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Influence of two different fluoride compounds and an *in vitro* pellicle on the amount of KOH-soluble fluoride and its retention after toothbrushing

FLORIAN J. WEGEHAUPT, VERA SCHNEIDERS, ANNETTE WIEGAND, PATRICK R. SCHMIDLIN & THOMAS ATTIN

Clinic for Preventive Dentistry, Periodontology and Cariology University of Zurich, Zürich, Switzerland

Abstract

Objective. To determine the influence of two different fluoride compounds and an *in vitro* pellicle on KOH-soluble fluoride formation – its retention and resistance to toothbrushing. ***Material and methods.*** Forty bovine incisors were randomly assigned to four groups (A–D). Of five samples prepared per tooth, one remained untreated and served as a baseline control. Groups A and B were pretreated with artificial saliva and groups C and D with human saliva. Groups A and C were treated with amine fluoride and groups B and D with sodium fluoride. After treatment, samples were brushed with 25, 50, and 75 brushing strokes. The amount of KOH-soluble fluoride formed on the enamel samples was measured at baseline, after application, and after 25, 50, and 75 brushing strokes. Fluoride uptake was calculated by unpaired *t*-tests and fluoride retention by paired *t*-tests. ***Results.*** No statistically significant differences in the KOH-soluble fluoride uptake of the groups that were pretreated (A vs B and C vs D) or treated equally (A vs C and B vs D) were observed. Retention of the KOH-soluble fluoride in the brushed samples was higher when the samples were pretreated with human saliva and treated with sodium fluoride than when the samples were pretreated with artificial saliva and treated with sodium fluoride. ***Conclusions.*** The fluoride compound and the acquired human *in vitro* pellicle have no influence on the uptake of KOH-soluble fluoride, but show a significant influence on abrasion resistance.

Key Words: *Abrasion, fluoride, pellicle*

Introduction

The prevalence of dental hard tissue loss due to caries has declined in recent decades [1]. A review article [2] has shown that during the same time period there has been a significant increase in dental hard tissue loss due to erosion. In the light of this finding, erosion has become a focus in dental research [3,4]. Erosion is defined as dental hard tissue loss due to dissolution by acids or by chelators in the absence of micro-organisms. Depending on the origin of the acid, it is possible to classify erosion according to extrinsic or intrinsic factors [5,6]. Extrinsic factors include exposure to acid fumes (by workers in factories) [7], acidic foods and drinks [8,9], and some types of medication, such as acetylsalicylic acid [10]. Intrinsic erosion is mainly caused by gastric acid [9,11]. In order to prevent tooth wear due to erosion, different approaches have been discussed, e.g. the improvement of remineralization of softened enamel, an increase in the acid resistance of the enamel, and a reduction of the erosive potential of acidic products [12,13]. The effectiveness of the reduction in erosive tooth wear by fluoride is controversial [14–16] and might be explained by differences in the type of fluoride used, its pH, or the concentration of the applied fluoride components. However, there is broad consensus as to how fluoride may protect the enamel against erosion. It is assumed that the application of highly concentrated fluoride on enamel leads to the formation of a calcium fluoride-like layer on the enamel surface, the amount of which depends upon the pH of the fluoride applied, its concentration, and the time and frequency of application [17]. Fluoride is released from this calcium fluoride-like precipitate over an extended period of time [18]. The precipitate can also be seen as a barrier hampering acid

Correspondence: Florian Wegehaupt, Clinic for Preventive Dentistry, Periodontology and Cariology, University of Zurich, Plattenstrasse 11, CH-8032 Zürich, Switzerland. Tel: +41 44 634 3354. Fax: +41 44 634 4308. E-mail: florian.wegehaupt@zzmk.uzh.ch

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diffusion to the underlying dental hard tissue [19], although this effect is limited as it is very readily dissolved at a low pH. A more plausible mechanism as to how the calcium fluoride-like precipitate protects the enamel is that this layer provides more minerals for the acid to dock on than the uncovered enamel, and so more of the acid is depleted before it reaches the enamel [20]. These proposed protective mechanisms only work so long as a calcium fluoride-like precipitate remains on the enamel surface. It has been shown that the amount of precipitate is reduced under abrasive conditions. In this study, the resistance of the precipitate to abrasion depended upon the type of fluoride component applied.

Additionally, a diffusion inhibitory effect of the salivary acquired pellicle formed by absorption of saliva proteins has been reported as protecting the enamel against erosion [21,22]. It is conceivable that the presence of the salivary acquired pellicle might influence the formation and stability of the calcium fluoride-like precipitate.

Therefore, the aim of this study was to evaluate the influence of amine fluoride, sodium fluoride, and saliva pretreatment of the enamel on the amount of calcium fluoride-like precipitate formed, and on its susceptibility to toothbrushing. It is hypothesized that the amount of KOH-soluble fluoride is reduced when the enamel is pretreated with human saliva before the application of amine fluoride or sodium fluoride.

Material and methods

Forty freshly extracted bovine lower incisors were used in this study. The crowns were separated from the roots at the cemento-enamel junctions and five cylindrical enamel samples (1–5) were drilled from the buccal surface of each tooth with a trephine mill. The samples had an outer diameter of 3 mm and were marked to ensure that the five samples from one tooth could be identified as such after further preparation. The enamel cylinders were embedded in acrylic resin (Palavit G; Kulzer Wehrheim, Germany) in steel molds with an inner diameter of 5 mm, so that only the enamel surface was free of resin. After removing the samples from the molds, the enamel surface was ground with abrasive paper (800, 1000, 1200, 2400, and 4000 grit; Water Proof Silicon Carbide Paper, Struers, Erkrath, Germany), thereby removing the outermost 200 μm of enamel. This enamel loss was controlled with a micrometer (Mitutoyo, Tokyo, Japan). Grinding was done in an automatic grinding machine with running tap water as coolant.

The samples "1–5" from 10 teeth were then assigned to 1 of 4 treatment groups (A–D). An overview of the treatment in the different groups is given in Table I. All samples in groups A and B were stored in artificial saliva for 1 h [23]; artificial saliva has a mineral content similar to the human saliva used for groups C and D. The samples in groups C and D were stored in pooled human saliva for 1 h, which in this case resulted in the formation of an *in vitro* pellicle. The stimulated human saliva was gained from three healthy volunteers over a 15-min period. Samples "1" received no further treatment in this study and served as a baseline for the other samples from the same tooth. Samples "2–5" in groups A and C were treated with an amine fluoride (Olaflur; Therwil, Switzerland) solution, while samples "2–5" in groups B and D were treated with a sodium fluoride solution. On each sample, 0.1 g of the assigned fluoride solution was applied for 4 min. The solutions (experimental solutions; GABA International AG, Therwil, Switzerland) had a fluoride concentration of 1% and were adjusted to pH 3.1 with the use of HCl and NaOH. The samples were rinsed under tap water for 1 min after application of the fluoride to remove any excess solution. Toothbrush abrasion was then performed in an automatic brushing machine applying reciprocating linear motion to the toothbrushes (ParoM43; Esro AG, Thalwil, Zürich, Switzerland), with a constant brushing frequency of 100 strokes per minute and a constant brushing load of 2.5 N. A toothpaste slurry was prepared by mixing 100 ml fluoride-free toothpaste (Sensodyne Classic; GSK, Bühl, Germany) with 300 ml of artificial saliva. While samples "2" were exposed to the toothpaste slurry for 30 s, samples "3–5" were brushed with the slurry. Samples "3" were brushed for 25 strokes, samples "4" for 50 strokes, and samples "5" for 75 strokes.

The amount of KOH-soluble fluoride on samples "1–5" of all groups was analyzed following the method described by Caslavska et al. [24]. Each sample was stored in an Eppendorf Tube (Eppendorf International, Hamburg, Germany) with 1 ml 1 mol/L KOH for 24 h under constant motion. The KOH solution was neutralized with 1 ml 1 mol/L HNO_3 and buffered with 1 ml of TISAB II (Orion Research, Cambridge, USA). The fluoride content in the solution was measured with a fluoride electrode.

Statistical analyses were done with Stat View (version 5.0.1; SAS Institute Inc., Cary, N.C., USA). The uptake of KOH-soluble fluoride after application of the fluoride solutions (samples "2") and the retention of KOH-soluble fluoride after toothbrushing (samples "3–5") were calculated by subtracting the baseline fluoride content (sample "1") of each tooth from the measured values. Mean KOH-soluble fluoride contents and standard deviations were calculated. The uptake of KOH-soluble fluoride (subgroup 2) between the different groups A–D was compared by unpaired *t*-tests. To calculate the resistance of the KOH-soluble fluoride, the amounts of KOH-soluble fluoride in subgroups 3–5 were compared with the amounts found in subgroup 2 by paired *t*-tests. The significance level was set at $p \leq 0.05$.

Table I. Pretreatment and treatment in groups A–D.

	Treatment group			
	A ($n=50$)	B ($n=50$)	C ($n=50$)	D ($n=50$)
Pre-treatment	Artificial saliva	Artificial saliva	Human saliva	Human saliva
Treatment	Amine fluoride	Sodium fluoride	Amine fluoride	Sodium fluoride

Results

The amount of KOH-soluble fluoride for the different groups (A–D) and subgroups (2–5) is given in Table II.

The unbrushed samples (subgroup 2) treated with amine fluoride and pretreated with either artificial or human saliva (groups A and C) showed higher amounts of KOH-soluble fluoride compared to the same tooth samples treated with sodium fluoride (B and D). However, these differences were not statistically significant ($p=0.09$ and $p=0.18$). For the samples in subgroup 2, the pretreatment with human saliva led to slightly lower amounts of KOH-soluble fluoride compared to the same tooth samples pretreated with artificial saliva, but this reduction was not statistically significant for treatment with amine fluoride or sodium fluoride.

In group D (pretreatment with human saliva and treatment with sodium fluoride), the amounts of KOH-soluble fluoride after 25, 50, and 75 brushing strokes (subgroups 3, 4, and 5) showed no statistically significant difference to the amount of KOH-soluble fluoride in the unbrushed samples (subgroup 2). The paired *t*-test used to evaluate the resistance of the KOH-soluble fluoride indicated a statistically significant difference ($p<0.05$) in the amounts of KOH-soluble fluoride between subgroup 4 (50 brushing strokes) and subgroup 2 (0 brushing strokes) of group A (pretreated with artificial saliva and treated with amine-fluoride). In group B (pretreatment with artificial saliva and treatment with sodium fluoride), the toothbrush abrasion with 25, 50, and 75 brushing strokes (subgroups 3, 4, and 5) led to a statistically significant reduction of the amount of KOH-soluble fluoride compared to the amount found in subgroup 2 ($p<0.05$, respectively). A statistically significant difference was observed in the amount of KOH-soluble fluoride in group C for the comparison of subgroups 3, 4, and 5 with subgroup 2 ($p<0.05$, respectively).

Discussion

While some investigations on fluoride uptake have employed human enamel samples [27], this study used bovine enamel as a substitute in accordance with the methodology described in various other studies [25,26]. This decision was based on the fact that it is easier to obtain a sufficient number of bovine teeth [28] and that the chemical composition of human and bovine enamel is similar. Furthermore, the size of a bovine tooth allows for harvesting multiple samples from a single tooth. In this case, one bovine tooth was large enough to provide samples for five different groups.

The amount of calcium fluoride-like precipitate was determined by the method described by Caslavska et al. [24]. This method has been used in a number of studies [26,27] dealing with the topic of fluoride uptake. Generally, the amount of calcium fluoride-like precipitate can also be measured by Raman spectroscopy [29]. However, since Raman spectroscopy was not sensitive enough to detect the calcium fluoride-like precipitate formed after treatment with sodium fluoride [30], the method established by Caslavska et al. [24] was chosen for use in the present study.

A pooled human saliva was used in this study to form an *in vitro* pellicle (groups C and D) in order to reflect the large number of diverse proteins usually found in an acquired pellicle [31]. The *in vitro* pellicle was formed by immersing the enamel samples for 1 h in pooled human saliva. Some studies show that the composition of *in vitro* and *in vivo* pellicle differ

Table II. Mean amount of KOH-soluble fluoride ($\mu g/cm^2$) for the different groups and after different numbers of brushing strokes. Standard error of means is given in parentheses. Amounts of KOH-soluble fluoride (subgroups 2) that show no statistically significant difference are marked with the same superscript letters (read vertically). Additionally, statistically significant differences in the comparison of subgroups 3, 4, and 5 with the respective subgroup 2 (read horizontally) are marked with an asterisk.

	Subgroup			
	2 (0 strokes)	3 (25 strokes)	4 (50 strokes)	5 (75 strokes)
Group				
A	$175.90^{A\ C}$(20.76)	141.92(24.28)	123.51*(16.12)	155.29(28.86)
B	$125.50^{A\ D}$(19.54)	84.96*(13.04)	86.70*(14.96)	90.63*(10.53)
C	$151.39^{B\ C}$(28.07)	113.32*(15.07)	105.25*(19.07)	129.01*(31.90)
D	$103.20^{B\ D}$(20.37)	84.84(11.70)	85.01(16.53)	82.08(21.97)

significantly, especially in enzymatic activity [32]. Other studies have found that *in vivo* formed pellicle is subject to high intra-individual and inter-individual variability and different modulating factors, such as varying salivary flow, nutrition, bacteria in the oral cavity and soft tissues [33]. As there is no study showing that the differences in enzymatic activity have an influence on the protective effect of the pellicle against erosion, an *in vitro* pellicle was used in this study to avoid possible problems caused by inhomogeneous experimental conditions. Finally, pellicle formation through the immersion of test samples in human saliva was the method of choice in some previous studies [34,35]. The exposure time of enamel to saliva varied from 5 min [36] up to 24 h [21]. Immersion time of 1 h was chosen, as a recent study found statistically significant protection against erosion after pretreatment of enamel with human saliva of ≥ 1 h [35].

In other studies, artificial saliva was used for simulation of the conditions given by natural human saliva. The mineral content of the artificial saliva used in the present study was similar to that of human saliva [23]. However, the saliva substitute used contains only a single protein component, namely mucin, derived from cattle. Thus, the protein content and type of protein in the artificial saliva is different from that of human saliva.

Comparison of the two different types of saliva in the present study should clarify whether the interaction of artificial saliva with the formulation and stability of the KOH-soluble fluoride fraction is similar to that of human saliva.

Since the results of the present study show differences for KOH-soluble fluoride retention between the two kinds of saliva, human natural saliva should be used in future studies dealing with the interaction of fluoride and dental hard tissues to simulate formation of the acquired pellicle.

The finding in this study that pretreatment of enamel with human saliva does not lead to higher amounts of KOH-soluble fluoride concurs with the results of Rosin-Grget et al. [36]. In their study, no statistically significant influence of saliva pretreatment was observed when the fluoride solutions were highly acidified (pH$<$4.0). In the present study, it appears that the *in vitro* pellicle formed with human saliva caused a lower amount of KOH-soluble fluoride to precipitate, irrespective of the type of fluoride used, in comparison with the samples pretreated with artificial saliva. It might be possible to explain this observation by the findings of Moreno & Zahradnik [37], namely that the acquired human pellicle can be seen as a barrier for acids. This diffusion inhibitory effect of the acquired human pellicle has also been found in other studies [21]. It might be assumed that the barrier effect is more pronounced, such that the contact time of the fluoride solutions with the enamel is reduced, since the fluoride solutions used in this study are purported to be more viscous than acidic solutions.

When the samples were treated with amine-fluoride (groups A and C), a lower resistance of the KOH-soluble fluoride to toothbrushing was recorded when the samples were pretreated with human saliva (group C). This finding might be explained by the interaction of the amine-fluoride with the *in vitro* pellicle. Amine-fluoride can be adsorbed into the acquired human pellicle by electrostatic interaction [38]. This fluoride fraction, which is bonded to the *in vitro* pellicle, will also be detected following the method of Caslavska et al. [24]. When the samples are brushed, and the *in vitro* pellicle with the adsorbed fluoride is removed, only a lower amount of KOH-soluble fluoride will be detected compared to the amount from the samples with no pretreatment in human saliva. Therefore, only the particular fraction of KOH-soluble fluoride that has passed the acquired human pellicle, and come into contact with the underlying enamel, will be detected. This reduced amount of KOH-soluble fluoride might be the result of a limited contact time between the amine-fluoride and the enamel due to a hampering effect of the *in vitro* pellicle.

When the samples were treated with sodium fluoride (groups B and D), the opposite finding was observed. The KOH-soluble fluoride showed better retention after bushing when the samples were pretreated with human saliva (group D). This observation might be explained by two assumptions. First, the *in vitro* pellicle provided some sort of protection for the KOH-soluble fluoride during brushing [39] compared to the samples that were not covered with the *in vitro* pellicle (group B). Second, when the sodium fluoride diffuses through the *in vitro* pellicle and reacts with the enamel, the diffusion barrier of the *in vitro* pellicle prevents rapid depletion of the dissolved calcium and phosphate needed for formation of the calcium-fluoride-like precipitate. This means that a more stable KOH-soluble fluoride layer might be formed due to the presence of an acquired salivary pellicle as compared to when no pellicle is present.

The finding that the *in vitro* pellicle leads to a better retention of KOH-soluble fluoride when the samples were treated with sodium fluoride after formation of the *in vitro* pellicle, and a lesser retention when the samples were treated with amine-fluoride after formation of the *in vitro* pellicle, might be explained by the difference in the molecule size of amine and sodium fluoride. It is suggested that the sodium fluoride can diffuse better through the *in vitro* pellicle because its molecules are much smaller in size then those of amine-fluoride and allows for better interaction with the enamel.

Thus, it might be concluded that the applied fluoride compound and the *in vitro* pellicle have no influence on the amount of KOH-soluble fluoride

formed. For the resistance of KOH-soluble fluoride to toothbrushing, an influence of the *in vitro* pellicle and the applied fluoride compounds was observed. The samples pretreated with human saliva and treated with sodium fluoride showed no reduction of KOH-soluble fluoride due to toothbrushing compared to the unbrushed samples. The same finding was observed when the samples were pretreated with artificial saliva (no *in vitro* pellicle) and treated with amine-fluoride.

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