Cytotoxic Effects of Acidulated Phosphate Fluoride on Oral Mucosal Cells of Orthodontic Patients

ความเป็นพิษของแอซิดดูเรตฟอสเฟตฟลูออไรด์เจลต่อเซลล์เยื่อบุกระพุ้งแก้มในผู้ป่วยจัดฟัน

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ABSTRACT

The aim of this study was to evaluate the cytotoxic effect in oral mucosal cells from orthodontic patients with three fluoride products. Fifteen patients requiring fixed orthodontic therapy were treated with acidulated phosphate fluoride gel (APF), neutral fluoride gel (NGel), or fluoride varnish (Fva). Five patients without any fluoride treatment were control. Buccal mucosal cells were collected before treatment (T1) and 3 months after appliance placement (T2). The cells were prepared for cell viability test (trypan blue exclusion test) and stained with Papanicolaou (PAP) staining. Degenerative nuclear alterations were scored under a light microscope. One-Way ANOVA statistical analysis indicated that APF showed a decrease of cellular viability and significant increase of morphological signs of cytotoxicity (P<.05). Therefore, application of APF gel in orthodontic patients could induce cytotoxicity. FVa and NGel are recommended during orthodontic treatment.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความเป็นพิษของฟลูออไรด์ต่อเซลล์เยื่อบุกระพุ้งแก้มในผู้ป่วยจัดฟัน ผู้ป่วยจัดฟันจำนวน 20 คนถูกแบ่งเป็น 4 กลุ่มตามชนิดฟลูออไรด์ที่ได้รับ ได้แก่ แอซิดดูเรตฟอสเฟตฟลูออไรด์เจล โซเดียมฟลูออไรด์เจล ฟลูออไรด์วาร์นิช และไม่ได้รับฟลูออไรด์ซึ่งถือเป็นกลุ่มควบคุม เซลล์เยื่อบุกระพุ้งแก้มถูกเก็บจากผู้ป่วยแต่ละกลุ่มจำนวน 2 ครั้ง คือ ก่อนติดเครื่องมือจัดฟันชนิดติดแน่นและหลังจากติดเครื่องมือจัดฟันชนิดติดแน่นเป็นเวลา 3 เดือน นำเซลล์เยื่อบุกระพุ้งแก้มไปทดสอบความมีชีวิตของเซลล์และการทดสอบแพป แล้วนับจำนวนเซลล์ที่มีสัณฐานวิทยาเปลี่ยนแปลงภายใต้กล้องจุลทรรศน์ ทำการวิเคราะห์ผลโดยใช้สถิติการวิเคราะห์ความแปรปรวนแบบทางเดียว พบว่า ความมีชีวิตของเซลล์เยื่อบุกระพุ้งแก้มลดลงและมีสัณฐานวิทยาแสดงความเป็นพิษภายในเซลล์เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในผู้ป่วยจัดฟันกลุ่มที่ได้รับแอซิดดูเรตฟอสเฟตฟลูออไรด์เจล จึงสรุปว่า การเคลือบแอซิดดูเรตฟอสเฟตฟลูออไรด์เจลทำให้เกิดความเป็นพิษต่อเซลล์ได้ จึงแนะนำให้ใช้โซเดียมฟลูออไรด์เจล หรือฟลูออไรด์วาร์นิชระหว่างรักษาทางทันตกรรมจัดฟัน

Keywords: Acidulated phosphate fluoride, Cytotoxicity, Fixed orthodontic appliances

คำสำคัญ: แอซิดดูเรตฟอสเฟตฟลูออไรด์ ความเป็นพิษต่อเซลล์ เครื่องมือจัดฟันชนิดติดแน่น

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Introduction

The orthodontic appliances remain in the mouth for several years in the oral condition which is the potentially corrosive environment. Many factors act as corrosion conductors, e.g., saliva, the fluctuation of pH and temperature, the enzymatic and microbial activity (Eliades, Bourauel, 2005). In many *in vitro* (Agaoglu et al., 2001; Matos de Souza, Macedo de Menezes, 2008; Menezes et al., 2007; Petoumenou et al., 2009) and *in vivo* (Barrett et al., 1993; Eliades et al., 2003) studies, the metal ions have been demonstrated to be released from orthodontic alloys by chemical reactions. This gradual destruction increased the roughness of the appliance surface which can lead to decrease the mechanical properties of metal alloys (Alavi, Farahi, 2011; Costa et al., 2007; Nanjundan, Vimala, 2016). Moreover, metal ions released from orthodontic appliances were found in the tissues and fluids of patients wearing them (Agaoglu et al., 2001; Amini et al., 2008; Faccioni et al., 2003; Menezes et al., 2007). It has been shown that these released metal ions are cytotoxic, mutagenic and allergic agents. A recent *in vivo* study reported that the metal ions from orthodontic appliances decreased cellular viability and induced DNA damage (Faccioni et al., 2003; Hafez et al., 2011).

Active orthodontic treatment is a factor increasing risk of developing caries (Chaussain et al., 2010; Mattousch et al., 2007). Fixed orthodontic appliances create an oral environment that is conducive to increase plaque retention and develop suboptimal oral hygiene status (Karadas et al., 2011). Professionally applied topical fluoride is recommended in high-risk caries orthodontic patients to prevent developing white spot lesion and carious lesion (American Dental Association Council on Scientific Affairs, 2006).

Among different fluoride-containing products, acidulated phosphate fluoride (APF) gel is commonly used because the acidity of APF provides more calcium fluoride in the enamel (Fejerskov, Kidd, 2009; Saxegaard, Rolla, 1988). However, corrosion of orthodontic metal appliances is strongly related with the acidic environment. Many *in vitro* studies have demonstrated that corrosion susceptibility of metals increased in fluoridated acidic environment (Kuhta et al., 2009; Schiff et al., 2002; Walker et al., 2007). A recent cell culture study indicated that metal ion remarkable increased and cell viability markedly decreased when the cells were incubated with culture media immersed with metal appliances and APF (Yanisarapan et al., 2018). So, using APF with fixed orthodontic appliances could lead to enhance the metal corrosion. Neutral fluoride gel (Ngel) and fluoride varnish (Fva), other types of professional fluoride-containing products which do not decrease pH of oral environment, have shown the similar amount of fluoride uptake in enamel as APF (Lee et al., 2010). These neutral topical fluorides are suggested to be product of choices in high caries orthodontic patients to reduce the metal corrosion in oral environment (Boere, 1995). To our knowledge, no longitudinal controlled clinical study has investigated which fluoride-containing product is safe when applying with orthodontic appliances in terms of metal corrosion.

Objectives of the study

The purpose of this study was to investigate the *in vivo* corrosion of fixed orthodontic appliances caused by APF, NGel and Fva by evaluating the cytotoxicity of orthodontic patients' oral mucosal cells.

Methodology

Subjects

The protocol was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Thailand (HREC-DCU 2018-064). The objectives of the study and the method of cell collection were explained to all subjects and written consent to participate was obtained. The subjects of this study consisted of 20 healthy adults with a mean age of 24.60 $\pm$ 11.01 years who had submitted to the orthodontic treatment at the Department of Orthodontics at Faculty of Dentistry, Chulalongkorn University, Thailand.

The inclusion criteria for subject selection were (1) permanent dentition with all second molars erupted in the oral cavity, (2) clinically healthy oral mucosa (2) no previous orthodontic treatment, (3) no occupational exposure to metals, (4) no amalgam fillings and metal restorations, (5) no palatal or lingual appliances soldered to the bands or extraoral auxiliary orthodontic appliances. The exclusion criteria were (1) smoking, (2) pre-existing systemic diseases or medication associated with oral mucosal changes and (3) allergy to nickel or chromium.

All patients were bonded with fixed orthodontic appliances in both arches. The appliances consisted of at least 4 tubes and 16 bonded brackets. Brackets and tubes are made of stainless steel (Omniarch, Tomy, Tokyo, Japan). The archwires used in the study were 0.014-in and 0.016-in nickel-titanium alloys throughout the observed period (ClassOne Orthodontics, Ortho Organizers, USA).

Buccal cell collection and fluoride application

The oral mucosal cells were collected at 2 times; before bonding of the fixed orthodontic appliances (T1) and three months after bonding the fixed orthodontic appliances (T2). The patients were randomly divided into four groups of 5 each according to the type of professional fluoride application. Group 1 received an application of 1.23% acidulated phosphate fluoride gel (APF group). Group 2 received an application of 2% neutral sodium fluoride gel (NGel group). In APF and NGel group, 2/5 of tray was filled with 1.23% APF or 2% NaF gel (Pascal International Inc., Bellevue, WA). The fluoride tray was placed in the patient's mouth for four minutes. Following the gel application, the patient was allowed to expectorate for 30 seconds and instructed not to eat, drink or rinse anything for 30 minutes. Group 3 received an application of 5% sodium fluoride vanish (FVa group). 5% sodium fluoride varnish (Duraphat, Colgate-Palmolive, USA) was applied around the brackets and tubes using microbrush. After 5 minutes of hardening time, the patient is asked not to eat or drink for 2 hours. Group 4 served as the control group which did not receive any type of professional fluoride application. All subjects were instructed to continue brushing with fluoride toothpaste twice a day, not to use any kind of mouthwash and to avoid carbonated drink and acidic food.

The cells were collected according to the standard protocol (Thomas et al., 2009) after rinsing the mouth with tepid water for 1 minute to remove the exfoliated dead cells. A soft interdental brush was used to collect the cells by gentle scraping of the surface of the right and left buccal mucosa. The brush was agitated in a tube prefilled with cold phosphate-buffered saline solution (PBS) to detach the cells. The cell suspensions were stored on ice in a closed isolated container and immediately transported to the laboratory. The cell suspensions were centrifuged, suspended in PBS, and

pass through a 100 μm nylon filter. The cells were counted to obtain a concentration of 10^5 cells/ml. Cell viability was determined by using trypan blue exclusion test and the percentage of viable cells was calculated.

Evaluation of the slides

One hundred and twenty μl of the cell suspension was dropped onto clean glass slides. The slides were air-dried and stained with Papanicolaou (PAP) method according to Ayyad et al. (2006). One thousand cells from each subject for each sampling time scored under a light microscope at the magnification of 1000x to determine the frequency of cellular death parameters (binucleated, pyknotic and karyolytic cells) as recommendation described by Tolbert et al. (1992). A single experienced investigator performed blinded analysis and one slide from each subject was analyzed twice by the same investigator for evaluating repeatability.

Statistical analysis

All data were analyzed with SPSS statistic software version 22.0 (SPSS, Chicago, IL). The mean and standard deviation were calculated in each study group. The normality of the data was tested in each group by using the Kolmogorov-Smirnov test. The Wilcoxon signed rank test was performed to evaluate the significance of differences in cellular viability and frequency of cellular alterations between T1 and T2 in each group. The differences in each value were compared among 4 groups by using the One-Way ANOVA test. $P<0.05$ was considered to be the level of significance.

Results

The general characteristics of the subjects in this study regarding age and gender was not significantly different between groups (Table 1). Among 20 patients, 13 were female (65%) and 7 were male (35%). The mean age was 24.60± 11.01 years. All subjects reported non-smokers and were not alcohol users. None of them used oral antiseptic solutions.

Table 1 General characteristics of subjects

Parameters	Control	APF group	NGel group	Fva group	Total
Number of subjects	5	5	5	5	20
Male/female	3/2	2/3	1/4	1/4	7/13
Age (y)	25.00±13.64	20.20±8.98	20.00±2.65	33.20±12.64	24.60± 11.01

The cytotoxicity of fluoride in the subjects with fixed orthodontic appliances was evaluated by cellular viability and frequencies of degenerative nuclear alterations indicative of apoptosis. The cell viability was not significant different between groups at T1($P=0.386$) but at T2 APF group showed the lowest cell viability. The results indicated a decrease of cellular viability in APF group from T1 to T2 whereas NGel, Fva and control group showed slightly higher percentage of cell viability. In APF group, the viability % were 8.83±3.47 at T1 and decrease to

5.58±2.89 at T2. Although changes in viability % were not statistically significant between groups, a decreasing trend of cell viability in APF group was noticed (Figure 1).

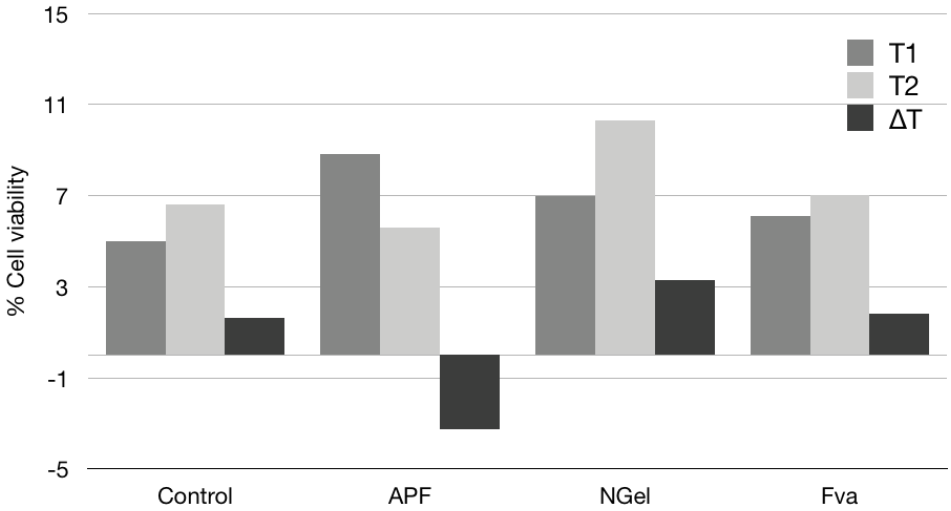

Figure 1 Cellular viability at T1 and T2 among different fluoride groups.

The occurrence of apoptosis, depicted by the frequency of karyolysis (KL), significantly increased between T1 and T2 in APF group. No statistically significant difference was noticed between time points in NGel, Fva and control groups. The mean change of KL in APF group was significantly higher than control group ($P<0.05$). In contrast, the changes of KL in NGel and Fva were not significantly different from control group (Figure 2). Similarly, an increase of other nuclear alterations was not observed throughout the study. Figure 3 showed a karyolytic cell.

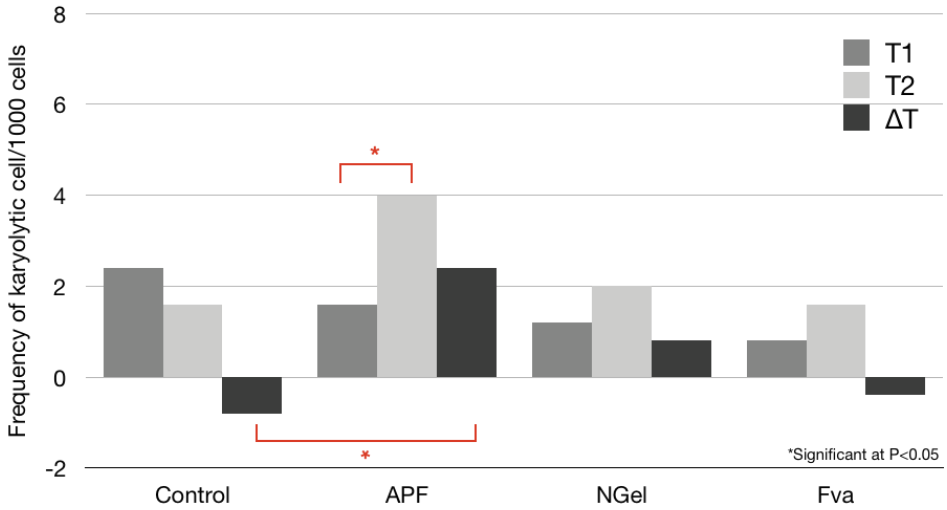

Figure 2 Karyolysis frequencies at T1 and T2 among different fluoride groups.

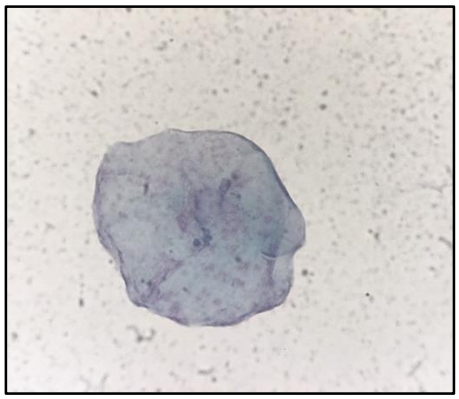

Figure 3 Karyolytic cell. X1000 magnification, PAP stain

Discussion

Orthodontic fixed appliances are manufactured from metal alloys which undergo corrosion in the extremely corrosive environment of the oral cavity. In addition, fluoride ions release from fluoride product and acidity environment can promote the rate of metal corrosion. The alloys exposed to these aggressive events result in the release of metal ions and reduction of their corrosion resistance leading to cause adverse biological effects on adjacent oral tissue. Therefore, in the present study, we aimed to evaluate the cytotoxic effects of orthodontic treatment with various fluoride products on human oral mucosal cells.

To monitor cytotoxic effects, the frequency of cellular death parameters was similar between T1 and T2 in NGel and Fva groups including control group. The similar number of cellular alterations between the time points indicates that orthodontic treatment was not able to induce the cytotoxicity of buccal mucosal cells. Similarly, Angelieri et al. (2011) demonstrated that orthodontic therapy may not be a factor to promote cytotoxicity as depicted by no significant difference in the number of nuclear alterations. On the other hand, cytotoxicity was also denoted by a decrease in cellular viability. Our findings showed no difference in cellular viability between NGel, Fva and control group. Similar findings revealed by Hafez et al. (2011) that patients undergoing orthodontic therapy did not showed a significant decrease in cellular viability during 3-month period. Nevertheless, some authors have shown high frequencies of apoptotic cells and a significant decrease in cellular viability in patients undergoing orthodontic therapy (Faccioni et al., 2003). The previous *in vitro* study demonstrated that high concentration of fluoride from professional fluoride-containing products was able to impair cellular function and was cytotoxic to human oral mucosal fibroblasts (Jeng et al., 1998). Moreover, Alavi, Farahi (2011) reported that fluoride ions reacted with hydrogen ions in the oral cavity resulting in hydrofluoric acid (HF) formation. HF is able to damage the oxidized layer formed on orthodontic metal alloys and causes the metal corrosion. However, our results pointed out that control group with regular use of fluoride toothpaste, fluoride varnish group and neutral fluoride gel group showed the similar cytotoxicity. Hence, only fluoride ions were not able to worsen the cytotoxic effect on human buccal mucosal cells of orthodontic patients. In accordance with Yanisarapan et al. (2018) who evaluated the cytotoxicity on cultured cells using MTT assay, the fluoride concentration in toothpaste was not enough to increase the corrosion of orthodontic appliances and did not diminish the cell viability. Therefore, both frequently use a low concentration of fluoride by daily brushing with fluoride

toothpaste and occasionally use of high concentration of fluoride with neutral fluoride gel or fluoride varnish are biologically acceptable to apply with fixed orthodontic appliances.

On the contrary, we found that cellular viability, evaluated after sampling, was lower only in APF group and the differences in the frequency of KL of APF group was statistically significant higher than the others. APF showed much greater cytotoxic effect. This finding may be attributed to the acidity of the fluoride product. Kuhta et al. (2009) documented that the low pH had a strong effect on the release of ions of metal appliances. At pH 3.5, the released ions remarkable increased several-fold higher than pH 6.75. The fluoride concentration and pH of commercial APF gel are approximately 10,000 ppm and 3.5, respectively (Rozier et al., 2010). APF gel is acidulated to facilitate fluoride uptake in the enamel (Brudevold et al., 1963). Such an environment, high concentration of fluoride and low pH, produces a higher amount of HF and promotes the corrosion of metal alloys (Castro et al., 2015; Matono et al., 2006). Previous studies evaluated the cytotoxicity when metal appliances immersed in APF solution (Kao et al., 2007; Yanisarapan et al., 2018). APF gel caused a higher significant metal release and reduced cell viability to only 30% which was considered as a cytotoxic level. Compared to fluoride toothpaste, APF solution showed up to 3-fold lower percent cell viability. (Yanisarapan et al., 2018)

Although significant cytotoxic effects of APF in exfoliated mucosal cells during 3-month period were observed in the present study, other studies that evaluated the cytotoxic effect of orthodontic appliances without fluoride treatment over longer periods showed that the changes induced by appliance probably decrease with time. The epithelial cells of buccal mucosa underwent rapid turnover and regenerate usually every 7-14 days (Thomas et al., 2009). The effects may be reversible in the long term. However, early cytotoxic effects after fluoride application should be considered in patients predisposed to additional cytotoxic damage due to the individual's lifestyle (Angelieri et al., 2011).

To our knowledge, this is the first study that describes the effect of fluoride application in fixed orthodontic patients. Due to possible adverse biological effects, research efforts should focus on assessing the long-term effects of fluoride products during orthodontic treatment. The relatively small sample size in this study might be resulted in no statistically significant difference of decreased cell viability. Further studies with a larger sample size might show the significant relationships.

Conclusions

Fixed orthodontic appliances applied with APF showed greater cytotoxicity than fluoride varnish and neutral fluoride gel during the 3-month period. Therefore, neutral fluoride products, fluoride varnish and neutral fluoride gel, are recommended to be products of choices during orthodontic treatment.

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