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Frequency of micronuclei and apoptosis in exfoliated buccal cells in chronic in flammation of oral mucosa

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Abstract

Chronic inflammations of the oral mucosa (CIOM) induced by biological , chemical , and physical factors and are in turn associated with an increased risk of oral cancers. The objective of the current study was the investigate the spontaneous genetic damageusingthe micronuclei (MN) and apoptosis test on exfoliated cells from (CIOM) patients. Cytological tests included, in addition to (MN),counting apoptosis(karyorrhexis, condensed chromatin , and, pyknosis). The study sample comprised 42 individuals aged (20–40) years including twenty two patients (13 males and 9 females) revealed (CIOM) and the another 20 (12 males and 8 females) were healthy control group. MN frequency was significantly increased ($P<0.001$) in buccal mucosa cells obtained from lesion area (LA) and normal area (NA) from chronic inflammation patients with mean value (7.53 ± 1.49) and (5.02 ± 1.47) respectively, when compared with the healthy control group (2.40 ± 1.40). Lower frequency of apoptosis observed in (LA) from chronic inflammation patients compared with control group, was ($P=0.06$) more over apoptosis was significant increased ($P<0.001$) in NA of chronic inflammation patients when compared with cells of control group. The MN was regarded with the gender, age, mouthwashes uses , and , oral hygiene of the patients. The frequency of MN was significantly higher in the females than males in both patients and control groups ($P< 0.01$), whereas non-significant differences in MN occurrence was observed in relation to age.

However MN frequency was significantly higher in poor oral hygiene and mouthwash users ($P<0.01$) patients group but shown non-significant differences in poor and good oral hygiene in control group . Also the significantly higher frequency was increased MN in mouthwashes user in control group, when compared with non-users. The results suggest that increased MN and decreased apoptosis in the long periods chronic inflammation of the oral mucosa patients could be consider as a useful diagnostic bio-monitoring assay to prevent the risk from transformation of chronic inflammation to pre cancers lesions.

Key Words :Micronuclei ,Apoptosis, Chronic inflammation, Oral mucosa exfoliated.

Introduction

Buccal cells form the first barrier of the inhalation or ingestion route and are capable of metabolizing and inflammation proximate carcinogens to reactive products (1-2-3).There-for,it could be argued the oral epithelial cells represent a preferred target sit for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion (4). The oral epithelium maintains itself by continuous cell renew, whereby new cells produced in the basal layer by mitosis migrate to the surface replacing those that are shed (5).How-ever the oral mucosa, in addition to its unique micro environmental niche fueled by food residues; microbial flora; and saliva, has also been recognized for its sensitivity to inflammation, fibrosis, response and proneness to drug, carcinogenic and mutagenic agents (6-7). Oral mucosa mainly exposed to inflammation, the sources of inflammation are wide spread and include microbial and viral infections, exposure to allergens , toxic chemicals, consumption of alcohol and tobacco (8–9).

In general, the longer the inflammation persists, the higher risk of cancer. Two stages of inflammation exist; acute and chronic inflammation. Acute inflammation is an initial stage of inflammation, which is mediated through the activation of innate immune system, this type of inflammation persists only for a short time, the second stage of inflammation, or chronic inflammation, sets in and may predispose the host to various chronic illnesses, including cancer (10). During inflammation, mast cells and leukocytes are recruited to the site of damage, which lead to a "respiratory burst" due to an increased uptake of oxygen, and thus, an increased release and accumulation of reactive oxygen species ROS at the site of damage (11-12) when (ROS) produced over long time, and thus significant damage may occur to the cell structure and functions and may induced somatic mutations and neoplastic transformation in site of chronic inflammation(13-14).

Molecular epidemiology research focuses on the biomarkers of exposure (e.g., cytoge- netic endpoints– chromosomal aberrations, micronuclei, sister chromatid exchanges, and, apoptosis) (5–15–16). MN assay has been applied to evaluate chromosomal damage for biological monitoring of human population exposed to variety of mutagenic and carcinogenic agents (17-18-19).

Buccal cells not only offer the clinician opportunities for early diagnosis but also provide a unique model for mutation research that permits correlating genetic alteration with histopathologic changes and for drug discovery investigations (4-20). The objective of the current study was to investigate the spontaneous genetic damage in exfoliated cells of the buccal mucosa from chronic inflammation oral mucosa patients and healthy controls by the abnormalities cells (MN and apoptosis) assay with exfoliated buccal mucosa cells regarding the factors that might affect MN and apoptosis frequency (i.e.,ch-ronic inflammation,

gender, age, oral hygiene and mouthwash use).

Materials and Methods

Samples: Twenty- two patients (13 males and 8 females) revealed clinical signs of chronic inflammation of the oral mucosa were used in this study.In addition,20 healthy (12 males and 8 females) were used and considered as control group. The patients and control group individuals were aged (20- 40) year's. A written consent was taken from each individual and the samples were taken from AL-Muthanna Dental Specialization Center. The period of study was extended for 6 months .This study was approved by the hospital ethical committee approved the human study.

Micronuclei and Apoptosis Assay: The samples of exfoliated cells were collected from buccal mucosa in area with lesions and area without lesions (normal area)from patients and from control group. The participants were also asked to rinse the oral cavity for 1 minute with 10 ml. of sterilized distilled water and exfoliated cellsof buccal mucosa were obtained by a light and gently pressure was applied, while scraping the buccal mucosa with cytobrush moistened with buffer (21–22-23) for each individual, cytobrush used to collected buccal cells was shaken in a centrifuge tube containing saline solution(Hank's basic or other buffer solution) to release the cells and the tube was then centrifuged to wash the cells in the buffer solution twice, The supernatant was discarded and pellets were re-suspended in 0.75 M Kcl and fixed by a cold methanol- glacial acid mix (3:1), then the tube stored at room temperature until investigation of MN and apoptosis. Slides were prepared by adding one drop of fixed cells solution onto center of clean glass slides and spreading on the slides. Afterwards ,the glass slides wasdried in air. Staining was carried out with 2%giemsa solutionfor a period of 10 minutes. Afterwards , the glass slide was rinsed with distilled water and dried in the air. The criteria of MN evaluation were those suggested by This study was approved by the hospital ethical committee Tolbert *et.al.(*1992) and Titenko-Holland 1998 (12). Screened for cell abnormalities, addition to counting MN, nuclear alterations suggestive of apoptosis were also investigated under oil immersion lens (1000x), followed by phase contrast microscopy for counting of MN and apoptosis according to established methods (22- 23- 24). At least 1000 intact epithelial cells per individual were scored to achieve the average percent of MN cells and apoptosis. Statistical analysis was done by SPSS version 15, comparison by (ANOVA-LSD) and correlation by Spearman correlation.

Results :

The sample characteristics ate represented, as that shown in The (table-1), the mean age ± SD of the whole sample was (35.22± 3.07) for (CIOM) patients and healthy control with means were (38.55±3.49) and (34.64±2.44) respectively which

indicated no statistically significant differences between the groups.

Table 1: shows Sample characteristics.

characteristic	Group		P value
	Patients	control	
	No. = 22	No. = 20	
Gender	No.%	No.%	
Female	9 40.9%	8 40%	P<0.01*
Male	13 59.1%	12 60%	0.040
Oral hygiene			
Good	3 13.7%	5 25%	
Poor	19 86.3%	15 75%	
Mouthwash use			
Use	5 22.7%	9 45%	
Non	17 77.3%	11 55%	0.012
Age (years)			
20-30	14 63.6%	9 45%	
30- 40	8 36.4%	11 55%	0.013

* Significant p<0.01

Micronuclei analysis , results showed (Table -2, Fig-2a,2b) the MN frequency was significantly higher (P<0.001) in buccal mucosa cells obtained from lesion area (LA) than those of normal area(NA) in chronic inflammation patients with mean SD value (7.53 ±1.49) and (5.02 ± 1.47) respectively in comparison with their healthy control group with mean ± SD was (2.40±1.40).

Table 2: Micronuclei cell frequency in buccal mucosa exfoliated cells of (CIOM) patients and healthy controls.

Subject	No.of individual	MN-Range	MN(%) (Mean SD/1000cells)	Comparison	P value
Patients Lesion area (LA)	22	1 - 20	7.53 ±o.78	LA versus control	P <0.001*
Patients Normal area (NA)	22	1 - 18	5.02± 0.533	LA versus NA	P =0.796
Control	20	0 - 6	2.40 ±0.40	NA versus control	P <0.001*

*Significant at P<0.001 compared with control

LA with control Significant.NA with control Significant.LA with NA Non-Significant.

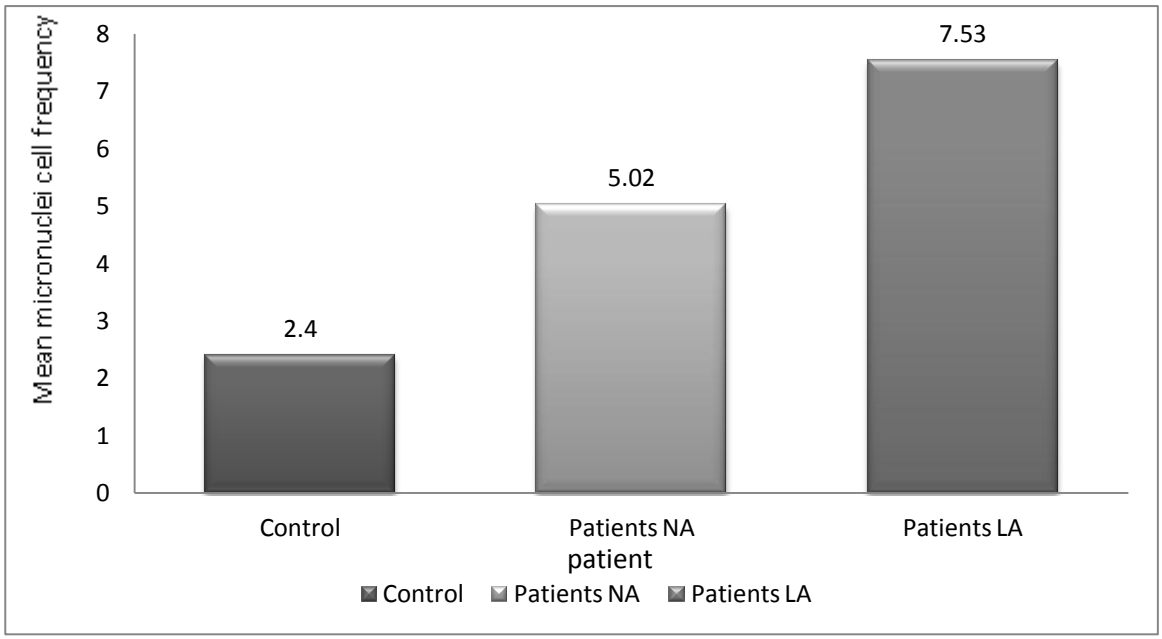

Figure 2a:- shows Micronuclei cell frequency in buccal mucosa exfoliated cells of chronic inflammation oral mucosa patients and healthy controls.

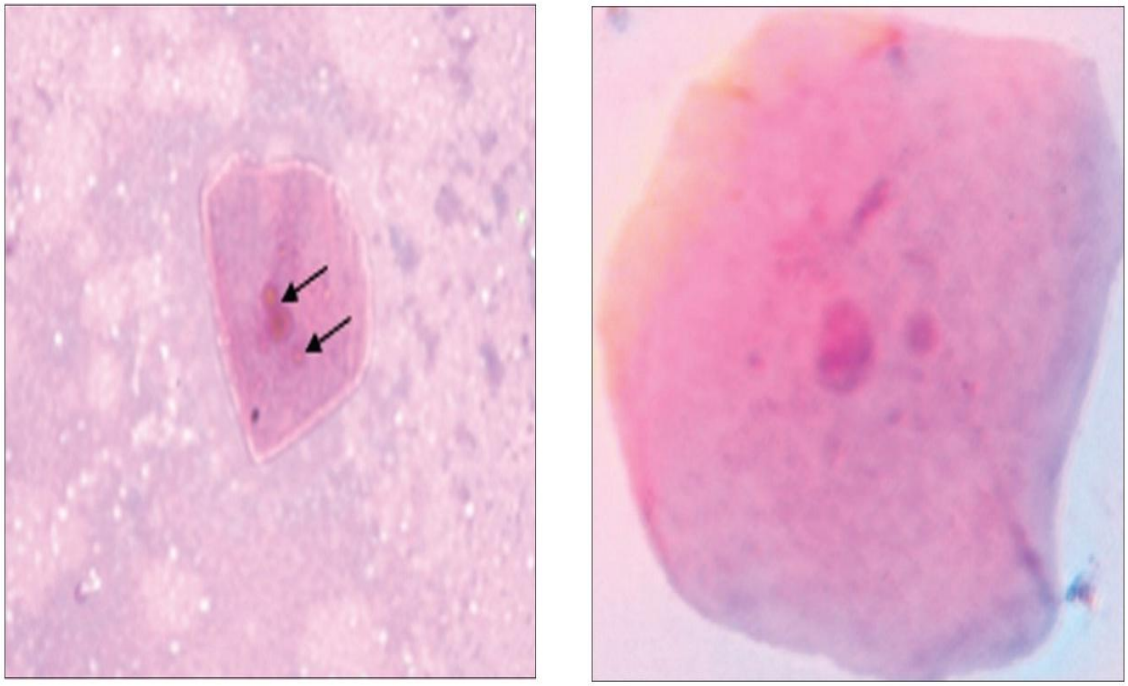

Figure 2 b: shows epithelium of buccal cell

Apoptosis, degenerative nuclear alterations indicative of apoptosis observed, includes (karyorrhcetic, condensed chromatin, and, pyknosis), As observed in (Table 3), the frequency of apoptosis did not show significant difference between buccal cells obtained from (LA) of chronic inflammation patients compared with cells of healthy control group (P=0.061), whereas apoptosis frequency was significantly increased in buccal cells obtained from (NA) of chronic inflammation patients compared with cells of control group (P< 0.001).

Table 3 : Frequency apoptosis in exfoliated buccal cells of chronic inflammation oral mucosa patients and healthy controls.

Subject	No. of individual	Total cells	Karyorrhcetic	Condensed chromatin	Pyknosis	P value
Patients Lesion area (LA)	**22**	**48.01**	**322**	**439**	**170**	**0.061**
Patients Normal area (NA)	**22**	**51.92**	**393**	**580**	**136**	**P < 0.001***
Control	**22**	**44.32**	**402**	**429**	**77**	**0.063**

*Significant at p< 0.001 compared with control.

The comparison mean ± SD apoptosis analysis in buccal mucosa cells shown in (Table 4 and fig 4), there was no significant difference in the frequency of apoptosis between cells obtained from (LA) in patients compared with apoptosis in cells of the control group with mean ± SD (27.81±3.45) and (28.08±4.65) respectively It was less apoptosis mean in the (LA) cells from patients than cells of control group . But,it showed significant increase in the apoptosis (P<0.001) in cells obtain from (NA) in patients compared with cells of the control group with mean (32.49±5.60) and (28.08±4.65) respectively.

Table 4: Comparison Apoptosis analysis(Karyorrhcetic, condensed chromatin and pyknosis) in buccal mucosa exfoliated cells of chronic inflammation oral mucosa patient sand healthy controls.

Subject	No. of individul	Apoptosis number	Apoptosis (%) (Mean ±SD / 100 cells)	Total cells	P value
Patients Lesion area	**22**	**931**	**27.81 ±3.45**	**48.01**	**0.478**
Patient Normal area	**22**	**1.109**	**32.49± 6.60**	**51.92**	**P < 0.001****
control	**20**	**908**	**28.08± 4.65**	**44.32**	**0.579**

** Significant at p < 0.001 compared with control.

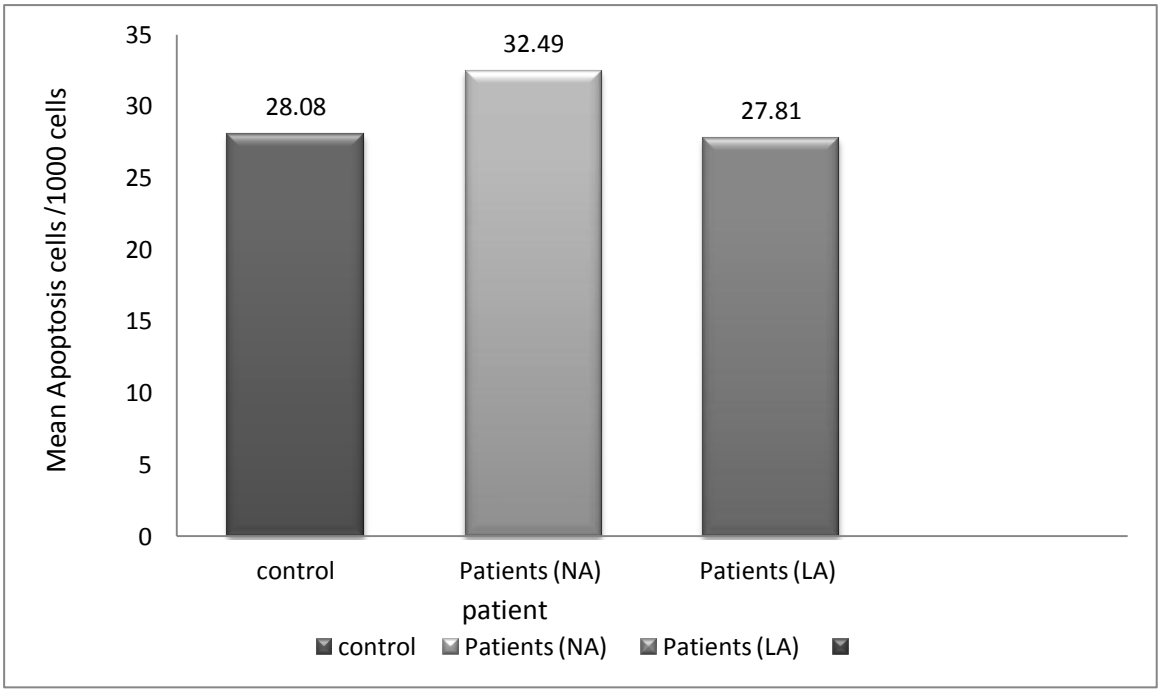

Figure 4:shows the Comparison between the apoptosis in buccal mucosa exfoliated cells of chronic inflammation oral mucosa patients and healthy controls.

The comparison between patients cell control group, revealed, non-significant differences in MN mean value occurrence, in relation to age. However MN occurrence was significantly higher in females, poor oral hygiene and mouthwash users ($P<0.01$). Table (5) and figure (5), showed the significantly increased MN mean ± SD in females than males in both buccal cells from patients and control groups with mean ± SD, in females (2.99±1.48) and (8.61±3.22), when in males (1.88±1.33) and (6.45±2.99) respectively. However, MN occurrence was significantly higher in poor oral hygiene and mouth- wash users ($P<0.01$) in chronic inflammation patients, whereas the frequency of MN was significantly higher in mouthwash use of control group, when compared with non-user with mean ± SD (2.78±1.46) and (1.99±1.88) respectively.

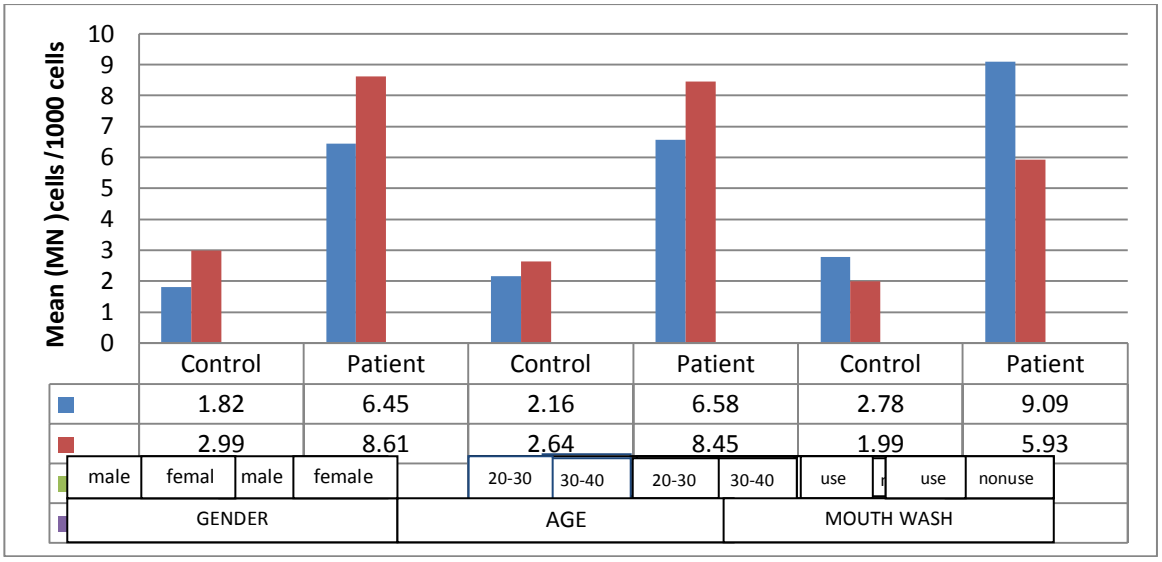

Figure 5: shows The comparison Mean (MN%) cells inbuccal exfoliated cells of chronic inflammation oral mucosa patients and healthy controls, by gender, age, and mouth wash use a variables.

Table 5: shows The comparison between the value Mean (MN%) in buccal mucosa exfoliated cells of chronic inflammation oral mucosa patients and healthy controls, by gender, and mouthwash use a variables.

Characteristic		MN (Mean SD/1000 cells)		P value
		Control	Patients	
Gender	Male	1.82± 1.33	6.45± 2.99	P < 0.01*
	Female	2.99± 1.48*	8.61± 3.22*	
Oral hygiene	Good	2.11 ± 1.97*	6.58± 2.69	P < 0.01*
	Poor	2.64 ±1.24*	8.45± 3.48*	
Mouthwash use	Use	2.78 ±1.46*	9.09± 4.33*	P < 0.01*
	Non	1.99± 1.88	5.93± 2.75	

Significantly different compared between characteristics in groups P< 0.01.*

Discussion

The micronuclei, is a recently upgraded topic, especially in the oral cancer filed (25), the cytogenetic biomarker of genome damage (e. g., MN, nuclear bud) and cell death (e. g. ,karyolysis, apoptosis) can be observed in both the lymphocytes and buccal mucosa cellssystems, and provide a more comprehensive assessment of genome damage of these MN and apoptosis in the context of cytotoxicity and cytostatic effects (26-27).

The significantly higher frequency of MN in exfoliated oral buccal cells from chronic inflammation patients with oral mucosa observed in this study, corroborate the results from some studies, The hypothesis of a direct association between the frequency of MN in target or surrogate tissues and cancer development, is supported by the finding like in clear increase in the frequency of MN in target tissue as well in peripheral lymphocytes in cancer patients (6-25-28-29). Al though this

association has been described by other worker indicated the MN in exfoliated oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents (30–27) Various studies have shown the correlation of frequency of MN an severity of this genotoxic damage (29–27). Also the results are in agreement with those of other workers, suggest in the site of chronic inflammation such as when occur in the oral mucosa .Indeed cancer initiation and progression has been linked to oxidative stress by increasing DNA mutation or inducing DNA damage ,genome instability and cell proliferation (27–31- 32-33).

The increase of MN frequency had been found associated with gender , we noted an increase in MN in female of chronic inflammation of oral mucosa patients , and in control groups, this increase may occur according to normally female have inactivated X-chromosome (Barr body) occupy site neighbor to nucleus in cytoplasmic of the cells, that lead to increase the counting number of MN in female samples than male, these result covenant with other workers indicated increased frequency of MN in female than male (19-34–35).

The significant increase of MN in mouthwash users in patients and control groups was also observed in this study that evaluated the genotoxic effect of risk factor for oral cancer development these occur according to components of mouthwash, ethanol is still a component of oral- care products (36–37). However, recent study showed that the genotoxicity of mouthwashes is caused by ethanol and not by any other ingredient (38), the mucosa may be damaged by ethanol, which leads to the stimulation of cell regeneration and genetic changes may then cause the development of dysplasia, or leukoplakia, and, finally cancer (38).The possibility of damage to the oral muosa exists with the use of mouthwashes (39), the risk was confined to users of mouthwash high in alcohol content (>25% Vol.) (40-41), from these results, It may be hypothesized that the use of mouthwashes could have a threshold for adverse effects.

The poor oral hygiene increased MN frequency, these observations suggest that there may be an influence on risk for oral cancer (39), poor oral hygiene increased the acetaldehyde production in oral micro flora, however, the metabolic acetaldehyde production directly affect the mucosa by alcohol dehydrogenase and the salivary acetaldehyde represents mostly microbial acetaldehyde formation in the oral cavity which increases the risk of genotoxicity to oral mucosa and may lead to genotoxic events induced by carcinogenic agents (25–42).

Therefore the lower frequency of apoptosis observed in lesion of patients group, indicate that with evolution of malignant transformation, the apoptotic response fails, as also bserved in precursor lesions of cervical cancer (43–44). The possible mechanisms include induction of genomic instability, alteration in epigenetic events and subsequent in appropriate gene expression, enhanced proliferation of initiated cells, resistance

to apoptosis, aggressive tumor new vascularization , invasion through tumor–associated basement membrane and metastasis (29–44–45).

The results, obtained in the present study showed that oral chronic inflammation is associated with higher frequency of chromosomal damage and suggests that apoptosis in decreased frequency is associated with evolution of malignant transformations. Additionally, they suggest that mouthwashes and poor oral hygiene are effective in inducing chromosomal damage.

In conclusion, the degenerative nuclear alteration could be consider as indicative of apoptosis .Beside MN can be se as a useful assay for bio-monitoring to increased risk of transformation of chronic inflammation of oral mucosa to cancer.

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تردد الانوية الصغيرة والاستماتة الخلوية في الخلايا المقشرة لمخاطية الفم لمرضى الالتهاب المزمن لمخاطية الفم

الخلاصة:

الالتهاب المزمن للبطانة المخاطية للفم والذي يحدث بواسطة عوامل بايلوجية او كيميائية او فيزيائية وارتباطه بزيادة مخاطر تحوله الى سرطان الفم.

الهدف من هذه الدراسة تقييم الضرر الوراثي التلقائي الحاصل في خلايا البطانة المخاطية للفم لمرض الالتهاب المزمن وذلك بواسطة اختبار النويات الدقيقه Micronuclei (MN) واستماتة الخلايا (Apoptosis) الحاصل في الخلايا المقشرة للبطانة المخاطية للفم.

تتضمن الدراسة الخلوية اضافة الى عد الانوية الصغيرة، عند الاستماتة الخلوية (تمزق الانوية،تكثيف الصبغي،تغلظ) شملت الدراسة (42) فرداً وبعمر يتراوح بين (20_40) سنة منهم (22) فرداً (13 ذكور،9 اناث) مصابين بالتهاب مزمن للبطانة المخاطية للفم اضافة الى (20) فرداً (12 ذكور،8 اناث) سليم كمجموعة سيطرة.

حيث كانت زيادة معنوية في تردد (MN) ($P<0.001$) في خلايا البطانة المخاطية للفم المأخوذة من مناطق الافة (Lesion area) والمناطق غير المتضررة (Non_Lesion area) في مرض الالتهاب المزمن للفم حيث كانت المتوسطات (5.49 ± 7.53) و(4.74 ± 5.02) وعلى التوالي مقارنة بمجموعة السيطرة حيث كان المتوسط (1.40 ± 2.40)، ولوحظ انخفاض في تردد الاستماتة الخلوية في مناطق الافات للمرضى مقارنة بمجموعة السيطرة ($P=0.06$) بينما كانت هنالك زيادة معنوية في الاستماتة الخلوية في العينات المأخوذة من المناطق غير المتضررة للمرضى مقارنة بمجموعة السيطرة ($P<0.01$).

اما في ما يخص تردد الانوية الصغيرة فيما يتعلق بأرتباطها بمتغيرات (العمر، الجنس، استخدام غسول الفم،صحة الفم).

كان هناك ارتفاع معنوي في تردد (MN) في الاناث مقارنة بالذكور في كلاً من مجموعة المرض والسيطرة ($P<0.01$) ولم يلاحظ هنالك اي فروق معنوية في وجود (MN) في الفئات العمرية المختلفة.

علاوةً على ذلك كان هنالك زيادة معنوية في تردد (MN) في الاناث اللاتي يعانين من صحة فم غير جيدة واللاتي يستخدمن غسول الفم ($P<0.01$) اضافة لذلك لوحظ هنالك زيادة في تردد (MN) في عينات المرضى الذين يستخدمون غسول الفم ويعانون من صحة فم غير جيدة،كذلك هنالك ارتفاع معنوي في تردد (MN) عند مستخدمي غسول الفم مقارنة بالذين لايستخدمون غسول الفم في مجموعة السيطرة ($P<0.01$).

من خلال النتائج نقترح ان زيادة (MN) وتثبط الاستماتة الخلوية ربما ترتبط مع تطور سرطان الفم في مرضى الالتهاب المزمن للبطانة المخاطية للفم.