

LETTER to the EDITOR

Editorial Process: Submission:01/22/2022 Acceptance:03/22/2022

Re: Evaluation of Micronuclei and Cytomorphometric Changes in Patients with Different Tobacco Related Habits Using Exfoliated Buccal Cells

Asian Pac J Cancer Prev, 23 (3), 755-757

Dear Editor

Kokila et al., (2021) evaluated micronuclei (MN) in buccal cells obtained from smokers, smokeless tobacco and combined tobacco users (i.e. chewers and smokers). Based on the obtained results they stated that “*tobacco in any consumable form is genotoxic*”. As for smokeless tobacco users and combined tobacco users, the statement is correct (Chandirasekar et al., 2013; Nersesyan et al., 2019b). But genotoxic action of tobacco smoking on buccal cells is questionable (Bonassi et al., 2011; Metgud and Neelesh, 2018; de Geus et al., 2019; Nersesyan et al., 2019a; Nersesyan, 2020). This is not the only weak point of this publication. Careful reading of it shows that the data presented by the authors are not reliable due to several serious reasons.

It is well known that exists a standardized and validated protocol for evaluation of MN in buccal cells (Thomas et al., 2009). According to it, MN should be scored in 2,000 buccal cells. In addition, not only MN but also other than MN nuclear anomalies should be considered in 1,000 cells. The authors evaluated ONLY 100 cells, i.e. 20-fold less that recommended number of cell and nuclear anomalies were not considered. Kokila et al. (Kokila et al., 2021) stated that they applied the criteria of Tolbert et al. (Tolbert et al., 1992) for MN scoring. We hesitate if the authors read carefully the paper by Tolbert et al., (1992). Otherwise they ought to score 2,000 – 3,000 cells because it is clearly written that “*if less than 5 MNC are observed after counting 1,000 cells, an additional 1,000 cells are scored, and so on up to a maximum count of 3,000 cells*”. Also Tolbert et al. described criteria for evaluation of so-called nuclear anomalies for the first time. But this important point was also disregarded by Kokila et al. It is notable that in the first, pioneer studies of MN in buccal cells, 50 cells per individual were evaluated (Stich et al., 1982), then the number of cells was increased to 500/individual (Rosin and Ochs, 1986).

The authors have also serious problems with calculations. Indeed, in the Table 1 are presented mean number of cells with MN (micronucleated cells), mean number of MN and mean of MN per cell. Let us, for example, check the controls. Corresponding numbers are following: 1.20, 0.60 and 0.305. There is a fatal mistake since number of MN should not be lower that mean number of cells with MN (since cell with MN can have

several MN). Since Kokila et al. declared that they scored only 100 cells, the numbers of mean MN per cell must be equal to mean numbers of MN divided by 100. But this is not the case. In group IV mean number of cells with MN is 1.20, mean number of MN is 0.6; hence, corresponding number in Table 2 must be $0.6 / 100 = 0.006$. In the Table is indicated 0.305. No one number is correct in other cases! Another example with group III, the most exposed: the mean number of MN is 15.77, the mean MN per cell is 1.469 (instead of 0.1577). Again number of cell with MN is higher that mean number of MN!

Less serious but important gaps in the study are following. The title of the article is not correct. The authors declare that the cells were obtained from patients. But in “*Materials and methods*” section is written following: “*a total number of 120 individuals without oral lesions were included in the study*” and “*Individuals with any history of systemic diseases and recent history of any viral infection or hospitalization, recent exposure to radiologic investigations, habituated with alcohol were excluded from the study*”. So, why word “patients” is used in the title of the paper?

The authors mentioned that the slides were stained with Feulgen and Pap stain, possibly for comparison (reason is not mentioned by the authors). In the Results section they stated that “*Results obtained were similar using either PAP or Feulgen stain in almost all the parameters evaluated*” but no data were presented to support this statement. It should be noted that DNA-non-specific stains (Pap stain in this case) visualize keratin bodies in buccal cells which mimic MN (Nersesyan et al., 2006). That is why “*non-DNA-specific stains give high false-positive results*” (Metgud and Neelesh, 2018; Juneja et al., 2019).

The quality of photographic images (Figure 2 and Figure 3) is not satisfactory, especially Figure 3. In photos of such quality is not possible to evaluate MN.

A lot of important information is missing. For example, demographic data and description of microscopic examination. Demographic data are very important because MN formation can be influenced by sex, age, nutritional habits and body mass index (Nersesyan et al., 2022). Moreover, the authors did not presented data on smoking – how many cigarettes were consumed by each participant, what kind of cigarettes (content of nicotine and tar). This information is very important since MN formation depends on these factors (Bonassi

et al., 2011; Nersesyan et al., 2011). Important also is information concerning microscope, i.e. producer and used magnification.

We may propose that serious errors in the publication could be due to technical reasons. But abovementioned points must be clarified by the authors to avoid confusion of the readers. In present form the results presented by Kokila et al., (2021) are misleading.

Keywords: Tobacco- genotoxic- micronucleus- oral cells- other nuclear anomalies

Conflict of interest

The authors declare that there is no conflict of interest.

References

- Bonassi S, Coskun E, Ceppi M, et al (2011). The Human MicroNucleus project on exfoliated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. *Mutat Res*, **728**, 88-97.
- Chandirasekar R, Suresh K, Sasikala K, et al (2013). Genotoxicity assessment in smokeless tobacco users: a case-control study. *Toxicol Ind Health*, **29**, 216-23.
- de Geus JL, Wambier LM, Loguercio AD, et al (2019). The smokeless tobacco habit and DNA damage: A systematic review and meta-analysis. *Med Oral Patol Oral Cir Bucal*, **24**, e145-e55.
- Juneja S, Katyal S, Rathore AS, et al (2019). Utility of DNA-specific stains in micronuclei assay as a marker of genotoxicity in oral potentially malignant disorders and oral squamous cell carcinoma. *J Cytol*, **36**, 111-5.
- Kokila S, Prasad H, Rajmohan M, et al (2021). Evaluation of micronuclei and cytomorphometric changes in patients with different tobacco related habits using exfoliated buccal cells. *Asian Pac J Cancer Prev*, **22**, 1851-5.
- Metgud R, Neelesh BT (2018). Effect of staining procedures on the results of micronucleus assay in the exfoliated buccal mucosal cells of smokers and nonsmokers: A pilot study. *J Cancer Res Ther*, **14**, 372-6.
- Nersesyan A (2020). Letter to Editor: The smokeless tobacco habit and DNA damage: A systematic review and meta-analysis. *Med Oral Patol Oral Cir Bucal*, **25**, 150-3.
- Nersesyan A, Kundi M, Atefe K, et al (2006). Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. *Cancer Epidemiol Biomarkers Prev*, **15**, 1835-40.
- Nersesyan A, Kundi M, Fenech M, et al (2022). Recommendations and quality criteria for micronucleus studies with humans. *Mutat Res - Reviews in Mutat Res*.
- Nersesyan A, Mišik M, Knasmüller S (2019a). Effects of tobacco Smoking on Micronucleus Frequency. In 'The Micronucleus Assay in Toxicology', Eds Royal Society of Chemistry, Croydon, UK, pp 355 - 72.
- Nersesyan A, Muradyan R, Kundi M, et al (2011). Impact of smoking on the frequencies of micronuclei and other nuclear abnormalities in exfoliated oral cells: a comparative study with different cigarette types. *Mutagenesis*, **26**, 295-301.
- Nersesyan A, Wultsch G, Mišik M, et al (2019b). Khat, Betel, Coca and Tobacco Chewing: Genotoxic Effects in Micronucleus Assays. In 'The Micronucleus Assay in Toxicology', Eds Royal Society of Chemistry, Croydon, UK, pp 373 - 86.
- Rosin MP, Ochs HD (1986). In vivo chromosomal instability in ataxia-telangiectasia homozygotes and heterozygotes. *Hum Genet*, **74**, 335-40.
- Stich HF, Stich W, Parida BB (1982). Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk for oral cancer: betel quid chewers. *Cancer Lett*, **17**, 125-34.
- Thomas P, Holland N, Bolognesi C, et al (2009). Buccal micronucleus cytome assay. *Nat Protoc*, **4**, 825-37.
- Tolbert PE, Shy CM, Allen JW (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat Res*, **271**, 69-77.

Rafayel E Muradyan¹, Gohar Parsadanyan², Armen Nersesyan^{3*}

¹Institute of Fine Organic Chemistry, 0014 Yerevan, Armenia.

²Yerevan State Medical University, 0025 Yerevan, Armenia.

³Center for Cancer Research, Medical University of Vienna, 1090 Vienna, Austria. *For Correspondence: anersesyan@yahoo.com

Response to Letter to the Editor

We would like to thank you for providing us the opportunity to respond to the queries raised by Muradyan et al in their letter, and also to clarify certain aspects of the methodology we had adopted in our previously reported article. We would also like to thank Muradyan et al for taking the time to raise these queries after doing a critical analysis of our paper. We have attempted to respond to their queries on a point by point basis below.

Muradyan et al. state that the “genotoxic action of tobacco smoking on buccal cells is questionable” along with references. However, there have been many publications in the past that have concluded that tobacco in smoked form can cause genotoxic changes in buccal cells (de Geus et al., 2018; Gopal KS et al., 2018; Upadhyay et al., 2019; DehghanNezhad et al., 2020). In fact, the meta-analysis by de Geus et al., (2018) synthesized the results of 16 reported studies and concluded that a higher frequency of micronuclei was noticed in the exfoliated cells of smokers when compared to non-smokers. de Geus et al, in their response to a letter to the editor (2019), noted that they observed similar results even after removing “weak” studies and performing the meta-analysis again. Therefore, smoking cannot be ruled out as a potential genotoxic agent for buccal mucosal cells.

The authors of the letter also point out that counting 100 cells alone is not sufficient, and at least 1,000 to 3,000 cells need to be counted for micronuclei assessment. While we do agree that counting more number of cells would give more reliable results, our methodology follows the procedure adopted by several researchers in the past who have counted only 100 cells for assessment of micronuclei (Dayanand et al., 2017; Upadhyay et al., 2019; Singam et al., 2019; Yoithappabhunath et al., 2021). There seems to be no worldwide consensus on the minimum number of cells that need to be counted. We have therefore used the criteria suggested by Tolbert et al., (1992) to identify and score micronuclei, in 100 cells per slide.

Muradyan et al. also state that the values provided in the Tables were wrong and that there were serious errors in calculations, with detailed explanations about the same. We would like to thank them for bringing this to our attention, since it is in fact an oversight that escaped many eyes. The third section of Table 1 of our published study under the subheading “*Frequency of cells showing micronuclei*” have errors in the values, most probably due to a bad copy paste while editing the manuscript. It is a gross error, and we sincerely apologize for missing it while scrutinizing the prepared manuscript. The correct values of the same are provided in Table 1 below. We would also like to point out that while the values were incorrectly copied, the final results and conclusions were drawn from the correct data, and they are the same as reported in the erroneous table. The number of cells showing micronuclei varied significantly among the different groups studied.

Regarding the title wrongly using the word “*patient*” while in fact none of the participants were suffering from any local or systemic illness, we agree with Muradyan et al. Using the term “*subjects*” or “*individuals*” would have been better, although it has no bearing on the results reported.

Frequency of cells showing Micronuclei		
Group (Mean ± SD)	Compared with	p-value
Group I (6.37±2.399)	Group II (8.8±3.537)	0.003*
Group I (6.37±2.399)	Group III (10.83±2.995)	<0.001*
Group I (6.37±2.399)	Group IV (0.53±0.937)	<0.001*
Group II (8.8±3.537)	Group III (10.83±2.995)	<0.018*
Group II (8.8±3.537)	Group IV (0.53±0.937)	<0.001*
Group III (10.83±2.995)	Group IV (0.53±0.937)	<0.001*

We have reported in our methodology that both Pap and Feulgen stains were used. We have however used the Feulgen stained smears for micronuclei evaluation, and Pap stained smears for cytomorphometric measurements. While we have not explicitly mentioned the same in the methodology, we have indicated the same in the titles of the tables. We did compare both stains for micronuclei evaluation in curiosity, but found no significant difference between both. We would be more than willing to share the data with anyone interested.

Muradyan et al, also have raised an issue with the quality of the photographs. The actual analysis was done directly on the computer attached to our Olympus BX43F research microscope, using ProgRes Jenoptik image analysis software. The photographs are screenshots of the same and hence appear to be poor in quality.

Finally, we have collected detailed demographic

data from all participants involved in the study using a structured proforma. However, we could draw no meaningful conclusions from them. Again, we would be willing to share the raw data with anyone interested in the same.

From the critical analysis made by Muradyan et al, it is clear that they are experienced and very interested in studies on micronuclei. We appreciate the efforts made by them to identify the lacunae and errors in our study. We have made a genuine attempt to answer all the queries, and hope that clears any confusions about the study. We also stand by the findings that we have reported.

Respectfully,

**Sivakumar Kokila, Harikrishnan Prasad*,
Muthusamy Rajmohan, Kenniyan Kumar
Srichinthu, Loganathan Mahalakshmi,
Sivanandhan Shanmuganathan, Perumal
Prema**

Department of Oral and Maxillofacial Pathology, KSR Institute of Dental Science and Research, Tiruchengode, Namakkal, India.

**For Correspondence: prasad@ksrids.edu.in.*

References

- Dayanand V, Kempegowda SKH, Raju PH, Muniyappa B, Rao SS (2017). Comparison of micronucleated cell in buccal smears among smokers and non-smokers. *J Evid-Based Med*, **4**, 100-3.
- de Geus JL, Wambier LM, Bortoluzzi MC, et al (2018). Does smoking habit increase the micronuclei frequency in the oral mucosa of adults compared to non-smokers? A systematic review and meta-analysis. *Clin Oral Invest*, **22**, 81–91.
- de Geus JL, Wambier LM, Bortoluzzi MC, et al (2019). Letter to the Editor replying to Armen Nersesyan about the article published in *Clinical Oral Investigations* titled “Smoking increases the frequency of micronuclei in the oral mucosa of adults relative to non-smokers-a systematic review and meta-analysis”. *Clin Oral Invest*, **23**, 503-5.
- DehghanNezhad M, Naderi NJ, Semyari H (2020). Micronucleus assay of buccal mucosa cells in waterpipe (Hookah) smokers: A Cytologic Study. *Iran J Pathol*, **15**, 75-80.
- Gopal KS, Padma M (2018). Evaluation of cytogenic damage in the form of micronuclei in oral exfoliated buccal cells in tobacco users. *Indian J Dent Res*, **29**, 773-80.
- Singam PK, Majumdar S, Uppala D, et al (2019). Evaluation of genotoxicity by micronucleus assay in oral leukoplakia and oral squamous cell carcinoma with deleterious habits. *J Oral Maxillofac Pathol*, **23**, 300.
- Tolbert PE, Shy CM, Allen JW (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat Res*, **271**, 69-77.
- Upadhyay M, Verma P, Sabharwal R, et al (2019). Micronuclei in exfoliated cells: A biomarker of genotoxicity in tobacco users. *Niger J Surg*, **25**, 52-9.
- Yoithapprabhunath TR, Ganapathy N, Renugadevi S, et al (2021). Cytogenetic analysis of genetic damage in young individuals exposed to chronic mechanical irritation in the lateral border of the tongue due to sharp teeth – a novel study. *Med Pharm Rep*, **94**, 88-93.