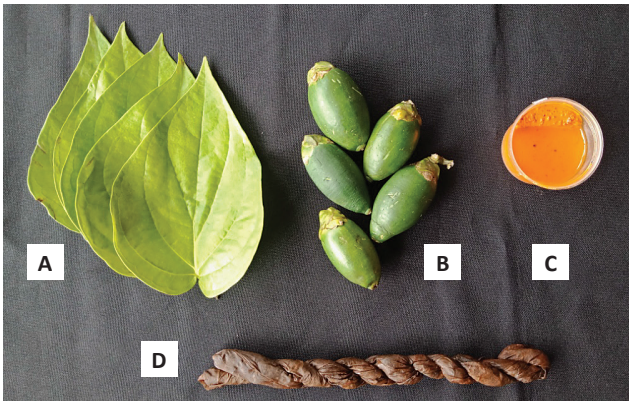

Figure 1. Components of betel quid; **A.** betel piper vine leaf, **B.** Areca nut, **C.** lime from ground and burnt sea shells, **D.** tobacco.

properties of other added substances.[3] The withdrawal among betel quid chewers has been observed to be similar to those seen among users of nicotine and caffeine.

There are people who chew betel quid as alternative for smoking, because of their perception that it has no negative effect on a person's health. Thus, some people would prefer to chew betel quid than to smoke because of the belief that it is safer.

Betel quid chewing has been linked to cause oral cancer in several studies. Frequent chewing of betel quid leads to oral submucous fibrosis which is attributed to the presence of the active alkaloid arecoline present in the betel nut.[4] Leukoplakia is also a common finding. Individuals with oral submucous fibrosis are at high-risk for precancerous conditions[5] which may develop into malignancy at a rate of 7.6%.[6]

The betel quid being sold in Zamboanga city has 4 main ingredients; 1) betel piper vine leaf, 2) betel nut, 3) calcium hydroxide, a lime made from ground and burnt sea shell and, 4) tobacco leaves. Among the major ingredients, 2 of these, betel nut and tobacco, have been reported to have harmful compounds that are deleterious to a person's health, specifically the oral parts due to its direct exposure from chewing. A betel nut contains alkaloids in which arecoline is the most abundant. When arecoline undergoes the process of nitrosation, it gives rise to betel quid specific nitrosamine which is reported to have carcinogenic properties.[7] Tobacco also contains nitrosamines which have clastogenic and mutagenic properties which cause the induction of chromatid and chromosomal aberrations giving rise to micronuclei in cells.[8] The lime consists mainly of calcium hydroxide which stimulates oral mucosal fibroblast proliferation but doesnot contribute to genotoxicity by means of DNA strand break.[9] However, slaked lime has been shown to promote carcinogenesis by inducing generation of reactive oxygen free radicals from betel nut. This makes both ingredients a toxic combination. On the good side, betel piper vine leaf is devoid of mutagenic and carcinogenic properties. The betel piper vine leaf possesses cancer-preventive properties. It contains various phytochemicals. In one study, aqueous extract of betel leaf did not induce tumor in mice by which they have concluded that is not carcinogenic.[10] Other studies have shown that betel leaf is effective for prevention of tobacco-specific nitrosamines that causes cancer.

Despite the advances in research on treating oral cancer, the outcome of such disease has not improved. Oral cancers are often diagnosed at advanced stages. Oral carcinogenesis involves multiple processes that progressively cause genetic damage. Early detection of oral cancer is an important factor in having a good prognosis for patients affected.

Oral cytology may aide in detecting patients with high risk for genotoxicity. One of the biomarkers described is the micronucleus. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei arising from either lagging chromosomes at anaphase or from acentric chromosome fragments.[11] These structures can be visualized in buccal epithelial cells using scrapings or brushings from the oral mucosa. Howel and Jolly were the first to mention and describe about micronuclei in the late 1800s and early 1900s.[8] Its presence indicates mutagenetic stress in an individual.There are several factors contributing to the formation of micronuclei in cells such as genetic makeup, exposure to physical or chemical substances and habits such as chewing betel quid, tobacco use and alcoholic drinking.

METHODOLOGY

Research design

This is cross-sectional study comparing the presence of micronuclei in buccal epithelial cells between betel quid chewers and non-betel quid chewers in Zamboanga City.

Population and sampling design

Betel quid chewers from selected areas in Zamboanga City were selected based on the following characteristics: male or female 18 years old and above, without any apparent oral lesions and must have been chewing at least 1 packet of betel quid per day for duration of at least 1 year or more.During the period of recruitment of participants, no female chewers were encountered. Thus, the betel quid chewer group was comprised totally of male participants. The control group was selected based on the following criteria: male or female 18 years old and above, has not been chewing betel quid in his/her entire lifetime and without any apparent oral lesions. The following were excluded: individuals taking medications (e.g. antibiotics, NSAIDS, or steroids for systemic diseases), alcoholic beverage drinkers, those who have undergone radiation therapy, and tobacco cigarette smokers.

Purposive sampling was used in enrolling the participants. A total of 52 betel quid chewers were recruited. For the control group, 52 participants were also recruited using the same method and from the same setting to match the exposed group.

Personal data sheets or questionnaires were used in recording the demographic data and chewing habits of participants who consented for the study. Materials needed prior to doing the staining technique were brought to collect and preserve the viability of the collected specimens

(e.g. tongue depressors, clean gloves, glass slides, tap water and 95% ethyl alcohol for fixation). An electric light microscope was used in viewing the stained smears. Micronuclei frequency was quantified using a tally counter and recorded in a tally sheet. A camera attached to the microscope was used in taking photomicrographs.

Data gathering procedure

Collection of specimen

The collection of the buccal specimens was adapted from the paper of Celik et al.[12] Each participant was interviewed and his/her buccal cell specimens were collected. Participants rinsed with tap water twice by gargling. Sterile and dry disposable tongue depressors were used to scrape off the buccal cells from each individual. The collected specimens were directly smeared onto pre-cleaned glass slides and were submerged immediately in 95% ethyl alcohol to prevent air drying (may cause enlargement of the nucleus). The fixation period was for at least 6-8 hours. One slide per participant was prepared.

Staining procedure

The slides were stained using regressive Papanicolaou staining technique by the medical technologists at Ciudad Medical Zamboanga Laboratory. The sequence of reagents or steps used were as follows: 1) 95% ethyl alcohol (fixed for at least 30 minutes), 2) 75% ethyl alcohol (10 dips), 3) 50% ethyl alcohol (10 dips), 4) distilled water (10 dips), 5) Harris hematoxylin (13 minutes), 6) rinsed with tap water (1 minute), 7) 0.25% HCl, 8) rinsed with tap water (1 minute), 9) blueing reagent (1 minute), 10) rinsed with tap water (1 minute), 11) 50% ethyl alcohol (10 dips), 12) 75% ethyl alcohol (10 dips), 13) 95% ethyl alcohol (10 dips), 14) OG-6 (10 dips), 15) 95% ethyl alcohol (10 dips), 16) 95% ethyl alcohol (10 dips), 17) EA-65 (5-7 minutes), 18) 95% ethyl alcohol (10 dips x 2), 19) 100% C_2H_3OH (1 minute), 20) Air dried, 21) Xylene (1 minute x 2), 22) mount.

Quantification of micronuclei

Each slide was viewed under a light microscope at 1000x magnification. The slides were read following a zigzag pattern; cells were counted from right to left as done in the paper by Anila et al.[13] A tally counter was utilized to quantify micronuclei seen and the data were recorded in a tally sheet. Micronuclei frequency was measured as the number of micronucleus per 1000 cells seen for each slide. Two pathologists separately validated the micronuclei seen by the researcher through the photomicrographs taken. Validation was performed as to whether the micronucleiseen wereactually micronuclei. Criteria set by Tolbert et al. (1992) (Table 1) were used. The following degenerative nuclear changes were not counted and included for the analysis of data: binucleated cells, nuclear bud, karyolysis, pyknosis, and karyorrhexis.

Table 1. Criteria for identification of micronuclei by Tolbert et al.[14]

Parameters for cell inclusion in cells to be scored	Suggested criteria for identifying micronuclei
Intact cytoplasm and relatively flat cell position	Rounded, smooth perimeter
Little or no overlap of cells	Less than a third of the diameter of the nucleus but big enough to discern the shape
Little or no debris	Staining intensity similar to nucleus
Nucleus normal and intact	Same focal plane as nucleus
Nuclear perimeter smooth and distinct	Absence of overlap with or bridge of the nucleus

Cut-off value for micronuclei frequency

For this study, the cut-off value was computed using the receiver operating characteristic (ROC) test. This test shows the graphical connection between clinical sensitivity and specificity to determine the most appropriate cut-off value for micronuclei frequency. The test revealed that the appropriate cut-off value for micronuclei frequency was 56.5. However, we cannot directly state that genotoxicity is directly related to frequency.

Ethical consideration

This study was approved by the Ethical Review Board of Ateneo de Zamboanga University Research Center.

Data analysis

Data were analyzed using Mann-Whitney U test to determine the significant difference in frequencies of micronucleus between the two groups at $\alpha=0.05$. Intraclass correlation coefficient was used to determine the inter-rater reliability. Pearson's correlation coefficient was used to determine the relationship between length of time of betel quid exposure and Micronuclei frequency among the betel quid chewers group.

RESULTS AND DISCUSSION

A total of 104 participants were enrolled in this study. The demographic characteristics of the participants are summarized in Table 2.

Table 2. Demographic characteristics of the participants

Characteristics	Betel Quid Chewers (n=52)	Non-betel Quid Chewers (n=52)
Age		
18-25 years old	22 (42.3%)	26 (50%)
26-35 years old	13 (25%)	11 (21.2%)
36-45 years old	9 (17.3 %)	9 (17.3%)
46-55 years old	3 (5.8%)	2 (3.8%)
>55 years old	5 (9.6%)	4 (7.7%)
Mean age±Standard Deviation	32±11.92	30 ±10.93
Sex		
Female	0	24 (46.2%)
Male	52 (100%)	28 (53.8%)
Religion		
Islam	52 (100%)	40 (76.9%)
Protestant	0	1 (1.9%)
Roman Catholic	0	9 (17.3%)
Ethnicity		
Bisaya	1 (1.9%)	3 (5.8%)
Chavacano	0	6 (11.5%)
Ilokano	0	1 (1.9%)
Maranao	1(1.9%)	0
Sama	2 (3.8%)	1 (1.9%)
Tagalog	0	1 (1.9%)
Tausug	47 (90.4%)	40 (76.9%)
Yakan	1(1.9%)	0

Majority of the participants belonged to the age group of 18-25 years old for both groups (22 or 42.3% for betel quid chewers and 26 or 50% in the non-betel quid chewers). Thus, it is important to educate the people especially the younger ones on the harmful effects of betel

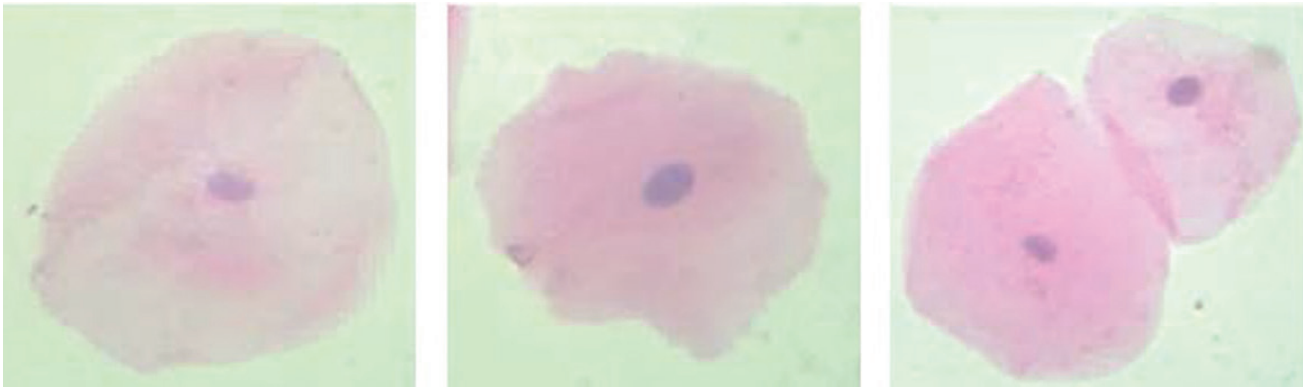

Figure 2. Normal buccal cells without micronuclei taken from non-betel quid chewers (Papanicolau stain, 1000x).

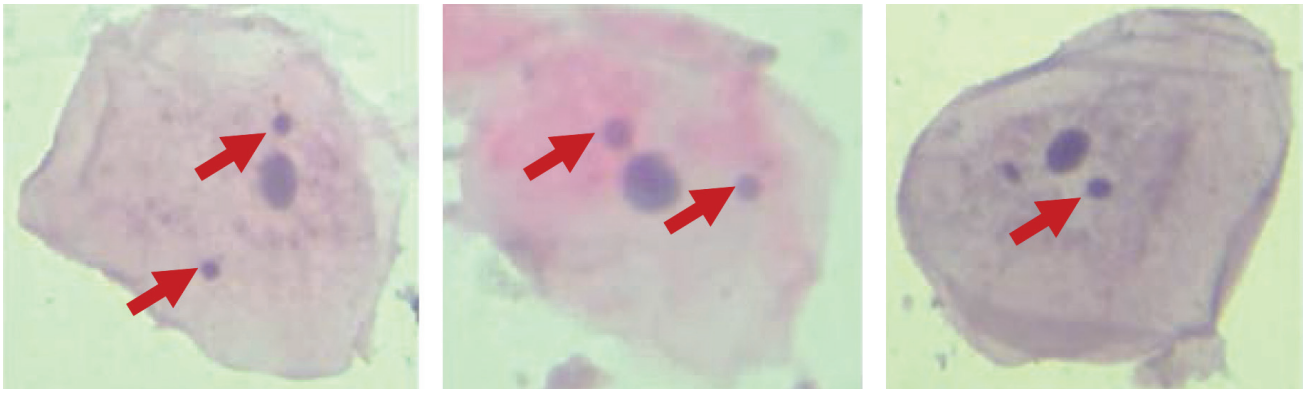

Figure 3. Buccal cells with micronuclei taken from betel quid chewers (red arrows point toward micronuclei), (Papanicolau stain, 1000X).

quid chewing. Oral cancer is increasing in young adults and most records state that 6% of all oral cancers in young people are under the age of 45 years.[15]

All 52 participants among the betel quid chewers were male; there were no females. On the other hand, among the 52 non-betel quid chewers, 24 were females. Majority of the chewers belong to the male gender in the early to mid 20's. Accordingly, in most countries, oral cancer is more common among men than in women.[15]

Islam is the religion of the majority for both groups (52 or 100% for betel quid chewers and 40 or 76.9% for control). This is because the participants were gathered in barangays that are populated mostly by Muslims. Participants were mostly Tausug for both groups (47 or 90.4% for betel quid chewers and 40 or 76.9% for control) since Muslims in Zamboanga City mostly belong to this ethnicity.

Inter-rater reliability

Intraclass correlation coefficient was used to determine the inter-rater reliability among the 3 observers which revealed a value of 1. This value indicates that there is high inter-rater reliability among the 3 observers.

Frequencies of micronuclei

The frequencies of micronucleus were determined in the 2 groups. Each group having 52 slides prepared and examined. A total of 1000 buccal cells per slide were examined. Table 3 shows the Micronuclei frequency/1000 cells of the participants.

The betel quid chewers group has a greater median of 56.5 compared to the non-betel quid chewers group with 36.

The results show consistency in relation to similar studies such as in the paper of Fareed et al., in which there is greater micronuclei frequency among betel quid chewers compared to non-chewers (Figures 2 and 3).[16]

Betel quid chewing has been linked to development of oral malignancies. Buccal cancer is more common among the Asian population due to the practice of betel quid chewing.[15] In Sri Lanka, where betel quid chewing is very popular, 40% of cancers of oral cavity are found on the buccal mucosa. Based from the results, the practice of betel quid chewing must be avoided just like cigarette smoking. The findings further support the previous studies that betel quid chewing is linked to micronucleus formation.

Among the betel quid chewers group, 19 out of 52 have values above the cut-off level. On the other hand, among the non-betel quid chewers group, 8 out of 52 had a value more than the cut-off (Figure 4). There are more participants from the betel quid chewers who have Micronuclei frequency above the cut-off value compared to the non-betel quid chewers. The Micronuclei frequency among non-betel quid chewers who scored values above the cut-off may be due to their exposure to other physical or chemical substances with carcinogenic properties.

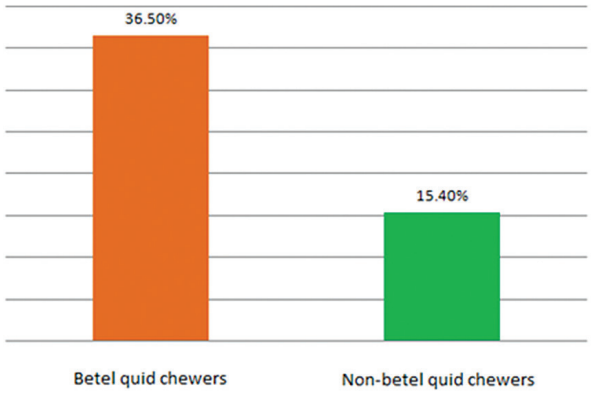

Figure 4. Bar graph of number of participants for each group who had Micronuclei frequency above the cut-off level of 56.5

Comparison of micronuclei frequencies between the groups

The micronucleus frequencies between the 2 groups were analyzed using Mann-Whitney U test. It is shown in Table 3 that there is a significant difference in the frequencies of micronucleus between betel quid chewers and non-betel quid chewers (p *value*=0.031) at α=0.05.

Table 3. Comparison of frequencies of micronucleus between betel quid chewers and non-betel quid chewers using Mann-Whitney U test at α=0.05

Group	N	Median	p value
Betel Quid Chewers	52	56.5	0.031*
Non-betel Quid Chewers	52	36	

*with significant difference: p ≤ 0.05

This significant difference further supports the previous findings that betel quid chewing promotes micronuclei formation in buccal cells.

Relationship between micronuclei frequency and length of time of betel quid exposure (LBE)

The betel quid chewing habits of the betel quid chewers group in terms of duration in years and the number of betel quid chewed per day is seen on Table 4. These numbers however are approximations due to difficulty among the participants in recalling their exact values. The length of time of betel quid exposure (LBE) was computed by multiplying the duration and number of betel quid chewed per day.

The scatter plot below (Figure 5) demonstrates the relationship between the Micronuclei frequency and length of time of betel quid exposure (LBE) variables. A very weak negative relationship is shown.

For this group of samples, the relationship between Micronuclei frequency and length of time of betel quid exposure (LBE) was analyzed using Pearson's correlation coefficient which revealed a very weak negative relationship (r=-0.072). Although it is well established that the degree or intensity of consumption of betel quid increases the risk of developing oral malignancies which have been reported by several studies, there is a weak correlation between Micronuclei frequency and LBE in this study. This coincides with the study done by Nair et al., wherein there was no correlation between the number of betel quid chewed per day, the number of years of chewing and the frequency of micronucleated oral mucosal cells.[17] The same goes for the cases of Stich et al. and Suhas et al., wherein there was a very weak or no simple relationship observed between the number of betel nuts chewed and the frequency of micronucleated mucosa cells.[18, 19]

Table 4. Chewing habits and length of time of betel quid exposure among betel quid chewers

Duration (years)	No. of betel quid chewed per day	LBE = duration x no. of betel quid chewed per day	Micronuclei frequency/ 1000 cells
1	3	3	63
8	10	80	7
3	10	30	13
13	7	91	70
19	10	190	33
15	30	450	107
10	4	40	65
2	5	10	197
5	10	50	11
4	8	32	125
9	10	90	159
9	20	180	29
6	20	120	39
3	2	6	90
5	20	100	16
13	15	195	58
7	5	35	58
10	10	100	45
5	6	30	39
8	10	80	13
2	5	10	76
3	3	9	109
20	10	200	9
10	5	50	67
10	3	30	118
3	10	30	99
20	6	120	68
4	1	4	59
6	10	60	111
5	5	25	44
2	10	20	13
6	5	30	78
12	7	84	4
10	50	500	60
10	8	80	68
18	10	180	11
8	8	64	12
10	7	70	77
10	4	40	63
6	5	30	67
2	10	20	11
5	10	50	23
3	20	60	46
1	4	4	51
3	3	9	88
10	4	40	34
15	5	75	8
1	6	6	58
13	10	130	9
3	11	33	39
13	7	91	55
2	20	40	18

Nair et al. suggested that it would be more valid to link the micronucleus test to the *cotinine* (an alkaloid found in tobacco and a predominant metabolite of nicotine) levels in saliva or urine which is a more reliable marker of actual betel quid exposure.[17]

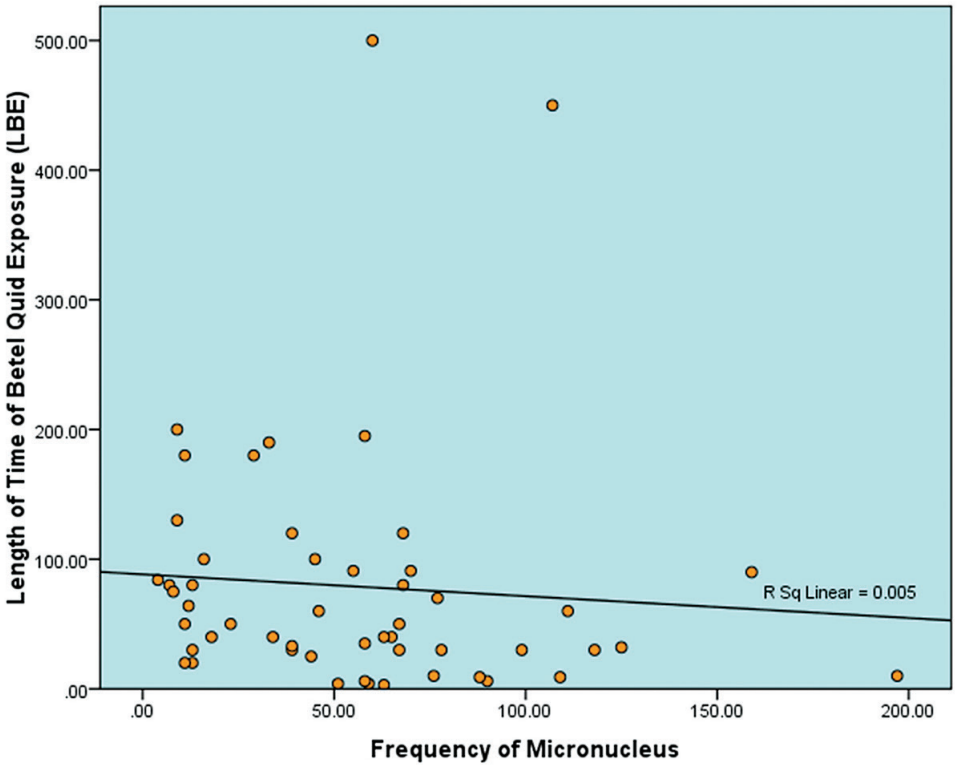

Figure 5. Scatter plot diagram showing the relationship between Micronuclei frequency and length of time of betel quid exposure (LBE).

The micronucleus test is a marker of the extent of chromosome breakage a few days to weeks previously, when the cells currently being exfoliated were dividing in the basal layer of oral mucosa.[20] A betel chewer's mucosa is said to be hyperplastic which means that there is an increase in the rate of proliferation in dividing cells.[21] Thus, if there is an increase in the proliferation rate in dividing cells, there is an increase in the micronuclei frequency. With continuous exposure of the oral mucosa to betel quid, there is also continuous formation of micronucleus. Hence, an inference that the genotoxicity in the oral epithelium is local and acute due to the short turn-over period of 25 days in buccal cells from the basal layer to the epithelium.[19] Furthermore, Nair et al. confirmed that this test can only reflect the current risk but not the cumulative risk over the years.[17] Therefore, a person with a lower LBE may exhibit a higher micronuclei frequency than a person with a higher LBE at the time of sample collection of exfoliated buccal cells or may result the other way around or even equal. The only difference between the 2 individuals is that, the person with a higher LBE has higher number of oral lesions that developed over the years due to betel quid chewing, putting him at higher risk for developing oral malignancies. Furthermore, the average frequencies of the micronucleus are increased in precancerous lesions when compared to oral mucosa, with further increase in carcinomas, suggesting that micronucleus is a biomarker of neoplastic progression.[19] This explains why there is a very weak or absence of correlation between the micronuclei frequency and LBE but when compared to non-betel quid chewers, betel quid chewers consistently exhibit higher average of micronuclei frequency as reported by several studies mentioned earlier.

To further determine the causal relationship between the carcinogenicity of betel quid chewing and its effect on the oral mucosa of individuals, studies such as ploidy studies and DNA adducts are highly recommended.

CONCLUSION

Between the 2 groups, the betel quid chewers exhibited significantly higher micronuclei frequencies compared to the non-betel quid chewers. The results of this study further support previous studies that the practice of betel quid chewing is associated with micronuclei formation and development of oral malignancies.

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STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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