Effect of silver diamine fluoride (SDF) on the dentin-pulp complex. 
Ex vivo histological analysis on human primary teeth and rat molars

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ABSTRACT
The aim of this study was to determine the effect of SDF on the dentin-pulp complex using two models: teeth after SDF application (ex vivo) and experimental animal molars. A descriptive study was performed using two models. In the first model, primary teeth (ex vivo) with enamel-dentin caries, without pulp involvement and previously treated with 38% SDF, were evaluated by means of two techniques: (a) Scanning Electron Microscopy (SEM) and energy-dispersive X-ray detector (EDS) to determine qualitative and quantitative composition, and (b) brightfield optical microscopy (OM) after decalcification. The second model used laboratory animal molars from 12 male Wistar rats. Standardized enamel-dentin cavities approximately 0.5 mm deep were made the distal fossa of the occlusal face of both first lower molars, to one of which a 38% SDF solution was applied, while the other was used as a control. Histological sections were prepared and dental pulp was evaluated qualitatively in both groups. SEM on ex vivo teeth showed areas of hypermineralization in the intertubular dentin and few blocked tubules, while EDS detected Ag in the center of the lesion (7.34%), its concentration declining at the edges (1.71%), with none in the areas farthest from the lesion. OM showed SDF sealing the tubules only at the site where it had been placed, with limited penetration beneath, the tubules appeared normal and the pulp tissue associated to treated caries showed chronic inflammatory infiltrate and formation of tertiary dentin, with no Ag precipitate. In the experimental animal model, pulp histology was not significantly altered in the molar cavities exposed to SDF. The observations using the different techniques on dental tissues suggest that SDF causes minimal adverse effects. The results of this study may contribute to further studies on the suitability of SDF as a cost-effective strategy for treating caries.

Key words: Dental caries; fluorides; silver diamine fluoride.

Efecto del diamino fluoruro de plata (DFP) sobre complejo dentino-pulpar. 
Análisis histológico ex vivo en dientes primarios humanos y molares de rata

RESUMEN
El objetivo del trabajo fue determinar del efecto del DFP en complejo dentino-pulpar aplicando dos modelos: piezas dentarias luego de su aplicación (ex vivo) y en molares de animales experimentales. Se realizó un estudio descriptivo aplicando dos modelos: en piezas dentarias primarias (ex vivo) con caries amelodentinarias sin compromiso pulpar que hayan sido sometidas previamente con DFP 38%, mediante dos evaluaciones: (a) Microscopía electrónica de barrido (MEB) y Detector de energía dispersiva de rayos X (EDS) a fin de determinar su composición cualitativa y cuantitativa y Microscopía óptica de campo claro (MCC) mediante la técnica descalcificación y en molares de animales de laboratorio donde se utilizaron 12 ratas Wistar macho. La técnica fue estandarizada en la fosa distal de la cara oclusal del primer molar inferior, se realizó una cavidad amelodentínaria aproximadamente 0.5 mm de profundidad, en ambos molares. En un molar se aplicó la solución DFP al 38 % y el opuesto como control. Se realizaron cortes histológicos y se evaluó en forma cualitativa la pulpa dental en ambos grupos. En las piezas ex vivas mediante MEB se observaron áreas de hipermineralización en la dentina intertubular y escasos conductillos obliterados y por EDS se detectó Ag en el centro de la lesión (7.34%), disminuyendo su concentración en los límites (1.71%) y no se detectó en las zonas más alejadas de la misma. En MCC se observó DFP sellando los conductillos sólo en sitio de colocación, con una penetración limitada, los conductillos se observaron de aspecto normal y el tejido pulpar asociado a caries tratada ha mostrado un infiltrado inflamatorio crónico y formación de dentina terciaria, sin observarse precipitado de Ag. En el modelo experimental en las cavidades expuestas con DFP en molares no se alteró en forma relevante la histología pulpar. Las observaciones realizadas con las diferentes técnicas y en tejidos dentarios sugieren que el DFP genera mínimos efectos adversos. Los resultados de este estudio pueden contribuir a continuar con investigaciones que permitan recomendar el producto como una estrategia costo efectiva para el tratamiento de la enfermedad.

Palabras clave: Caries dental; fluoruros; diamio fluoruro de plata.
INTRODUCTION
The antimicrobial agent silver nitrate (AgNO₃) was used industrially for over 100 years to make water potable. AgNO₃ is used medically in eye drops to prevent infections in newborns, and in dentistry it is often used in stomatological treatments for mouth ulcers. In 1969, silver diamine fluoride [F(NH₃)₂Ag] (SDF) solution was synthesized for dental treatments. Since then, it has been used in Japan as Saforide® Solution (J Morita Company, Japan) for application to caries lesions due to its capacity as an antimicrobial agent and to stabilize caries processes, particularly in primary teeth, thanks to which it has an important role in pediatric dentistry.

SDF is a colorless solution which is used at 38-40%, pH 8-10. On contacting the caries surface it produces calcium fluoride (CaF₂) and silver phosphate (Ag₃PO₄). The F:Ag ion ratio is 44,800:255,000 ppm. SDF is manufactured and marketed in South America as Fluoroplat® (Laboratorios Naf, Buenos Aires, Argentina), in Australia (Creighton Pharmaceutical, Sydney, Australia) and in Brazil as Safluoride di Walter® in 10% solution (Polidental, Rio de Janeiro, Brazil).

The mechanism of action of SDF on caries has been related to the formation of silver phosphate by reaction with the tooth enamel surface. When the dentin is compromised, the compound penetrates the tubules, partially or totally, blocking their lumen. In addition, it has an antimicrobial effect, inactivating cariogenic bacteria it contacts.

Silver fluoride (AgF₂) is much more soluble in water than other silver halides. Silver diamine fluoride (SDF) contains ammonium in addition to AgF₂. The ammonium ions combine with the silver ions to produce a complex ion called silver diamine ion [Ag(NH₃)₂], which is reversible and more stable than AgF₂. It can thus be kept at a constant concentration for a longer time.

Craig et al. and Gotjamanos showed that silver fluoride (AgF₂) is effective in arresting caries in primary molars in children. Different studies have evaluated the potential toxicity of SDF in children. Gotjamanos and Afonso report that commercial 40% AgF₂ preparations contain high concentrations of fluorides and if used for treatment of young patients may cause fluorosis. Western Australia Dental Health Services conducted a study using AgF₂ and found no evidence of its appropriate use causing fluorosis. There is no clinical report of fluorosis as a result of using SDF.

Chu et al. conducted an 18-month study on 375 children and reported 70-83% effectiveness of SDF applied on primary central incisors. Llodra et al. found similar effectiveness in a controlled cohort clinical study on a Cuban population of 373 6-year-old schoolchildren over 36 months, finding 80% effectiveness on canines and primary molars, and 65% effectiveness on permanent first molars.

Chu and Lo and Zhi et al. report that SDF application once or twice a year significantly reduces the incidence of caries and reduces the substantial risk of adverse events. Llodra et al. and Chu et al. report that SDF applications produce reversible gingival irritation, although this disadvantage is minimized when SDF is applied using an adequate relative isolation protocol. Sharma et al. claim that SDF is efficacious for arresting caries lesions.

Gao et al. and Mei et al. conclude that professional use of 5% sodium fluoride varnish can remineralize enamel caries and that 38% SDF can arrest dentin caries.

In vitro studies on the penetration of SDF into the tooth structure found that it penetrates approximately 2 µm into enamel and 50-200 µm into dentin, while in arrested lesions it reaches a thickness of approximately 150 µm.

Different authors have reported the antimicrobial effect of SDF. Among the most relevant studies is Chun et al., reporting that SDF has antimicrobial effect against cariogenic S. mutans or A. naeslundii biofilm on dentin surfaces. These findings agree with Mei et al., who report the same conclusions with 38% SDF solution.

De Almeida et al. confirmed the antimicrobial effect of SDF at commercial concentrations of 12% and 30%, which are lower than the concentration used in our study.

One of the main drawbacks of SDF is esthetic because of the dark stain it produces on the tooth surface. Knight et al. therefore conducted in vitro studies combining SDF with potassium iodide (IK), which lessened the stain while preserving the antimicrobial properties. Although the product containing SDF and IK emerged on the Australian market, it has not become well known in the rest of the countries that use SDF.
SDF is considered a simple, low-cost therapeutic alternative which does not require training for application by health professionals and has a significant benefit for individuals and populations, based on biological sealing\textsuperscript{22,28-29}.

Although satisfactory results have been obtained using SDF, to date, its potential toxic action and mode of interaction with dental tissues have not been fully elucidated.

Thus, the \textit{aim} of this study was to determine the effect of SDF on the dentin-pulp complex by using two models: (a) \textit{ex vivo} teeth after SDF application and (b) molars in experimental animals.

\section*{MATERIALS AND METHODS}

We performed a descriptive study on the effect of SDF on the dentin-pulp complex by using two models: \textit{ex vivo} human teeth after application and molars in experimental animal.

\section*{Histological Study of human teeth treated with SDF}

We used 8 human primary teeth obtained by exfoliation or indicated extraction. Inclusion criteria were: teeth with dentin-enamel caries without pulp involvement and previously subject to SDF treatment prior to exfoliation or extraction due to persistence (approximately 1 year after SDF application).

The following protocol was used for applying 38\% SDF:

- Relative isolation of the lesion, removal of affected dentin using hand instruments, application of 38\% SDF by rubbing for 1 minute with a manual applicator soaked in the solution, followed by rinsing with distilled water.

This study was partly performed within the framework of the project “Strategic approach for reconsideration of barriers to access to dental care in highly vulnerable groups”, which was reviewed and approved by the Ethics Committee at the Buenos Aires University School of Dentistry (UBACYT U20020120100324BA). In order to include children in this study, we obtained informed consent from their legal guardians and formal acceptance from each child. An authorization for donation was attached to the consent forms describing how the tooth would be used, the research aims, and a statement that refusal to participate would not generate any conflict with participation in the project.

Four teeth were cut in half using a diamond disc for Scanning Electron Microscope (SEM) observations. The other four teeth were decalcified for observation under brightfield microscopy.

In each histological analysis of human teeth (\textit{ex vivo}) the zone opposite the treated lesion was used as control.

\subsection*{Evaluation using Scanning Electron Microscopy (SEM)}

Sections were prepared from the 4 primary teeth which had been previously cut to expose the lesion. Residue was removed from the sections by ultrasound and they were dehydrated in an alcohol concentration gradient (100, 96, 70 and 50\%). Samples were sputter-coated with gold-palladium (using a Termo VG Scientific SC 7620 sputter coater) for SEM observation (Scanning electron microscope model SUPRA 40 Gemini II, Carl Zeiss). One of the teeth was also studied using an energy-dispersive X-ray detector (EDS) in order to determine its qualitative and quantitative composition.

\subsection*{Evaluation by brightfield optical microscopy (OM): Decalcification technique}

Teeth were fixed in 10\% formalin buffer for at least 48 hrs, after which they were decalcified in 7.5\% nitric acid for 7 days. Then they were embedded in paraffin, and mesiodistal histological sections approximately 8 \(\mu\)m thick were cut. Sections were stained with hematoxylin and eosin and a qualitative histological evaluation of dental pulp was performed under brightfield optical microscopy.

\section*{Experimental study on laboratory animals}

Twelve male 2-month-old Wistar rats weighing 300-350 grams were used. They were anesthetized (ketamine 50ml/kg and xylazine 15 ml/kg i.p.) and placed on an adapted operating table. The lower jaw was isolated and the following standardize technique applied. An enamel-dentin hole about 0.5 mm deep was made in the distal fossa of the occlusal face of each lower first molar (left and right) using a ¼ carbide drill bit at medium speed. The 38\% \(\text{F(NH}_3\text{)}_2\text{Ag}\) solution was applied to the left molar using a paper point, while the right molar was used as a control (cavity only).

Seven days after treatment the animals were euthanized using 0.2 mg/weight sodium pentobarbital (euthanyle).
Lower jaws were extracted and fixed in 10% buffered formalin for 48 hs, decalcified in 10% EDTA pH 7.2 for 30 days, processed histologically and embedded in paraffin. Histological mesiodistal sections were prepared and stained with hematoxylin and eosin, and qualitative histological evaluation of the dental pulp was performed on both treated and untreated molars under brightfield optical microscopy.

The experimental protocol is in keeping with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The procedure described above is shown in Fig. 1.

RESULTS
Histological study on human teeth treated with SDF

Scanning electron microscopy (SEM) - EDS

Fig. 2 shows the lesion treated with SDF, evaluated by EDS within the lesion, outside the lesion. EDS values for the Ca/P ratio were: within the lesion, only Ca was recorded. At the edge of the lesion, the Ca:P ratio was 3.51, and outside the lesion it was 2.275. Ca percentage was 4.07 within the lesion, 27.74 at the edge and 25.63 outside the lesion. Silver (Ag) was recorded within the lesion (7.34%) and at the edge (1.87%), but none was recorded outside the lesion.

Cross sections of dentin tubules in healthy/untreated dentin vs. treated dentin were analyzed for each portion of the selected primary teeth. The tubules corresponding to dentin treated with SDF showed areas of hypermineralization of the intertubular dentin and few blocked tubules (Fig. 3).

Brightfield optical microscopy (OM): Technique using decalcification

In the histological sections, SDF was observed sealing the tubules and there was limited microscopically visible penetration (Fig. 4). Beneath the treated lesion, the tubules appeared normal and the pulp tissue associated with the treated caries showed chronic inflammatory infiltrate and formation of tertiary dentin, with no silver precipitate (Fig. 5).

Experimental study on laboratory animals

Analysis of the histological sections of Wistar rat molars with and without SDF treatment showed a precipitate inside the dentin tubules at the application site of the molars treated with SDF. No silver was found in the pulp, and evaluation of pulp in both groups showed well-organized dental pulp with good vascularization and mild inflammatory infiltrate, with no relevant histological change (Fig. 6).

DISCUSSION

Historically, several lines of work have questioned the idea of surgical treatment of dental caries as the only therapeutic alternative, and reported protocols and results of the use of chemical inhibition of the caries process using different resources 30-34.

Some of these positions were based on the need to respond to the issue of dental caries in countries with high prevalence of the disease and low capacity for its resolution, whether as a result of the healthcare model in place or the incompetence of any of its components 35.
Nevertheless, before recommending extensive clinical use of SDF on the dentin-pulp organ, its safety must be established. Mei et al. observed that SDF application on patients with high caries prevalence generates formation and precipitation of silver phosphate. They also found calcium fluoride, silver phosphate and less soluble proteins with silver forming a protective layer that may reduce the loss of calcium and phosphorus from the carious lesion.

![Fig. 2: SEM images show enamel prisms and lesions. Insets show EDS determination inside (left) and outside (right) of the treated lesion. The graphs beneath the figure show the concentrations of elements present in each zone. Note that Ag is only present within the lesion.](image1)

![Fig. 3: SEM images showing healthy/untreated dentin tubules (left) and tubules treated with SDF (right). The healthy dentin (left) shows a dentin tubule and intertubular dentin. The treated dentin (right) shows a partially blocked tubule and the intertubular dentin appears to be hypermineralized.](image2)
The results of our study showed that surfaces treated with 38% SDF and observed using SEM had areas of hypomineralization in the intertubular dentin and few blocked tubules. EDS studies showed that the proportion of silver content is minimal with relation to the composition of the dentin structure. In an in vitro study, Mei et al.\textsuperscript{36-37} used 38% SDF and found partially blocked dentin tubules, similar to the results of our study\textsuperscript{12, 27,36,37}. In another study, Yu et al.\textsuperscript{38} found precipitates with high silver and phosphorus content blocking dentin tubules. Rosenblatt et al.\textsuperscript{1} published a review of the literature and report results similar to those of Yu et al.\textsuperscript{38} and of our study.

Our observations found hypermineralization at the edge of the treated lesion, reflected by the difference in Ca concentration, which was 4.07% within the lesion and 27.74% at the edge. The Ca:P ratio just beyond the edge the lesion dropped to 2.27, increasing to 2.4 as distance increased. It may be assumed that the value would increase up to normal values if it were measured at points increasingly distant from the lesion. These results are similar to those of Mei et al.\textsuperscript{24}, who observed a highly mineralized zone rich in calcium and phosphate in primary teeth dentin lesions which had been arrested with a single application of SDF. However, they analyzed the lesion as a whole according to depth and did not distinguish the two zones within it. Moreover, the protocol used by Mei et al.\textsuperscript{25} applied SDF twice a year, whereas our study examined a single application. They reported that the mineral density was higher in the outer layer of the active lesion than within the body of the lesion, finding a distinct layer 150 μm thick in the arrested lesion, with greater density than the unaffected dentin, which could be considered to be the hypermineralization zone. They did not find very high fluoride levels in the lesion, probably because it was rinsed away with water. For silver, the highest absorption capacity was within the lesion, decreasing noticeably at the edge, with no silver absorption outside the lesion.
In another study, Mei et al.\textsuperscript{12} concluded that application of 38% SDF arrests the caries process by reducing demineralization and collagen destruction. In addition, the presence of high fluoride and silver concentrations may inhibit microbial growth of the species present in cariogenic biofilm. It has also been suggested that SDF has an inhibitory effect on metalloproteinases, thereby protecting collagen from destruction in carious lesions, and acting as another form of protection against dentin degradation. In another in vitro study, Mei\textsuperscript{2} et al. found that the primary components of SDF appear to react with dentin tissues, forming calcium fluoride, a compound that protects against caries. Dentin microhardness changes according to its mineral content. Any changes produced by applying SDF may thus also be evaluated by microhardness, and it would be useful to supplement our observations with mechanical determinations on dentin in order to assess its potential correlation\textsuperscript{36, 37}.

The evaluation of experimental animal molars showed that SDF has limited penetration. We found no major alteration to dental pulp or presence of silver in the pulp of either human teeth or experimental animal molars. These findings agree with Korwar et al.,\textsuperscript{39} who reported absence of inflammatory symptoms in \textit{ex vivo} teeth with cavities treated with SDF. Our paper also describes the presence of tertiary dentin adjacent to the treated cavity.

Internationally, Japan has marketed products containing SDF for over 80 years and the US Food and Drug Administration (FDA) approved its use in the year 2014\textsuperscript{40}. Dental caries is a highly prevalent disease in children in developing countries\textsuperscript{41}. Under many circumstances, conventional methods for prevention and treatment of caries are unavailable or unaffordable to communities in those regions. The use of unconventional protocols for treatment of caries lesions, including agents that stabilize the process, is essential to the development of dental care programs for highly vulnerable sectors of society and/or sectors with barriers to healthcare access. Traditional approaches for treating caries in populations with these barriers provide temporary benefits due to the high relapse rates in individuals with greater burden of disease.

The results of our study may contribute to establishing the absence of potential adverse effects in dental tissues subject to topical application of SDF and thereby enable continued research which may ultimately enable SDF to be recommended as a low-cost, highly effective strategy for treating caries. Through the use of different histological study models (tooth substrates and observation techniques) this study suggests that SDF produces minimal adverse effects on the structures described. However, the toxicity and biocompatibility of silver compounds require further evaluation before its safety can be established and its application recommended as a therapeutic measure in programs intended for populations with barriers to conventional dental care.

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