ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 4, Issue – 1, February - 2021, Page No. : 414 - 422

Evaluation of combined genotoxicity of mouth washes and orthodontic appliances in patients with fixed orthodontic appliance - An ex vivo study.

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Citation of this Article: Dr. Babu Salam C, Dr. Midhun S Kishor, Dr. Shruti Jain, Dr. Radhika B, Dr. Sheetal S Menon, "Evaluation of combined genotoxicity of mouth washes and orthodontic appliances in patients with fixed orthodontic appliance - An ex vivo study", IJDSIR- February - 2021, Vol. – 4, Issue - 1, P. No. 414 – 422.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

The physical and chemical characteristics of oral environment are favourable for the biodegradation of metal alloys. The discharged ions like Ni was reported to be a strong immunologic sensitizer. Hence we designed this study, where micronuclei frequency will be evaluated using a DNA specific stain (fuelgen stain) from the buccal mucosal cells of fixed orthodontic patients using mouthwashes. Buccal cells are collected by rubbing the buccal mucosa with hard bristled tooth brush or wooden spatula and processed by Fuelgen staining technique. A significant increase in Mn frequency in buccal mucosal cells of cases compared to controls. This shows that subjects with fixed orthodontic appliance using chlorhexidine mouthwash have increased incidence of localized genotoxicity.

Introduction

Fixed orthodontic appliance consists of bands, brackets and wires made of metal alloys containing nickel, chromium, cobalt, iron and titanium. The physical and chemical characteristics of oral environment are favourable for the biodegradation of metal alloys. The discharged ions like Ni was reported to be a strong immunologic sensitizer. They can trigger hypersensitivity reaction like contact dermatitis and asthma. Since metal ions are non biodegradable, their sustained release can result in cumulative accumulation on adjacent tissues. This results in toxic effects on cellular metabolism and DNA stability. [1,2]

Although mechanical plaque control can be an effective strategy for preventing the progression of periodontal diseases, most individuals do not adequately brush their teeth, and use a dental floss on a daily basis. The daily use of an effective mouth rinses are generally considered a simple strategy and most patients can easily incorporate this into their home care routine. There are many studies that have suggested a possible connection between the usage of alcohol-based mouthrinses and the development of oropharyngeal cancer, questioning the daily use of alcohol based mouthwashes. The genotoxicity can be evaluated by well established end points like micronuclei assay (Mn) from exfoliated buccal mucosal cells.[3]

Even though some studies have been done in patients with fixed orthodontic appliance where genotoxicity was evaluated using micronuclei from buccal mucosal cells, most of these studies have used non DNA specific stains for micronuclei. This can increase the false positive results thus affecting the validity of the results.[2,4]

Hence we designed this study, where micronuclei frequency will be evaluated using a DNA specific stain (fuelgen stain) from the buccal mucosal cells of fixed orthodontic patients using mouthwashes. Aim of this study is to evaluate and compare the genotoxicity in patients with fixed orthodontic appliance with and without using mouthwashes controls using buccal mucosal micronuclei assay.

Materials And Methods

Study design: Case control

Study setting: Private dental Hospital

Study period: 50 days

Sample size: n = 500 for case and control groups. Mean difference and standard deviation were referred from literature.[4] The sample size was calculated for 80% power and 5% α error using nMaster™ sample size calculation software.

Study groups

- **Case group:** Patients with fixed orthodontic appliance (n=500)
- **Control group:** Patients without any orthodontic appliance (n= 500)

Inclusion criteria of cases

- Subject with orthodontic appliance for a period of 6 months to 3 year.
- Subject of age group between 16 to 25 years.
- Subjects who are using mouthwashes (Chlorhexidine) for oral hygiene maintanence[5]

Exclusion criteria of cases

- Patients with metallic restorations
- Patients with tobacco or alcohol related habits
- Patients who had taken radiograph since last 3 months.
- Patients with oral lesions or systemic illness like diabetes or HIV infection.
- Patients being treated for malignancies.

Inclusion criteria of controls

- Subject with orthodontic appliance for a period of 6 months to 3 year.
- Subject with no previous history of treatment with orthodontic appliance.
- Subject of age group between 16 to 25 years.

Exclusion criteria of controls

- Patients with metallic restorations
- Patients with tobacco or alcohol related habits
- Patients who had taken radiograph since last 3 months.
- Patients with oral lesions or systemic illness like diabetes or HIV infection.
- Patients being treated for malignancies.
- Patients using chlorhexidine mouth rinses since 1 month.[6]

Technique used[3]

a) Buccal mucosal micronuclei assay

Specimen collection[3]

Buccal cells are collected by rubbing the buccal mucosa with hard bristled tooth brush or wooden spatula. The cells are directly spread on a clean glass slide. The slides will be fixed in methanol acetic acid (3:1) for 20 minutes.[4]

Fuelgen staining technique[11]

Smear is denatured by exposing to

1) Deionised water at 37°C for 1 minutes 2)1N HCl at 37°C for 2 minutes, 3)IN HCl at 60°C for 8 mnutes,4) IN HCl at 37°C for 2 minutes ,5) Deionised water for 1 min.

Following which the smear is flooded with Schiff's reagent for 20 minutes. The smear is counter stained with 1%Light green for 10 seconds. The slide is washed in deionised water and blotted gently to dry. Slide is mounted and observed.

Scoring technique

A minimum of 1000 cells are counted under 400x magnification. The Mn recordings were standerdized using the published photomicrographs.[3] Two evaluators recorded the Mn frequencies. The evaluators simultaneously recorded Mn using multiheaded microscope order to reduce the inter observer variability.

MN scoring criteria[3]

The diameter of MN should be 1/3rd or 1/6th of the size of the main nucleus. MN should be separated from the main nucleus with clear nuclear boundaries. MN should have similar staining like the main nucleus. MN should be on the same plane of focus as main nucleus.[8]

Statistical analysis

The data will be entered in SPSS software v.16. Mean with standard deviation will be calculated. Comparison between the groups will be done by paired t test. p value less than 0.05 will be considered as statistically significant.

Results

Patients with orthodontic appliance using mouthwashes were selected as cases (n=500) and those with appliance, not using any mouthwashes were selected as controls (n=500).Cases and controls were age and sex matched.

1. Demographics

The average age of subjects in cases and controls were 25.20 ±1.05.case and control group was comprised of 70% females and 30 % males. (Graph 1)

2. Comparison of Nuclear abnormalities between cases and controls

a) Micronuclei (Mn) frequency (Table 1,Graph 2)

The mean micronuclei frequency among cases was 3.5±0.89 and in controls were 1.3±0.97.

There was a statistically significant increase in the micronuclei frequency in cases compared to controls ($p<0.001$)

b) Nuclear bud (Nu Bd) frequency (Table 1,Graph 3)

The mean nuclear bud frequencies among cases were 0.9±0.96 and in controls were 0.7±0.92.

There was no statistically significant difference in the nuclear bud frequency between cases and controls ($p=0.541$)

c) Binucleate cell (Bi Nu) frequency (Table 1,Graph 3)

The mean binucleate cell frequencies among cases were 3.15±2.47 and in controls were 1.15±1.136.

There was a statistically significant increase in the binucleate cell frequency in cases compared to controls ($p=0.002$)

d) Total nuclear abnormality frequency (Table 1,Graph 4)

The mean nuclear abnormality frequency among cases was 7.55±2.87 and in controls were 3.19±1.9.

There was a statistically significant increase in the nuclear abnormality frequency in cases compared to controls ($p<0.001$)

Figures

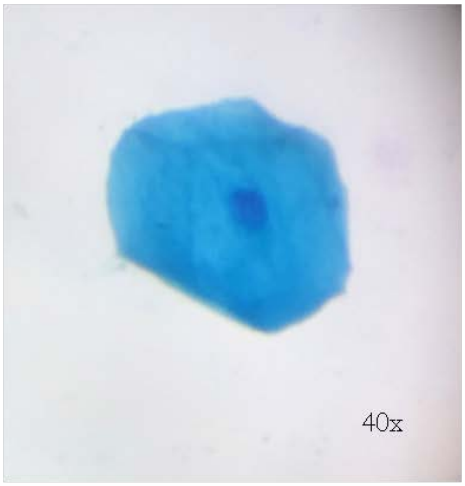


Fig 1: Normal Epithelial Cell

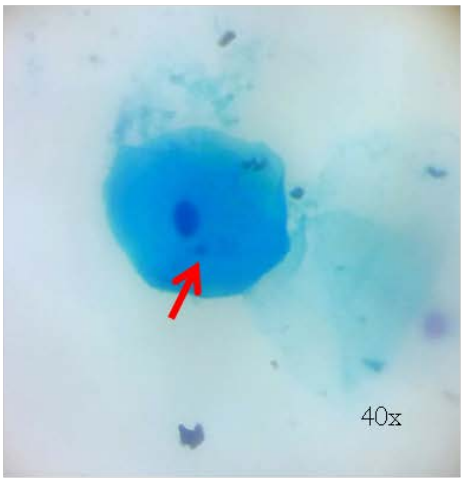


Fig 2:Epithelial Cell with micronuclei (red arrow)

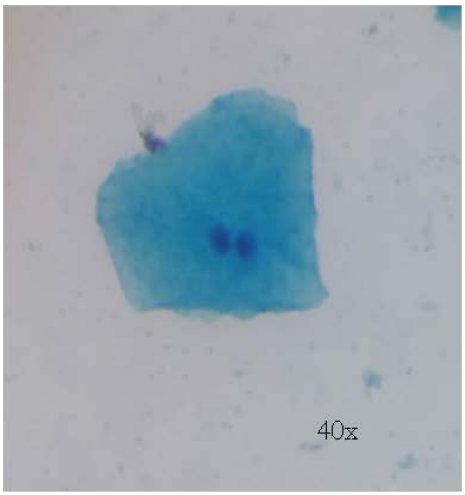


Fig 3:Epithelial cell with nuclear budding

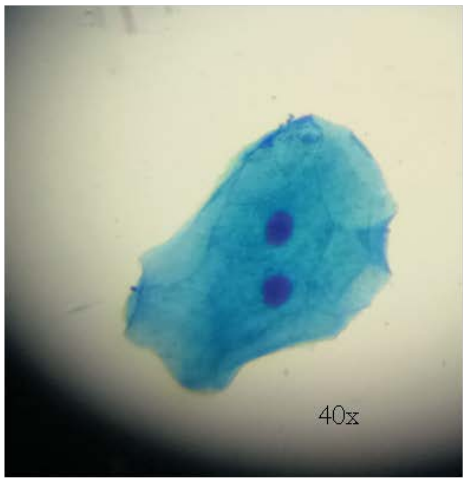


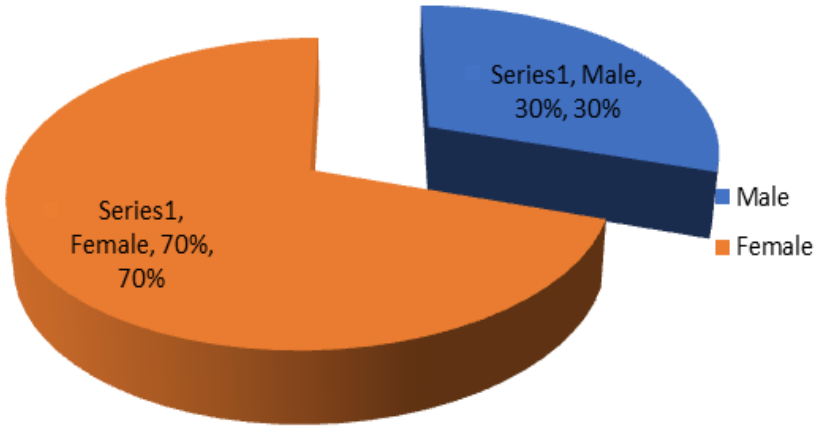
Fig 4:Binucleate epithelial cell

Table 1: Comparison of nuclear abnormalities between cases and controls

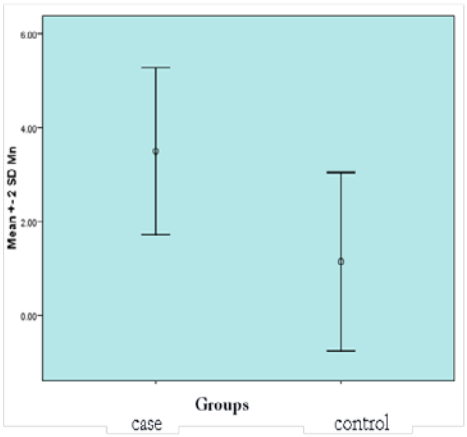
Si No	Parameter	Category	Mean ±SD	t (df)	p value
1	Micronuclei (Mn)	Cases	3.5±0.89	8.45 (19)	<0.001*
		Controls	1.3±0.97		
2	Nuclear bud (NuBd)	Cases	0.9±0.96	0.623 (19)	0.541
		Controls	0.7±0.92		
3	Bi nucleate cells (BiNu)	Cases	3.15±2.47	3.53 (19)	0.002*
		Controls	1.15±1.136		
4	Total nuclear abnormalities (Mn+NuBd+BiNu)	Cases	7.55±2.87	6.525 (19)	<0.001*
		Controls	3.19±1.9		

*p value ≤0.05 is considered as statistically significant

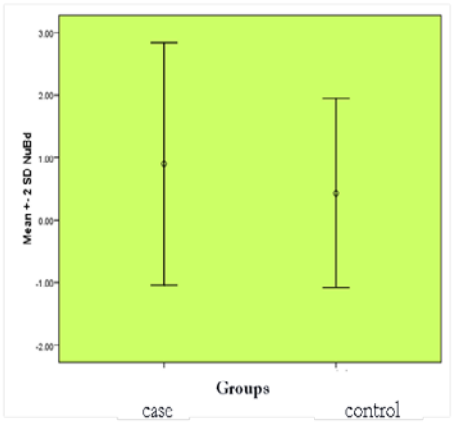
Graph 1: Sex distribution among cases and controls.



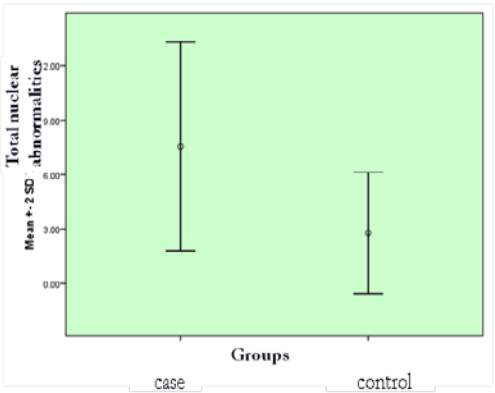
Graph 2: Comparison of mean micronuclei frequency between cases and controls.



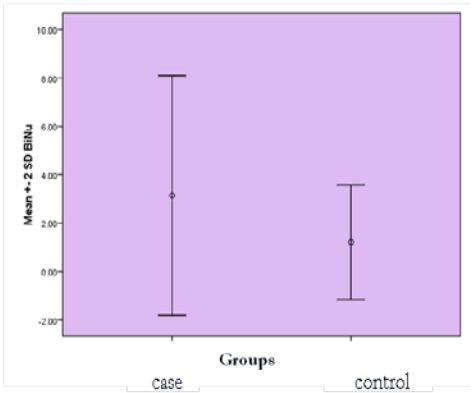
Graph 3: Comparison of mean nuclear bud frequency between cases and controls.



Graph 5: Comparison of mean total nuclear abnormality frequency between cases and controls.



Graph 4: Comparison of mean binucleate cell frequency between cases and controls.



Discussion

Orthodontic appliance used in dentistry is made of alloys containing nickel, chromium, cobalt, iron and titanium.[5]Various studies have found that oral environment provides ideal condition for the degradation of these alloys and release of metal ions. Saliva acts as an electrolyte with the fluctuation in pH, temperature, enzymatic activity, the food and drinks introduced to the oral cavity all contributes to metal corrosion. [6]The metals ions released from the orthodontic appliance are reported to be taken up by the tissues which are in direct contact with them. There will be a cumulative effect on these cells due to metal release.[12] Mekulewicz M *et al* and Langes BR *et al* has reported that significant sustained release of Ni and Cr ions from orthodontic appliance in the oral environment. Ni ions are reported to act as an allergen and mutagenic agent. They were found to act by suppressing the promoter sites of the genes by hypermehylation.[5,11,13]

The genotoxicity of chronic metal ion exposures on adjacent cells were evaluated by many researchers. They have used surrogate markers for genotoxicity like micronuclei assay and comet assay on the buccal mucosal cells. [1,2,4,9] Westphalen GH *et al* found that micronuclei was more reliable in recording the genotoxic events in fixed orthodontic appliance toxicity.[1] Micronuclei (Mn)assay is based on the frequency of chromosomal fragments or whole chromosomes which are not included in the main daughter nuclei during cell division.This arises due to DNA breakage leading to chromosomes or chromatin lagging during anaphase. [1]The frequency of micronuclei is being established as reliable marker for genetic damage due to low doses of mutagen exposure.[13]Other nuclear features which indicate genetic and cellular damages are nuclear buds and binucleate cells. Binucleate cells are considered occour due to a defect in the cytokinesis [3,14]

Age and sex of the subjects are reported to be confounding factors that can influence the Mn frequency.[3] Ferreira *et al* has reported an increase in the frequency of Mn as age advances.[10,15] In order to avoid this confounding effect, we selected age and sex matched controls. Hafez HS *et al* reported the cell viability was significantly lower in buccal mucosa of patients with 6 months of orthodontic appliance theraphy. There was also a significant increase in the Ni and Cr content in the buccal mucosal cells during this time.[12] So in our study, in order to understand the toxic effects of the metal release from orthodontic appliance we selected cases with a minimum of 6 months orthodontic appliance use.

In our study we found a significant increase in the Mn frequency in cases compared to controls($p<0.0001$) (Fig 2, Table 1,Graph 2)This increase in Mn frequency was similar to previous studies done by Westphalen GH *et al* and Natarajan M *et al*.[1,4] But the mean Mn frequency was much lower in our study (3.5±0.89) compared to these previous studies (259±233)[4].These previous studies have used non DNA specific stains like Geimsa and PAP in order to evaluate Mn frequency. It has been established that the use of non DNA specific stains increase false positive results because non nuclear material like keratin bodies in degrading cells can resemble like micronuclei.[10]

Nuclear bud (Nu Bd)is reported to be another indicator of genetic damage. They are nuclei with a sharp constriction at one end suggestive of a budding process. It is an indication of elimination of amplified nuclear material.[3]In our study we found Nu Bd formation in both cases and controls, but there was no significant difference in its frequency between cases and controls.(Fig 3,Table 1,Graph 3) In our study we recorded binucleate (Bi Nu) epithelial cells in both cases and controls.(Fig 4) We found a statistically significant increase in the mean frequency of Bi Nu cells in cases compared to controls

(p=0.002) (Table 1,Graph 4) Philips *et al* has reported that Bi Nu cells are indicative of failed cytokinesis following last nuclear division. This was found to be a checkpoint mechanism for aneuploid cells.[3] Bi Nu frequencies were found to be higher in patients with higher rates of aneuploidy.[15]To our knowledge this is the first time Bi Nu cell frequencies were recorded in buccal mucosal cells of subjects using orthodontic appliance.

We also evaluated total nuclear abnormality (Mn+NuBd+Bi Nu) frequency between cases and controls. We found a significant increase in nuclear abnormalities in cases compared to controls ($p<0.0001$) (Table 1,Graph 4) Mn,Nu Bd and Bi nu are all established nuclear alterations indicative of genotoxicity.[3] Hence this increase in nuclear abnormality frequency in cases is an indication of genotoxicity in orthodontic patients using chlorhexidine mouthwash. Most mouthwashes available in market contains ethanol which allows the penetration of carcinogenic compounds, which induce DNA damage to epithelial tissue, since alcohol has the capacity to dissolve the lipid portion of cell membranes promoting increased permeability. The proportion of basal cells and differentiated cells in cellular death is an indication of tissue renovation capacity. However, 90 % of all known cancers seem to have epithelial origin, indicating that this proportion may become an unbalanced front of exposure to genotoxic agents. Thus, the buccal mucosa cells can be used for monitoring the first genotoxic event as result of potential carcinogens that reach the tissues

Conclusion

We found a significant increase in Mn frequency in buccal mucosal cells of cases compared to controls. This shows that subjects with fixed orthodontic appliance using chlorhexidine mouthwash have increased incidence of localized genotoxicity. This can be probably due to exposure to the bicationic chlorhexidine molecule and metal ions released from the orthodontic appliance. Other celluar markers of genotoxicity like Bi Nu cell frequency was also found to be raised in cases compared to controls. Bi Nu cells are an after effect of aneuploidy state of the affected cell. These results will help in sensitizing the clinicians regarding the possible biological effects of long tern fixed orthodontic therapy. Emphasis should be placed in regular patient monitoring and follow up. More research should be focused in developing better biocompatible dental and therapeutic materials.

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Legend Table

Questionnaire for Micronuclei Study

Serial .No **Date:**

Op.No:

Personal Details

Name: **Age :** **Sex** :

Address:

Questionnaire

Q.No	Questions	Answers
1.	Duration of orthodontic appliance (in Months)	
2.	Type of appliance used :	

3.	Tooth paste used		
4.	Frequency of brushing		
5.	Mouthwash	Yes	No
6.	If yes ; type of mouthwash		
7.	Radiographic exposure OPG	Yes	No
8.	Smoking	Yes	No
9.	Tobacco	Yes	No
10.	Alcohol	Yes	No
Systemic Illness			
11.	Diabetes	Yes	No
12.	Any long term medication	Yes	No
13.	Any metal restorations	Yes	No
14.	Other restorative procedure	Yes	No
15.	Oral Hygiene status Plaque /calculus (+)		
16.	Oral lesions		
17.	If yes , specify		