

AgNPs: The New Allies Against *S. Mutans* Biofilm - A Pilot Clinical Trial and Microbiological Assay

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The purpose of this study was to evaluate the antimicrobial properties of a new formulation containing silver nanoparticles, named Nano Silver Fluoride (NSF), to inhibit *Streptococcus mutans* biofilm formation on children's dental enamel. The variations in dental biofilm pH and in the Simplified-Oral-Hygiene-Index (OHI-S) also were evaluated after the treatment with NSF. This was a randomized, double-blind, crossover and prospective pilot clinical trial study in which 12 schoolchildren, aged between 7-8 years, had their dental enamel treated with two solutions: S1 - Nano Silver Fluoride and S2 - negative control (saline solution), in different experimental moments. The dental biofilm adhered to enamel treated with NSF had lower values of *S. mutans* viability (absorbance) and colony forming units (CFU) than the S0 (baseline) and S2. There was a statistically significant difference between the OHI-S mean values of S0 and S1. There were no differences between the biofilm pH (both before and after the use of the test substances) and among the different groups. These properties suggest that NSF has bactericidal effect against *S. mutans* biofilm and it may be used for clinical control and prevention of dental biofilm formation.

Key Words: silver nanoparticles, antimicrobial activity, biofilm, cariostatic agents, *S. mutans*.

Introduction

Dental caries remain a major public health issue that affects 95% of the world's population, mostly in developing countries, which lack access to dental care (1). Owing to its acidogenic and aciduric characteristics that contribute to colonization of the tooth surface, dental plaque formation and to demineralization of hard dental tissues, *Streptococcus mutans* is regarded as the main bacterium related to the initiation of dental caries (2).

A new experimental formulation containing silver nanoparticles, chitosan and fluoride, called Nano Silver Fluoride (NSF), which arrests and prevents caries in children, claims to have ideal chemical, physical and antibacterial properties. Silver nanoparticles (AgNPs) interact with organic and inorganic molecules depending on both their size and shape, since the nanoparticles have a large surface area that allows broad interactions (3).

In dentistry, 38% Silver Diamine Fluoride (SDF), $\text{Ag}(\text{NH}_3)_2\text{F}$, has been used to arrest caries since 1969 (4). Laboratory studies have reported that SDF prevents the formation of *Streptococcus mutans*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* biofilms (5), but its use for dental purposes is generally

not encouraged, because it induces black tooth discoloration. However, the use of nanoscale has eliminated this problem, either in caries lesion or in healthy dental tissue, in addition to enhancing chemical and antimicrobial properties and reducing cell toxicity (6-11). AgNPs also have little or no cytotoxicity (6,7) and clinical application has become possible without risks.

The bactericidal activity and stability of silver nanoparticles are believed to be influenced by the nature of the stabilizing agent used in the formulation, which must allow constant interaction between the silver nanoparticles and bacteria (11-15). Bactericidal effects have also been observed for cells organized in biofilms, due to nanoparticle interference in "cell to cell" aggregation and "cell-surface" adherence (6,15). It has also been reported that the silver particles may inhibit free glycosyltransferases, the enzymes responsible for promoting glucose and cell adhesion that enables the production of glycans and extracellular polysaccharides. The described mechanism may disturb cell respiration and change cell metabolism (13).

The properties of this novel product suggest its potential as an effective cariogenic biofilm inhibitor, with both aesthetic and antimicrobial advantages over traditional silver fluoride products. These characteristics

suggest clinical trials for AgNPs products to prove their *in vivo* antimicrobial action against *S. mutans* biofilm. This study evaluated the antimicrobial efficacy of Nano Silver Fluoride on *S. mutans* growth in children's dental biofilms, variations in dental biofilm pH after product application and the Simplified-Oral-Hygiene-Index (OHI-S) of the treated tooth surfaces.

Materials and Methods

Ethical Aspects

The study was approved by the Research Ethics Committee of Universidade de Pernambuco University (UPE), protocol (CEP-in 265-619). The project was registered at ClinicalTrials.gov under protocol NCT01950546. All caregivers for the children signed a free and informed consent form to allow their participation in study.

Nano Silver Fluoride Formulation

The experimental NSF solution was synthesized according to Targino et al. (7). The silver nanoparticles were characterized by UV-Visible (UV-vis) in a DH-2000 Mikropack Transmission Electron Microscopy (TEM) on a FEI-Tecnaï20 at an accelerating voltage of 200 kV. Tem analysis revealed that 97% of the silver nanoparticles were spherical and particle size was 8.7 ± 3.1 nm.

Clinical Assay

Sample

The sample was comprised of 12 schoolchildren of both genders, aged between 7 and 8 years and regularly enrolled in the Municipal School Anita Trigueiro do Valle in João Pessoa, PB, Brazil.

Inclusion Criteria

The volunteers should be children whose parents had signed the informed consent form for their participation in the study and who were not using antimicrobial substances such as mouthwashes and antibiotics or immunosuppressing drugs for at least one month before the study began. The volunteers were required to have the following index teeth: permanent maxillary right central incisor (8), permanent mandibular left central incisor (24), permanent maxillary and mandibular first molars (3,14,19); to attend classes during the week of collection; not having systemic health involvement; who did not use braces and without carious lesions (white spot or cavitated), supra-gingival calculus, gingivitis or malocclusions in the index teeth.

The study was randomized, double blind, crossover and prospective. The children's dental enamel was treated with two solutions - Nano Silver Fluoride (S1)

and negative control (saline solution - S2) at different experimental moments. Neither the researcher nor the participants were aware of the treatment delivered at each experimental period. Each subject received both treatments. Microsoft Excel 2003® software was used to randomly allocate the selected group volunteers in two groups, according to the two solutions: S1 and S2. Codes were assigned by a biostatistician and decoded only at the end of the study.

Sample Standardization

All the volunteers received a hygiene kit containing soft toothbrush (Condor Trip®, Condor, São Bento do Sul, SC, Brazil), toothpaste without fluoride (Cocoricó, Tutti-Fruti, Sem Flúor, Bitufo Hypermarcas®, São Paulo, SP, Brazil) and oral hygiene guidelines. At the beginning of the study, all participants received a professional prophylaxis to remove plaque and to standardize the oral cavity with the lowest possible amount of biofilm. To complete the standardization, each volunteer used the dentifrice kit for 7 days (cleaning or "wash-out" time), from before the study beginning until the end of the study. The volunteers were told not to use any product containing antimicrobial substances (mouthwash, toothpaste, gel) during the experiment days, but just to brush their teeth 3 times a day with the kit provided by the researcher.

Applications of the Solutions

The study was randomized, double blind, crossover and prospective. The subjects used two solutions and neither the researcher nor the participants were aware of which treatment was delivered. Microsoft Excel 2003® software was used to randomly allocate the selected group volunteers into two distinct groups: S1- nanosilver fluoride, and S2 - negative control (saline solution). There were two experimental times of solution application. , in one there were children treated with S1 and children treated with S2. In the second experimental time, after one week without solution application (wash out period) the volunteers were treated with a different solution from the one they were treated in the first experimental time. All volunteers used both solutions. Codes were kept for assignment by a biostatistician and decoded only at the end of the study. Five-milliliter portions of S1 and S2 solutions were applied using a disposable micro-brush (KG Sorensen®, Cotia, SP, Brazil), on the buccal surfaces of permanent maxillary right central incisor (8), permanent maxillary first molars (3,14), permanent mandibular left central incisor (24) and lingual surfaces of the permanent mandibular first molars (19).

Biofilm pH

The measurements of dental biofilm pH were performed with a Beetrode (WPI Inc., Sarasota, FL, USA) microelectrode connected to a potentiometer (Orion 230 A). Its calibration was maintained between 4 and 7 pH. The standard measurement times were: "baseline" (T0 - before product application), T1 (30 min after product application), and T24 (24 h after product application). Only the buccal surfaces of the permanent maxillary molars (3 and 14) were measured to assess whether the products caused some change in the biofilm pH in the experimental period.

OHI-S Index

A single examiner examined all subjects. The examinations were performed in a classroom with adequate artificial illumination, using latex gloves and dental mouth mirrors. The Simplified Oral Hygiene Index (OHI-S) was used to analyze the clinical biofilm parameters (16). It assessed the amount of visible biofilm with a plaque disclosing agent on the surface of index teeth: the buccal surfaces of permanent maxillary right central incisor (8), permanent mandibular left central incisor (24), permanent maxillary first molars (3,14) and lingual surfaces of the permanent mandibular first molars (19,30). The OHI-S was measured at the following study times: T0 (baseline) and T24 hours, always after pH measurement and before biofilm collection.

Each tooth was scored according to the condition found: 0=absence of colored biofilm; 1=colored biofilm surface corresponding to 1/3 of the tooth surface; 2=colored biofilm surface corresponding to 2/3 of the tooth surface; 3=colored biofilm corresponding to the complete tooth surface.

Biofilm Collection

The dental biofilm was fully collected after staining with a sterile dental curette and transferred to a tube containing 1.0 mL of sterile phosphate-buffered saline (PBS) at T0 (baseline) and T24. The tubes containing

the biofilm were refrigerated (a portable cooler with ice) and immediately sent to the laboratory.

Laboratory Phase

This phase was performed in the Oral Biology Laboratory (Universidade Federal da Paraíba). The tubes were submitted to sonication in an ultrasonic apparatus (UltrasonicCleaner®, Odontobrás, Ribeirão Preto, SP, Brazil) for 2 min to release the adhered bacteria. Next, they were centrifuged in a micro-centrifuge for 10 min to precipitate the dispersed biofilm. The bacterial growth was then measured by spectrophotometry using OD. The use of OD technique for monitoring growth kinetics is widely used because of its speed and simplicity (17). The supernatant was discarded from the tubes and the biofilm was re-suspended in 1 mL of sterile phosphate-buffered saline. Then the tubes were vortexed and 100 µL were transferred to a microplate, where the absorbance values were measured. Then, the suspensions were serially diluted (10^{-1} , 10^{-2} , 10^{-3}) and 20 µL of each dilution were seeded in Petri plates containing MSKB medium (in triplicate), a modification of *Mitis salivarius* medium supplemented with 15% sucrose and a 1% solution of potassium tellurite. To this medium were added 500 µg/mL of kanamycin and 50 IU/mL of bacitracin. MSKB is specific for isolation of *S. mutans* (18). The plates were incubated at 37 °C for 48 h, under microaerophilic conditions (jar with candle system). A single trained investigator performed the counting of colony forming units (CFU) using a counter (Phoenix CP608®, Phoenix Lufesco, Araraquara, SP, Brazil). The means of the triplicate counts (multiplied by the dilution factor) resulted in the final bacterial count (expressed in colony forming units - CFU/mg). The calibration performed for this step showed an excellent intra-examiner agreement (Kappa =0.91) was achieved.

Statistical Analysis

Microsoft Excel 2010® software was used for statistical analysis of the data. The CFU count, the supernatant and biofilm absorbance, the biofilm pH and the OHI-S index data for the different groups, NSF and negative control were analyzed by ANOVA test for repeated measures, because the data were normally distributed. The T-test was carried out to analyze the difference between variations in S1, S2 and the baseline group values. A Pearson's correlation coefficient was performed to analyze the relationships between the CFU count and the OHI-S values. The

Table 1. The averages of *S. mutans* colony forming units (CFU) counting, *S. mutans* viability values in dental biofilm (absorbance) and the OHI-S averages values that were measured in the standard times: baseline and 24 h after the solutions applications on dental biofilm

Group	CFU/mg (mean/ SD)	<i>S. mutans</i> viability (absorbance)	OHI-S values Mean/SD
Baseline	34 x 10 ⁴ ± 4.3 x 10 ⁴	0.237	1.75±0.32
S1	8.6 x 10 ⁴ ± 2.7 x 10 ⁴	0.130	1.35±0.13
S2	26.7 x 10 ⁴ ± 3.6x10 ⁴	0.239	1.72±0.11
	ANOVA (p≤0.05)	ANOVA (p≤0.05)	ANOVA (p≤0.05)

significance level for all tests was 5% ($p \leq 0.05$).

Results

When the CFU counts between the groups and different dilutions were compared, there were statistically significant differences among the baseline, S1 and S2 groups ($p=0.004$). The samples treated with NSF revealed lower CFU counts compared to the other groups (Table 1). There was a statistically significant difference of the CFU counts of the groups ($p=0.0097$), with statistically significant differences ($p=0.004$) between the values at baseline and S1 (Table 1). The dental biofilm absorbance after 24 h of solution application revealed that values for S1 were lower than those found for S2 ($p=0.0007$). There was a real reduction in the *S. mutans* growth in enamel treated with the NSF (Table 1). When the biofilm pH values were compared (at different times, as well as between different groups), there was no statistically significant difference between them ($p=0.27$) (Table 2). There was a statistically significant difference between the OHI-S mean values at baseline and S1 ($p=0.02$) (Table 1). The Pearson's correlation showed no correlation between the CFU counts and OHI-S values ($r=-0.13$).

Discussion

A pilot study allows to test the instruments, ensuring that each one will yield its own results to answer the research questions and it may anticipate results. This test evaluated the feasibility and usefulness of the collection methods in each execution phase, in order to review and improve the necessary points. Finally, the pilot study is a valuable instrument, since it allows the researcher to reach the context of his research more experienced and with more refined methodological choices. For these reasons, the present study did not show experimental limitations.

The CFU count showed that AgNP product reduced *S. mutans* growth compared with the baseline and the negative control group. The same bactericidal effect was observed by absorbance of the collected biofilm, in

which the S1 values were lower than in the other groups. These results agree with those reported in previous studies (7,13-15,19) performed in a static biofilm model, which verified greater *S. mutans* adherence inhibition zones (non-stained zones) and antimicrobial effects on dental enamel surfaces treated with spherical AgNPs. Similar results were found by Freire et al. (6) in a dynamic biofilm model, where four AgNP colloids presented better bactericidal effects against *S. mutans* than those produced by 30% SDF. The antimicrobial activity of silver nanoparticles is influenced by the size: the smaller the particles, the greater antimicrobial effect. This suggests the possibility that the AgNPs may also penetrate inside the bacteria. Nanoparticles sizes from 1 to 10 nm attach to the cell surface and drastically disturb membrane permeability and respiration functions. The nanoparticles are also able to penetrate the bacteria and cause further damage, possibly by interacting with sulfur- and phosphorus-containing compounds such as DNA (3,20,21). Targino et al. (7) verified that the MIC value of NSF was lower than the ones found to 30% SDF over *S. mutans* biofilm, which indicated that the new product could replace the traditional silver compound as a bactericide and bacteriostatic agent. In the same way, the NSF formulation was effective to arrest active dentin caries in children after one year of application and did not stain teeth (8). These results suggest NSF as a promising product for controlling the cariogenic process and to avoid cariogenic biofilm formation on tooth surface. Silver nanoparticle products involve very low metal concentrations, which are enough to inhibit the development of *S. mutans* strains. Due to the low silver concentrations, no color changes were observed on the teeth treated with NSF (6,8). Zhang et al. (22) suggest the use of silver nanoparticles in adhesives, composites, cements and sealants to inhibit biofilms and caries.

Biofilms consist in agglomerates of food debris and bacteria, not only microorganisms. To analyze the clinical biofilm parameter was used the Simplified Oral Hygiene Index OHI-S (16), which assessed the amount of visible biofilm by a plaque disclosing agent on the surface of the teeth. The study revealed a decrease in OHI-S values for the S1 group. There was also no relationship between the OHI-S values and CFU count. Thus the index does not provide the visible biofilm composition and is much more related to dental hygiene and dietary habits than to the amount of live bacteria.

In the present study there were no differences between the values of biofilm pH (both before and after the use of the test substances) and between the different groups. The presence of NaF and Ag in the

Table 2. Biofilm pH mean values at different study times

Group	pH values (Mean \pm SD)
pH T0	6.22 \pm 0.06
pH T1 S1	6.39 \pm 0.13
pH T24 S1	6.15 \pm 0.17
ph T1 S2	6.26 \pm 0.04
pH T2 S2	6.09 \pm 0.16

ANOVA $p \leq 0.05$.

NSF did not change biofilm pH. These results disagree with those found by Bradshaw et al. (23), where fluoride inhibited critical metabolic processes (direct effect) and also reduced environmental acidification (indirect effect) in biofilms. This is justifiable as the degree and rate of pH decline increases with carbohydrate concentration in the food, particularly refined sugars, so that plaque pH can reach values less than 5.0 and remain low for some time (24). In the present study, there was no control over the children's carbohydrate ingestion. There was no significant decrease in biofilm pH in S1 and this may be explained by decreases in the number of *S. mutans*. This was evidenced by the CFU counts and the absorbance analyses, because this species degrades carbohydrates rapidly, with formation of large amounts of acid that reduce the biofilm pH (25).

Nanosilver fluoride was an effective dental biofilm inhibitor because it reduced the *S. mutans* CFU counts and absorbance values. The product had no effect over biofilm pH and it reduced IHO-S values. More studies are required to understand the NSF action mechanism in dental biofilm and in dental structure.

Resumo

O objetivo do presente estudo foi avaliar as propriedades antimicrobianas de uma nova formulação composta por nanopartículas de prata, denominada nano silver fluoride (NSF), na inibição de biofilme de *Streptococcus mutans* sobre a superfície do esmalte dentário de crianças. Variações no pH do biofilme dental e nos valores do índice de Higiene Oral Simplificada (IHO-S) também foram avaliadas após o tratamento com NSF. Trata-se de um estudo piloto, randomizado, duplo cego, cruzado e prospectivo. A amostra foi composta por 12 crianças, de ambos os gêneros, com idades entre 7 e 8 anos, as quais utilizaram as duas soluções testes, S1- NSF e S2- controle negativo (solução salina). O biofilme dental tratado com NSF apresentou menores valores de viabilidade de *S. mutans* (absorbância) e de unidades formadoras de colônias (UFC) do que o biofilme baseline e o biofilme tratado com S2. Houve diferença estatisticamente significativa entre os valores médios de IHO-S dos grupos baseline e S1, com uma redução dos valores. Não houve qualquer variação nos valores de pH do biofilme dental, antes e depois do tratamento com S1 e S2 e entre os diferentes grupos. Estas propriedades sugerem que NSF possui efeito bactericida sobre o biofilme de *S. mutans*, podendo ser utilizado clinicamente para o controle e prevenção da formação do biofilme dentário.

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