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ORIGINAL RESEARCH

Assessment of Metal Ion Toxicity, Cellular Viability, and Deoxyribonucleic Acid Damage induced by Orthodontic Appliances

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ABSTRACT

Aim: To determine the salivary levels of the metals ions (Ni, Cr, Fe, and Co), and also the cytotoxicity and genotoxicity induced by these metal ions in the buccal mucosal cell of patients being treated with fixed orthodontic appliances.

Materials and methods: A total of 50 patients aged between 14 and 30 years of age were included in the study. Four samples of saliva were collected from each orthodontic patient at the following times: T0 – before insertion of the appliance, T1 – 1 hour after insertion of the appliance, T2 – 3 months after insertion of the appliance, T3 – 6 months after insertion of the appliance. Three samples of buccal cells were collected from each orthodontic patient at the following times: T0 – before insertion of the appliance, T1 – 3 months after insertion of the appliance, T2 – 6 months after insertion of the appliance. Metal ion concentrations in saliva and buccal cells were evaluated using atomic absorption spectrophotometer. The buccal cells were also evaluated for genotoxicity (deoxyribonucleic acid [DNA] damage) and cytotoxicity (cellular viability) using alkaline comet assay (CA) and trypan blue exclusion dye test respectively.

Statistical analysis: The results of the cellular ion concentrations were statistically evaluated using paired t-test. The cellular viability results were evaluated using the Tukey's multiple comparison test. A significance level of $p > 0.05$ was considered as statistically insignificant.

Results: It was noted that there was a significant ($p < 0.001$) increase in the metal ion concentrations in both saliva and buccal cell samples. The CA results showed significant DNA damage in the buccal cells, whereas the cellular viability showed a decrease over the study period but the decrease was insignificant.

Conclusion: The findings indicate that measurable amounts of nickel, chromium, iron, and cobalt can be found in the saliva of the patients wearing fixed orthodontic appliances over a period of 6 months but the increase in values does not reach toxic levels. The buccal mucosa cells of patients treated with fixed orthodontic appliances over a period of 6 months showed significant increases in nickel, chromium, iron, and cobalt content, with significant DNA damage and insignificant decrease in cellular viability. Further studies should be carried out to evaluate the effects of these changes over the course of the treatment.

Keywords: Buccal cell, Cytotoxicity, Genotoxicity, Metal ions.

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INTRODUCTION

In orthodontics, most of the fixed metallic appliances comprise metals like chromium (Cr), nickel (Ni), and cobalt. These metals are widely used since they can withstand various physical, mechanical, and biological insults but they also should be equally biocompatible in the oral environment (Bourauel et al).[1] Since oral tissues experience long-term exposure of these fixed appliances which are not biodegradable and which show sustained release of metals over time, they are expected to produce irreversible toxic effects on the tissues. It is difficult to assess the exact level of metals that produce toxicity or cellular damage since metal toxicity is governed by various factors.[2] The corrosion of an alloy releases free ions from the metals which may have significant influence on surrounding tissues, such as toxicity, allergy, mutagenicity, and carcinogenicity. However, there is also evidence available regarding the systemic toxicity caused by the elements which are released from casting alloys due to slow release of free ions over a prolonged period of time.[2] Saliva acts as an electrolyte for electron and ion conduction, and the fluctuation of pH and temperature, the enzymatic and microbial activity, and the various chemicals introduced into the oral cavity through food and drink are all corrosion conductors. The inherent heterogeneity of each metal alloy and its use with other

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alloys, the microsurface discontinuity, the forces acting on the appliances, and the friction between wires and brackets also add to the corrosion process.[2] The most commonly used materials for orthodontic fixed treatment are stainless steel and nickel titanium (NiTi) alloys. The major corrosion products include nickel (Ni), chromium (Cr), and iron (Fe). Although all three elements potentially have adverse effects, Ni and Cr have received most attention because of their reported potential for producing allergic, toxic, or carcinogenic reactions, and are classified as chemical carcinogens. Cobalt (Co) is also a minor product of corrosion and has been found to impair the phagocytosis of bacteria by human polymorphonuclear leucocytes *in vitro* (Haynes et al).[3] Among the metals, nickel is considered as strong immunologic sensitizer since it promotes the expression of intercellular adhesion molecule-1, activates certain immune cells like monocytes, and also activates endothelial cells. Arsenide and sulfide complexes formed by nickel also cause changes in deoxyribonucleic acid (DNA) morphology like base damage, site-specific DNA scission, and prevents the DNA repair by inhibiting various DNA repair enzymes which promotes microsatellite mutations and increases total genomic methylation contributing to genetic instability, whereas chromium and cobalt long-term exposure leads to certain side effects like hypersensitivity, dermatitis, and asthma.[4] The term genotoxicity indicates either mutagenic or carcinogenic processes related to DNA. Thus, the genotoxic properties of metals from orthodontic appliances are defined as an essential criterion to select these materials in a safe biological manner for patients.[5] Since DNA is most vulnerable to damage during mitosis due to environmental exposure to genotoxins, micronutrient deficiency (e.g., folate), lifestyle factors (e.g., alcohol, smoking, drugs, and stress), medical procedures (e.g., radiation and chemicals), and genetic factors, such as inherited defects in DNA metabolism or repair, the epithelial stem cells, or progenitor cells play a key role in safeguarding the genetic information of the tissues. The stratified squamous oral epithelium is divided into various layers, with the deepest layer containing progenitor cells that show mitotic division. These cells migrate toward the surface and shed off. Thus, the turn over time of the epithelium is the time that a cell takes to divide and pass through the entire epithelium. Reports suggest that the median value of 14 days is considered as the turnover time for human buccal epithelium. So, a biomarker which is minimally invasive and reliable is essential to improve the implementation of biomonitoring, diagnosis, and treatment of diseases caused by, or associated with, genetic damage.[6] Very few studies have reported an association of genotoxicity with fixed orthodontic appliances since normal cells repair these lesions, but inhibition of enzymes that promote the reparative process or loss of reparative capacity might be the initiating episode of undesirable biologic effects. Various assays that can assess the genotoxic agents through the application of some well-established endpoints are available like the micronucleus (MN) frequency, as determined by the MN assay, or primary DNA damage, as accessed by the comet assay (CA). Among them, CA is regarded as the most simple, quick, sensitive, reliable, and fairly inexpensive way of measuring DNA damage. It measures through cell approach, the single- and/or double-strand breaks in a cell.[6] Though orthodontic appliances release very minimal levels of metal ions which fall way below the recommended daily dietary intake, there are certain *in vivo* studies which reported biologic toxicity in orthodontic patients due to chronic low levels of metal ions like alteration in cellular metabolism and morphology, and exaggerating the inflammatory response and even DNA instability.[2] Thus, the purpose of this study is to determine the concentrations of these corrosion products in saliva and buccal cells and to evaluate the DNA damage induced by them in the mucosal cells.

MATERIALS AND METHODS

This study was carried out in the Department of Orthodontics and Dentofacial Orthopaedics, A B Shetty Memorial Institute of Dental Sciences, Mangaluru, India. Buccal mucosal cell preparation was done at the Nitte University Centre for Science Education and Research (NUCSER), K.S. Hegde Medical Academy (KSHEMA), Mangaluru, India. Spectrometric analysis was done for metal ion concentrations in buccal cells at the University Science Instrumentation Centre, Mangalore University, Mangaluru. The buccal cells were evaluated for DNA damage and cellular viability at NUCSER, KSHEMA. This study used salivary and buccal cell samples from new patients starting orthodontic treatment. Samples from a total of 50 patients between the age group of 14 and 30 years were collected. The brackets used were standard stainless steel MBT brackets and arch wire materials used were NiTi and stainless steel. The patients were nonsmokers, had no previous history of orthodontic treatment, and did not use any medicine or supplements. Subjects were thoroughly examined for the absence of oral disease, systemic disease, oral restorations, or prosthesis. There was no known allergy to jewelry, watches, or any other sources of nickel, chromium, cobalt, and iron. Salivary samples were collected in four stages from each orthodontic patient at the following times: T0 – before insertion of the appliance, T1 – 1 hour after insertion of the appliance, T2 – 3 months after insertion of the appliance, T3 – 6 months after insertion of the appliance. The patients initially rinsed their

mouth thoroughly with a mouthful of distilled, deionized water for 30 seconds. Approximately 5 mL of unstimulated whole saliva was collected in polypropylene test tubes and the samples were stored at –20°C before they were processed. The metal ion concentrations of saliva are stable for 6 months when stored at –20°C. The use of an atomic absorption spectrophotometer (Fig. 1) permits the analysis of metals in biological samples without any separation of the metal ion from its biological matrix. By using spectrophotometric method, there is no necessity for extraction procedures to analyze the elements, only dilution of the samples was enough to eliminate the interference and effects of the biological matrix. Before the analysis, samples were centrifuged at 3000 rpm for 10 minutes to settle the particulate matter (Fig. 2). A total of 1 mL of saliva samples of each patient was treated with nitric acid (2 mL, 0.5%), heated in sand bath to get a clear solution, and then diluted to 10 mL with deionized distilled water. To measure the amount of nickel, chromium, cobalt, and iron release, atomic absorption spectrophotometry was used. Standard solutions of nickel, chromium, cobalt, and iron were prepared with concentrations between 0.1 and 0.8 ng/L. Each test was analyzed three times and the average was used as the result. Before each test, one distilled water sample was processed in order to prevent possible contamination. The insoluble precipitate was not included in the analysis because of the problem of particles causing variation in the results. The samples were analyzed and the nickel, chromium, cobalt, and iron concentrations were calculated as parts per billion (ppb). Three samples of buccal cells were collected from each orthodontic patient at the following times: T0 – before insertion of the appliance T1 – 3 months after insertion of the appliance T2 – 6 months after insertion of the appliance. Before the study, all subjects were instructed to continue brushing but not use toothpastes and mouthwashes containing chlorhexidine because it has been reported to cause DNA damage. The buccal mucosal cells were harvested by gentle scrapping of the internal part of the right and left cheeks with a wooden tongue depressor. Gentle scrapings are required to prevent heterogeneous cell sample. The depressors were stirred in a 15 mL tube prefilled with 1 mL phosphate-buffered solution diluted up to 10 mL. Ten strokes on each side of the buccal mucosa was enough to ensure adequate cell density in the suspension. The buccal cell samples were evaluated for cellular viability using trypan blue exclusion dye test and DNA damage was assessed using alkaline CA. Cellular concentrations of Ni, Cr, Fe, and Co were assessed using atomic absorption spectrometer.

CELL VIABILITY ASSAY (CYTOTOXICITY)

The estimation was carried out by following Hartmann and Speit[7] method. An aliquot of cell suspension being tested is taken and centrifuged for 5 minutes at 100 × g for viability and discard supernatant. The approximate number of cells present would decide the size of the aliquot being tested. Sufficient number of cells should be present in the aliquot to count in a hemocytometer when suspended in 1 mL phosphate-buffered saline (PBS) and then diluted again by mixing with 0.4% trypan blue (e.g., 5×10^5 cells/mL). The cell pellet was resuspended in 1 mL PBS or serum-free complete medium since serum proteins can produce false results when stained with trypan blue. Determinations must be made in serum-free solution. One part of 0.4% trypan blue is mixed with 1 part cell suspension (dilution of cells) and the mixture is allowed to incubate for 3 minutes at room temperature. Cells should be counted within 3 to 5 minutes of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts. In a well of microtiter plate or a small plastic tube, mixing is done using 10 to 20 µL each of cell suspension and trypan blue. A drop of trypan blue/cell mixture is taken and applied to a hemocytometer and now this hemocytometer is placed on the stage of a binocular microscope and the cells are viewed. All the viable and nonviable cells, i.e.,

Fig. 1: Atomic absorption spectrophotometer

Fig. 2: Centrifuge

the unstained and stained cells respectively, are separately counted on a hemocytometer.

To obtain the total number of viable cells per mL of aliquot, multiply the total number of viable cells by 2 (the dilution factor for trypan blue). To obtain the total number of cells per mL of aliquot, add up the total number of viable and nonviable cells and multiply by 2.

Calculation:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per mL of aliquot}}{\text{Total number of cells per mL of aliquot}} \times 100$$

EVALUATION OF GENOTOXICITY (COMET ASSAY)

The genotoxicity estimation assay was carried out in accordance with the method given by Singh et al.[8] A total 400 µL of 0.5% agarose is placed onto a frosted slide at one end and the agarose is spread quickly to the other end of the slide using a cover slip. These slides are kept on ice for instant drying. This can also be done in advance and called as slide precoating. Buccal cell suspension of 20 µL is taken in an eppendorf tube and mixed with 100 µL of 0.5% agarose. Now this mixture is placed onto the precoated slide and the cover slip is kept immediately and allowed to dry for few minutes. Then, after the slide is dried up, the cover slip is slowly removed and a final layer of 100 µL of 1% agarose solution is placed onto a slide and immediately covered with cover slip. Then, 50 µL of trypsin solution is poured onto the gel and cover slip is placed for uniform distribution and this is kept in a hot air oven at 37°C for 30 minutes. The cover slip is then removed and the trypsin solution is discarded. A total of 50 µL of ice cold proteinase K is poured onto the gel and cover slip is placed and kept in refrigerator for 1 hour at 0 to 4°C. After an hour, the cover glasses are removed, they are immersed in lysing solution, and kept overnight in dark at 20°C. Then next day the slides are washed in PBS and kept for electrophoresis. The slides are kept in electrophoretic unit and immersed in electrophoretic buffer for 10 minutes and then electrophoresed at 12 V for 18 minutes. The slides are then taken out and immersed in neutralizing solution at room temperature for 5 minutes. Slides were then immersed in 1× ethidium bromide solution for 1 minute and observed under fluorescent microscope for comets. The slides are analyzed using fluorescent microscope under a magnification of 40× using a green filter (Fig. 3). Further comet software is used for analysis of comet scores (Fig. 4). The average tail length, tail area, and olive tail movement of the observed is calculated to study the extent of DNA damage in buccal mucosa cells.

Statistical Analysis

The following analysis was employed to statistically evaluate the results:

- Paired t-test
- Tukey's multiple comparison test

The results of the salivary and cellular metal ion concentrations and the CA results were statistically evaluated using paired t-test. The cellular viability results were evaluated using Tukey's multiple comparison test. Significance level of $p > 0.05$ was considered as statistically insignificant.

RESULTS

Nickel

A variation in the concentration of nickel in saliva was observed. On examining the concentration of nickel in saliva released over a period of 6 months, it was seen that the concentrations increased from T0 (30 ± 2.3 ppb) to T3

Fig. 3: Fluorescent microscopy with green filter

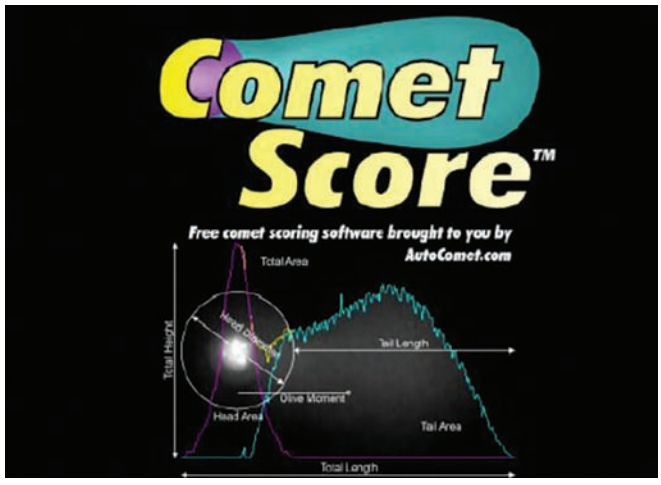


Fig. 4: Comet score software

Table 1: Comparison of the mean salivary nickel ion concentration using paired t-test

	Group				*Paired differences*				
	Salivary nickel	*Mean*	*n*	*Standard deviation*	*Mean*	*Standard deviation*	*t-value*	*df*	*p-value*
Pair 1	T0	30.002	50	2.36828	–15.262	3.57251	–30.208	49	<0.001
	T1	45.264	50	2.67999					
Pair 2	T0	30.002	50	2.36828	–30.686	2.63028	–82.494	49	<0.001
	T2	60.688	50	2.67228					
Pair 3	T0	30.002	50	2.36828	–50.164	2.74105	–129.408	49	<0.001
	T3	80.166	50	2.54587					
Pair 4	T1	45.264	50	2.67999	–15.424	4.11283	–26.518	49	<0.001
	T2	60.688	50	2.67228					
Pair 5	T1	45.264	50	2.67999	–34.902	4.01134	–61.524	49	<0.001
	T3	80.166	50	2.54587					
Pair 6	T2	60.688	50	2.67228	–19.478	3.14333	–43.817	49	<0.001
	T3	80.166	50	2.54587					

df: degree of freedom

Table 2: Comparison of the mean salivary chromium ion concentration using paired t-test

	Group				*Paired differences*				
	Salivary chromium	*Mean*	*n*	*Standard deviation*	*Mean*	*Standard deviation*	*t-value*	*df*	*p-value*
Pair 1	T0	3.472	50	0.67098	–4.676	1.03816	–31.849	49	<0.001
	T1	8.148	50	0.76618					
Pair 2	T0	3.472	50	0.67098	–11.44	1.11429	–72.596	49	<0.001
	T2	14.912	50	1.11733					
Pair 3	T0	3.472	50	0.67098	–17.232	1.60782	–75.785	49	<0.001
	T3	20.704	50	1.5036					
Pair 4	T1	8.148	50	0.76618	–6.764	0.99668	–47.988	49	<0.001
	T2	14.912	50	1.11733					
Pair 5	T1	8.148	50	0.76618	–12.556	1.81897	–48.81	49	<0.001
	T3	20.704	50	1.5036					
Pair 6	T2	14.912	50	1.11733	–5.792	2.13693	–19.166	49	<0.001
	T3	20.704	50	1.5036					

df: degree of freedom

(80.1 ± 2.5 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when salivary concentrations were compared at different time intervals with each other (Table 1).

Chromium

On examining the concentration of chromium in saliva released over a period of 6 months, it was seen that the concentrations increased from T0 (3.4 ± 0.67 ppb) to T3 (20.7 ± 1.5 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when salivary concentrations were compared at different time intervals with each other (Table 2).

Iron

On examining the concentration of chromium in saliva released over a period of 6 months, it was seen that the concentrations increased from T0 (51.9 ± 6.8 ppb) to T3 (100 ± 4.3 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when salivary concentrations were compared at different time intervals with each other (Table 3).

Cobalt

On examining the concentration of chromium in saliva released over a period of 6 months, it was seen that the concentrations increased from T0 (4.9 ± 1.1 ppb) to T3 (14.6 ± 1.1 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when salivary concentrations were compared at different time intervals with each other (Table 4 and Graph 1).

ALKALINE COMET ASSAY

The potential genotoxic effects of metals on buccal mucosa cells were evaluated by CA, which can show DNA damage. The assay is named for the characteristic shape when the DNA exits the nucleus and the cell body. In this study, three parameters characterizing DNA strand breaks were evaluated: Tail length, the percentage of DNA in the tail or tail intensity, and tail moment.

Tail Length

On examining the results, it was seen that the tail length increased from T0 (6.9 ± 1.2) to T2 (8.3 ± 2.5). The increase

Table 3: Comparison of the mean salivary iron ion concentration using paired t-test

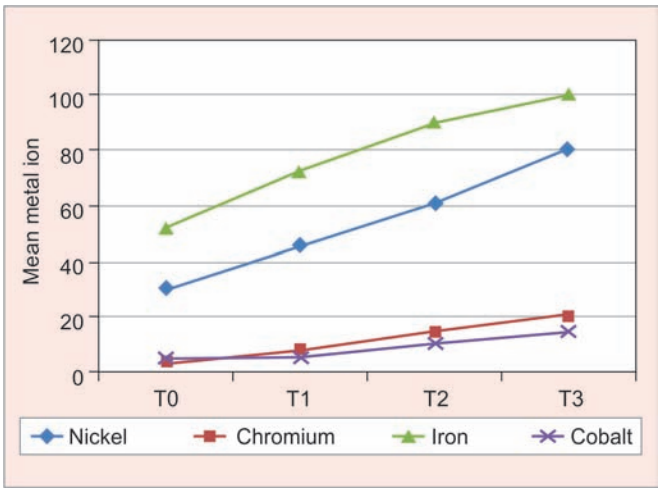
	Group				Paired differences				
	Salivary iron	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	51.96	51	6.86405	–20.428	7.08875	–20.58	50	<0.001
	T1	72.388	51	5.58117					
Pair 2	T0	51.96	51	6.86405	–37.8	6.92682	–38.971	50	<0.001
	T2	89.76	51	3.64675					
Pair 3	T0	51.96	51	6.86405	–48.074	7.91034	–43.401	50	<0.001
	T3	100.034	51	4.39225					
Pair 4	T1	72.388	51	5.58117	–17.372	5.9645	–20.8	50	<0.001
	T2	89.76	51	3.64675					
Pair 5	T1	72.388	51	5.58117	–27.646	6.92067	–28.528	50	<0.001
	T3	100.034	51	4.39225					
Pair 6	T2	89.76	51	3.64675	–10.274	3.47228	–21.131	50	<0.001
	T3	100.034	51	4.39225					

df: degree of freedom

Table 4: Comparison of mean cobalt ion concentration using paired t-test

	Group				Paired differences				
	Salivary cobalt	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	4.968	50	1.17133	–0.492	0.48058	–7.239	50	<0.001
	T1	5.46	50	1.16479					
Pair 2	T0	4.968	50	1.17133	–5.326	1.61281	–23.351	50	<0.001
	T2	10.294	50	1.18415					
Pair 3	T0	4.968	50	1.17133	–9.688	1.81554	–37.732	50	<0.001
	T3	14.656	50	1.16936					
Pair 4	T1	5.46	50	1.16479	–4.834	1.67717	–20.38	50	<0.001
	T2	10.294	50	1.18415					
Pair 5	T1	5.46	50	1.16479	–9.196	1.87823	–34.621	50	<0.001
	T3	14.656	50	1.16936					
Pair 6	T2	10.294	50	1.18415	–4.362	1.6886	–18.266	50	<0.001

df: degree of freedom



Graph 1: Representation of mean metal ion concentration in saliva at various time intervals

from T0 (6.9 ± 1.2) to T1 (7.8 ± 2.4) was found to be statistically significant (p < 0.02), whereas the increase from T1 (7.8 ± 2.4) to T2 (8.3 ± 2.5) was found to be statistically highly insignificant (p = 0.2). However, the increase from T0 (6.9 ± 1.2) to T2 (8.3 ± 2.5) was found to be statistically highly significant (p < 0.001) (Table 5 and Graph 2).

Percentage of DNA in Tail

On examining the results, it was seen that there was a slight increase in the DNA % from T0 (9.2 ± 0.8) to T2 (10.3 ± 1.3). The increase from T0 (9.2 ± 0.8) to T1 (9.8 ± 2.6) was very minor, was found to be statistically insignificant (p = 0.1), the increase from T1 (9.8 ± 2.6) to T2 (10.3 ± 1.3) was also found to be statistically insignificant (p = 0.2). However, the increase from T0 (9.2 ± 0.8) to T2 (10.3 ± 1.3) was found to be statistically highly significant (p < 0.001) (Table 6 and Graph 3).

CELLULAR VIABILITY

On examining the cellular viability of the buccal cells, it was observed that the percentage of viable cells decreased from T0 (18.6 ± 1.67) to T2 (9.3 ± 4.21). The comparison between the time periods over 6 months showed that even though there was a decrease in the percentage of viable

Table 5: Comparison of the mean tail length using paired t-test

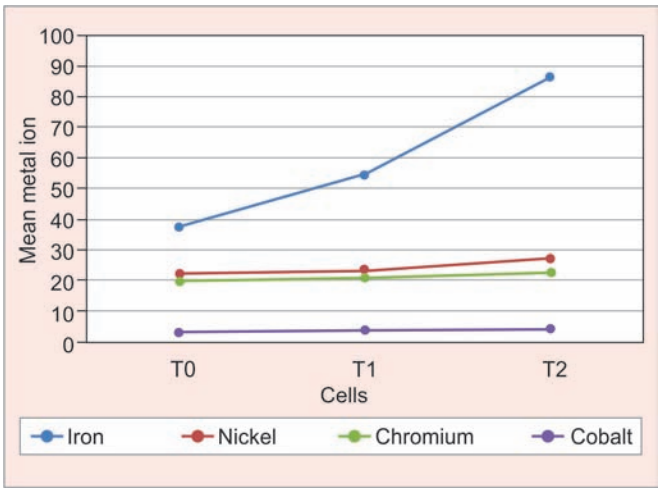
	Group				Paired differences				
	% DNA	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	9.2224	49	0.81758	–0.57959	2.70678	–1.499	48	0.14
	T1	9.802	49	2.64287					
Pair 2	T0	9.2224	49	0.81758	1.12041	1.59569	–4.915	48	<0.001
	T2	10.3429	49	1.30464					
Pair 3	T1	9.802	49	2.64287	–0.54082	2.95289	–1.282	48	0.206
	T2	10.3429	49	1.30464					

df: degree of freedom

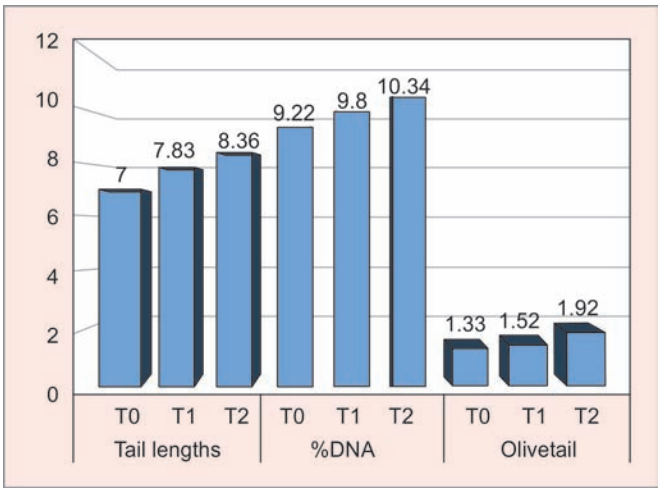
Table 6: Comparison of the mean olive tail moment using paired t-test

	Group				Paired differences				
	Olive tail movement	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	1.326	50	0.18273	–0.192	0.24146	–5.623	49	<0.001
	T1	1.518	50	0.16986					
Pair 2	T0	1.326	50	0.18273	0.596	0.21089	–19.984	49	<0.001
	T2	1.922	50	0.12002					
Pair 3	T1	1.518	50	0.16986	–0.404	0.203	–14.073	49	<0.001
	T2	1.922	50	0.12002					

df: degree of freedom



Graph 2: Representation of mean metal ion concentration in buccal mucosal cells at various time intervals



Graph 3: Representation of the mean tail length, % DNA, and olive tail moment of the alkaline CA at various time intervals

cells, it was statistically insignificant ($p > 0.05$) (Table 7 and Graph 4).

CELLULAR CONCENTRATIONS OF METAL IONS

Nickel

On examining the concentration of nickel in buccal cells over a period of 6 months, it was seen that the concentrations increased from T0 (21.9 ± 1.1 ppb) to T2 (26.9 ± 1.1 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when cellular concentrations were compared at different time intervals with each other (Table 8).

Chromium

On examining the concentration of chromium in buccal cells over a period of 6 months, it was seen that the concentrations increased from T0 (20 ± 1.1 ppb) to T2 (22.7 ± 1.2 ppb). A statistically significant increase was observed between T0 and T1. The comparison between T1 and T2, T0, and T3 showed a statistically highly significant p-value ($p < 0.001$) (Table 9).

Iron

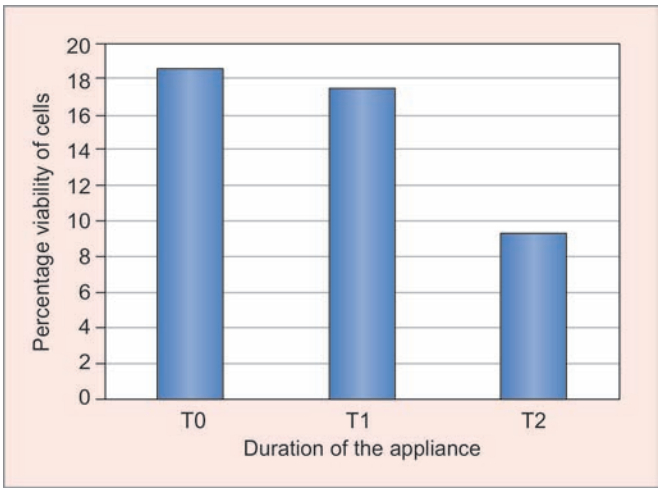
On examining the concentration of iron in buccal cells over a period of 6 months, it was seen that the concentrations greatly increased from T0 (37.6 ± 1.9 ppb) to T2 (86.3 ± 2.7 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when cellular concentrations were compared at different time intervals with each other (Table 10).

Cobalt

On examining the concentration of cobalt in buccal cells over a period of 6 months, it was seen that the concentrations greatly increased from T0 (3 ± 0.63 ppb) to T2

Table 7: Comparison of the mean % cellular viability using Tukey's multiple comparison test

	Group				Paired differences	
	% Cellular viability	Mean	n	Standard deviation	Tukey's multiple comparison test	p-value
Pair 1	T0	18.6	50	1.67	T0	>0.05
	T1	17.4	50	6.31	T1	
Pair 2	T0	18.6	50	1.67	T0	>0.05
	T2	9.3	50	4.21	T2	
Pair 3	T1	17.4	50	6.31	T1	>0.05
	T2	9.3	50	4.21	T2	



Graph 4: Representation of mean % viability of cells at various time intervals

(4.1 ± 0.5 ppb). The results showed a statistically highly significant p-value ($p < 0.001$) when cellular concentrations were compared at different time intervals with each other (Table 11).

DISCUSSION

This is a longitudinal clinical evaluation which was carried out for a period of 6 months to evaluate genotoxicity, cytotoxicity, and the metal ions released by various metallic fixed orthodontic appliances on saliva and buccal mucosal cells. This study also evaluated the effects of the corrosive by-products produced by the orthodontic appliances present in the oral cavity on the buccal mucosal cells of the treated patients.[1]

Table 8: Comparison of the mean cellular nickel ion concentration using paired t-test

	Group				Paired differences				
	Salivary nickel	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	21.978	50	1.17583	–1.44	2.05198	–4.962	49	<0.001
	T1	23.418	50	1.56384					
Pair 2	T0	21.978	50	1.17583	–4.94	1.76601	–19.78	49	<0.001
	T2	26.918	50	1.15382					
Pair 3	T1	23.418	50	1.56384	–3.5	1.94044	–12.754	49	<0.001
	T2	26.918	50	1.15382					

df: degree of freedom

Table 9: Comparison of the mean cellular chromium ion concentration using paired t-test

	Group				Paired differences				
	Salivary chromium	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	20.082	50	1.16841	–0.784	1.90695	–2.907	49	0.005
	T1	20.866	50	1.2295					
Pair 2	T0	20.082	50	1.16841	–2.62	1.69537	–10.928	49	<0.001
	T2	22.702	50	1.26499					
Pair 3	T1	20.866	50	1.2295	–1.836	1.73073	–7.501	49	<0.001
	T2	22.702	50	1.26499					

df: degree of freedom

Table 10: Comparison of the mean cellular iron ion concentration using paired t-test

	Group				Paired differences				
	Salivary iron	Mean	n	Standard deviation	Mean	Standard deviation	t-value	df	p-value
Pair 1	T0	37.652	50	1.99082	–16.71	2.7824	–42.466	49	<0.001
	T1	54.362	50	1.93189					
Pair 2	T0	37.652	50	1.99082	–48.742	3.21051	–107.353	49	<0.001
	T2	86.394	50	2.75936					
Pair 3	T1	54.362	50	1.93189	–32.032	3.23068	–70.109	49	<0.001
	T2	86.394	50	2.75936					

df: degree of freedom

Table 11: Comparison of the mean cellular cobalt ion concentration using paired t-test

	Group				*Paired differences*				
	Salivary cobalt	*Mean*	*n*	*Standard deviation*	*Mean*	*Standard deviation*	*t-value*	*df*	*p-value*
Pair 1	T0	3.002	50	0.63229	–0.616	0.82322	–5.291	49	<0.001
	T1	3.618	50	0.57096					
Pair 2	T0	3.002	50	0.63229	–1.134	0.86958	–9.221	49	<0.001
	T2	4.136	50	0.5989					
Pair 3	T1	3.618	50	0.57096	–0.518	0.83169	–4.404	49	<0.001
	T2	4.136	50	0.5989					

df: degree of freedom

Water ionizes into hydrogen (H^+) and hydroxyl (OH^-) ions. When these ions are in equal proportions, the pH is neutral, 7. When there are more H^+ ions, then the water is said to be "acidic." If OH^- ions outnumber H^+ ions, then the water is said to be "alkaline." The pH scale ranges from 0 to 14 and is logarithmic, which means that each step is 10 times the previous one. In other words, a pH of 4.5 is 10 times more acidic than 5.5, 100 times more acidic than 6.5, and 1,000 times more acidic than 7.5.

Minerals with a negative electrical charge that are attracted to the H^+ ions are called acid minerals. Acid minerals include chlorine, sulfur, and phosphoric acid. Minerals with a positive electrical charge that are attracted to negatively charged OH^- ions are called alkaline. Nutritionally important alkaline minerals include calcium, potassium, magnesium, and sodium.

Petoumenou et al[4] in their study reported that such a short period is not sufficient to effectively evaluate the salivary metal ion content of orthodontic patients. However, the present study was done over 6 months and the salivary concentrations of the metal ions were much higher than the concentrations reported in other studies done over a short period and were also statistically significant. This study in general showed an increase in salivary Ni, Cr, Fe, and Co concentrations in patients with fixed orthodontic appliances over a period of 6 months.

According to the World Health Organization guidelines (2003), normal ranges of metal ions assessed in this study are <1 mg/L nickel (<1,000 ppb), 0.05 mg/L of chromium (50 ppb), 0.3 mg/L of iron (300 ppb), and cobalt 0.05 mg/L (50 ppb). According to the study at the end of 6 months, the concentrations of various metal ions studied were as follows: Ni – 80.1 ± 2.5 ppb, Cr – 20.7 ± 1.5 ppb, Fe – 100 ± 4.3 ppb, and Co – 14.6 ± 1.1 ppb. So it is seen that even though there is an increase in metal ion concentrations (Ni, Cr, Fe, and Co) seen over the study period, these values were well within the normal limits and none of the metal ion concentrations reached toxic levels. This study showed significant changes occurring in orthodontic patients treated with fixed appliances over a 6-month period. These changes included increases in cellular nickel, chromium, iron, and cobalt content.

Evaluation of these by-products in saliva might have limitations, as the saliva is continuously washed and swallowed, and will give information at the moment of sampling only.

Allergy has been a documented reaction in some orthodontic patients. Although a problem, the true concern should be the possible cytotoxicity or, even more importantly, the genotoxicity of orthodontic appliances since damage to DNA can lead to mutations. In tissues, such as buccal mucosa, where there is high mitotic capacity, cellular proliferation of a single damaged cell leads to production of many defective cells, which ultimately leads to defective function and reparative capacity of cells.[2]

Cytotoxicity was denoted by a significant decrease in cellular viability. Cellular viability at T0 in the treatment group was high. When compared with the T0 value, the viability decreased significantly at T2. Faccioni et al[9] reported similar cytotoxicity for the treated group in their study, when the cellular viability decreased significantly. Studies done by Kasacka et al[10] and Pereira et al[11] also reported reduced or decreased cellular viability and metabolism in patients undergoing orthodontic treatment.[9] Few cellular alterations occur, which include change in metabolic activity of cells, increased irregularity of cells, increase in nuclear cytoplasmic ratio of cells and morphologic changes of nuclei, such as pyknotic and vacuolated nuclei.[2] Although it was observed that there is decrease in cellular viability, it was not found to be statistically significant.

Several studies have been conducted both *in vivo* and *in vitro* to study the release of metals from fixed orthodontic appliances into various biological fluids like saliva, blood, and urine. These metals were released during the first 4 or 5 months of orthodontic therapy. Studies reported that the released metals were absorbed systemically by most of the patients and the amount of metal ions released in biological fluids were significantly below the average dietary intake and did not reach toxic concentrations. But, it cannot be excluded since even nontoxic concentrations might be sufficient to induce important biologic effects in cells of oral mucosa. Even if there is a tendency for genotoxicity of cells induced by metals, the mechanism underlying this is not clearly

understood, and several studies have reported possible pathways, such as the interaction of metals with DNA (cross-links), the generation of oxidative DNA damage, or interference with DNA repair and replication processes.[7]

On evaluation of the CA for the genotoxic effects in the buccal mucosa, it was observed that DNA damage increased from T0 to T2 after evaluation of all the parameters of the assay (% DNA, tail length, and olive tail moment) and was found to be statistically significant ($p < 0.001$).

The result obtained was in accordance with studies done by Hafez et al[2] and Faccioni et al.[9] A study done by Westphalen et al[5] in 30 patients after orthodontic treatment also recorded DNA damage with MN test. However, the CA showed insignificant changes.

The cellular concentrations of the metal ions (Ni, Cr, Fe, and Co) also showed an increase from the time intervals T0 to T2 and were statistically significant ($p < 0.001$) in relation to studies done by Hafez et al[2] and Faccioni et al.[9] However, a study done by Amini et al reported that there was only significant increase of Ni and that of Cr and Co were insignificant. So it is seen that even though there is an increase in metal ion concentrations (Ni, Cr, Fe, and Co) seen over the study period, these values were well within the normal limits and none of the metal ion concentrations reached toxic levels.

In this study, the patients treated with fixed orthodontic appliance over a period of 6 months showed significant changes of increased metal ion concentrations of cellular nickel, chromium, iron, and cobalt content, decreases in cellular viability, and evidence of DNA damage. When DNA damage occurs, various reparative mechanisms come into play to maintain normal integrity of DNA. So the patients should be followed up since repair of biologic changes is possible. However, the persistence of DNA damage will lead to genetic instability and DNA mutations. Many studies have reported that metal ions interfere with cellular reparative and protective capacities that maintain homeostasis and integrity of DNA. Based on all these evidences and findings, it would be wise to reduce insult induced by orthodontic appliances. This can be achieved by introducing newer treatment techniques that reduce the time lapse of treatment and also enforcement of higher standard metals by manufacturers for orthodontic appliances that are resistant to corrosion.[2]

CONCLUSION

- The buccal mucosa cells of patients treated with fixed orthodontic appliances over a period of 6 months showed significant increases in nickel, chromium, iron, and cobalt content, with significant DNA damage and insignificant decrease in cellular viability.
- Further studies should be carried out to evaluate the effects of these changes over the course of the treatment.

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