

APPLICATION OF NANOSILVER FOR PREVENTION OF RECURRENT DENTAL CARIES IN PATIENTS SUFFERING FROM XEROSTOMIA.

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ABSTRACT

Objective: Prevention of recurrent caries due to decreased oral clearance and an abnormal microbial milieu in patients suffering from xerostomia poses a significant challenge in restorative dentistry. The present study was designed to control Gram negative bacilli and *S. aureus* which comprise a majority of such atypical populations by using nanosilver modified dental composite.

Methods: Silver nanoparticles were synthesized by the chemical reduction process using polyvinyl pyrrolidone as a stabilizer and ethylene glycol as the reducing agent. Nanoparticles were characterized using UV visible spectroscopy and a scanning electron microscope equipped with an energy dispersive X-ray analyser. Additionally a nanoparticle analyzer was used to determine the particle size and zeta potential of the nanosilver synthesized. The minimum inhibitory concentration of nanosilver for the test organisms was determined. Further, nanoparticle impregnated Anterior/ Posterior Nano-Hybrid composite was used to evaluate microbial biofilm inhibition.

Results: The average size and charge of the nanoparticles were confirmed to be 80 nm and -10mV respectively. Atomic absorption spectroscopy revealed the concentration of the nanoparticles in solution to be 998.5 ppm. The minimum inhibitory concentration of nanosilver for *E. coli* and *Ps. aeruginosa* was found to be 0.49 ppm and 0.975 ppm respectively. A slightly higher concentration of 1.95 ppm nanosilver was required to inhibit *S. aureus*. The minimum bactericidal concentration/minimum inhibitory concentration ratio was ≤ 4 which indicated that nanoparticles displayed a predominant bactericidal activity against the test organisms. The Nano-Hybrid composite studies demonstrated a 10^2 to 10^5 fold decrease in the viable count of bacteria as compared to the control.

Conclusion: To our knowledge, this is the first study to demonstrate the potential of silver nanoparticles, for controlling the formation of secondary dental caries due to pathogenic oropharyngeal colonization in patients suffering from xerostomia.

Keywords: Xerostomia, Nanosilver, Dental composite, Abnormal oral milieu, Biofilm.

INTRODUCTION

The term xerostomia is used to encompass a spectrum of oral complaints which become apparent when more than 50% reduction in salivary secretion occurs [1, 2]. Hyposecretion of saliva is most commonly seen as a manifestation of Sjogren's syndrome, a chronic autoimmune disorder leading to the destruction of secretory acini of salivary glands [3]. Acute clinical symptoms of pain in the salivary glands, swelling and general dryness of the mouth have been reported to occur within minutes to several hours of radiation therapy. Additionally, xerostomia is also associated with other conditions such as HIV infection, vasculitis and bone marrow transplantation. Salivary hyposecretion is also observed commonly in a majority of the geriatric population.

The decreased salivary production in xerostomia patients causes reduction in oral irrigation and an inability to clear foods rapidly from the buccal cavity. This leads to changes in the oral microbial milieu. Abnormalities in clearing allow colonization of the oral cavity by potentially pathogenic organisms such as *E. coli*, *S. aureus*, *Pseudomonas*, *Klebsiella*, *Enterobacter* and yeast [4]. Hence a major complication of xerostomia is the promotion of rapid dental caries and aphthous ulcers. When salivary function is compromised there is an increase in the demineralization of the teeth, speeding the loss of tooth structure. Development of rampant caries has been observed within a few weeks after radiation therapy to the head and neck [5]. There is marked increase in the erosion and subsequent formation of dental caries, particularly observed on root surfaces and even cusp tips. In some patients, the decay becomes progressive even in the presence of vigilant oral hygiene.

Formation of secondary dental caries after tending to primary ones, remain an unresolved problem in xerostomia patients [6]. New caries develop adjacent to or beneath the restorative filling of an old cavity. Microbes on the walls of the restorative filling can gain access

to the cavity when microleakage occurs in the cementing material [7]. Ideally luting cements used as filling material, should possess bactericidal properties to limit adhesion and proliferation of pathogens at a very early stage [8]. Currently used glass ionomer endodontic cements are designed to release fluorides to suppress caries formation [9]. Numerous efforts have been made to improve the antimicrobial activity of dental restoratives with most of them focusing on slow releasing antibacterial agents such as zinc and silver ions [10]. Recently the use of nanosilver modified dental cements was explored for controlling *S. mutans*; an organism associated with tooth decay in otherwise healthy individuals [11,12,13]. The current study was designed to investigate whether a similar inhibition could be obtained against *E. coli*, *Pseudomonas* and *S. aureus*, selected as model organisms representing atypical pathogenic oropharyngeal colonization. This study could thus help to establish a method to prevent secondary dental caries, a major complication occurring in patients suffering from xerostomia.

MATERIALS AND METHODS

Culture and reagents

Cultures of *Pseudomonas aeruginosa*, *Escherichia coli* and *S. aureus* were obtained from a local hospital. Microbial suspensions were obtained from a single colony isolated on agar plates and inoculated in nutrient broth (HiMedia) for overnight cultures. After incubating microbial cells at 37°C overnight, optical density (OD) of the suspension at 600 nm was adjusted to 1.0 using a spectrophotometer (ErmaInc. Colorimeter).

The suspension was diluted with phosphate-buffered saline (pH 7.4) to 1:100 and suspended to the final concentration of 1.0×10^7 cells/mL. Silver nitrate was purchased from SD Fine Chemical Ltd. Mumbai, while ethylene glycol and polyvinyl pyrrolidone were procured from Research Lab Fine Chemical Industries, India.

Preparation of silver nanoparticles

13.5g of polyvinyl pyrrolidone (PVP) was dissolved in 75 ml of ethylene glycol (EG). 50 ml of this solution was transferred to another beaker to which 0.3g silver nitrate was added [14]. The mixture was agitated using a magnetic stirrer for 24 h at room temperature. Formation of a clear homogenous brown solution indicated the formation of the chemically synthesized silver nanoparticles (CAGNPs).

Quantification and characterization of silver nanoparticles

Characterization of the nanoparticles was done using UV visible spectroscopy (ELICO SL 207 MINI). For this 0.1 ml of test solution was diluted with 10 ml of ethylene glycol whereas 0.1 ml of PVP-EG diluted with 10 ml of ethylene glycol served as the blank solution.

Quantification of the prepared silver nanoparticles was carried out by atomic absorption spectrophotometer (Perkin Elmer AA700) using air-acetylene flame [15]. Prior to analysis, the sample was digested using nitric acid on the hot plate for 3 hrs. The dry residue obtained was diluted with 5% nitric acid and filtered through 0.45 μ filter. Further, the morphology of the CAGNPs was studied using Scanning electron microscope equipped with Energy Dispersive X ray Analyzer (Icon Analytical Pvt. Ltd). Each specimen was dispersed ultrasonically to separate individual particles before analysis. The zeta potential and the particle size distribution were confirmed with a nanoparticle analyzer (HORIBA SZ 100Z).

Determination of minimum inhibitory concentration of CAGNPs against the test bacteria

Using stock solution of the prepared silver nanoparticles, various dilutions were prepared. 0.1 ml of 24hr old test cultures *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* were added to all dilutions. Positive and negative controls were maintained. Tubes were incubated at 37°C for 24 hrs and the lowest concentration that did not show growth corresponded to the minimum inhibitory concentration (MIC).

Determination of minimum bactericidal concentration of CAGNPs against test bacteria

0.1 ml of culture from the tubes indicating minimum inhibitory concentration and all higher concentrations beyond it were surface spread on nutrient agar and incubated at 37°C for 24 hrs. The minimum bactericidal concentration (MBC) endpoint was the minimum concentration of CAGNPs at which at least 99.9% of the initial inoculum was eradicated and at which only one or no colonies could be seen on the agar.

Preparation of silver nanoparticle impregnated dental composites

For the study Anterior/ Posterior Nano-Hybrid composite (ICE A2, SDI Ltd, Australia) was used. The composite was a restorative which was composed of 22.5% wt (39% volume) multifunctional methacrylic ester and 77.5% wt (61% volume) inorganic filler (40 nm-1.5 micron). The ingredients of the composite material were removed from the syringe (1g at a time) on to the mixing pad. To this silver nanoparticle solution was added and the dough mixed together using a shaping tool to prepare small square blocks 1 cm x 1 cm in size. To harden the composites they were photocured by a LED unit (Mako T-hand held wand) with a controlled wave length of 450-470 nm for 60 seconds at one point.

Susceptibility of biofilm to silver nanoparticles

Blocks of dental composite impregnated with bactericidal concentrations of nanosilver (as determined previously) were placed in fresh nutrient broth with the test culture for 48 h. The incubation temperature was maintained at 37°C. Controls were maintained and consisted of blocks of dental composite without incorporating nanosilver.

Evaluation of biofilm

Each composite block was first dip-washed in sterile phosphate buffer saline (PBS) and gently swirled to remove any adherent

medium. They were then placed in 2 ml PBS and the adherent cells harvested by scraping with the sterile scalpel. For determining the viable count, the homogenized cell suspension was serially diluted with PBS and 0.1 ml of the culture was surface spread on nutrient agar. Plates were incubated at 37 °C for 24h and colonies counted.

Experiments were performed in triplicate and repeated three times. Six blocks and eighteen plates of viable count were prepared for each set. The data was statistically analysed using student's t-test. Values were considered significant when p value was less than 0.05.

RESULTS

Synthesis of silver nanoparticles was observed within 24 h and was indicated by the formation of a homogenous brown solution (Fig. 1).



Fig. 1: Shows brown coloured chemically synthesized nanosilver solution.

Particles exhibited maximum absorption at 420-440 nm on analysis with UV-visible spectroscopy corresponding to their surface plasmon resonance. The energy dispersive spectroscopic analysis of the nanoparticle dispersion confirmed the presence of elemental silver in the sample with trace amounts of oxygen (Fig. 2).

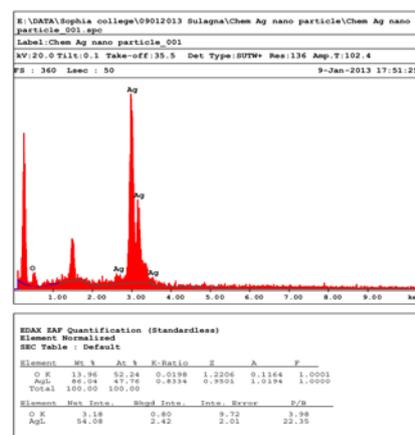


Fig. 2: EDS spectra of CAGNPs with presence of silver and oxygen.

Scanning electron micrograph revealed nanoparticles ranging in size from 60-80 nm. SEM studies showed presence of nanoparticles possessing two distinct morphologies, spherical and triangular, in solution (Fig. 3).

Further characterization using Nanoparticle Analyzer based on Dynamic Light Scattering (DLS) confirmed the size of the particles to be 80 nm.

Concentration of AgNPs in solution was found to be 998.5 ppm by atomic absorption spectroscopy. The mean surface charge of the synthesized CAGNPs was found to be -10mV. To examine the

bactericidal effect of CAgNPs, isolates of *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* were analyzed under the same growth conditions, bacterial density, incubation time and temperature. There was no difference in the median MIC and MBC values obtained in three separate experiments conducted in triplicate. As shown in Table 1 the concentration of nanosilver required to inhibit as well as kill the organisms was the same.

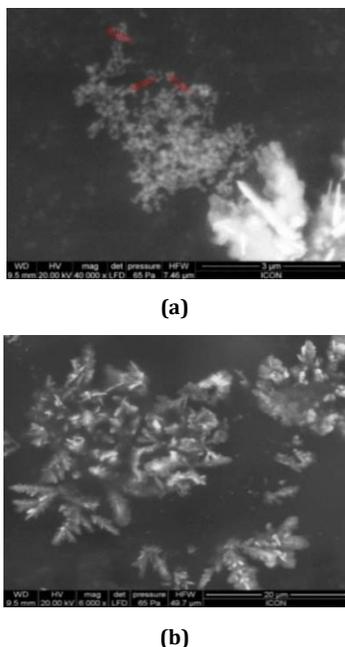


Fig. 3: SEM images of CAgNPs possessing (a) spherical as well as (b) triangular morphologies.

Table 1: MIC and MBC of CAgNPs for the test organisms in the planktonic state.

Test organism	MIC	MBC
<i>Escherichia coli</i>	0.49 ppm	0.49 ppm
<i>S. aureus</i>	1.95 ppm	1.95 ppm
<i>Pseudomonas aeruginosa</i>	0.975 ppm	0.975 ppm

Table 2: Viable count of cells present within the biofilm formed on dental composite

	Test organism		
	<i>E. coli</i>	<i>Ps. aeruginosa</i>	<i>S. aureus</i>
Test (cfu/ml)	1.1x10 ⁵	2.2x10 ⁶	2.5x10 ⁸
Control (cfu/ml)	2.8x10 ¹⁰	3.2x10 ⁸	4.1x10 ¹⁰

The experimental data show that for all organisms tested, the CAgNP-containing composites exhibited a significant decrease in the number of viable cells within the biofilm as compared to the controls. It was evident that the addition of silver nanoparticles to the Anterior/ Posterior Nano-Hybrid composite rendered it with good antibacterial property effective against the test organisms.

DISCUSSION

The current study explores the use of nanosilver impregnated dental composite as a restorative material for the control of atypical oral microbial populations. These organisms are responsible for the increased tendency in patients suffering from xerostomia, to develop severe untreatable caries. Bactericidal property is an important feature to be considered while selecting a restorative material. Silver, known for its antimicrobial properties exerts its action by causing cell lysis or inhibition of cell transduction [16]. It has a far lower propensity to induce microbial resistance than antibiotics [17]. Compared to the original bulk material nanosilver possesses a

higher surface area to volume ratio which provides an enhanced bactericidal effect. Silver nanoparticles can be made by both chemical and biological synthesis methods [18,19].

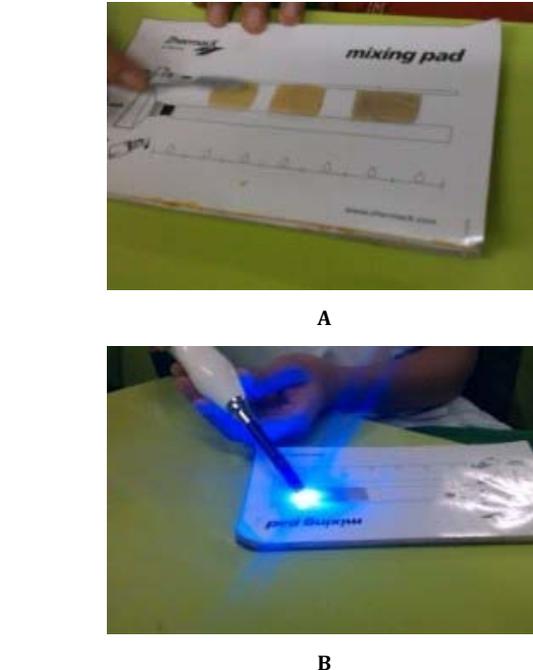


Fig. 4: a) A shaping tool being used to make blocks of 1 cm x 1 cm. (b) Using a hand held curing unit, blue light was used to form hardened blocks.

The ability of nanosilver to control atypical dental plaque microcosm growing in the form of a biofilm on the surface of dental composite was checked. The mean values of viable count are mentioned in Table 2.

In the current study, silver nanoparticles were synthesized by the chemical reduction method involving polyvinyl pyrrolidone as a stabilizer and ethylene glycol as the reducing agent. The mean size of the prepared particles was 80 nm.

In general, nanosilver have been observed to have the greatest antibacterial effect with the smallest particle sizes, with average diameters under 10 nm being most effective[11]. The hydrodynamic diameter of the nanoparticles measured by dynamic light scattering refers to how a particle diffuses within a fluid and is always greater than the size measured by electron microscopy. Previously it has been reported that nanosilver undergoes shape-dependent interaction with bacterial cells and that nanoparticles with the same surface area but with different shapes may also have different effective surface areas in terms of active facets [17]. Zeta potential

analysis indicated stability and the interaction between particles and microbial cell surfaces. Typically, nanoparticles with zeta potentials greater than 20 mV or less than -20mV have sufficient electrostatic repulsion to remain stable in solution. While the mechanism of the interaction between these particles and the constituents of the outer membrane of bacterial cells also known to possess a negative surface charge, is unfortunately still unresolved, it would appear that, despite their negative surface charge, the nanoparticles somehow interacted with the "building elements" of the bacterial membrane, causing structural changes and degradation and finally, cell death. Previous researchers too have observed similar anomaly in the surface charge and interaction between nanoparticles and bacterial cells [16]. The MIC and MBC values were determined using broth microdilution assay with the end point at which 99.9% bacteria were inhibited or killed, respectively. The MBC/MIC ratio was ≤ 4 which indicated that the nanoparticles displayed a predominant bactericidal activity.

Though the biofilm phenotype has been recognized only relatively recently in medical history, it is clear that the development of many, if not the majority of bacterial infections depends upon the formation of a biofilm. Ventilator associated pneumonia is a major complication occurring due to xerostomia and atypical dental biofilm formation in critical care patients [20]. The inhibitory action of nanosilver modified composite could mainly be attributed to the release of silver ions from the composite upon its immersion in a liquid medium. Biofilms exhibit tolerance to biocides and chemotherapeutic agents and subsequently, biofilm-associated infections are extremely difficult to treat, making them a confounding clinical problem. It is known that all microbial cells within a biofilm express increased resistance and that the concentration of an antimicrobial compound required for eliminating them is considerably higher than that required for planktonic cells [21]. In our current study, though silver nanoparticles showed a significant decrease in the number of viable cells, complete inhibition of biofilm formation was not achieved. Hence it is suggested that further experiments may be devised employing composites with higher concentrations of nanoparticles.

In conclusion, CAgNP-containing Anterior/ Posterior Nano-Hybrid composite displayed good antibacterial property. It could be used to inhibit biofilm production by organisms constituting an atypical oral milieu and prevent formation of secondary dental caries in xerostomia patients. However, further tests need to be performed to ensure that impregnation of nanosilver and increasing its concentration would not change the mechanical characteristics of the modified material and compromise its strength. Despite the effectiveness that silver nanoparticles have shown in dental practice, they remain a controversial area of research with respect to their toxicity in biological and ecological systems. Therefore any application of nanosilver in dentistry requires more study.

CONFLICT OF INTERESTS

Declared None

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