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ORIGINAL ARTICLE

Assessment of DNA Damage of Oral Mucosa due to Ceramic Bracket Using Comet Assay and Mutagenicity of Orthodontic Bonding System Using Ames Test

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ABSTRACT

Objective: The objective of this study was to determine the DNA damage caused by ceramic bracket (Clear II, SIA Orthodontic Manufacturer Srl, Italy) on human buccal mucosal cells using comet assay and the mutagenicity of orthodontic bonding system (Transbond XT; 3M Unitek) by Ames test. **Methods:** In this study, twenty orthodontic patients were recruited from Specialist Orthodontic Clinic, Hospital Universiti Sains Malaysia. The Buccal mucosal cell sample was obtained from each patient at three time points-before (TO), after one month (T1) and after two months (T2) of ceramic bracket placement for performing comet assay. The spot test version of Ames test was performed using four *Salmonella Typhimurium* (*S. Typhimurium*) tester strains (TA 98, TA100, TA1535 and TA1537) for mutagenicity testing. Total comet score (TCS) and damage frequency (DF) were used to determine the DNA damage using non-parametric Friedman test followed by multiple pairwise comparison. The Ames test was analysed by a non-statistical method based on revertant growth ring formation. **Results:** There was no significant change of both TCS and DF between T0 and T1, but both parameters increased significantly from T0 to T1 and from T1 to T2. Non-statistical analysis was carried out to evaluate the results of Ames test based on the formation of revertant colony growth ring. None of the *S. Typhimurium* tester strains showed any revertant growth ring formation around the light cure adhesive primer. **Conclusion:** Ceramic bracket does not cause any DNA damage on human buccal mucosal cells and light cure adhesive primer is non mutagenic under the present test conditions.

Key words: Ames test, ceramic brackets, DNA damage, mutagenicity of orthodontic bonding system

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INTRODUCTION

Ceramic brackets were introduced to meet the increasing demand for more esthetic appliances by adults seeking orthodontic care.[1] Various orthodontic ceramic brackets and bonding agents are being utilized by clinicians in daily practice. Many studies mainly focus on their physical properties such as shear bond strength and biological and microbiological changes that accompany orthodontic treatment compared with their biocompatibility.[2,3]

The duration for orthodontic treatment with fixed appliances may take an average of two years with the appliances remaining in direct or indirect contact with the buccal mucosal cells. Biodegradation of orthodontic appliances due to intra-oral thermal, microbiological, and enzymatic properties can lead to various adverse effects like cellular and genetic toxicity.[4] Perusal of literature review shows that there is a large controversy and limited knowledge about the biocompatibility of orthodontic appliance materials especially on its intraoral ions' compatibility released from ceramic brackets and their effect on buccal mucosal cells. A few studies have reported positive effects on the biocompatibility and safety of ceramic brackets while other findings have reported that these appliances need to be further evaluated to ensure their biosafety. The purpose of mutagenicity testing is to identify substances that can cause genetic alterations in somatic

or germ cells, and this information is used in regulatory decision-making. Mutagenicity of orthodontic bonding system also requires further investigation because only a few studies[5,6] have examined the genetic and cytological effects over time after light-induced polymerization. Assessment of DNA damage in a single cell have extensively used comet assay to test genotoxic properties owing to its advantages which includes sensitivity of low-level DNA damage detection, requirement of only minimal amount of cells/sample (<10000), competency to assay using test substance in less quantity and completion of the assay within a shorter period.[7]

Mutation can be defined as alterations of gene which are expressed at the phenotypic level and modification of DNA such as changes in the specific base pairs or shifting of chromosomal location.[8] The Ames test is a short-term bacterial reverse mutation assay that can distinctively detect a broad spectrum of mutagens responsible for genomic impairment leading to gene mutation.[9] The test uses different strains of *Salmonella typhimurium* for detecting and classifying various mutagens. At present, Ames test is a popular mutagenicity testing assay for initial screening of mutagenic potential of biomaterial commonly used worldwide.[9,10] An extract from a medical device or material is tested using the Ames method to see if it has any potential mutagenic properties. Moreover, this test is carried out as a component of the battery of genotoxicity studies to establish whether leachables from a medical device or materialare mutagenic. Bearing this in mind, this study aimed to assess the DNA damage of ceramic brackets on buccal mucosal cells by comet assay and to evaluate if the light cure adhesive primer of orthodontic bonding system induced any mutagenic effect using Ames test.

METHODS

Subjects and sample

Ethical approval was obtained from Human Research Ethics Committee Universiti Sains Malaysia (USM/JEPeM/17010066) (IRB number: IRB00010568). The study protocol complied with the Helsinki Declaration on human experimentation guidelines. The study comprised twenty patients (11 females and 9 males) with a mean age of 22.15±1.6 years. The inclusion criteria of the patients included those with permanent dentition with healthy oral mucosa requiring fixed orthodontic treatment in both jaws. The exclusion criteria comprised those with past orthodontic treatment history, presence of any existing dental restoration, presence of any dental prosthesis, any existing systemic disease, regular intake of any supplement or medication and presence of any existing health condition that may restrict patients from maintaining good oral hygiene. Based on inclusion and exclusion criteria, screening of the patient was done by the orthodontist in Specialist Orthodontic Clinic, Hospital Universiti Sains Malaysia. At the beginning of the orthodontic treatment procedure, ceramic brackets (Clear II, SIA Orthodontic Manufacturer Srl, Italy) were placed after initial treatment planning and obtaining informed consent from the patient. Prior to placement of ceramic brackets, the first buccal swab was collected (T0). The ceramic brackets were kept without activation for 1 month and a second buccal swab (T1) was collected after the 1 month of ceramic bracket placement. For the activation of the brackets buccal tube (BioMIM, Orthoclassic, USA) was bonded in all first molar in both arches followed by 0.14 Niti archwire (aesthetic tooth color coated, SIA Orthodontic Manufacturer Srl, Italy) placement and modules application. One month from the day of ceramic bracket activation, the third buccal swab (T2) was collected.

During the recruitment of the patients for the study, the participating patients were supplied with non-fluoridated tooth paste for their routine brushing activities until the study period finished and were advised to refrain themselves from using any kind of fluoride or chlorhexidine containing mouthwash or toothpaste. This is because high buccal mucosal cell's DNA damage have been reported due to the use of fluoride or chlorhexidine containing mouthwash.[11]

Buccal mucosal cell sample collection

Prior to sample collection, patients were instructed to rinse with temperate water to wash out mucosal exfoliated dead cells. The site of sample collection was interior middle part of right and left cheeks by means of gentle scraping with a sterile interdental brush.[12] After that, the interdental brush was vigorously agitated into ice cold 5 ml of PBS (free from calcium and magnesium ions at pH7.4) in a sterile plastic tube (15ml) which facilitated detachment of cells from the interdental brush. Samples were then immediately transferred to the laboratory in a closed icebox for performing comet assay.

Comet assay

In this study, comet assay protocol was followed according to the guidelines proposed by Tice *et al.*[13] with modification as suggested by Szeto *et al.* [14] in lysis steps using CometAssay® kit (Trevigen, USA). Once the samples were transported to the laboratory, the cell suspension was centrifuged at 15ºC for 10 minutes at 1500 rpm and a cell suspension of 10000 cells/ml was prepared.[12] 8 µl from the prepared cell suspension was taken into two 1.5 ml centrifuge tubes each and 72 µl of molten Low Melting Agarose (LMA) were added into each tube. Onto the agarose layer of CometSlide™, it was then dispersed by pipetting and refrigerated for solidification (4ºC for 10 minutes). Once the slides were solidified, they were treated for 45 minutes with 100 µl of 1 mg/ml of Proteinase K (Qiagen, Germany).[13]

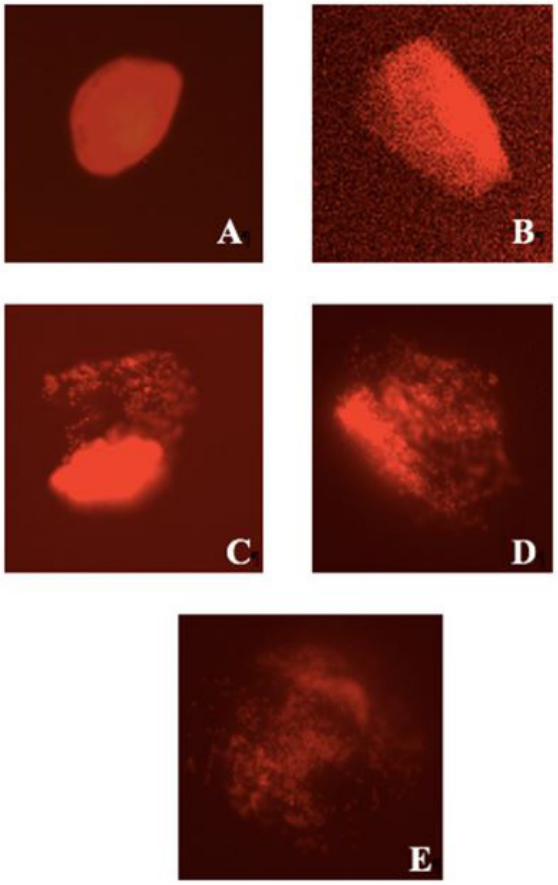

Figure 1. Cells (Buccal mucosal cells) showing varying amount of DNA damage A) Class-0, score 0, B) Class-1, score 1; C) Class-2, score 2; D) Class-3, score 3; and E) Class-4, score 4. (BX41 Epifluorescence Microscope and XC50 Digital Microscope Camera; Olympus, Japan).

After the proteinase K treatment, slides were placed in lysis buffer (Trevigen, USA) (4ºC for 1 hour) and then placed in freshly prepared alkaline unwinding solution (sterile distilled water 49.75 ml, NaOH 0.4 g, 200 mM EDTA 250 µl) for 20 minutes in a dark room. Following this, electrophoresis was performed in a horizontal electrophoresis chamber (Cleaver Scientific, USA) attached with PowerPac™ Electrophoresis Power supply (Bio-Rad Laboratories, USA) containing electrophoresis solution (1000 ml sterile distilled water, 8 g NaOH, 2 ml of 500 mM EDTA). The slides were placed in the electrophoresis tray side by side submerged in the electrophoresis solution and run at 25 V, 300 mA for 20 minutes. Consequently, neutralization of slides was done by rinsing the slides thrice in neutralization buffer (five minutes each time). In an attempt to dehydrate the slides, they were placed in 95% of ice-cold ethanol solution for 10 minutes. Following this, slides were stored in a dark container overnight at room temperature prior to staining with 20 µg/ml ethidium bromide (Promega, USA) for 15 minutes.

Comet visualization was done in a dim room by placing the slides under an epifluorescence microscope (BX41; Olympus, Japan) at 40x magnification. The images were captured using XC 50 digital microscope camera (Olympus, Japan) supplied with AnalySIS FIVE (Olympus Soft Imaging Solution, Japan) host imaging software. Two slides were scored from each sample where 50 randomly selected nucleoids were scored from each slide totaling 100 nucleoids from each sample. Visual scoring method was used in this study for scoring comets.[14] For scoring, the comets were classified into five classes (class 0 to class 4)[15,16] based on their tail intensity and each type was given a value in arbitrary unit according to their DNA fragmentation in tail (Figure 1). Total comet score (TCS) was then calculated for each sample from the two slides. This calculates the DNA damage by multiplying the number of nucleoids in each class of damage by its value and summing these values; thus, for each patient at a given sampling time, the TCS was between 0 and 400. Also, the damage frequency (DF) was calculated; this represents the number of comets per 100 examined nucleoids. H2O2 was used as positive control during each comet assay performance to validate the study. A group of cells was treated by 500µl of 100µM of H2O2 and incubated at 4ºC for 30 minutes.

Ames test

In this study, Ames test (spot test version) was carried out following the method that was proposed by Maron and Ames[17] to assess the mutagenicity of the light cure adhesive primer of orthodontic bonding adhesive (Tranxsbond™ XT, 3M Unitek, USA).

Bacterial tester strains

In this study, four bacterial tester strains of *Salmonella Typhimurium* (TA98, TA100, TA1535 and TA1537) (Moltox, USA) were used. Genetic analysis was carried out forthe *Salmonella Typhimurium* (*S. Typhimurium*) tester strains in order to check the genetic integrity and spontaneous mutation rate, prior to performing Ames test.

Bacterial tester strains

In this study, four bacterial tester strains of *S. Typhimurium* (TA98, TA100, TA1535 and TA1537) (Moltox, USA) were used. Genetic analysis was carried out for the *S. Typhimurium* tester strains to check the genetic integrity and spontaneous mutation rate, prior to performing the Ames test.

Positive and negative controls

Due to the absence of cellular toxic effect, as negative control for all four tester strains,sterile distilled water was used. 4-nitro-*o*-phenylenediamine (ACROS Organics, USA) was used as positive control for tester strain TA98, 9-aminoacridine (ACROS Organics,USA) for tester strain TA1535 and TA100 and sodium azide (ACROS Organics, USA) for tester strain TA1537.

Spot test procedure

The plate incorporation Ames test has different variants and spot test is one of them in which the test material is applied directly to the top agar surface seeded with tester strains of bacteria. The *S. Typhimurium* tester strains (TA98, TA100, TA1535, and TA1537) culture were inoculated in nutrient broth and incubated overnight at 37ºC with shaking. The top agar was melted and maintained at 43º–48ºC. Prior to its use, the already prepared Glucose Minimal (GM) agar plates were warmed to room temperature that were stored at 4ºC earlier. Then, the modified top agar solution was prepared by adding 5ml of histidine biotin solution (0.5 mM) and 2.5 ml of overnight bacterial culture into 50 ml of top agar and mixed thoroughly. 2 ml of this modified top agar solution were delivered onto the GM agar surface of each plate. For assuring the uniform distribution of top agar onto GM agar, surface plates were swirled properly. Once the top agar was solidified (within 2-3 minutes), 10 µl of the test material was delivered in the center of the plate and covered with plastic top. After that, at 37ºC for 48 hours, the plates were intubated in an upside-down manner. Each tester strain was subjected to triplicate plating.

Evaluation of spot test result was done by means of visual inspection and colony growth was scored as 'negative' if there was no revertant colony growth ring seen, 'weakly positive' if a narrow and dense revertant growth ring was seen and 'positive' if the revertant colony growth ring was denser more than 1 mm in width around the test material.[18]

Statistical analysis

IBM SPSS version 22 software was used for statistical analyses. The non-parametric Friedman test was used as per Kolmogorov-Smirnov test results. Differences between groups were further tested by multiple pairwise comparisons and the *p* value were set at <0.05 to be considered as statistically significant.

RESULTS

The results of Friedman test and multiple pairwise comparisons are given in Table 1 and Table 2. All results are expressed as median (Inter Quartile Range). Comet assay showed that the increase of both TCS and DF from before to one month after ceramic brackets placement was not significant ($p > 0.05$). However, the increase of both TCS and DF after ceramic brackets placement was significant ($p < 0.05$) from before to two months after and from one month to two months. Figure 2 shows the overall median increase of both TCS and DF from before, one month after and two months after bonding ceramic brackets.

Table 3 shows the result of mutagenicity of light cure adhesive primer (Transbond™ XT, 3M Unitek, USA) by spot test version of Ames test. The revertant colony growth was scored by visual inspection as negative, weakly positive and positive based on the formation of revertant growth ring around the tested material. According to Mortelmans and Zeiger,[9] single bacterial strain of *S. Typhimurium* is sufficient for the

Table 1. Changes of total comet score in test group.

Group	Median (IQR)	Chi square	p
T0	28.50 (8.25)		
T1	32.00 (10.00)	38	$p < 0.05$
T2	59.00 (13.25)		

*Friedman test, **IQR=Interquartile Range,
Multiple pairwise comparison:
T0 vs T1- not significant ($p = 0.114$);
T0 vs T2 and T1 vs T2 - significant ($p < 0.05$)

Table 2. Changes of damage frequency (DF) in test group.

Group	Median (IQR)	Chi square	p
T0	18.50 (5.75)		
T1	20.50 (5.5)	37.130	$p < 0.05$
T2	39.00 (8.50)		

*Friedman test, *IQR=Interquartile Range,
Multiple pairwise comparison:
T0 vs T1- not significant ($p = 0.082$);
T0 vs T2 and T1 vs T2 - significant ($p < 0.05$)

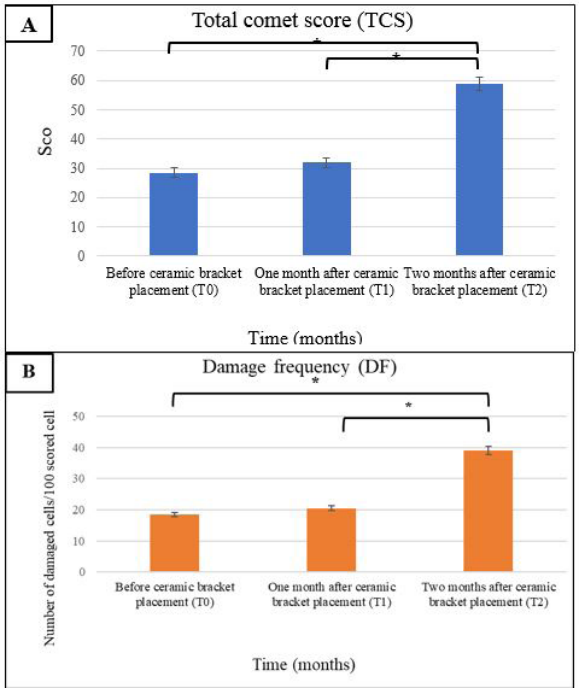

Figure 2. DNA damage assessment of buccal mucosal cells by comet assay. A) Total comet score (TCS), B) Damage frequency (DF). TCS and DF are reported as median compared to the different time points of ceramic bracket placement. Multiple pairwise comparison test showed that the increase of median values of both TCS and DF at two months after ceramic bracket placement were significant. *significant at $p < 0.05$.

Table 3. Spot test result of Light cure adhesive primer (Transbond XT®; 3M Unitek, USA).

Plate number	*Salmonella Typhimurium* tester strains			
	TA98	TA100	TA1535	TA1537
Plate 1	(-)	(-)	(-)	(-)
Plate 2	(-)	(-)	(-)	(-)
Plate 3	(-)	(-)	(-)	(-)

(-) Denotes Negative

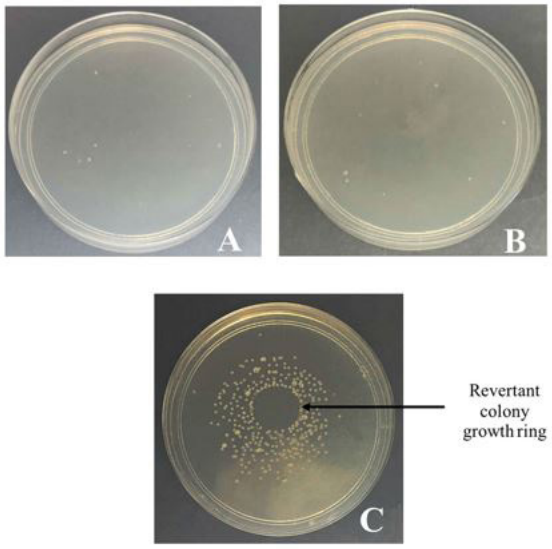

Figure 3. Revertant colony growth of *Salmonella Typhimurium* TA98. A) Negative control (distilled water); B) Test material - light cure primer adhesive (Transbond™ XT; 3M Unitek, USA) and C) Positive control (4-nitro-o-phenylenediamine); revertant colony growth ring positive.

determination of mutagenic effect of testing material. Nevertheless, negative mutagenic results with 4-5 tester strains are accepted generally. In the present study, four *S. Typhimurium* tester strains, namely TA98, TA100, TA1535 and TA1537 were selected for spot test. It is recommended to include at least two strains of which one is from frameshift mutation and the other is from base pair mutation.[19] As per this recommendation, among the selected strains in this study, TA98 and TA1537 had frameshift mutation and TA100 and TA1535 had base pair mutation. Moreover, this selection was made as the histidine operon of selected strains contain different mutations and differ genetically from each other. The result showed negative score for the revertant colony growth ring formation in all three plates for each tester strain of S. Typhimurium. Figure 3 shows the formation of revertant colony growth ring with positive control for the tester strain TA98.

DISCUSSION

This is an *in vivo* prospective longitudinal study comprising orthodontic patients treated at Orthodontic Specialist Clinic Hospital Universiti Sains Malaysia by using comet assay and *in vitro* experimental study on orthodontic bonding system by using spot test version of Ames test to assess the mutagenicity. The study followed the method of Fernández-Miñano *et al.* to keep metallic brackets passive without any activation for 30 days. The aim was to affirm the DNA damage effect of ceramic bracket only by using comet assay.[20] Ceramic brackets were activated after one month after archwire insertion and buccal mucosal cell sample was collected from interior middle part of cheeks by gentle scraping according to methods suggested by Besaratinia *et al.*[11] and Fernández-Miñano *et al.*[19] again to assess the DNA damage effect of ceramic brackets after one month of activation. *In vivo* method was chosen as *in vitro* testing may not always simulate intra-oral multifactorial environment.[14] Mimicking a corrosive environment and appropriate cell type selection as in oral cavity is also challenging. Buccal mucosal cells have the advantages in contrast to other body samples because buccal mucosal cells are in closest proximity with orthodontic appliances thus allowing to uptake and accumulate ions released by adjacent orthodontic appliances thereby helping to demonstrate damaging effects of those appliances.[21]

The findings from comet assay study showed no significant results although a slight increase of DNA damage was seen during this one-month period. Vitral and colleagues reported that the reason for the slight increase of DNA damage could be due to cellular adaptation with newly introduced materials intraorally.[22] Intermodal cytotoxicity from polycrystalline ceramic brackets can cause low level of toxic effects to cells which can also explain the reason of slight increase in DNA damage in this present study. Composition and surface texture of different orthodontic appliance components used may be the factors for a significant increase of DNA damage in buccal mucosal cells compared to before and after one month of ceramic bracket placement.[23] Intraoral corrosive environment and composition of appliances are the factors for ions released from orthodontic appliances as reported by Westphalen *et al.*[24] An increase in Ni ions release and DNA damage after 1.5 years of orthodontic treatment also has been reported.[25] This statement was also supported by Hafez *et al.*[14] where a significant increase of Ni ions at 3 months was noticed in their patients. Facciono *et al.*[4] reported a correlation between Ni ions release and the increase in the number of comets in their study. They also found that both Ni and chromium ions released 3.4 folds

compared to that of control group.[4] Among the ions released by orthodontic alloys, Ni ions are considered as the major cytotoxic agents and is also considered as a known carcinogenic and mutagenic agent which in higher levels could be responsible for the innate immune response to metallic orthodontic alloys.[26] This finding may explain the result of the present study as NiTi archwires containing Ni were used in this study thus resulting in significant DNA damage to buccal mucosal cells. It has also been reported that Ti alloy is considered as the highest biocompatible material[27] However, according to the report by Bakhtari *et al*., Ti alloy when used in the presence of other metal alloys can cause high level of toxic cellular damage because of galvanic current influence.[28] This is also in agreement with the results of the present study as stainless-steel alloy made of buccal tube was used. The biocompatibility of conventional aesthetic coated NiTi archwires and Teflon coated NiTi archwires has been compared by Rongo *et al*.[29] They reported that conventional aesthetic coated (a resin-polymer coat) NiTi archwires were more toxic to cells. This statement supports the present results as conventional aesthetic coated archwires were also used in this study.

Tomakidi *et al*.[30] conducted a study to compare biocompatibility of different fixed orthodontic appliance components and reported no DNA damage based on comet assay. Similar results have been reported by other researchers. Mockers *et al*.[1] concluded that metallic bands, buccal tubes, brackets and archwires were found to be non-cytotoxic in their study. They explained that the toxic metallic eluates found in their study was lower than the accepted non-toxic levels, but it is difficult to rule out that even non-toxic concentration of metallic ions may be sufficient to induce any biological toxic effect to buccal mucosal cells.[1]

Due to continuous friction with archwires, biodegradation of ceramic brackets may occur once they are activated or by chemicals attached from intraoral corrosive environment or both. Inorganic ions released in aqueous media varies depending on the ceramic composition and surrounding environment.[31,32] The chemical durability reduction of dental ceramics is important because the increased susceptibility to chemical attacks leads to impairment of Si-O-Si bond. This can cause ions to be released from elements ($K_2O.Al_2O_3.4SiO_2$) which is unwanted from a biocompatibility perspective.[32] Sjögren *et al.* in 2000 tested release of different ions from various dental ceramics by inductively coupled plasma optical emission spectrophotometry and found release of aluminium (Al), silicon (Si), sodium (Na), potassium (K) and calcium (Ca).[31] This finding was in line with Milleding *et al.* regarding the release of different ions from the dental ceramics.[32] Both studies have concluded that ions released from crystalline alumina type of dental ceramic was significantly lower than other types such as glass phase ceramics. Thus, based on the significant result from the findings, prior to two months after bonding of the ceramic brackets (polycrystalline ceramic), it can be assumed that ceramic brackets itself had little influence and that the significance could have resulted from the metallic components of appliances present during the second month.

The spot test was implicated for mutagenicity assessment of liquid component of orthodontic bonding system.[18,33] The liquid component (light cure adhesive primer) of Transbond XT® was evaluated qualitatively. This process was recommended to be performed prior to performing plate incorporation assay. A positive result by spot test helps in determination of bacterial strain for dose-response mode of plate incorporation assay.[17] No bacterial tester strain yielded any positive revertant colony growth ring formation around the light cure adhesive primer in this study. However, the positive revertant colony growth ring was seen around the positive control agent which validated the current study. Fredricks in 1981 used spot test version of Ames test to test liquid component of eight different orthodontic bonding systems. The result showed that only one liquid component had positive result which was then further investigated by dose-response method.[14] Six different brands of orthodontic bonding system using spot test was tested by Cross and colleagues, in 1983.[33] Only two brand showed positive results which were further investigated by dose-response method.[33] Light cure adhesive primer of Transbond™ XT did not have any mutagenic effect in this present study.

There are some differences in findings for biocompatibility assessment on Transbond™ XT regarding the cytotoxicity. A few studies concluded the Transbond™ XT as an acute cytotoxic agent in their studies.[2,5,34,35] The result showed less cytotoxicity of Transbond™ XT when compared with chemical cured orthodontic adhesives.[36] This finding was not in line with the study by Huang *et al*.[37] Their study found that the primer component of Transbond™ XT was cytotoxic.[37] However, in the current study, light cure adhesive primer of Transbond™ XT was found to be non-mutagenic.

CONCLUSION

Ceramic bracket can be considered as non-genotoxic based on the comet assay result in the current study as it did not cause DNA damage to human buccal mucosal cells. However, the combination of ceramic brackets with other metallic components of fixed orthodontic appliances may not be considered as biocompatible. In addition, as the light cure adhesive primer did not exhibit any revertant colony growth ring formation in spot test version of Ames test, it can be considered as non-mutagenic under the present test conditions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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