Evaluation the Gold Nano Particles' Different Sizes Effect on Dental Plaque Isolated Streptococcus Spices

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KEY WORDS
Gold nanoparticle; Streptococcus mutans; Streptococcus sanguinis; Streptococcus salivarius;

ABSTRACT
Statement of the Problem: Dental caries is considered as an important problem of public health in which Streptococcus mutans, Streptococcus sanguinis and Streptococcus salivarius have been verified as its most common etiologic bacteria.

Purpose: This study aimed to compare the antibacterial effect of chlorhexidine and three sizes of gold nano particle (25, 60, 90nm) against clinical and standard strains of Streptococcus mutans, Streptococcus sanguinis, and Streptococcus salivarius.

Materials and Method: The specimens were collected from 75 children aged 3-5 years old. These bacteria were detected by PCR and were exposed to three sizes of gold nano particle. Serial dilution method was used to compare their antibacterial efficacy. The effect of chlorhexidine and three sizes of gold nano particle were investigated by evaluating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against three bacterial strains.

Results: The MIC and MBC of different sizes of gold nanoparticles against different bacteria were statistically different (p< 0.001). For Streptococcus mutans, Streptococcus sanguinis and Streptococcus salivarius, The MIC and MBC of all sizes of gold nano particles (25, 60, 90 nm) were significantly different (p<0.001). In comparison the MIC and MBC values of patient derived bacteria and standard species in all evaluated ones and all gold nano particle sizes, significant (p<0.001) differences were reported except the MIC and MBC of 60 and 90 nm gold nano particles for Streptococcus mutans and Streptococcus salivarius.

Conclusion: The results of this study confirmed the significant size-dependency of gold nano particles for antibacterial activity. As the size gold nano particles decrease, the antibacterial properties enhance.

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Introduction
Nano is derived from the Greek word which means ‘dwarf’; nanotechnology is a science that deals with manipulation of matter at the atomic level [1-2].

Nanobiotechnology is a field of applying nano scale techniques and biomaterials for inventing new treatments, medications, drug delivery systems [3-4], enzyme immobilization, and DNA transfection [2]. This science has been developed greatly. The efficacy of nano-particles can be affected by their size.

Dental caries is the most common human infectious disease in which diverse pathogenic factors and microorganisms have been identified such as streptococcus mutans (S. mutans), streptococcus salivarius (S. salivari-
ius) and streptococcus sanguinis (S. sanguinis), salivary related disorders and individual diet [5-6].

Different treatments or preventive protocols have been introduced for dental caries. For centuries, metals have been proposed as antibacterial agents. Silver, gold, zinc, platinum [4-5] are the most common metallic agents. The antibacterial properties of metals can be affected by their contact area; larger surface of metals nanoparticles may cause more potent interactions with other molecules, which have not yet been determined [5, 7]. Recently gold nanoparticles (AuNPs) have been introduced as a novel platform for new applications including nanobiotechnology and nanobiomedicine. Gold nanoparticles have convenient surface bio-conjunction and noticeable Plasmon resonance optional properties. In addition, they have antimicrobial effect and cause bacterial membrane damage, toxicity and aggregation interference [8]. To the best of our knowledge, there were few studies about comparing different sizes of AuNPs. On the other hand, many evaluations have been confirmed the nanoparticles antibacterial effect.

Martínez-Castañón et al. [9] evaluated the antibacterial properties of silver nanoparticles (7, 29, 89nm) against Escherichia coli (E.coli) and S.aureus. Decreasing in nanoparticle size, the antibacterial activity increased in the mentioned study. Smaller silver nanoparticles can present greatest surface area, interact with bacteria in a broader surface, and reach the nuclear contact more easily.

Hernández-Sierra et al. [5] have assessed the effect of silver, zinc oxide and gold nanoparticles with average sizes of 25, 125, 80nm on S.mutans. They have confirmed the increase in contact surface by reduction of nanoparticles size.

A study has reported antibacterial effect of gold and silver nanoparticles against E.coli and Bacillus Calmette-Guerin (10). In addition, this was confirmed for silver and AuNPs against E.coli and S.aureus [3].

According to these researches, we aimed to evaluate antibacterial effects of different sizes of AuNPs against dental biofilm bacteria (such as S.mutans, S.salivarius, and S.sanguinis).

Materials and Method
In this study, 75 children aged 3-5 years old referred to Shiraz Dental Faculty were enrolled in this study during 6 months. The Ethics Committee of Shiraz University of Medical Sciences has been approved this study (IR.SUMS.REC.1395.S1017). This study has been conducted according to the Declaration of Helsinki (1975). One of the participant’s parents signed the written consent form.

Dental caries of children was assessed by using dental explorer and bitewing radiographs [11]. A total of 75 specimens from teeth plaque with dental caries were achieved by a sterile toothpick. In addition, a sterile cotton swab was employed for collecting unstimulated saliva from sublingual region. The samples were inserted into separate 1.0-mL reduced transport fluid vials [12] and sent to the microbiologic center (located in Jahrom, Fars province, Iran) for processing and laboratory evaluations. The saliva and plaque samples were diluted and placed on MM10-sucrose agar [13].

The cultures were incubated anaerobically (85% N2, 10% CO2, and 5% H2) and S. sanguinis colonies were selected based on their firm, adherent, star-shaped colony morphology [14-15], also those colonies with spherical and gram-positive anaerobic bacteria that were catalase and oxidase negative were S.salivarius.

These discrete colonies were placed on proper medium in order to detect the hydrolysis of arginine and lack of mannitol fermentation in order to differentiate S.mutans from S. sanguinis.

The prototype strain of S. sanguinis (ATCC 10556), S. mutans (ATCC 25175), and S.salivarius (ATCC9759) were used as standard species.

Polymerase chain reaction (PCR) was used for biochemical tests confirmation of all obtained specimens and detection of the S. mutans, S. sanguinis, and S. salivarius by primers pairs [16]. These primers were 5-GqAGCACCCACAACTTTGGGAAGCTCATGTT and 5-GGAATGGCCTAACTGCAACAGGAT for S. mutans (433bp) and GGATAGTGGCTCAGGGCCAGGCAGTT and GAACAGTTGCTGGACTTTGTC for S. sanguinis and MMK-GTGTGCGCCACATACCTCGTTCGG and MKK-GTGTGCGCCACATACCTCGTTCGG for S. salivarius (544 bp). The amplicons were 433bp, 313bp and 544bp size (respectively Figures 1, 2 and 3). Blast analysis was used for assessing the candidate primers sequences in the database (http://www.ncbi.nlm.nih.gov/GenBank).
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Figure 1: PCR amplification of patient isolated *S. mutans* species in this study. The electrophoresis agarose gel was stained with 0.5 µg/ml ethidium bromide and the figure was prepared by UV gel documentation system. Positive control *S. mutans* (ATCC 25175) (433bp) is also seen in this figure.

Figure 2: PCR amplification of patient isolated *S. sanguinis* species in this study. The electrophoresis agarose gel was stained with 0.5 µg/ml ethidium bromide and the figure was prepared by UV gel documentation system. Positive control (313bp) *S. sanguinis* (ATCC 10556) is also seen in this figure.

Figure 3: PCR amplification of patient isolated *S. salivarius* species in this study. The electrophoresis agarose gel was stained with 0.5 µg/ml ethidium bromide and the figure was prepared by UV gel documentation system. Positive control *S. salivarius* (ATCC9759) (544bp) is also seen in this figure.

The genomic DNA was extracted according to the manufacture direction (kit: Thermo Science, Vilnius, Lithuania). DNA ladder (Ampliquou, Denmark) PCR was performed. DNA amplification in temperature gradient thermal cycler (Biometra-T Gradient, Whatman Biometra, Göttingen, Germany).

PCR amplification of patients isolated *S. mutans*, *S. sanguinis* and *S. salivarius* species in this study are shown in Figure 1, 2 and 3. The electrophoresis agarose gel was stained with 0.5 µg/ml ethidium bromide and the figure was prepared by UV gel documentation system.

The antibacterial assessment was conducted on clinical isolated bacteria (*S. mutans*, *S. sanguinis*, and *S. salivarius*) and standard species including *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 10556) and *S. salivarius* (ATCC 9759) from the Pasteur Institute, Tehran, Iran.

They were sub-cultured in 5% sheep’s blood agar. At first, five to six colonies from an overnight culture were diluted in brain heart infusion broth and were incubated in an aerobic environmental condition for 1-2 hours at 35°C to reach the concentration of 1.5×10^8 CFU/ml. The final colonies concentration of 1.5×10^6 CFU/ml were achieved by saline solution.

The AuNPs with different sizes including 25, 60, and 90nm were selected for this study (Biometra-T Gradient, Whatman Biometra, Göttingen, Germany). According to the supplier, nanoparticles were more than 99% pure after ignition. A water- based solution of nanoparticles was prepared. The nanoparticles size distribution was confirmed by ultraviolet-visible spectroscopy (Shimatzu, Kyoto, Japan) and a particle size analyzer (Zetasizer, Nano-ZS, Malvern, Herrenberg, Germany) (Figure 4). Mean size ranged from 25 to 90nm for nanoparticles.

Colloidal solutions of nanoparticles with initial concentration of 500µg/ml, were sterilized in gravity autoclave before anti-microbial tests.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed in this study. The MICs for prepared solutions were assessed by spectrophotometric microdilution method (SMM) and turbidity. For each strain, we used a 96-well ELISA plate, and solutions were colored with resazurin (Sigma, St Louis, MO, USA). Row 1 was filled with chlorhexidine as control and 140 µL BHI (brain heart infusion agar), 50 µl of control solution and 10µl...
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Figure 4: Gold nano particles with size of 90, 60, and 25nm in a, b, c respectively

of bacterial culture (about 108 colony-forming units/mL). Pure culture media and bacterial solution were poured in a row as negative and positive control.

Three rows were filled with 100 µl of experimental solutions, 100 µl BHI, and 10 µl of culture. Then they were incubated at 37°C for 18 hours, and before and after incubation, automatic ELISA tray reader (Read-well Plate) adjusted at 524 nm specified the absorbance of each well, then to assure the true viability of antimicrobial activity, all wells were filled with the oxidation-reduction indicator resazurin [17].

A row for checking the viability of bacteria strains, and another one for assessing the sterility of experimental solutions, and the medium were considered and nanoparticles alone were added in another row.

MBC was specified when no visible bacterial growth on plates with Brain Heart Infusion Agar which had been incubated at 37°C for 24 hours was detected. All tests were conducted three times.

Evaluation the MIC and MBC values of AuNPs different sizes against standard species of S.mutans, S.salivarius, and S.sanguinis was also performed, data were analyzed by SPSS version 18. In order to compare the MIC and MBC values two way ANOVA and Poshoc Tukey were used.

Results
The MIC and MBC of AuNPs against patient isolated bacteria and standard species of S.mutans, S.salivarius, and S.sanguinis are reported in Table1 and 2 respectively. The MIC and MBC of different sizes (25, 60, 90nm) of AuNPs against different patient isolated bacteria and standard species (S.mutans, S.salivarius and S.sanguinis) were statistically different (p< 0.001). The only exceptions are the MIC and MBC of 60 and 90nm AuNPs against S.mutans and S.salivarius, which were not significantly different. For patient isolated bacteria and standard species, the reported MIC and MBC of 25 nm AuNPs against all three spices of streptococcus (S.mutans, S.salivarius, and S.sanguinis) were very lower than 60 and 90 nm AuNPs. In addition, S.mutans was more susceptible for AuNPs than S.salivarius and S.sanguinis.

Table 1: MIC and MBC mean of Au NPs against patient derived S.mutans, S.salivarius, S.sanguinis

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Bacteria</th>
<th>MIC mean(µg/ml)</th>
<th>MBC mean(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>S.mutans</td>
<td>1.73±1.23</td>
<td>4.05±2.68</td>
</tr>
<tr>
<td></td>
<td>S.sanguinis</td>
<td>3.17±1.50</td>
<td>6.46±2.98</td>
</tr>
<tr>
<td></td>
<td>S.salivarius</td>
<td>2.86±1.58</td>
<td>6.09±3.15</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2.51±1.55</td>
<td>5.41±3.09</td>
</tr>
<tr>
<td>60</td>
<td>S.mutans</td>
<td>91.61±46.39</td>
<td>184.65±91.37</td>
</tr>
<tr>
<td></td>
<td>S.sanguinis</td>
<td>148.21±64.46</td>
<td>289.28±124.15</td>
</tr>
<tr>
<td></td>
<td>S.salivarius</td>
<td>119.31±63.20</td>
<td>242.42±124.76</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>117.46±61.82</td>
<td>234.37±119.97</td>
</tr>
<tr>
<td>90</td>
<td>S.mutans</td>
<td>500</td>
<td>717.26±236.59</td>
</tr>
<tr>
<td></td>
<td>S.sanguinis</td>
<td>353.57±130.34</td>
<td>329.53±326.91</td>
</tr>
<tr>
<td></td>
<td>S.salivarius</td>
<td>329.54±124.28</td>
<td>302.53±141.42</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>299.10±136.42</td>
<td>277.47±294.03</td>
</tr>
</tbody>
</table>

Table 2: MIC and MBC of Au NPs against standard S.mutans, S.salivarius, S.sanguinis

<table>
<thead>
<tr>
<th>AuNPs</th>
<th>S. salivarius (ATCC 9759)</th>
<th>S. sanguinis (ATCC10556)</th>
<th>S. mutans (ATCC 25175)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBC (µg/ml)</td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
</tr>
<tr>
<td>25nm</td>
<td>1.95</td>
<td>0.97</td>
<td>3.9</td>
</tr>
<tr>
<td>60nm</td>
<td>125</td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td>90nm</td>
<td>500</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
For instance, 25 nm AuNPs had inhibitory effect at minimum concentration of 1.73±1.23 µg/ml against *S. mutans*, while MIC was 91.61±46.39 µg/ml for 60 nm and 232.95±124.53 µg/ml for 90 nm AuNPs (Table 1).

The patient isolated bacteria had higher level of MIC and MBC for AuNPs in comparison to standard species of streptococcus.

The MIC and MBC of chlorhexidine against all three patient isolated and standard spcies of *streptococcus* (Table 2), which have been evaluated in this study, were very higher than values that have been registered for 25 nm AuNPs. In addition, chlorhexidine affected all three evaluated species of bacteria similarly.

The MIC and MBC values of AuNPs against patient isolated *S. mutans*, *S. sanguinis* and *S. salivarius* are represented in Figure 5 and 6 respectively.

![Figure 5: The MIC value of AuNPs against *S. mutans* (1), *S. sanguinis* (2) and *S. salivarius* (3)](image)

![Figure 6: The MBC value of AuNPs against *S. mutans* (1), *S. sanguinis* (2) and *S. salivarius* (3)](image)

**Discussion**

The antibacterial properties of AuNPs were inversely size dependent. The smallest AuNPs (25 nm) was the most potent antibacterial agent (*p*<0.001) and the lowest MIC and MBC has been reported for 25 nm AuNPs against evaluated both patient isolated and standard bacteria. The 25 nm AuNP was more potent than chlorhexidine against evaluated standard species of *S. mutans*, *S. salivarius*, and *S. sanguinis*. The registered MIC and MBC against patient isolated bacteria were higher than values of standard species.

The antibacterial activity of nanoparticles has been investigated in many studies in which different nanoparticles ions showed diverse range of antibacterial activity against different bacteria, gram positive and gram negative [9-10, 18].

Agnihotri *et al.* [18] confirmed size-specific antibacterial efficacy of silver nanoparticles against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Silver nanoparticles smaller than 10 nm showed considerable enhancement in antimicrobial activity; moreover, the smallest size mediated the fast bactericidal activity.

Findings of another study about the effect of size and shape of silver nanoparticles were in consistent with previous researches. MIC of 7nm silver nanoparticles against *S. aureus* and *E. coli* were the lowest [2].

In accordance to these studies Zhou *et al.* [10] evaluated silver nanoparticles antimicrobial effect against both aerobic and anaerobic oral pathogen; confirming the size-dependency of antibacterial activity of nanoparticles, the reported MIC for 5 and 15nm silver nanoparticles against *S. mutans* and *S. sanguinis* were 50µg/ml.

Hernández-Sierra *et al.* [5] reported MIC of 4.86 µg/ml for 25nm silver nanoparticles against *S. mutans*.

These values are different from our reports for AuNPs against *S. mutans* (MIC 25nm AuNPs=1.73µg/ml) and *S. sanguinis* (MIC 25nm AuNPs=3.17 µg/ml).

These differences may be related to different concentration and type of nanoparticle in addition to the method of measuring MIC.

A study in 2009 has reported the higher susceptibility of standard *S. mutans* to silver nanoparticles in comparison to the clinical isolated strains [19]. This is in line with findings of Yamamoto *et al.* [20] and present study. However, for 90nm AuNPs there were some violated case. Although there are more reports
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about antibacterial effect of silver nanoparticles [5, 21], few studies have evaluated and confirmed antibacterial activity of other kinds of nanoparticles such as Zinc Oxide nanoparticles [20, 22-23], Zn nanoparticles, and AuNPs [5]. Raghupathi et al. [22] have confirmed size dependency of ZnO nanoparticles with bacterial growth inhibitory power. In spite of using different nanoparticles, the results of previous studies are consistent with our findings. Moreover, different size and concentration of nanoparticles can play an important role in determination of the inhibitory effect on microorganisms. Although the precise antibacterial mechanism of nanoparticles is unclear, there are some theories, which explain their mechanism. Nanoparticles can attach to the cell membrane and disturb the permeability of the outer membrane. Therefore, they can enter the inner layer of membrane and stop respiratory chain dehydrogenase [24-25], disassociate the respiratory chain and oxidative phosphorylation and disable proton- motive force via cytoplasmic membrane [26]. Diminishing the size of nanoparticles can lead to more surface area of interaction whit bacterial cell membrane and increasing gold ions release and better antibacterial properties [19].

Electrostatic attraction between bacterial cell membrane and nanoparticles, produce a tendency to enhance nanoparticles accumulation [27-28] on bacterial cell membrane, which can lead to high stress in bacterial membrane and penetration of nanoparticles to cytoplasm and finally cell lysis [10]. In theory, interaction of nanoparticles with thiol groups of bacterial proteins may affect the DNA replication [29].

Several researches have theorized two possible mechanisms of antibacterial activity including increase in reactive oxygen species (ROS) production (hydroxyl radicals and singlet oxygen) [30-31] and disruption of cellular function by accumulation of nanoparticles on bacterial cell wall, in the cytoplasm or periplasm region [32-33]. Some nanoparticles can affect the bacterial junction and expression of cytokine gene [34].

The effect of nanoparticles on bacterial respiration can be explained by more resistancy of anaerobic oral bacteria such as S.mutans, S.sanguinis, S.mitis, and Actinobacillus actinomyctetemcomitans. For anaerobic bacteria, the release of nanoparticles may be blocked by insufficient air; hence, the difference in releasing ions of nanoparticles makes the diversities in antimicrobial potencies for aerobic and anaerobic bacteria [35].

Beside this item, the effect of nanoparticles on gram-negative and gram-positive species is different because of different width of their cellular wall [36].

Although there is a controversy about the relation of concentration of nanoparticles and antibacterial effect [10, 35], all articles support the size - dependency in a similar manner; as the size of nanoparticles decrease, the antimicrobial effect increase [5, 9, 19, 35].

In the current study, the antibacterial potency of three different sizes of AuNPs was evaluated on both clinically isolated and standard species. This can help evaluate the trend of resistancy in oral pathogens.

According to findings of this study, unfortunately there is an increasing and concerning trend of antimicrobial resistancy in human isolated microorganisms. Therefore, the need for introducing new antimicrobial agents is completely necessary.

AuNP has been selected in our study for evaluation because of its solubility in water and in culture media. Using water as our solvent can eliminate the antibacterial effects of other kinds of solvents as a confounding factor. Reviewing the literature revealed that more studies have used AuNPs with larger size than presenting study. The method of preparing nanoparticles and their concentration for determination of AuNPs antibacterial efficacy was different. There is a concentration limitation for nanoparticles in order to show their best antimicrobial properties. More concentration may lead to nanoparticles agglomeration and diminishing their size dependent properties.

In this study, the small sizes of AuNP have been used for antibacterial assessment. Future belongs to new commercial nano containing antimicrobial and antibiotic film agents. Further in vivo evaluations for new designed nano products such as dentifrices and mouthwashes can be recommended for future studies.

Conclusion

The results of this study confirmed the significant size-dependency of AuNPs for antibacterial activity. As the size of AuNPs decrease, the antibacterial properties enhance. The patient isolated bacteria are more resistant to antibacterial effect of AuNPs.
Conflict of Interest
The authors declare that they have no conflict of interest.

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