Research

Cytotoxicity, genotoxicity, and cellular metal accumulation caused by professionally applied fluoride products in patients with fixed orthodontic appliances: A randomized clinical trial

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A B S T R A C T

Background: Corrosion of metal orthodontic appliances caused by professional fluoride products has been recently concerned. Therefore, the objective of this study was to evaluate the cytotoxic and genotoxic effect of these products on buccal mucosal cells from patients wearing fixed orthodontic appliances.

Methods: A total of 44 patients, aged 12 to 35 years, who began orthodontic treatment with fixed appliances were included in this single-center, prospective, randomized clinical trial. Patients were randomly allocated into 4 parallel groups according to the type of professional fluoride treatment applied after placing the appliances: acidulated phosphate fluoride gel (APF); neutral fluoride gel (NGel); fluoride varnish (FVa); and without fluoride treatment (control). Buccal cells were collected before treatment (T1) and 3 months after appliance placement (T2). The cells were assayed for cell viability and underwent Papanicolaou staining. Cells with micronuclei and degenerative nuclear alterations were scored using a light microscope. Cell metal content was quantified by inductively coupled plasma–mass spectrometry. The data were analyzed with the Kruskal–Wallis test.

Results: The intracellular nickel content in the APF group significantly increased ($P < 0.05$), whereas that of the control, NGel, and FVa groups did not. The changes in chromium concentration in all groups were not significantly different compared with control. Use of APF resulted in a significantly higher decrease in cell viability and increase in morphologic signs of cell death compared with control ($P < 0.05$). The change in frequency of micronucleated cells was not significantly different from that in the control group.

Conclusions: Applying APF gel on fixed orthodontic appliances increased the cell metal content and decreased cell viability; however, genotoxic effects were absent. FVa and NGel are suggested as the products of choice to use during orthodontic treatment.

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1. Introduction

Orthodontic fixed appliances composed of metal alloys commonly remain in the oral environment, which is particularly corrosive, for several years. Many factors act as corrosion inducers, e.g., saliva, microbial activity, and fluctuating pH and temperature [1].

Many in vitro and in vivo studies have demonstrated that metal ions are released from orthodontic alloys by chemical reactions [1,2]. This gradual destruction increases the roughness of the appliance surface, which can decrease the mechanical properties of metal alloys [3]. Moreover, metal ions released from orthodontic appliances were found in the tissues and fluids of patients wearing the appliances [2,4]. Therefore, the toxicity of orthodontic appliance corrosion is a major concern. These released metal ions are cytotoxic, mutagenic, and carcinogenic [5]. These ions decrease the enzyme and mitochondrial activity of the nearby cells in the oral cavity [5]. In vivo studies reported that the metal ions from orthodontic appliances decreased cell viability and induced DNA damage [4,6]. A longitudinal study revealed that these cytotoxic effects occurred in buccal mucosa cells after treatment with fixed...
orthodontic appliances for 3 months [6]. Moreover, ingested metal ions, such as chromium and nickel ions, impair immune cell function and may induce type IV hypersensitivity [5,7].

Active fixed orthodontic treatment increases the risk of developing caries and creates an oral environment conducive to increased plaque retention and development of suboptimal oral hygiene [8]. Professionally applied topical fluoride is recommended in orthodontic patients at high risk for caries, to prevent white spot lesion and serious lesion development [9].

Among fluoride-containing products, acidulated phosphate fluoride (APF) gel is commonly used because of its lower pH, which facilitates fluoride uptake in the enamel [10]. Orthodontic metal corrosion is strongly associated with an acidic environment. In vitro studies have demonstrated that the corrosion of metals increases in a fluoridated acidic environment [11]. Fluoride reacts with the hydrogen ions from bacterial products, resulting in the formation of hydrofluoric acid that damages the protective oxide layer on the surface of metal orthodontic appliances, releasing metal ions into the mouth. The level of nickel in gingival crevicular fluid from subjects undergoing orthodontic treatment with fluoridated agents was elevated 30 days after appliance placement [12]. Moreover, the cytotoxicity of an acidic fluoridated product was evaluated using gingival fibroblast cell viability in a released metal ion solution. This cell culture study indicated that the level of release of metal ions was highly increased and cell viability was decreased when cells were incubated with culture media containing APF immersed with metal appliances [13]. Thus, using APF on fixed appliances may result in enhanced metal corrosion.

Neutral fluoride gel (NGel) and fluoride varnish (FVa), which do not decrease the pH of the oral environment, have been found to have an effect similar to that of APF on enamel [14]. These neutral topical fluorides have been suggested to be the products of choice in patients at high risk for caries, to reduce metal corrosion in the oral environment [15].

To our knowledge, no longitudinal controlled clinical study has investigated the effect of applying different professional fluoride products to orthodontic appliances on buccal mucosal cell damage, in terms of metal corrosion. Therefore, 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 volume of metal ions in oral mucosal cells, their cytotoxicity, and their possible genotoxic effect.

2. Methods and materials

2.1. Trial design, subjects, eligibility criteria, and settings

The study was a randomized, single-center parallel-group clinical trial, with a 1:1 allocation ratio. The trial was registered on thaclinicaltrials.org (registration number: TCTR20210513003). The study protocol (Fig. 1A) was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand (HREC-DCU 2018-064). The Consolidated Standards of Reporting Trials (CONSORT) checklist, a guideline for conducting and reporting trials, is shown in Figure 1B. The sample size estimation was calculated based on the results of Faccioni et al. [4], who evaluated the metal content and cytotoxicity in the oral mucosal cells of patients wearing fixed appliances. The calculation indicated the need for a minimum of 11 patients per group, with a power of 0.8 and a type I error of 0.05. The subjects of this study comprised 44 healthy patients who received orthodontic treatment at the Department of Orthodontics at the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The objectives of the study and the cell collection method were explained to each subject before they provided written informed consent.

To be eligible for the study, patients had to be less than 35 years old [4] and about to start orthodontic treatment with fixed appliances in both arches. The inclusion criteria for subject selection were as follows: 1) permanent dentition with all second molars erupted in the oral cavity; 2) clinically healthy oral mucosa; 3) no previous orthodontic treatment; 4) no occupational exposure to metals; 5) no amalgam fillings or metal restorations; and 6) no lingual appliances or extraoral auxiliary appliances. The exclusion criteria were as follows: 1) smoking; 2) pre-existing systemic disease or medication associated with oral mucosa changes.

The study group consisted of 44 patients (17 male and 27 female) recruited from the Postgraduate Orthodontic Clinic, Faculty of Dentistry, Chulalongkorn University, from September 2018 to January 2019. Recruitment and intervention administration dates were the same, and data for the last recruited participant were collected in April 2019.

The subjects were bonded with fixed orthodontic appliances on both arches. The appliances consisted of 4 tubes and at least 16 bonded 0.018 × 0.022-in slot MBT stainless steel brackets (Omniarch, Tomy, Tokyo, Japan). The archwires used in the study were 0.014-in and 0.016-in nickel–titanium alloys (ClassOne Orthodontics, Ortho Organizers Inc., Carlsbad, CA) and were ligated with an elastomeric ligature.

Sample randomization was implemented through a computer-generated randomization list. Each patient in the study was randomly allocated into 1 of the 4 groups using sequentially numbered, opaque, sealed envelopes. Each group consists of 11 subjects. A secretary was responsible for the randomization procedure and implementation.

2.2. Fluoride application

After placing the fixed metal orthodontic appliances, group 1 was treated with 1.23% APF gel (APF group, pH 3–3.5). Group 2 was treated with 2% neutral sodium fluoride (NaF) gel (NGel group, pH 6–7, Pascal International Inc., Bellevue, WA). A tray, 2/5 filled with fluoride gel, was placed in the patient’s mouth for 4 minutes. After gel application, the patient was allowed to expectorate for 30 seconds and was instructed not to eat, drink, or rinse with anything for 30 minutes. Group 3 was treated with 5% NaF varnish (FVa group: Duraphat, pH 7, Colgate-Palmolive Co., New York, NY) applied around the brackets and tubes using a microbrush. After 5 minutes of hardening, the patient was instructed not to eat or drink for 2 hours. Group 4 served as the control group and did not receive professional fluoride application. The patients were instructed to continue brushing with fluoride toothpaste twice a day, not use mouthwash, and avoid carbonated drinks and acidic food. Verbal and video oral hygiene instructions were given and reinforced throughout the 3 months of the trial.

2.3. Buccal cell collection

The oral mucosal cells were collected 2 times—before bonding of the fixed appliances (T1) and at 3 months after bonding (T2). After rinsing the mouth with water for 1 minute to remove the exfoliated dead cells, the cells were collected according to the standard protocol [16] by gently scraping the buccal mucosa using a soft interdental brush. The brush was agitated in a 2-ml tube pre-filled with 1 ml cold phosphate-buffered saline solution to detach the cells. The cell suspension from each patient was stored on ice in a separate closed container and immediately transported to
the laboratory. The cell suspensions were centrifuged, suspended in phosphate-buffered saline solution, and passed through a 100-μm nylon filter. The cells were counted to obtain a concentration of 10,000 cells/mL. Cell viability was determined using a trypan blue exclusion test [17], and the percentage of viable cells was calculated (viability % = number of viable cells/total counted cells x 100).

2.4. Metal ion measurement

The cellular nickel and chromium content was measured as previously described [4] using inductively coupled plasma–mass spectrometry (ICP-MS; NexION 350X, PerkinElmer, Waltham, MA). Of the cell suspension, 100 μL was treated with nitric acid (2 mL; 0.5%) and heated in a water bath at 80°C to dissolve the metal content. The cell lysate was diluted to 5 mL with deionized water and kept at –80°C until all the samples were collected. The instrument was calibrated with standard solutions ranging from 1 to 100 parts per billion of each metal prepared in 2% nitric acid. The sample concentrations were calculated from a standard curve. The results of the metal content in the buccal mucosal cells are presented as μg/L or parts per billion.

2.5. Buccal micronucleus cytome assay

Of the cell suspension, 120 μL was dropped onto clean glass slides. The slides were air-dried and stained using the Papanicolaou (PAP) method [18]. A single investigator (N.A.), who is a qualified medical technician, performed blinded analysis and scored 1000 cells. The cells from each patient, at each sampling time, which had an intact cytoplasm and lay relatively flat on the slide, and were not clumped, and did not overlap with adjacent cells, were scored using a light microscope at x1000 magnification to determine the frequency of cell-death parameters (binucleated, pyknotic, and karyolytic cells) as previously described [19], as well as the frequency of micronucleated cells (MN cells). The micronucleus

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**Fig. 1.** Schematic of the (A) experimental protocol and (B) consort flow diagram. ICP-MS, inductively coupled plasma–mass spectrometry; PAP, Papanicolaou; PBS, phosphate-buffered saline solution.
cells containing the main nucleus and micronucleus were scored using the criteria described by Bolognesi et al. [20], as a marker of DNA damage. The morphologic characteristics of the micronucleus were as follows: round or oval in shape with similar staining intensity as the main nucleus; 1/3–1/16 the diameter of the main nucleus; located within the cell cytoplasm; clearly identified boundary and not connected to the main nucleus.

2.6. Statistical analysis

The data were analyzed with SPSS statistical software version 22.0 (SPSS, Chicago, IL) and are shown as median and interquartile range. The normal distribution was evaluated by the Kolmogorov–Smirnov test. The Wilcoxon signed rank test was performed to determine significant differences in cell viability, the amount of metal ions, the frequency of cell-death parameters, and the frequency of micronucleus cells between T1 and T2 in each group. The differences in each value were compared among the 4 groups using the Kruskal–Wallis test. Group differences were investigated with Dunn’s post hoc test. Significance was defined as a P value < 0.05.

3. Results

A total of 44 patients were enrolled in the trial. One subject in the APF group was lost to follow-up. The details are given in the Consolidated Standards of Reporting Trials (CONSORT) flow diagram (Fig. 1B). The study participants’ demographic characteristics indicated that the mean age of the 43 patients was 21.3 ± 6.6 years. Baseline data on age and sex were collected for all patients (Table 1).

The median and interquartile range of the intracellular metal ion content, cell viability, and the frequency of cell death and micronucleated cells are presented in Table 2. We found that the nickel and chromium ion content in mucosal cells was not significantly different among the groups at T1. The APF group had the highest cellular nickel content at T2. Moreover, the nickel ion content significantly increased between T1 and T2 in the APF group (P < 0.05), whereas the NGel, FVa, and control groups showed a slight, nonsignificant decrease in nickel content. In the APF, NGel, and FVa groups, the change in chromium concentration was not significantly different compared with that in the control group (Fig. 2A and 2B).

The cytotoxicity of the fluoride products in the subjects was evaluated by cell viability and the frequency of degenerative nuclear alterations indicative of apoptosis. The results demonstrated a significant decrease in cell viability in the APF group from T1 to T2. The viability was 8.96 at T1 and decreased to 4.26 at T2 (Fig. 2C). The occurrence of apoptosis evaluated using the sum of the binucleated, karyolytic, and pyknotic cells (Fig. 3A–E) increased between T1 and T2 in all groups; however, the increase was significant in only the APF group (P < 0.05), which presented the highest number of apoptotic cells at T2, significantly higher compared with the control group (P < 0.05). However, in the FVa and NGel groups, the changes in these parameters were not significantly different from that in the control group (Fig. 2D).

The potential genotoxic effects of the metal ions on the buccal mucosal cells were evaluated using a buccal micronucleus cytome assay that indicates DNA damage as measured by frequency of micronucleus cells. There was no significant difference in frequency of micronucleus cells between the groups at T1. The change in frequency of micronucleus cells between T1 and T2 was not significantly different among the 4 groups (Fig. 2E).

4. Discussion

The present study evaluated the cytotoxic and genotoxic effects of treating orthodontic appliances with professional fluoride products on human buccal mucosal cells using a single-center, prospective, randomized clinical trial with control, NGel, APF, and FVa groups having similar criteria over a 3-month period. We found that the oral mucosal cells from orthodontic patients in the APF group had a significantly increased nickel concentration, whereas NGel and FVa did not lead to a significant increase in metal ion content.

A previous study reported that the corrosive effect of fluoride products on metal orthodontic appliances results from the chemical reactions between NaF and the protective oxide layers of the metals, which are chromium oxide (Cr₂O₃) and titanium oxide (Ti₂O₃, TiO₂) [3]. NaF reacts with the hydrogen ions (H⁺) in the oral environment and produces hydrofluoric acid (HF), a strong inorganic acid, which destroys the oxidative layers protecting the alloy surfaces and initiates the process of metal corrosion [3]. The chemical reactions are shown in the following equations [21]:

\[ \text{NaF} + \text{H}^+ \rightarrow \text{HF} + \text{Na}^+ \]

\[ \text{Cr}_2\text{O}_3 + 6\text{HF} \rightarrow 2\text{CrF}_3 + 3\text{H}_2\text{O} \]

\[ \text{Ti}_2\text{O}_3 + 6\text{HF} \rightarrow 2\text{TiF}_4 + 3\text{H}_2\text{O} \]

\[ \text{TiO}_2 + 4\text{HF} \rightarrow \text{TiF}_4 + 2\text{H}_2\text{O} \]

\[ \text{TiO}_2 + 2\text{HF} \rightarrow \text{TiF}_2 + \text{H}_2\text{O} \]

Our study demonstrated that APF gel induced greater metal corrosion from orthodontic appliances, as shown by increased metal ion release [13]. Kuhla et al. documented that low pH has a strong effect on the ion release from metal appliances [11]. Accordingly, our finding may be attributable to the acidity of APF from phosphoric acid (H₃PO₄). APF provides a higher amount of HF, a key factor in enhancing the corrosion of orthodontic metals, as shown by the following equation:

\[ \text{H}_3\text{PO}_4 + 3\text{NaF} \rightarrow \text{Na}_3\text{PO}_4 + 3\text{HF} \]

Therefore, with a high fluoride concentration and low pH, APF promotes more metal corrosion compared with the other 2 fluoride products [21].

The alloys exposed to corrosive conditions release metal ions that subsequently cause adverse biological effects on the adjacent oral tissue. Our findings indicate a significantly lower cell viability (approximately 45%) in the patients’ cells with APF gel application, and a significantly increased frequency of cell-death parameters compared with that in the control group. Pyknosis and karyolysis indicate cell apoptosis, and a binucleated cell is an indication of cytokinetic defects [16]. Similarly, a significantly higher
Table 2
Median and interquartile range of intracellular metal ion content, cellular viability, and frequency of cell death and micronucleated cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>APF</th>
<th>NGel</th>
<th>Fva</th>
</tr>
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<tbody>
<tr>
<td>Ni ion (µg/L)</td>
<td>0.05 (0.00–1.18)</td>
<td>0.06 (0.00–0.58)</td>
<td>0.41 (0.00–0.98)</td>
<td>1.11 (0.56–1.71)</td>
</tr>
<tr>
<td>Cr ion (µg/L)</td>
<td>0.19 (0.12–1.70)</td>
<td>0.14 (0.09–0.24)</td>
<td>1.03 (0.42–2.73)</td>
<td>0.78 (0.00–2.00)</td>
</tr>
<tr>
<td>% viability</td>
<td>5.56 (4.55–8.57)</td>
<td>6.45 (4.17–8.82)</td>
<td>8.96 (3.96–10.00)</td>
<td>4.26 (1.50–6.09)</td>
</tr>
<tr>
<td>Cell death</td>
<td>3.00 (2.00–4.00)</td>
<td>3.00 (2.00–4.00)</td>
<td>2.00 (2.00–4.00)</td>
<td>4.50 (2.75–5.25)</td>
</tr>
<tr>
<td>Frequency of micronucleus</td>
<td>0.00 (0.00–1.00)</td>
<td>1.00 (0.00–1.00)</td>
<td>0.5 (0.00–1.25)</td>
<td>1.00 (0.00–1.25)</td>
</tr>
</tbody>
</table>

APF, acidulated phosphate fluoride gel; Cr, chromium; FVa, fluoride varnish; NGel, neutral fluoride gel; Ni, nickel; T1, time 1 = before fixed appliance placement; T2, time 2 = 3 months after appliance placement.

![Fig. 2](image)

Fig. 2. Parameters indicating the cytotoxicity and genotoxicity of metal corrosion caused by professionally applied fluoride products at T1 and T2 in the different fluoride groups (*p < 0.05). (A) Nickel and (B) chromium ion content (µg/L) in the oral mucosal region. (C) Cell viability. (D) Cell-death parameters. (E) Frequency of micronucleus cells.

Metal release and reduced cell viability to 30% were observed in vitro in cultured cells when treating orthodontic appliances with APF [13]. Nickel released from orthodontic alloys is the main factor increasing oxidative stress in the oral cavity and induces apoptosis [22]. Moreover, the ratio of live/dead cells gradually decreased as the nickel concentration increased [23]. A study has found that chromium demonstrated less antigenicity in the metal form [7]. Thus, these data revealed that orthodontic alloys applied with APF release metal ions in sufficient quantities to induce cytotoxic effects. Conversely, we found no difference in cell metal ions, cell viability, or cell-death parameters among the NGel, FVa, and control groups. These results indicated that nonacidic fluoride did
not enhance metal corrosion enough to alter normal cell repair over 3 months [6]. In conclusion, routine application with FVa or NGel in orthodontic patients did not induce buccal mucosal cell cytotoxicity.

The genotoxic effects of fixed orthodontic appliances treated with fluoride products were assessed using the micronucleus assay, which is relatively easy to score, low-cost, and not time-consuming. Furthermore, several studies using the micronucleus test evaluated the genotoxicity induced by orthodontic appliances in exfoliated buccal mucosal cells [24,25]. Comparing the differences in the number of micronucleus cells within each fluoride group indicated no genotoxic effects during the 3-month period. Although apoptosis at higher than normal levels may serve as an indicator of genotoxic insult, our data imply that fluoride did not expose healthy orthodontic patients to an increased risk of genotoxic damage in oral mucosal cells.

Several in vitro studies have evaluated the cytotoxicity of orthodontic appliances from metal corrosion [11,13]. However, the corrosion process in the oral environment is multifactorial and difficult to simulate in the laboratory. An in vivo study in the natural environment of the oral cavity is more advantageous. Moreover, salivary clearance dilutes and continuously eliminates the metal ions released from orthodontic appliances by swallowing. Our in vivo study evaluated the effects of the corrosion products in the oral cavity, including the saliva, plaque accumulation, and the complex intraoral flora that may alter the microenvironment [5]. Compared with saliva, the buccal mucosal cells better represent the cumulative effects of metal release. Collecting buccal mucosal cells that are in direct contact with metal appliances allowed us to evaluate the biological consequences of metal corrosion more accurately [6].

There are some limitations when performing an in vivo metal corrosion study. Biological variations, and different diets, daily routines, and lifestyles among subjects can compromise the standardization of the study. Thus, a wide interquartile range and some outliers were found, and a significant difference was not detected in many study variables. Moreover, in an in vivo study, some factors cannot be controlled owing to individual variation. Thus, the patients were evaluated longitudinally to eliminate these individual variations and served as their controls. In addition, a control group without fluoride treatment was included in the experiment to contrast and clarify the effect of fluoride in the treatment groups.

Although APF demonstrated significant cytotoxicity in exfoliated mucosal cells during the 3-month study period, our results should be considered in light of the short observation period. Furthermore, because of the nature of longitudinal studies and the restrictive inclusion criteria in this preliminary study, a small patient sample size was used to evaluate the effect of professional fluorides in patients with fixed orthodontic appliances on the cytotoxicity, genotoxicity, and cellular metal accumulation in buccal mucosal cells. Therefore, the results should be evaluated with caution. Future research efforts should focus on assessing the long-term effects of fluoride products over the course of orthodontic treatment. Further studies with a larger sample size and a longer treatment period are also required to confirm and expand these findings. However, the early cytotoxic and possible genotoxic effects after fluoride application should be considered in patients genetically predisposed to additional genotoxic damage due to the individual’s lifestyle.

5. Conclusion

During the 3-month period of orthodontic treatment, applying APF in fixed orthodontic patients resulted in a significant increase in intracellular nickel content in buccal mucosal cells, with significant cytotoxic effects, whereas applying FVa and NGel did not cause an increase in cytotoxicity. None of the professional fluoride applications demonstrated a genotoxic effect.

Routine application of FVa or NGel is recommended in patients at high risk for caries during orthodontic treatment with fixed metal orthodontic appliances. Conversely, APF gel application is not recommended.

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