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ORIGINAL RESEARCH

In vitro efficiency of fluoride-containing compounds on remineralization of carious enamel lesions under cyclic pH conditions

Eficiencia in vitro *de compuestos fluorados en la remineralización de lesiones cariosas del esmalte bajo condiciones cíclicas de pH*

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ABSTRACT

Fluoride-containing products are mainly used due to their anti-cariogenic effect upon dental enamel. **Objective:** The aim of the present study was to determine the efficiency of three fluorinated compounds when used on demineralized enamel. **Method:** A demineralized surface was obtained by means of exposing the enamel of 120 permanent third molars to a demineralizing solution for 96 hours. Molars were randomly divided into four groups in order to receive topical treatment with the following fluorinated compounds: 1) silver ammonium fluoride, 2) difluorsilane, 3) acidulated phosphate fluoride and 4) a control group. All samples were immersed in a cyclic pH system (pH 4.4 to 7.0) and retrieved after 5, 10 and 15 days in order to be observed. Samples were examined under polarized light and Tholuet solution in a photomicroscope (Zeiss, Germany) in order to determine remineralization according to exhibited birefringence. **Results:** After 5 and 10 days, the group treated with silver ammonium fluoride showed significant difference with respect to other groups. Nevertheless, acidulated phosphate fluoride 10-day results remained equally unaltered at 15 days. **Conclusions:** The group treated with silver ammonium fluoride presented more significant changes than groups treated with silver ammonium fluoride, difluorsilane as well as the control group.

RESUMEN

Los productos fluorados son utilizados principalmente por su efecto anticariogénico en el esmalte dental. **Objetivo:** El propósito de este estudio fue determinar la eficiencia de tres compuestos fluorados en el tratamiento del esmalte desmineralizado. **Método:** Una superficie desmineralizada fue producida por exposición en solución desmineralizante en el esmalte de 120 terceros molares permanentes durante 96 horas. Se dividieron aleatoriamente en cuatro grupos para recibir tratamiento tópico con los siguientes productos fluorados: 1) fluoruro de plata amoniacal, 2) difluorsilano, 3) flúor fosfato acidulado y 4) un grupo control. Las muestras fueron inmersas en un sistema de pH cíclico (pH 4.4 a 7.0) y recuperadas a 5, 10 y 15 días para ser observadas bajo luz polarizada con solución de Tholuet en un fotomicroscopio y determinar la remineralización de acuerdo con la birrefringencia que mostraron. **Resultados:** El grupo tratado con fluoruro de plata amoniacal mostró una diferencia significativa con los otros grupos a 5 y 10 días. Por otra parte, los resultados a los 10 días para el flúor fosfato acidulado se mantuvieron iguales a los 15 días. **Conclusiones:** El grupo tratado con fluoruro de plata amoniacal presentó mayores cambios que los grupos tratados con flúor fosfato acidulado, difluorsilano y control.

Key words: Fluoride, enamel, remineralization, demineralization, caries, cyclic pH.
Palabras clave: Flúor, esmalte, remineralización, desmineralización, caries, pH cíclico.

INTRODUCTION

Dental enamel is made up of a set of prisms which extend from the dentin-enamel junction towards the external surface. These prisms have a 10,000 A thickness and can reach a length of up to 3 mm. Properties of hardness and density exhibited by these prisms are derived from their distribution as well as their structural characteristics.[1] Basic chemical composition of these prisms comprises Ca^{+2} P^{-4} and O^{-2}, all in different crystalline shapes (hydroxyapatite, fluorapatite, β-Whitlockite, etcetera). All these shapes, due to their crystallographic characteristics, have been called apatites. The composition of enamel, dentin, cement and bone differs in terms of: a) amount and type of present organic phases, b) type of inorganic phases, c) amount of water, d) concentration of minor elements in the inorganic phase and e) size, morphology orientation and organization of the biological apatite crystal with respect to the organic matrix.[2]

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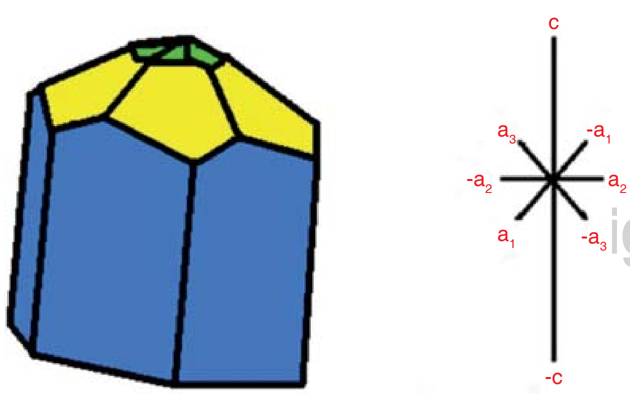
Enamel is the densest inorganic or mineral component: it contains amounts close to 95% of weight (87% volume) when compared to dentin and enamel, which present 70% (47% volume), and bone, with 65% (47% volume). Inorganic phases of enamel, dentin and bone differ in their molar radius and Ca/P weight, as well as in concentrations of minor elements such as CO_3, Mg^{+2}, Na^{2+} and Cl^{+5}. The amount of molar radius Ca/P of mature human enamel is close to 1.64.[2]

In the initial phase of enamel synthesis, Ca^{-2} and O^{-2} ion precipitation, as well as PO_4 molecules and crystalline growth are expressed in long and thin ribbons. These incipient crystals are disoriented, and follow ondulating routes. They lack the straight borders and flat surfaces of well-ordered crystals. Mature crystals develop through the slow thickening of the aforementioned ribbons. Nevertheless, it is probable that some defects present in the ribbons remain trapped within the mature crystals' nuclei and therefore impact on the stability of these crystals as well as the acid dissolution models of the enamel.[1]

Crystal morphology is hexagonal in shape. This is due to the fact that cell axes a and b form a 120° angle and present equal lengths: c axis is perpendicular to the other two *(Figure 1)*.

Longitudinal dimension of an enamel apatite crystal is corresponding with the c axis of the apatite unit cell.[1]

Apatite composition changes intervene within the scope of chemical aspects involved in enamel solubility in acid solution. These changes are induced by the ion exchange between crystalline and liquid phases. It therefore can be said that apatite does not have a constant solubility product. Solubility increases with the decrease of pH and is similar to that of calcium phosphate secondary to pH = 6.0 and to that of calcium phosphate primary to pH = 4.0. The presence of carbonate tends to increase the solubility of enamel apatite, whereas fluoride tends to decrease it. In acid solutions, enamel apatite solubility is affected by concentration and viscosity of available buffers, by the relation of buffer/mineral volume as well as the inter-ionic activity occurring during the dissolution process.[3]



1a **1b**

Source: direct

Figure 1. Schematic representation of hydroxyapatite cristal (a) and axes whereon those crystals are oriented (b).

Chemical kinetic studies show that diffusion of hydrogen ions and non-dissociated acid molecules in the enamel, as well as reaction velocity between mineral and acid are paramount for the control of speed and degree of acid attack. Barriers to diffusion in the surface of the tooth or in the enamel external layer decrease acid dissolution speed and delay surface de-mineralization. Once they break through the protective superficial layer, acid ions are free to react with the tooth structure and thus dissolve it. Cyclic repetition of these regulated diffusion processes leads to the deep decalcification of the tooth's structure.[3]

The preventive effect exerted by fluoride on the presence of dental caries has already been documented for several decades. This effect is due to the rapid F incorporation into the apatite crystals of the tooth's hard tissues, resulting in a less soluble-to-acid structure.

Scientific literature reports that in an incipient carious lesion, its progress can be stopped or even reversed. This process is known as «remineralization».[4-7]

The term «remineralization» was originally used to describe the complete reparation of the de-mineralized dental region. This phenomenon was clinically observed as the disappearance of incipient white lesions in the enamel. Alternatively, the term «re-mineralization» can be used to describe the process of mineral deposit.[8]

Remineralization acts through two processes: a) reduction of the size of the lesion and b) increase of resistance to caries progression.[8]

During the acid attack, fluoride increase results in a fluorhydroxyapatite precipitation, which mainly occurs in the superficial layer of the enamel. The slight increment in fluoride concentration provides strong remineralization.[9,10] Therefore, it can be stated that fluoride action is exerted through:

a) Its antibacterial properties during the formation of hydrofluoric acid, which interferes with enzymes involved in glycolysis.
b) Inhibits calcium and phosphate dissolution in the enamel sub-surface in incipient carious lesions during acid exchange (demineralization).

c) Promotes remineralization, assisting calcium and phosphate to precipitate on the enamel surface in order to crystallize into a more acid-resistant surface.[11,12]

Knowledge of fluoride effect on demineralization and remineralization was extended when it was studied with the method called cyclic pH. In this method, enamel or dentin samples are subjected to pH changes, as occurs in the oral cavity.[13]

A wide selection of chemical resources have been used for the preventive treatment of carious lesions. These resources were of varied composition and presentation; nevertheless, their common trait was their capacity to scale down the advance of destructive lesions such as caries.

In the dental clinic, gel presentation of topical fluoride is the most used compound for caries prevention. The formula of this gel is 1.23% sodium fluoride and 0.1 M phosphoric acid, with 3.2 to 3.4 pH. This presentation is known as acidulated phosphate fluoride (APF). This formula provides high fluoride concentrations in an acid environment which promotes fluoride uptake.[5] Resulting fluorapatite is more stable and exhibits greater retention. Application time is 4 minutes in isolated dry teeth having been subjected to prophylaxis .[3,14,15]

A system involving polyurethane containing 0.1% fluoride in the form of difluorsilane (DS) was introduced in 1975 under the commercial name of Protective Fluoride ™ (Vivadent, Liechstenstein).[16] Its mechanism was based upon the following factors: it controls the remineralization process, it incorporates fluoride into the enamel layers, and therefore remineralizes incipient carious lesions. Protective Fluoride™ usually adheres to the dental surface for over 24 hours,[14,17] optimum results are obtained when performing periodic applications every 6 months.[18]

A silver ammonium fluoride-based compound (SAF) (380 mg per 1 mL)[19,20] was first used in 1970 in order to arrest carious lesions progress. It bore the commercial name Saforide™ (J. Morita, Japan). Scientific literature mentioned the fact that caries inhibition and arrest promoted by SAF was the result of the following factors:[21]

a) Bonding of proteins and silver ions (bacterial proteins and infected carious dentin proteins), which promote instant clotting and form silver proteins.
b) Bacterial enzyme inhibition (trypsin, collagenase) consisting in proteins, therefore, a later dentin collagen denaturalization is prevented.
c) The process of affected dentin into infected dentin is arrested.
d) Fluoride contributes to the remineralization of affected dentin.

In order to assess remineralization under polarized light, the body of the lesion was observed as the region showing positive birefringence, and was represented in green color. Superposition of the light beam was the area of the body's region after having been exposed to oral fluids; this was represented in red. After exposition to oral fluids containing aggregated fluoride ions, the region of the body was superposed to a diagram in a blue outline.[5]

The aim of the present study was to determine the efficiency of three fluoridated compounds in the inhibition of incipient caries lesions progression, under cyclic pH conditions *in vitro*.

METHODOLOGY

Sample preparation

120 third molars were included in the sample. Molars had to comply with established participation criteria: (healthy, not erupted, with crown integrity and lacking visible structural defects in the enamel. Teeth were prepared following the method described by Klein and his group.[22] This method is now briefly described: root soft tissue was removed, and prophylaxis was performed[23] with fluoride-free prophylactic paste (QOM, Mexico). Molars were then sectioned[24] with a diamond disk (Allied High Tech Products, USA) in a mesio-distal direction and at the longitudinal axis, under constant irrigation. All specimens were coated with acid-resistant varnish (Revlon, France), with the exception of one area, measuring 3.0 x 6.0 mm, located at the labial or lingual surface *(Figure 2)*.

Specimens were immersed in a demineralizing solution composed of 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 0.05 M acetic acid and pH adjusted to 4.4 through the addition of KOH at 1 M during 96 hours in order to produce the first lesion[8,24-27] in the enamel, at a constant 37 ºC temperature. Four groups of 30 teeth each were randomly selected. In these groups, one the following compounds would be applied:

1) Silver ammonium fluoride SAF (Saforide™, J. Morita, Japan).
2) Difluorsilane (DFS) (Protective Fluoride™ Ivoclar, Germany).
3) Acidulated phosphate fluoride APF (Sultan™, USA).
4) Control group (no treatment).

Source: direct

Figure 2. Sample preparation. A 3.0 x 6 mm working window was established which was delimited with acid-resistant varnish.

Source: direct

Figure 3. Specimen preparation in diamond disk trimmer.

All specimens were placed into the following cyclic pH model:[4,28,29]

- 3 hours in demineralizing solution.
- Placement (application) of fluoridated compound (SAF, DFS, APF and CG) and a 4-minute waiting period.
- 21 hours immersed in remineralizing solution.

The remineralizing solution was composed of: 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 mM KCl at pH = 7.0.[8,24-27] This solution was similar to the apatite mineral super-saturation found in saliva, and was similar to that used by Ten Cate & Duisters as reported by Itthagarun.[8]

Containers holding the samples were kept at constant temperature (37 °C) and absolute humidity during the 15 days of the cyclic pH model.[29] Solutions were prepared and changed every second day.

Lesion assessment

In order to assess remineralization, every 5 days,10 samples of each group were randomly selected. Samples were set into self-curing acrylic resin blocks to facilitate longitudinal cuts (100-120 μm). A diamond disk trimmer was used to this effect *(Figure 3)*.

Determination of the effect exerted by fluoride-containing materials on the enamel was assessed through observation of samples. To this effect, an Axiophot Photomicroscope was used, with 5x and 10x work objectives, following the polarized light and grey and blue light filters technique. Means used for birefringence were deionized water and Tholuet solutions with 1.41 and 1.47 refraction indexes (RI). Individual photographic records were obtained and birefringence changes were assessed.[8,22,26-28]

Table I.

Scale	Remineralization grade
0	No change
1	Superficial change
2	Sub-superficial change
3	Deep change

Source: Direct.

A data capture sheet recorded data concerning number of samples, tincture used, as well as valuation scale. *(Table I)*.

Obtained data were captured and organized in a data base (Excel, Microsoft Inc, USA) and statistically treated with the program Sigma Stat for Windows, version 2.0 (Jandel Corporation Software Inc, USA) in order to be analyzed with application of variance analysis (ANDEVA).

RESULTS

All 120 teeth were cut into 4 sections, yielding thus 480 specimens. Each specimen was randomly and blindly examined. In order to standardize observation examination was conducted by one single, previously gauged observer.

Data were organized in function of the time and treatment type according to previously established criteria.

Table II reflects changes in the enamel's appearance. This table depicts observation results under polarized right of specimens treated with fluorinated compounds.

When observing specimens 5 days after treatment with fluorinated solutions *(Figure 4),* the following observations could be made: SAF group exhibited the highest average (1.1 ± 0.876), followed by the DFS group (0.8 ± 0.632) and the APF group (0.6 ± 0.516) *(Figure 5)*. The CG exhibited the lowest average of all groups (0.2 ± 0.422). Data statistical analysis revealed that SAF and CG were groups where the greatest difference of values were found. Comparison among these groups revealed a statistical significant difference ($p = 0.024$). Nevertheless, this was not the case when an association with CG was sought between DFS and SAF groups.

10 days after treatment initiation, SAF group once more showed the highest average with value of 1.5 ± 0.850, this was followed by APF group (0.8 ± 0.789), DFS group exhibited (0.6 ± 0.699) and finally, CG with (0.3 ± 0.483) *(Figures 6 and 7)*. Data analysis allowed to establish a statistically significant difference ($p = 0.013$). Nevertheless, in the inter-group difference determination, only the difference between CG and SAF groups could be established ($p < 0.05$).

15 days after treatment initiation, averages obtained for groups SAF, DFS and APF were 1.1 ± 0.316, 1.0 ± 0.816 and 0.8 ± 0.79, respectively. Mean for CG was 0.4 ± 0.516 *(Figures 8 and 9)*. No statistically significant differences ($p = 0.083$) could be established with the variance analysis.

Specimens treated with SAF at 5, 10 and 15 days showed a mean of 1.1 ± 0.876, 1.5 ± 0.850 and 1.1 ± 0.316, respectively. These figures indicated that the highest values were obtained 10 days after treatment initiation. Nevertheless, no statistically significant difference could be established in samples included in this group ($p = 0.401$).

After 5 days of treatment, specimens treated with DFS showed a mean of 0.8 ± 0.632, after 10 days of treatment, values decreased, showing a mean of 0.6 ± 0.699; after 15 days of treatment values were 1.0 ± 816 *(Figure 10)*.

In group APF, after 5 days of treatment, specimens showed mean of 0.6 ± 0.516; after 10 and 15 days of treatment, they showed a mean of 0.8 ± 0.789; this indicated that APF behavior remained constant. Statistical analysis in this group did not reveal statistically significant differences ($p = 846$).

Control Group CG exhibited a mean of 0.2 ± 0.422 at 5 days, 0.3 ± 0.483 at 10 days and 0.4 ± 0.516 at 15 days at the different treatment times.

Table II.

Group	T x 5 days mean ± SD	T x 10 days mean ± SD	T x 15 days mean ± SD
Control	0.2 ± 0.422	0.3 ± 0.483	0.4 ± 0.516
SAF	1.1 ± 0.876*	1.5 ± 0.850*	1.1 ± 0.316
DFS	0.8 ± 0.632	0.6 ± 0.699	1.0 ± 0.816
APF	0.6 ± 0.516	0.8 ± 0.789	0.8 ± 0.789

* Statistically significant difference.
Source: Direct

DISCUSSION

Scientific literature reports many methods to assess caries progression. Among them we can count the cyclic

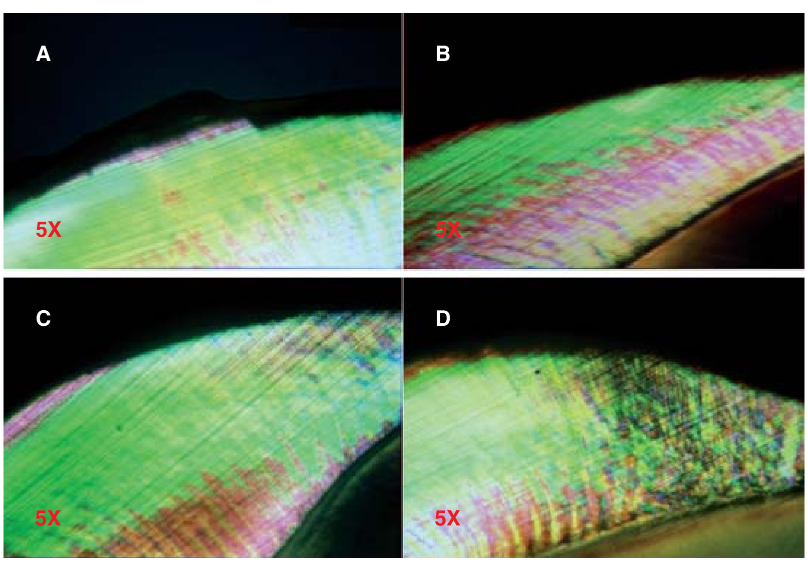


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Figure 4. Effect of fluorinated compounds 5 days after treatment (A: SAF, B: DFS, C: APF, D: CG). Samples were observed with 5 X polarized light technique with photomicroscope, Zeiss, Germany).

pH model as referred by Itthagarun, Klein, Ivancakova and Orth.[8,22,26,27,29] The aim of the cyclic pH method is the reproduction of the demineralization and remineralization circumstances present in the mouth. Several versions of the cyclic pH method have been described.[4,8,26,27,30] The differences among these methods are mainly the time intervals during which the specimens are subjected to remineralization and de-mineralization periods.

In 1995, Seppa et al conducted a study in which they compared the anti-cariogenic effect of fluoride, in gel and varnish presentation (APF and FNa respectively). Results revealed there were no statistically significant differences, thus, it was concluded that fluoride varnish was as effective as fluoride gel.[31]

Dr De la Cruz conducted in 2001 an *in vivo* study to assess dental enamel resistance to acid attack before and after topical application of different fluorinated agents (FFA, FNa and fluoride varnish). Dr De la Cruz determined that all treatments elicited increased resistance to acid attack. Results equally revealed that the group treated with fluoride varnish exhibited greater effect of homogeneity on the dental enamel.[32]

In 2002, Dr Chu et al assessed the effect of SAF and FNa in the treatment of caries arrest. They determined that yearly SAF application elicited more satisfactory results than FNa. They therefore concluded that SAF was more efficient for treating caries arrest.[33] This could be explained due to the fact of the formation of silver phosphates on the tooth`s surface after application of SAF.[34]

Dr Nelson, through the use of proton microprobe, measured with high resolution in a cross-section profile, calcium and fluoride through the artificial lesion. To this effect, he used amorphous carbon supported by scanning electron microscope.[30]

Drs Ten Cate[4,24] and Mukai[28] assessed enamel lesions with the help of microradiography. Assisted by this technique they determined Ca, P and fluoride levels present in a medium after application of a fluorinated agent. Their results showed Ca and F increase only 15 days after treatment. When this technique is followed, the studied sample is lost.

Dr Dammen, in order to measure increases in calcium levels, used atomic absorption spectrometry and gas chromatography in order to measure fluoride levels in acid-dissolved samples.[26,27]

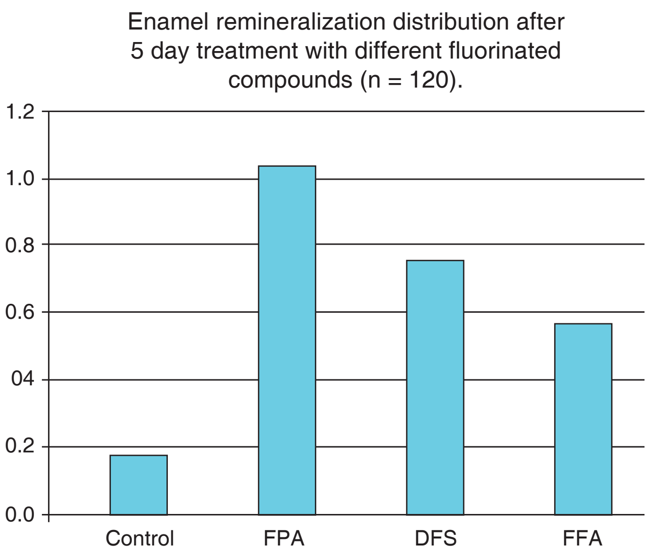

Figura 5. Source: direct.

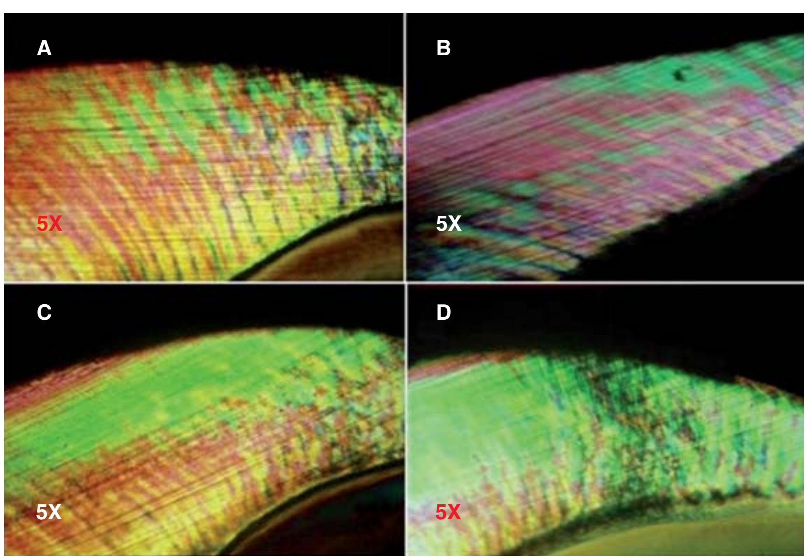

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Figure 6. Effect of fluorinated compounds 10 days after treatment (A: SAF, B: DFS, C: APF, D: CG. Cuts observed with 5 X polarized light technique with photomicroscope, Zeiss, Germany).

In the previously mentioned quantitative procedures, sophisticated instrumental techniques were required for sample characterization. Due to this situation, determination of remineralizing effect by means of polarized light image interpretation (PL) offers advantages when compared to techniques employed by other researchers. In our present research, the use of PL (qualitative technique) allowed to establish, in a simple manner, the effect of different fluorinated compounds on enamel. This has also been reported by Drs Klein and Orth,[22,26,27] although it is nevertheless important to mention that the use of quantitative techniques provides more specific results of elemental behavior in the remineralization process.

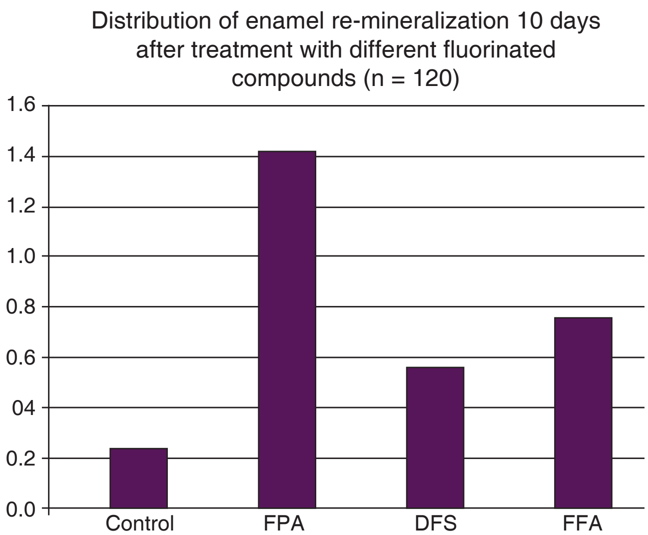

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Figure 7. Enamel remineralization distribution after 10 days treatment with several fluorinated compounds.

Among tested fluorinated compounds APF and DFS have been widely studied and their results are well known. Nevertheless, reports on SAF are scant in scientific literature. This is a product considered as a resource to arrest caries progression in pedodontics as well as medication to be used within the canals in endodontic treatments. With the exception of reports submitted by Dr Chu and his group,[33] there is no information with respect to comparison between SAF and FNa.

Effect of SAF on demineralized enamel is manifest not only in carious lesions,[33] but also in re-mineralization processes. This can be interpreted as an increase in enamel resistance to acid attack or in the better performance of automatic reconstruction treatment in pedodontic practice.

In the present work, SAF exhibited better performance on demineralized enamel than APF and FNa. This could be due to factors associated to high concentrations of F- (38%)[34] and the great reactivity of F- with respect to the hydroxyapatite chemical structure, due to the substitution of functional OH- groups for F-.

Even though no differences were observed between APF and FNa during the experimental period, SAF did show different behavior at 5 and 10 days of treatment. Nevertheless, when reaching 15 days after treatment, behavior of all groups was similar. This could be due to a saturation of the medium where SAF acts, which is not necessarily negative if we take into consideration the fact that continuous and prolonged applications can elicit negative effects such as fluorosis.[35]

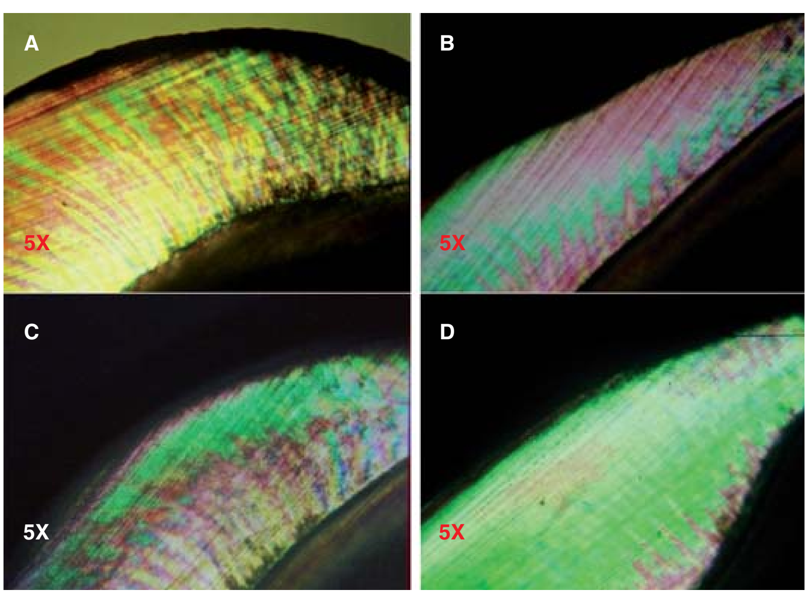

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Figure 8. Effect of fluorinated compounds at 15 days of treatment (A: SAF, B:DFS, C:APF, D:CG). samples observed with 5X polarized light technique with photomicroscope, Zeiss, Germany).

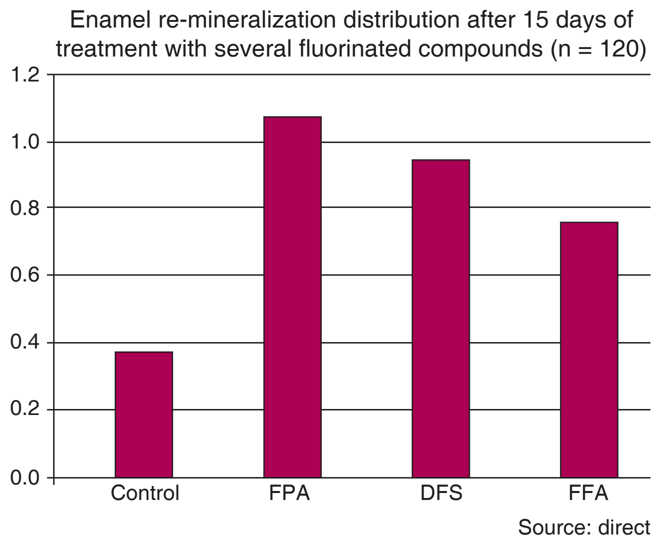

Figure 9. Enamel remineralization distribution after 15 days treatment with several fluorinated compounds.

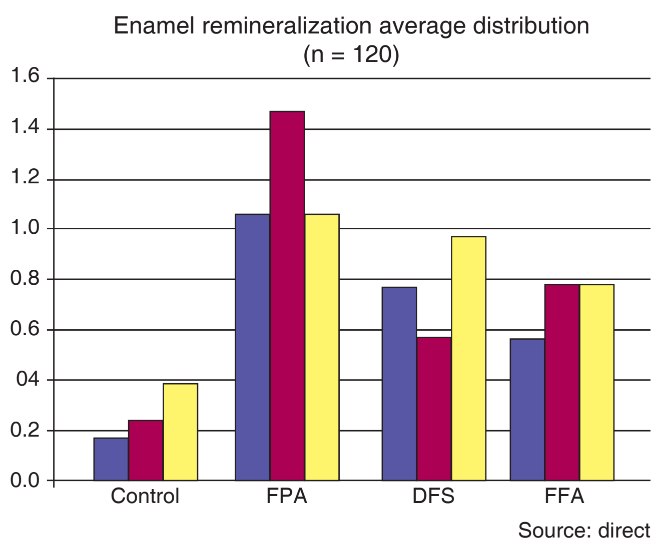

Figura 10. Distribution of averages at 5, 10, 15 days after enamel treatment with fluorinated solutions.

CONCLUSIONS

The present study revealed that SAF was the most effective fluorinated compound for treatment of incipient caries.

The group treated with FPA exhibited greater changes than groups treated with APF, DFS and control.

The cyclic pH model provided controlled constant changes by simulating circumstances present in the oral cavity.

In incipient carious lesions, enamel demineralization and remineralization could be effectively assessed with the help of polarized light.

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