RESEARCH ARTICLE

The Release of Total Metal Ion and Genotoxicity of Stainless Steel Brackets: Experimental Study Using Micronucleus Assay

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Abstract

BACKGROUND: Stainless steel brackets are composed of various metal that may corrode in oral cavity. Corrosion is caused by the release of metal ions such as chromium, nickel, and iron. The release of metal ions can cause adverse effects such as toxicity, allergic, and mutagenicity. To evaluate the biocompatibility of stainless steel brackets, micronucleus assay as one of genotoxicity assay is used in this study. To determine the differences and the correlation of metal ions release and genotoxic activity among three brand stainless steel brackets.

METHODS: Three brands of brackets were immersed in artificial saliva for 672 hours and the release of ion chromium, nikel and iron were examined. The cytokynesis block micronucleus assay (CBMN) using lymphocytes was performed as well.

RESULTS: The highest metal releasing were nickel, cromium, iron, respectively (30.5, 27.2, 23.4 ppb). There was a significant differences between total nickel and iron ion release among three brand brackets (p=0.04, p=0.02). Genotoxicity of metal ion released was correlated with durration of immersion brackets (p=0.01). Genotoxicity was significant correlated with the release of chromium (p=0.03) and nickel (p=0.01).

CONCLUSION: Genotoxicity of stainless steel brackets was influenced by duration of immersion but not influenced by brand brackets.

KEYWORDS: genotoxicity, stainless steel brackets, metal ion

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Introduction

Fixed orthodontic appliances are commonly made of metal and alloys, which composed of various metals substances. Orthodontic bracket is the main elements of the fixed appliance orthodontic which delivers the activated force from the wire to the teeth.(1) Alloy brackets can be made of stainless steel, nickel-free stainless steel, and nickel-titanium.(2)

Stainless steel brackets have certain limitation as it is prone to corrode thus releases metal ions.(3) Corrosion on stainless steel bracket will reduce its aesthetic and strength. From the stand point of biocompatibility, corrosion of metals can cause adverse biologic effect.(4) Exposure to metal ions will lead to accumulation of these ions on the soft tissue and cause toxicity.(5) Some of the metal ions, such as nickel and chromium, were found abundant in stainless steel brackets and were classified as chemical carcinogens.(4)

Some factors such as saliva and time expossure can influence corrosion in the oral cavity. The longer these metal exposed to corrosive environment, the more metal ions were released. The length of exposure time to metal ions in the body has been known to limit the ability of cells

to repair themselves.(4) According to Sfondrini, *et al*., the release of nickel from brackets occurred after 24 hours of exposure time and tended to increase until the end of their study, which was 120 hours.(6) According to research done by Park and Shearer, an average release of 40 µg Nickel was released from a simulated full mouth fixed appliances.(7)

The correlation between the amount of metal ions released from fixed orthodontic appliances and their systemic effect to human body has not yet been well addresed. Up to this point, not all orthodontic brackets brands on the market has been tested and known their effects on the tissues inside the mouth which had occasional contact with brackets.(8)

Biocompatibility of stainless steel brackets can be studied with cytotoxicity and genotoxicity assay. Genotoxicity test on genetically altered cells related to metal ions exposure released by orthodontic devices can be determined.(4) The genotoxic effects that occur in the oral cavity may not be clinically observed yet, but the genetic alterations occur continuously and this mutagenity may eventually lead into malignancy.(9) Genotoxicity of the human cells due to exposure of metal ions released from brackets need to get attention because of long contact between brackets with oral cavity tissues, *i.e.,* during the orthodontic treatment with fixed appliance.

The purposes of this study were to determine the difference between the ion release of three brand stainless steel brackets, to determine the differences of genotoxicity from three brands of stainless steel brackets and to determine relationship of genotoxicity with the release of metal ions.

Methods

In order to evaluate the release of metal ions, three brackets from different commercial brands were included in this study; bracket Orthox (lot JO 19/SR2, JJ Orthodontics Pvt Ltd., India), Protect (lot 21003-2, Zhejiang 2 Medical Equipment Co, Ltd., China), Forestadent (lot 212, Benhard Föster GmbH., Germany) were consecutively named as bracket 1, 2 and 3. All brackets were immersed in Fusama-Meyer artificial saliva (Chemical Laboratoy in University of Indonesia) pH 4 and pH 7 alternating each minute for 16 hours and then immersed for 8 hours in the saliva pH 7. Saliva were collected at this time points for 24, 72, 168, 336 and 672 hours.

The level content examination of the metal ions (nickel, chromium, and iron) were determined using Inductive Couple Plasma-Atomic Emission Spectrometry (ICP-AES) machine (Iris Intrepid II XSP, Thermo Electron Corporation, Germany). ICP-AES is a technique to determined metals in a variety of different sample, such as liquid. Saliva injected into a radiofrequency (RF)-induced argon plasma using nebulizers with high temperature which excites the atomic species in the aerosol. Ion and photon with variety characteristic wavelength is recorded by an optical spectrometer.(10)

Micronucleus test was carried out using peripheral blood lymphocyte culture. A total of 4 mL venous blood were collected from human donors of each sex, male and female under informed consent. The following solutions were added into culture blood: 4.5 mL of RPMI (Roswell Park Memorial Institute, Gibco) completed Hepes and L-glutamin, 1 mL of FBS (Fetal Bovine Serum, Gibco), 0.2 mL of Pen-Strep (Penicilin-Streptomycin, Gibco), 0.2 mL of blood, 0.2 mL of PHA (Phytohemagglutinin, Gibco) and brackets extract. The blood tube was then incubated at 37^{o}C and 5% CO_2. Forty four hours after PHA stimulation, 20µL Cyt-B (cytochalasin-B, Sigma) was added. Control treatment used blood cultures that were not given the extract brackets and 1% Dimethyl sulfoxide (DMSO) as solvent extract brackets.

The culture was terminated at 68-72 hours after the addition of PHA. Blood culture was centrifuged at 800 rpm for 10 minutes and supernatan was discarded. Hypotonic solution 0.075 M KCl was added and immediately centrifuged at 800 rpm for 8 minutes. Supernatan was then discarded and fixative solution was added (methanol:acetic acid = 10:1) to lyse red blood cells and Ringer's solution 1: 1 (4.5 g NaCl, 0.12 g KCl, 0.21 g CaCl2 in 500 mL H2O). Then lymphocytes were centrifuged 800 rpm for 8 minutes. Lymphocytes were washed 2-3 times with a fixative solution until the clear cell suspensions were obtained. Supernatan was removed and the cell pellet which located at approximately 1 cm from the bottom of the tube was dropped on glass objects for examination. The microscopic slides were stained with Giemsa reagent and a repetition of 5 slides was made for each treatment.(11) Micronuclei observations was done with the light microscope (Motic® BA310, China) with 1000 magnification (Figure 1).

Kolmogorov-Smirnov test was used to analyze the normality of all data. Two-Way ANOVA test or Kruskal Wallis test) could be used to analyze the significance of ion release among three brackets in different times. Since the data was normal, Two-Way ANOVA test was used in this study. Statistical analysis of the micronulei's frequency of the respective brackets, time points, and sex is done with Three-Way ANOVA. Honestly Significant Difference (HSD)

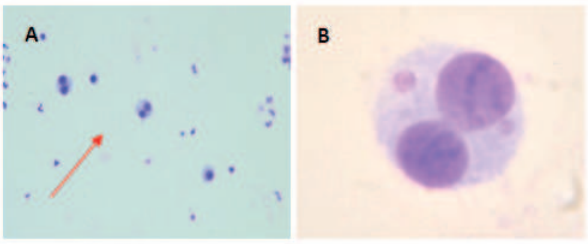

Figure 1. A. Binucleated cells (arrow) with 400x magnification, Giemsa stained. B. Binucleated cells with two micronuclei with 1000x magnification.10 µg/mL BJLE (F) for 24 hours. Cells were documented under inverted microscope. Size bars were provided as indicated in the picture.

Multiple and One-Way ANOVA were used to compare each sample groups. Correlation analysis between genotoxicity and ion release using Pearson test.

Results

The results of normality test, Kolmogorov-Smirnov, indicated that all group data had normal distribution (p>0.05). There were significant differences in total nickel release (p=0.04) and iron ion release (p=0.02) among three brands of stainless steel brackets but not with the release of chromium ions (p>0.05). There were significant differences in nickel ion release among different time points (p=0.02), but not with the release of total chromium and iron ions (Table 1).

The highest amount of total nickel ions released by brackets 1 (21.97±7.83) and the lowest by bracket 3 (13.77±6.42). Nickel ions released most at 168 hours (25.30±6.37) and least at 672 hours (11.13±4.06). The highest amount of iron ion released by bracket 2 (16.15±9.31) and the lowest by bracket 3 (2.61±3.51) as displayed in Table 2 and 3.

There was significant difference among the micronuclei's frequency of three brands of stainless steel brackets in different times (p=0.01), but there is no significant difference among three brands of stainless steel brackets (p>0.05) (Table 4). Statistical analysis by one way ANOVA and HSD-Tukey showed that the highest micronuclei's frequency occurred at 168 hours (53.33±5.28) and the lowest at 672 hours (34.00±5.25). Micronucleus frequency was higher in female gender (41.07±8.88) than male (50.07±10.65) (Table 4).

There were significant correlations between the frequency of micronuclei and the release of total chromium ions (F=0.39; p=0.03) and total nickel ions (F=0.46; p=0.01). However, there was no significant correlation between the frequency of micronuclei with total iron ion release (correlation coefficient=-0.01; p=0.99).

Discussion

This study used a method of immersing brackets in saliva at pH 7 and pH 4 for 16 hours and the saliva at pH 7 for 8 hours to simulate the actual conditions of oral cavity. To date, most of authors only applied static conditions in their in vitro studies. In fact, the release of ion metal is influenced also by the composition of saliva and changes of the flow rate in the mouth.(12,13,14) For example, a certain amount of passive films on the surface of brackets will be lost during tooth brushing. The acidity of the saliva will reduce the stability of passive film on the stainless steel brackets and its resistance from corrosion can therefore be compromised.(15)

In our study, we observed that the release of chromium started at 24 hours for bracket 1, 72 hours for bracket 2, and 336 hours for bracket 3 (Table 5). The release of these ions decreased gradually and reached 0 ppb at the end of the study (672 hours). Chromium ion formed chromium oxide which acted as a passive film on the surface of brackets that would effectively prevent the stainless steel they covered from oxidation. This oxide film would form again if brackets were exposed to corrosive environment in oral cavity due to saliva and the friction.(16) When the passive film was formed, there were some chromium ions that did not bind and eventually be lost.

The release of nickel ion occurred from the beginning of study (24 hours) and continued to fluctuate over time until the end of study (336 hours). This was probably due to the position of nickel ions which lied on the surface of brackets and helped increasing the corrosion resistance of brackets by competing with the chromium to form salts,

Table 1. Statistical analysis of total ion releases in three brand brackets with Two-Way ANOVA.

Groups	Mean	F
Chromium ion in Three Brand Brackets	791.67	1.43
Time	428.78	0.77
Nickel ion in Three Brand Brackets	87.22	4.73
Time	110.75	5.26
Iron ion in Three Brand Brackets	280.13	6.33
Time	42.48	0.96

Table 2. Comparison of total ion releases among three brand brackets.

Groups		F	Mean±SD	*p*
Total chromium ion of brackets	1	0.75	1.8±3.25	0.50
	2		7.89±11.43	
	3		2.77±6.08	
Total nickel ion of brackets	1	4.47	21.97±7.83	0.04*
	2		19.25±6.88	
	3		13.77±6.42	
Total iron ion of brackets	1	*6.33*	3.85±5,67	0.02*
	2		16.15±9.35	
	3		2.61±3.51	

*p<0,05

Table 3. Comparison total ion releases of three brands brackets at different time points.

Groups		F	Mean±SD	*p*
Total chromium ion at	24 hours	0.52	2.55±4.33	0.73
	72 hours		3.05±51.96	
	168 hours		9.12±15.70	
	336 hours		6.08±6.59	
	672 hours		0.05±0.00	
Total nickel ion at	24 hours	3.01	23.27±6.41	0.02*
	72 hours		15.77±4.97	
	168 hours		25.30±6.37	
	336 hours		15.27±7.22	
	672 hours		11.13±4.06	
Total iron ion at	24 hours	0.96	2.18±3.70	0.48
	72 hours		9.95±6.15	
	168 hours		6.42±11.03	
	336 hours		12.08±11.71	
	672 hours		7.05±12.12	

*p<0.05

allowing more chromium to be available to form the passive film.(2) However the union between nickel atoms and the intermetallic compounds was not strong so that the release of nickel ion would take place continuously until nickel on the surface has finished or new passive films on the surface has formed so that the nickel in brackets protected from the outside environment and the release of nickel ion decreased.(12)

The highest releasing of nickel ions is bracket 1 was occurred at 24 hours (Table 5). Our finding was not in accordance with the result of previous study published by Barrett, *et al*., which revealed that the highest release of nickel ion occurred on the day 7 (168 hours).(12) This may be due to the difference in treatment of the brackets and physical and chemical conditions at the time of testing by using a simulation of flow rate saliva and different pH of saliva that speeds up the corrosion process.

There was no significant difference between the frequency of micronuclei among three brackets (p>0.05). However, there was a significant difference between the frequency of micronuclei between time (p=0.01) (Table 4). This finding was on the contrary to the result of previous in vivo study done by Angelieri, *et al*.(8) The authors compared the frequency of micronuclei in buccal mucosal cells before orthodontic treatment, 170 days, as well as 6 month following the placement of brackets. It showed no significant difference inthe frequencies of the micronucleus formed between these time points. Similarly, the result of a study by Natarajan, *et al*.(17) also revealed no significant difference in the formation of micronucleus before and 30 days following the orthodontic appliance release. This may be because after 170 days, 6 months after the start of orthodontic treatment and orthodontic appliance release when it does happen the release of ions which stimulate the formation of micronuclei result of the stopping of the process of metal corrosion of orthodontic bracket.

This study found a correlation between the release of total nickel and chromium but not iron ions with the genotoxic activity. This finding may be associated with the amount of metal ions that can be tolerated by the body. The normal amount of chromium ions in the blood is 0.28 g/L of blood.(16) The release of excess of chromium ions may cause toxicity which affects cell viability and destructs DNA synthesis. The normal amount of nickel ions in the blood is 5 mg/L of blood.(16) Nickel ion with a high concentration will cause toxicity and become carcinogenic. However, iron ion is one of the constituents of red blood cells. The normal content of iron in the body is 5 mg and they are considered as non toxic metal for human body.(16) Therefore, the release of ion iron of stainless steel orthodontic brackets does not result in increasing genotoxic activity on cells. Meanwhile, release of chromium ion in spite of small amounts can increase the frequency of micronucleus in cells.

We also observed that the frequency of micronuclei on female was significantly higher than male (Table 4). Our finding is in accordance with the result of previous study by Wojda, *et al*., and Araujo, *et al*.(18,19) The difference in frequencies of micronuclei between male and female is likely due to higher susceptibility to malsegregation of chromosome X in comparison with autosome and distal

Table 4. Comparison of micronuclei's frequency among three brand brackets, time points, and gender.

Groups		F	Mean±SD	*p*
Micronuclei's frequency in brackets	1	0.1	45.10±9.33	0.97
	2		45.10±14.59	
	3		46.50±8.06	
Micronuclei's frequency at	24 hours	4.68	45.17±9.75	0.01*
	72 hours		48.67±14.38	
	168 hours		53.33±5.28	
	336 hours		46.67±7.50	
	672 hours		34.00±5.25	
Micronuclei's frequency on	male	9.24	41.07±8.88	0.01*
	female		50.07±10.65	

*$p<0.05$

Table 5. Univariate data of total metal ion released from three brands brackets (ppb).

Hours	Chromium Level			Nickel Level			Iron Level		
	Bracket 1	Bracket 2	Bracket 3	Bracket 1	Bracket 2	Bracket 3	Bracket 1	Bracket 2	Bracket 3
24	7.5	0.0	0.0	30.5	18.3	21.0	0.0	0.0	6.4
72	0.0	9.0	0.0	17.0	19.7	10.3	6.3	17.0	6.4
168	0.0	27.2	0.0	27.2	30.4	18.2	0.0	19.1	0.0
336	1.5	3.0	13.6	23.5	12.3	10.0	12.7	23.4	0.0
672	0.0	0.0	0.0	10.9	15.3	7.2	0.0	21.0	0.0

lagging behind in anaphase. Chromosome X sensitivity to aneuploidy was suggested to be due to premature centromere division and modified centromere function.(20)

Almost all of micronuclei's frequency from three brand brackets in different time points above the normal range (0.04).(11) Based on cytotoxicity study of three brackets conducted by Komaladi, the decrease of cell viability below normal, *i.e.,* 50% only occurred on bracket 2 at 72 hours.(21) This indicates that the genotoxicity assay is more sensitive in detecting cellular damage than cytotoxicity assay. The molecular changes may have occurred in the cells even when the cellular viability within normal range.

Of these three brackets are examined, among bracket 1, 2, and 3 indicating release of nickel ion in all the time points. This shows that although the content of nickel improves corrosion resistance on brackets, there will be the release of nickel ion because the bonds of nickel ions on brackets are not strong. The release of nickel ion had occurred in the first 24 hours and decreased at 168 hours (7 days). Genotoxicity of three brands brackets increased above normal from the beginning of study until 336 hours (14 days). This was in concordance with the amount of ions release. Therefore, dentists must be aware of the adverse effects related to the release of metal ions from orthodontic brackets particulary from the bracket placement to 14 days following.

Conclusion

There are significant differences of nickel ion release from three brands brackets in different times. Genotoxicity of stainless steel brackets is influenced by time and gender but not influenced by brand brackets. There is correlation between genotoxicity of stainless steel brackets and the release of metal ions, *i.e.,* chromium and nickel ions.

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