Original Article

An Assessment of Genotoxic Damage in Exfoliated Buccal Cells Using Saliva as a Tool

Sonia Jaiswal, P. K. Sharma

Department of Anatomy, Era's Lucknow Medical College, Lucknow, Uttar Pradesh, India

Abstract

Introduction: Exfoliated buccal cells serve as an ideal site for genotoxic events since they are the first cells to come in contact with anything ingested or inhaled. Micronuclei (MNs) are also known as Howell–Jolly bodies and are a result of some aneugenic and clastogenic events. The aim of the study was to assess the severity of genotoxic damage in the exfoliated cells of participants consuming smokeless tobacco and its products and those diagnosed with oral submucous fibrosis (OSMF). **Material and Methods:** Individuals addicted to alcohol, pleasure drugs, or smoked forms of tobacco were excluded from the study. Saliva was collected and centrifuged; smear was prepared using the pellet, while the supernatant was used to extract DNA. The cells collected were stained with Feulgen fast green and acridine orange. **Results:** A progressive increase in the number of MNs was observed from the control group to those who consumed smokeless tobacco and their products, while the frequency of MNs was found to be the highest in participants with Oral Submucous Fibrosis. **Discussion and Conclusion:** Micronucleus assay using saliva as a tool can be useful in detecting early genotoxic damage.

Keywords: *Acridine orange, DNA, Feulgen fast, micronuclei, submucous fibrosis*

Introduction

Saliva is rapidly emerging as a multipurpose diagnostic tool since its collection is noninvasive and easy to access. It plays an important role in the early detection and prevention of many oral lesions as it is in direct contact with the oral lesion.

DNA damage can be assessed by extracting and purifying it from biofluids such as saliva. DNA was discovered in the human circulatory system in 1948;[1] since then, various researchers have found DNA in other body fluids as well. Lo *et al* discovered free DNA in body fluids in the year 1997.[2] Later on, DNA was also detected in urine[3-6] and saliva.[7]

Early detection of oral lesions was possible using cytology, a method introduced by Papanicolaou in the year 1943. It is a simple method and noninvasive in nature and thus can be carried out in suspected lesions.[8] Cytological techniques have improved over the years which have led to the development of liquid-based preparations, thus using it as an auxiliary tool for the identification of oral lesions.[9] Genotoxins from various medical procedures, deficiencies, factors related to lifestyle, and genetic disorders produce genetic imbalance in cells.[10]

It is essential to have biomarkers which are reliable and are minimally invasive so as to improve their diagnostic potential in lesions associated with genetic damage. An assessment of micronuclei (MNs) is a good method to detect chromosome loss or malfunction.[11] Various studies have shown a correlation between frequency of MN and severity of genotoxic damage.

The aim of our study was to assess genotoxic damage in exfoliated buccal cells of tobacco users using liquid-based cytology where saliva was used as a tool.

Material and Methods

The study was given clearance by the ethical committee, and informed consent was taken from all the participants.

Five hundred participants were screened with the help of a structured questionnaire. The questionnaire was divided into two sections: one related to the oral hygiene status of the participants and their pattern

How to cite this article: Jaiswal S, Sharma PK. An assessment of genotoxic damage in exfoliated buccal cells using saliva as a tool. J Anat Soc India 2019;68:181-6.

Article Info

Received: 25 July 2019
Accepted: 04 September 2019
Available online: 07 January 2020

Address for correspondence:
Dr. Sonia Jaiswal, Department of Anatomy, Era's Lucknow Medical College, Lucknow, Uttar Pradesh, India. E-mail: jaiswalsonia2008@gmail.com

Access this article online

Website: www.jasi.org.in
DOI: 10.4103/JASI.JASI_99_19
Quick Response Code:

of the use of tobacco and its products and the other section was dedicated to the clinical examination of oral submucous fibrosis (OSMF). Participants consuming alcohol, smoked form of tobacco, and pleasure drugs were excluded from the study. All those participants who were suffering from debilitating diseases or exposed to radiation were also excluded from the study. The participants in the study belonged to the rural areas near Lucknow. Their main occupation was farming.

After a careful evaluation, 240 participants were selected and divided into four groups with 60 participants each in the following categories: those consuming tobacco, those who consumed gutkha (a preparation of areca nut with tobacco), a group comprising participants with OSMF, and a control group.

Collection of saliva

Saliva samples were collected early in the morning; participants were asked to rinse their mouths to remove any residual debris. Saliva was collected in a test tube through a passive drool method, in unstimulated conditions. We used sterilized glass test tubes to collect the samples from Group 1 (control group) and Group 2 (tobacco). We had limited Oragene saliva collection kits and hence used them to collect saliva samples from Group 3 (gutkha) and Group 4 (OSMF).

Preparation of smear and staining

The collected sample was centrifuged at 1500 rpm for 5 min. The supernatant was kept aside. A drop of normal saline was added to the pellet and vortexed to get a homogeneous mixture of the sample. Fifty microliters of diluted pellet was placed on clean slides with a drop of fixative solution. The smear was subsequently stained with acridine orange and Feulgen fast green.

Assessment of micronuclei

One thousand cells per individual were assessed using the parameters laid down by Tolbert *et al.*[12] who state that the diameter of the MN must be at least one-third of the diameter of the nucleus. The staining intensity and texture should be similar to the nucleus, and the MNs should lie in the same focal plane as the nucleus.

Purification and extraction of DNA from saliva

The reagents used were Prep IT and L2P. The sample was mixed with DNA Genotek kit. The procedure was done according to the protocol manual. The DNA pellet obtained was quantified using a NanoDrop.

Results

Micronuclei in buccal cells

The mean frequency/cell of MN formation was found to be highest in the submucous fibrosis group (2.37 ± 1.10), followed by the group of gutkha users (2.03 ± 1.16) and tobacco users (1.49 ± 0.77).

The mean frequency/cell of MN formation was minimum (1.02 ± 0.17) in the group of normal individuals.

According to the Kruskal–Wallis test, the difference in mean frequency/cell of MN formation among various groups is found to be highly significant ($P < 0.001$) Figure 1, Table 1.

Results using Feulgen fast and acridine orange

Feulgen fast staining technique

The DNA stained magenta and the cytoplasm light green when the cells were stained with Feulgen fast [Figure 2]. In this technique, there is a mild hydrolysis done with 1 molar hydrochloric acid at 60ºC breaking the purine-deoxyribose bond. Then, a reaction with Schiff's reagent gives DNA a magenta color. An analysis of MNs was easier with this nuclear-specific stain since the cytoplasm was clear.

Acridine orange staining technique

When acridine orange intercalates into double-stranded DNA it emits green fluorescence on the contrary it emits red when there is an interaction with single-stranded DNA (apoptotic cells have a larger fraction of DNA in the denatured form and displays an intense red fluorescence [Figure 3]. Green emission was seen in the control group and tobacco chewer [Figure 4] group, and red emission was seen in the gutkha group [Figure 5] and Oral submucous fibrosis group [Figure 6].

The purity of extracted DNA can be measured by calculating the absorbance ratio A260/A280. This ratio assesses the contamination of protein solutions because proteins absorb light at 280 nm. The absorbance ratio with commercial kits is around 1.6–1.9. The method of collection may, however, affect the purity of the extracted DNA. The purity of DNA collected in sterile tubes was lower compared to the purity of extracted DNA collected with commercial kits such as Oragene. The A260/A280 absorbance ratio obtained shows that saliva results in DNA of the highest integrity which can be used for genome sequencing, etc. [Table 2]

Discussion

MNs originate from whole chromosome or chromosomal

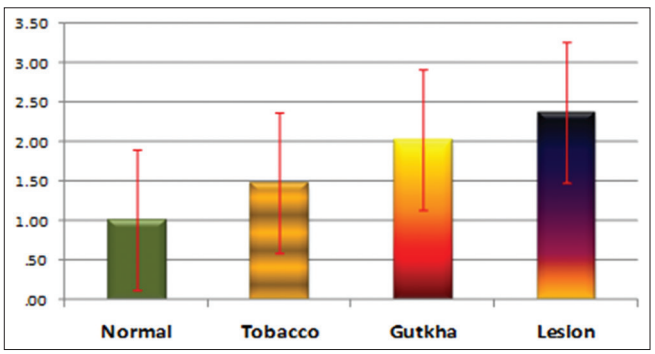

Figure 1: Intergroup comparison of micronucleus frequency/cell in exfoliated buccal cells

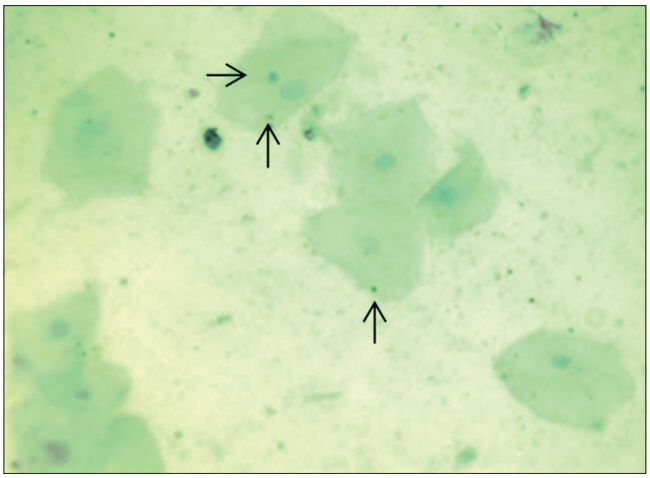

Figure 2: Cells stained with Feulgen fast green stain, arrow marked shows the micronuclei at ×40 magnification viewed under a light microscope

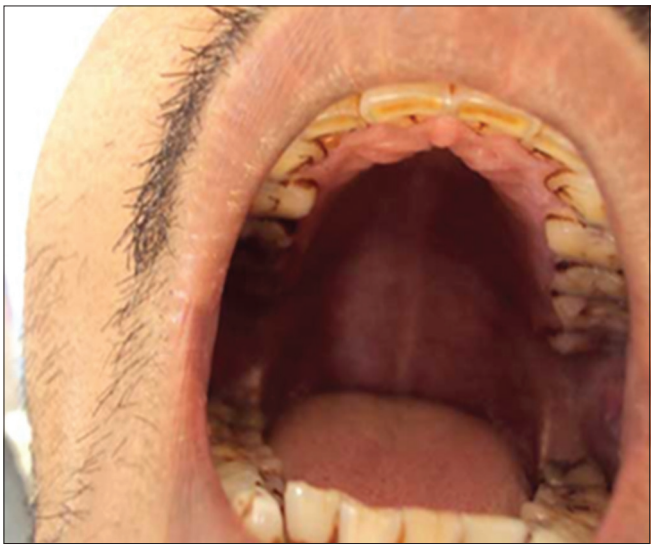

Figure 4: Tobacco chewer

fragments that fail to reach the pole during anaphase or telophase in the course of nuclear division. MNs can be easily assessed in erythrocytes, lymphocytes, and exfoliated cells, for example, oral epithelium, urothelium, and nasal epithelium to obtain a measure of genome damage induced *in vivo*. MN assesses the level of genomic damage in a cell, and an evaluation can be done from exfoliated apart from lymphocytes and can be done *in vivo*, whereas *ex vivo* assay can be performed after cell culture and measuring sister chromatid exchange.

Frequency of micronuclei in tobacco users

The application of MN assay is to measure tobacco-induced genotoxic damage, which was demonstrated by Stich *et al.*,[13] and subsequently, further evidence was put forward by Stich *et al.*[14] who used the MN assay in individuals belonging to Bihar. They particularly measured the genotoxicity in khaini-induced buccal cells.

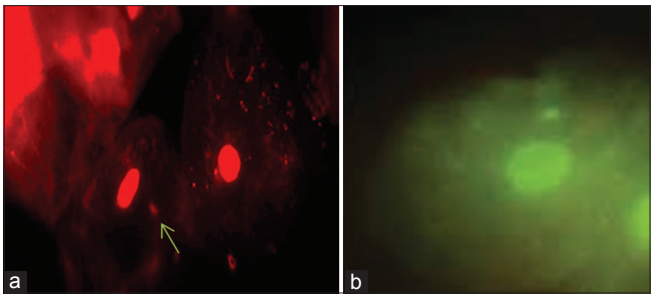

Figure 3: a) bright red emission b) green emission using acridine orange stain viewed under a fluorescent

Figure 5: Gutkha chewer

Patel *et al*. in 2009[15] conducted a study on tobacco chewers, and they measured genotoxic damage by performing a MN assay and also by evaluating sister chromatid exchange.

A study conducted by Sellappa *et al*.[16] assessed individuals who were regularly consuming tobacco and areca nut with paan. The MN frequency was increased in such individuals; hence, they concluded that tobacco is hazardous for oral and systemic health. Dash *et al*. in 2018 found the mean number of MNs to be 18.28 ± 10.0 in smokeless tobacco users,[17] while in our study, the frequency of MNs in tobacco chewers when compared with healthy controls was 1.49/cell, with a standard deviation of 0.771.

Frequency of micronuclei in gutkha chewers

According to Palaskar and Jindal, various studies from 1985 till date have shown that the frequency of MNs was increased in tobacco consumers as compared to healthy individuals.[18] Our study shows a higher frequency of MNs among gutkha users; this was also proved by previous studies by Nair *et al*. in 1991, Gandhi and Kaur in 2000.[19,20] Siddique *et al*. in 2008 and Nair *et al*. in 1999 found that carcinogens in gutkha are derived from areca nut alkaloids; there is also a formation of tobacco-specific nitrosamines due to chewing of gutkha that leads to

exposure of buccal cells to volatile nitrosamines derived from areca nut alkaloids.[21,22] According to Wenke *et al*. in 1984, the saliva of gutkha chewers shows that the nitrite and nitrate reductase activities are increased.[23] Murdia *et al*. in 1982 and Nair *et al*. in 1986 found that the acidic pH in the stomach causes nitrosation of secondary and tertiary amines once the quid is swallowed; therefore, the levels of nitrosoproline are increased in the urine 4–6.5 times in gutkha chewers.[24,25] Chakradeo *et al*. in a study in 1994 found that there is a release of reactive oxygen due to aqueous extracts of areca nut which causes damage to the buccal mucosa.[26] The frequency of MN depends on the ingredients of the quid and how many times quid is consumed and for what duration. Nair *et al*. in 1985 found that the frequency of MN is also affected by certain lifestyle factors.[25] In our study, we found that the frequency of MNs per cell in gutkha chewers was 2.03, with a standard deviation of 1.160.

Micronuclei and oral lesions

Many studies have shown that there is a formation of oxygen reactive species which is responsible for the formation of fibrous band in the buccal mucosa. The alkaloids present in areca nut also contribute to the formation of collagen bands. In OSMF patients, there was an increase in the total serum protein and iron. Although the mechanism of formation of collagenous band is not fully understood, some workers suggest the role of flavonoids, guvacoline, and tannins in areca nut. The formation of collagen bands increased with smoking. OSMF occurrence was more common in individuals who used both smoked and smokeless forms of tobacco.

Figure 6: Oral submucous fibrosis

Parvathi *et al*. in 2011[27] found an increase in the frequency of micronucleated cells, it was 0.06% in normal, in precancerous lesions it was 0.12%, and in cancerous lesions it was 0.45% thus showing cytogenetic damage within the epithelium. Halder *et al*. in 2004[28] found the MN frequency in precancerous lesions to be 0.63%, and in cancerous lesions, it was 1.36%. Palve and Tupkari in 2008 also concluded that the frequency of micronuclei was found to be 0.21% in normal cells while it was 1.84% in cancerous cells.[29] Casartelli *et al*. in 2000[9] compared MN frequencies in exfoliated cells from normal mucosa, in precancerous lesions, and squamous cell carcinoma and concluded that there was an increase in the frequency of MNs in malignant lesions when compared with normal buccal mucosal cells, thus indicating a shift toward malignancy.

Kalita *et al*. in 2013[30] found the MN count to be 4.2 ± 0.96 in females and 6.6 ± 1.95 in males consuming tobacco quid. Smita *et al*. in 2013[31] used acridine orange to measure MNs in oral OSMF cases and found the count raised when compared with normal individuals. In a study conducted by Kiran *et al*. in 2018, the number of MNs was found to be 60/500 cells in participants with epithelial dysplasia,[32] while in our study, the frequency of MNs per cell in participants with OSMF was 2.37, with a standard deviation of 1.100.

Nuclear-specific stains and micronuclei

Staining procedures affect the micronuclei count and according to the results of the detailed survey done during the eighth workshop of the HUMN (Human Micronucleus Project) more than 50% of the laboratories which participated in the survey used Feulgen stain and only one of the laboratories had used H and E stain. According to the same survey, the second most commonly used stain was May–Grünwald–Giemsa (MGG) stain; however, Ayyad *et al*.[33] found better results for counting of MNs using Pap stain as compared to MGG stain.

We used Feulgen fast and acridine orange staining procedures in our study. We found that identification and

Table 1: Intergroup comparison of micronucleus frequency/cell in exfoliated buccal cells

Group	Mean±SD	Minimum	Maximum	Kruskal-Wallis test	
				χ^2	*P*
Normal (Group 1)	1.02±0.139	1	2	168.783	<0.001
Tobacco (Group 2)	1.49±0.771	1	6		
Gutkha (Group 3)	2.03±1.160	1	6		
Submucous fibrosis (Group 4)	2.37±1.100	1	6		

SD: Standard deviation

Table 2: Quantification of salivary DNA

Group	Mean±SD	Median	*F*	*P*
Normal (Group 1)	1.116±0.0933	1.130	27.794	<0.0001
Tobacco (Group 2)	0.889±0.1144	0.890		
Gutkha (Group 3)	1.317±0.1033	1.335		
Oral lesion (Group 4)	1.671±0.4460	1.865		

SD: Standard deviation

counting of MNs were much easier with Feulgen fast green stain since it is a nuclear-specific stain. Using acridine orange was a cumbersome procedure since the slides can be viewed only under a fluorescent microscope and slides cannot be preserved for a long time.

Purity of extracted DNA from saliva

Source of genomic DNA in saliva is not only from epithelial cells but also from white blood cells.[34] When extracting DNA from saliva, bacterial DNA can also be recovered if methods such as oral swabs are used.[35] In our study, the absorbance ratio was higher when we used commercial kits such as Oragene, where the bacterial DNA content is much lower owing to its special design. Most scientists prefer large amounts of DNA for performing various applications, but with advanced technology available, even small amounts of DNA can be used for performing single-nucleotide polymorphism genotyping, etc., Collecting DNA from saliva is a noninvasive procedure. Daksis and Erikson[36] state that acquiring DNA from oral samples is clearly advantageous over blood samples and opens the path for point-of-care testing.

Clinical implications

1. Saliva is a versatile biofluid which is a storehouse of a variety of biomarkers including tumor markers. Participants readily agree to give saliva samples but are hesitant to give blood samples since it is an invasive procedure. We successfully extracted and purified salivary DNA which can give a variety of information about the well-being of the participant
2. Smears prepared using liquid-based cytology have less cellular clumping, a clear background with distinct cellular features
3. A progressive increase in the number of MNs from cells obtained from the control group to the ones obtained from participants with oral lesions shows that changes can be detected at cellular level rather than waiting for a full-blown lesion for obtaining biopsy. Hence, a cellular grading will help in the early detection of genotoxic events [Figures 4-6]
4. Staining techniques such as acridine orange show apoptosis at 480–490 excitation in cells of participants with oral lesions and gutkha chewers. It is a proven fact that areca nut is a major etiological factor in submucous fibrosis. A bright red fluorescence shows a larger fraction of DNA in the denatured form. This can also be proved by a clinical evaluation of OSMF which is a premalignant lesion.

Conclusion

Researchers and clinicians around the world prefer working with saliva since it is a high-performance biofluid, which is easy to collect and handle. Using liquid-based cytology, we collected not only epithelial cells for evaluation of MNs but also extracted and purified DNA; therefore, we conclude that saliva can be used as a tool for mass-screening purposes. From the present study, we also conclude that the number of MNs per cell increases in participants with oral lesions. The technique is simple and noninvasive. It can become a valuable tool and a prognostic indicator in patients who are suffering from potentially malignant disorders.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Mandel P, Metais P. Les acides nucleiques du plasma sanguin chez l'homme. C R Acad Sci Paris 1948;142:241-3.
2. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485-7.
3. Zhang J, Tong KL, Li PK, Chan AY, Yeung CK, Pang CC, *et al*. Presence of donor – And recipient-derived DNA in cell-free urine samples of renal transplantation recipients: urinary DNA chimerism. Clin Chem 1999;45:1741-6.
4. Garcia Moreira V, Prieto Garcia B, Baltar Martin JM, Ortega Suarez F, Alvarez FV. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. Clin Chem 2009;55:1958-66.
5. Su YH, Wang M, Brenner DE, Ng A, Melkonyan H, Umansky S, *et al*. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. J Mol Diagn 2004;6:101-7.
6. Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y, *et al*. Genetic analysis of DNA excreted in urine: A new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 2000;46:1078-84.
7. Jiang WW, Rosenbaum E, Mambo E, Zahurak M, Masayesva B, Carvalho AL, *et al*. Decreased mitochondrial DNA content in post treatment salivary rinses from head and neck cancer patients. Clin Cancer Res 2006;12:1564-9.
8. Barak V, Goike H, Panaretakis KW, Einarsson R. Clinical utility of cytokeratins as tumor markers. Clin Biochem 2004;37:529-40.
9. Casartelli G, Bonatti S, De Ferrari M, Scala M, Mereu P, Margarino G, *et al*. Micronucleus frequencie in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinonoma. Anal Quant Cytol Histol 2000;22:486-92.
10. Ogden GR, Cowpe JG, Wright AJ. Oral exfoliative cytology: Review of methods of assessment. J Oral Pathol Med 1997;26:201-5.
11. Ogden GR, Cowpe JG, Green NW. Detection of field change in 11. Oral cancer using oral exfoliative cytologic study. Cancer

1991;68:1611-5.
12. Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear anomalies in buccal smears: Methods development. Mutat Res 1992;271:69-77.
13. Stich HF, Rosin MP, Vallejera MO. Reduction with Vitamin A and betacarotene administration of proportion of micronucleated buccal mucosal cells in Asian betal nut and tobacco chewers. Lancet 1984;1:1204-6.
14. Stich HF, Curtis JR, Parida BB. Application of the micronucleus test to exfoliated cells of high cancer risk groups: tobacco chewers. Int J Cancer 1982;30:553-9.
15. Patel BP, Trivedi PJ, Brahmbhatt MM, Shukla SN, Shah PM, Bakshi SR. Micronuclei and chromosomal aberrations in healthy tobacco chewers and controls: A study from Gujarat, India. Arch Oncol 2009;17:7-10.
16. Sellappa S, Balakrishnan M, Raman S, Palanisamy S. Induction of micronuclei in buccal mucosa on chewing a mixture of betel leaf, areca nut and tobacco. J Oral Sci 2009;51:289-92.
17. Dash KC, Nishat R, Kumar H, Mishra S, Raghuvanshi M, Bajoria A. Comparative study of micronuclei count in patients with different tobacco related habits using buccal epithelial cells: A tool for assessment of genotoxicity. J Contemp Dent Pract 2018;19:1076-81.
18. Palaskar S, Jindal C. Evaluation of micronuclei using papanicolaou and may Grunwald giemsa stain in individuals with different tobacco habits – A comparative study. J Clin Diagn Res 2010;4:3607-13.
19. Nair U, Obe G, Nair J, Maru GB, Bhide SV, Pieper R, *et al*. Evaluation of frequency of micronucleated oral mucosa cells as a marker for genotoxic damage in chewers of betel quid with or without tobacco. Mutat Res 1991;261:163-8.
20. Gandhi G, Kaur R. Cytogenetic studies in exfoliated cells of high cancer risk groups, pan masala chewers. Hum Ecol 2000;9:221-8.
21. Siddique YH, Ara G, Beg T, Afzal M. Micronucleus investigation in oral mucosal cells of gutkha/pan masala chewers. Indian Biol 2008;40:1-5.
22. Nair UJ, Nair J, Mathew B, Bartsch H. Glutathione S-transferasen M1 and T1 null genotypes as risk factors for oral leukoplakia in ethnic Indian betel quid/tobacco chewers. Carcinogenesis 1999;20:743-8.
23. Wenke G, Rivenson A, Brunnemann KD, Hoffmann D, Bhide SV. A study of betel quid carcinogenesis. II. Formation of N nitrosamines during betel quid chewing. In: O'Neill IK, von Borstel RC, Miller CT, Long J, Bartsch H, editors. N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Lyon: IARC Scientific Publications; 1984. p. 859-66.
24. Murdia US, Mehta FJ, Bhide SV. Nitrate reductase activity and nitrite levels in the saliva of habitual users of various tobacco products. Food Chem Toxicol 1982;20:269-71.
25. Nair J, Ohshima H, Pignatelli B, Friesen M, Calmels S, Bartsch H. Modifiers of endogenous carcinogen formation: Studies on *in vivo* nitrosation in tobacco users. In: Mechanisms in Tobacco Carcinogenesis, Banbury Report 23. Cold Spring Harbour NY: Cold Spring Harbour Laboratory Press; 1986. p. 45-61.
26. Chakradeo PP, Nair J, Bhide SV. Endogenous formation of N-nitrosoproline and other N-nitrosamino acids in tobacco users. Cancer Lett 1994;86:187-94.
27. Parvathi D, Thimmarasa VB, Mehrotra V, Arora P. A micronucleus assay for evaluation of genotoxicity in potentially malignant and malignant disorders. J Indian Acad Oral Med Radiol 2011;23:97-100.
28. Halder T, Chakraborty K, Mandal P. Comparative study of exfoliated oral mucosal cell micronuclei frequency in normal, precancerous and malignant epithelium. Int J Hum Genet 2004;4:257-60.
29. Palve DH, Tupkari JV. Clinico-pathological correlation of micronuclei in oral squamous cell carcinoma by exfoliative cytology. J Oral Maxillofac Pathol 2008;12:2-7.
30. Kalita H, Boruah DC, Dutta K, Devi R. Genotoxic effect on buccal epithelial cells of betel quid chewers by micronuclei assay. Asian J Exp Biol Sci 2013;4:491-4.
31. Smita J, Saif K, Afzal M, Falaq N, Hasan SY. Evaluation of micronucleus frequency by acridine orange staining in buccal epithelial cells of oral submucous fibrosis (OSMF) patients. Egypt J Med Hum Genet 2013;14:189-93.
32. Kiran K, Agarwal P, Kumar S, Jain K. Micronuclei as a predictor for oral carcinogenesis. J Cytol 2018;35:233-6.
33. Ayyad SB, Israel E, El-Setouhy M, Nasr GR, Mohamed MK, Loffredo CA. Evaluation of Papanicolaou stain for studying micronuclei in buccal cells under field conditions. Acta Cytol 2006;50:398-402.
34. Thiede C, Prange-Krex G, Freiberg-Richter J, Bornhäuser M, Ehninger G. Buccal swabs but not mouthwash samples can be used to obtain pre-transplant DNA fingerprints from recipients of allogeneic bone marrow transplant. Bone Marrow Transplant 2000;25:575-7.
35. Human Genomic DNA Content of Saliva Samples Collected with the Oragene® Self-Collection Kit, DNA Genotek White Paper, PD-WP-011.
36. Daksis JI, Erikson GH. Heteropolymeric triplex-based genomic assay to detect pathogens or single-nucleotide polymorphisms in human genomic samples. PLoS ONE 2007;2:e305.