Materials Science and Engineering C 32 (2012) 896–901

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Antimicrobial sensibility of *Streptococcus mutans* serotypes to silver nanoparticles

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ARTICLE INFO

Article history:
Received 8 September 2011
Received in revised form 6 December 2011
Accepted 2 February 2012
Available online 10 February 2012

Keywords:
Streptococcus mutans
Silver Nanoparticles

ABSTRACT

Dental caries is a common infectious disease in humans all over the world and is considered a public health problem with an incidence and prevalence still high. The principal pathogen associated with the development of dental caries is *Streptococcus mutans* (*S. mutans*), a microorganism that has been widely studied. A new approach to treat dental caries is the use of silver nanoparticles (SNP) due to their antibacterial effect. It has been reported the effectiveness of SNP against *S. mutans*, but sensibility of *S. mutans* serotypes to SNP is still unknown. The aim of this study is to analyze the inhibitory effect of three different sizes of silver nanoparticles on *S. mutans* obtained from clinical isolates and one reference bacteria strain, determining sensibility differences between serotypes, which were determined by using PCR analysis. Silver nanoparticles with sizes around 9.3, 21.3 and 98 nm were prepared, characterized and used to find, with a microdilution method, the minimum inhibitory concentration (MIC) on *S. mutans*. SNP showed bactericidal effect on all strains tested with statistical differences between smaller nanoparticles and larger nanoparticles, 9.3 nm SNP showed a higher antibacterial activity than 21.3 and 98 nm SNP; however, sensibility was different among all clinical strains ($p<0.05$) being serotype *c* the most sensible and serotype *e* the most resistant to the three different sizes of SNP considering the inhibitory effect better when nanoparticle size is smaller.

1. Introduction

The study of *Streptococcus mutans* (*S. mutans*) has been very important in the last years due to their high association with the etiology of dental caries, a biofilm dependent infectious disease. This bacterium grows principally in presence of sucrose, making a complex micro community with several oral microorganisms under an acidic environment producing tooth demineralization [1–5]. Clark was the first researcher who isolated *S. mutans* from carious lesions in humans and also associated this microorganism with infective endocarditis in damaged heart valves [1]. *S. mutans* can be serologically divided into 4 different groups (*c*/*e*/*f*/*k*) according to their specific rhamnose-glucose polysaccharides, and it has been found that these groups can induce dental caries at different degrees of severity; serotype *k* has been identified in extirpated heart valve specimens from persons with infective endocarditis [6–9]. Clinical studies indicate that serotype *c* is the most prevalent one (70–80%), followed by serotypes *e* (20%), *f* (5%) and *k* (2 to 5%) [10–13].

In the other hand, about 95% of populations around the world have been affected by dental caries during their lives; this infectious disease has been a serious health problem for centuries. Industrialized countries have access to dental prevention programs and their population is less affected, but the prevalence and incidence are high in undeveloped countries, where the prevalence in urban zones is about 91.6% and 54.4% in rural areas [14,15]. It is necessary to find an alternative in order to attack this health problem.

In the last years, nanotechnology has had a serious impact in many applications in several areas; mainly, in the medicine field, where metals such as silver, copper and gold have been applied as an important tool for maintaining and preserving health. Specifically, Silver Nanoparticles (SNP) have been used and applied in burn injuries in hospitals due to its antibacterial activity on several gram positive and negative microorganisms. It has been demonstrated that in low concentrations, silver is not toxic to human cells and that the bactericidal effect of nanoparticles depends on the size and shape of the particle [16–23]. The aim of this study is to analyze the inhibitory effect of three different sizes of SNP on *S. mutans* from clinical isolates and a reference bacteria strains, determining sensibility differences between serotypes.

2. Experimental Procedures

2.1. Samples collection

83 children (47 males and 36 females) were included on this cross-sectional study. All of them collected in the Clinic of the

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doi:10.1016/j.msec.2012.02.009

Advanced Education General Dentistry Program at San Luis Potosi University, México. Children's parents completed a standardized health questionnaire, where medical and oral evaluations, and the last course with antibiotics were recorded. An informed and voluntary written consent was obtained from parents prior to clinical examination according to the ethical guidelines of the Helsinki Declaration (2008), and the protocol was approved by the Research Committee of the Master's Degree in Dental Science with Specialization in Advanced General Dentistry Program at San Luis Potosi University, Mexico (reference number 102009). A non-probabilistic consecutive sampling was performed and involved 83 children. Inclusion criteria were as follows: Caries-active (CA) children without restorations, 3 to 6 years old, and either gender. Exclusion criteria included children who had received antibiotics during the last three months before starting the study.

2.2. Saliva sampling

Saliva was taken from children over a 5 min period stimulated by paraffin, after that saliva was stored in a sterilized propylene tube; this procedure was made on mornings to minimize the circadian rhythm effects. During saliva collection the children were comfortably seated in a ventilated and lighted room along with their mothers. Saliva samples were stored at −40 °C until PCR evaluations were performed.

2.3. Microbiological analysis

Saliva samples and reference strain (*Streptococcus mutans* ATCC® 25175TM) were sonicated and dispersed for 10 seconds and 10 fold dilutions were made in saline solutions (0.9% NaCl). MSB (Mitis Salivarius Agar and Bacitracin) supplemented with 20% of sucrose and 1% of potassium tellurite was used as a specific medium, 100 μL of each dilution was placed and spread by spiral bacterial plater (by duplicate). Agar plates were stored and incubated at 37 °C for 48 h with 10% CO_2 atmosphere; agar plates were then placed for 24 h at room temperature. Finally, Colony Forming Units (CFU) were estimated and *S. mutans* morphology was determined by a Stereoscopic Microscope (Olympus, SD-ILK, Japan).

2.4. Identification of S. mutans by PCR

One UFC corresponding to *S. mutans* morphology was selected from each MSB agar plate and it was inoculated in BHI broth. It was incubated 24 h at 37 °C and centrifuged to obtain bacterial pellet. It was washed in 1 mL of PBS (pH 7.4), resuspended into 200 μL of cell lysis buffer (1.0% Triton X-100, 20 mM Tris–HCl, 2 mM EDTA, pH 8.0) and incubated at 85 °C for 10 min. After that, 100 μL with 200 Units/mL of mutanolysin (Sigma, St. Louis, MO, USA) were added and incubated at 50 °C for 1 h, followed by treatment with 80 μL of nuclei cell lysis solution at 80 °C for 10 min. Then, 60 μL of protein precipitation solution was added, and proteins were then removed by centrifugation (13 000 rpm for 10 min). DNA was purified by extraction with phenol-chloroform-isoamyl alcohol (25:24:1, v/v; Invitrogen, Carlsbad, CA, USA) and further isopropanol precipitation. The extracted DNA was dissolved in 100 μL of DNA hydration solution; this solution was used for PCR (Polymerase Chain Reaction) assay. All solutions were used according to the manufacturer's protocol (Puregene DNA isolation Kit, Gentra Systems, Minneapolis, MN, USA) for Gram-positive bacteria. PCR assays were carried out in 25 μL of a reaction mixture containing 0.5 U Taq polymerase (Roche), 0.5 μM of specific primers, 5 μL (10 ng/μL of DNA template) and 1.5 mM of $MgCl_2$, following the manufacturers protocol. The PCR reaction was performed in a thermal cycler (iCycler, BIO-RAD laboratories, Hercules, CA, USA) with the following cycling parameters: an initial denaturation at 98 °C for 3 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 70 °C for 1 min, extension at 70 °C for 1 min and a final extension at 70 °C for 4 min. GS5 serotype *c*, NG71 serotype *c*, LOMA13 serotype *e*, OMZ175 serotype *f*, and FT1 serotype *k* were used as a positive controls in each PCR set by using DNA from strains for *S. mutans*. Table 1 shows primers used in this study to identify *S. mutans* and *S. mutans* serotypes. The PCR products were analyzed by electrophoresis in a 2% agarose gel using Tris-acetate-EDTA buffer and a 100-bp DNA ladder marker (New England Biolab, Beverly, MA, USA) as molecular size. Each gel was stained with ethidium bromide (0.5 μg/mL) and photographed under UV illumination (ChemiDoc, BIO-RAD laboratories, Hercules, CA, USA) for final analysis. All samples were identified and classified by PCR as *Streptococcus mutans*; after that, they were subclassified by serotypes (*c, e, f,* and *k*).

Table 1
Streptococcus mutans and serotypes oligonucleotides used for PCR.

Table 1.

SEROTYPE	NAME	SEQUENCE 5'-3'	SIZE	REFERENCE
Serotype *k*	CEFK-F	ATTCCCGCCGTTGGACCATTCC	300 pb	Nakano et al., 2004
	K-R	CCAATGTGATTCATCCCATCAC		
Serotype *c*	SC-F	CGGAGTGCTTTTTACAAGTGCTGG	727 pb	Shibata et al., 2003
	SC-R	AACCACGGCCAGCAAACCCTTTAT		
Serotype *e*	SE-F	CCTGCTTTTCAAGTACCTTTCGCC	517 pb	Shibata et al., 2003
	SE-R	CTGCTTGCCAAGCCCTACTAGAAA		
Serotype *f*	SF-F	CCCACAATTGGCTTCAAGAGGAGA	316 pb	Shibata et al., 2003
	SF-R	TGCGAAACCATAAGCATAGCGAGG		
S. mutans	MKD-F	GGCACCACAACATTGGGAAGCTCAGTT	433 pb	Hoshino et al., 2004
	MKD-R	GGAATGGCCGCTAAGTCAACAGGAT		

PCR: Polymerase Chain Reaction.
S. mutans: Streptococcus mutans.
pb: pair bases.

2.5. Silver nanoparticles preparation

Silver nanoparticles with spherical and pseudospherical shapes and three different sizes (9.3, 21.3 and 98 nanometers) were synthesized. All preparations started with a 0.01 M $AgNO_3$ solution placed in a 250 mL reaction vessel. Under magnetic stirring, 10 mL of deionized water containing gallic acid (0.1 g for 9.3 and 98 nm samples and 0.5 g for 21.3 nm sample) was added to 100 mL of silver nitrate solution. After the addition of gallic acid the pH value of the solution was immediately adjusted (for 9.3 nm sample the pH was raised to 11 with NaOH 1.0 M and for 21.3 nm sample, pH was raised to 10 with NH_4OH). For 98 nm sample, after the addition of gallic acid the mixture was irradiated with UV light (254 nm, 15 W) during 30 min (pH was not modified). After that, the solution was heated during

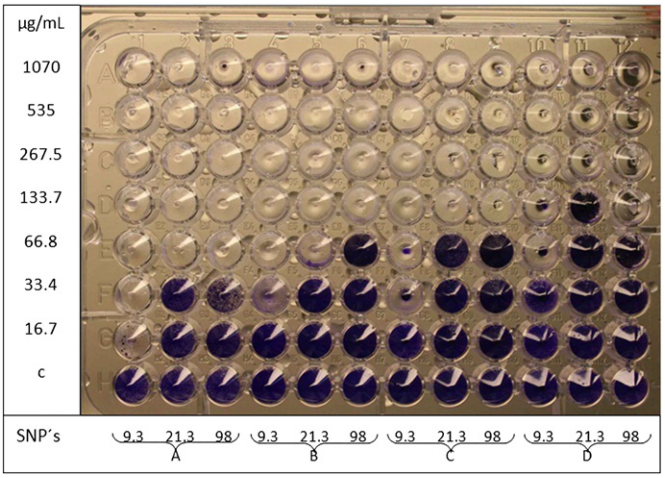

Fig. 1. MIC of silver nanoparticles with clinical and one reference strain of *Streptococcus mutans* from children. (A) serotype *c* clinical stock, (B) serotype *f* clinical stock, (C) serotype *e* clinical stock and (D) reference stock.