

King Saud University

Saudi Dental Journal





ORIGINAL ARTICLE

In vitro evaluation of antimicrobial property of silver nanoparticles and chlorhexidine against five different oral pathogenic bacteria



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Received 22 April 2018; revised 28 October 2018; accepted 28 October 2018 Available online 2 November 2018

KEYWORDS

Biofilm; Chlorhexidine; Oral bacteria; Pathogenic; Silver nanoparticles **Abstract** *Introduction:* Numerous antimicrobial agents are used to eliminate oral biofilm. However due to emergence of multi drug resistant microorganisms, the quest to find out biologically safe and naturally available antimicrobial agents continues.

Aim: To evaluate antimicrobial efficacy of silver nano-particles against five common oral pathogenic bacteria.

Objective: To determine antimicrobial activity of silver nanoparticles and chlorhexidine gluconate against oral pathogenic bacteria.

Material and Method: We used strains of Streptococcus mutans (MTCC 497), Streptococcus oralis (MTCC 2696), Lactobacillus acidophilus (MTCC 10307), Lactobacillus fermentum (MTCC 903), and Candida albicans (MTCC 183). We used commercially available silver nanoparticles (experimental group) and chlorhexidine gluconate (positive control). We determined minimum inhibitory concentration (MIC) minimum bactericidal concentration (MBC) of both agents and analyzed the data using paired 't' test, one way ANOVA and Tucky's post Hoc HSD.

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[★] Scientific Paper presented at 39th Indian Society of Pediatric and Preventive Dentistry conference, Chennai, Tamil Nadu, India – on 14th Sept – 2017.



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Peer review under responsibility of King Saud University.

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Result: Silver nanoparticles inhibited bacterial growth moderately. The mean MIC of AgNP against S. mutans was $60 \pm 22.36 \,\mu\text{g/ml}$, S. oralis $-45 \pm 11.18 \,\mu\text{g/ml}$, L. acidophilus $-15 \pm 5.59 \,\mu\text{g/ml}$, L. fermentum $-90 \pm 22.36 \,\mu\text{g/ml}$, Candida albicans $-2.82 \pm 0.68 \,\mu\text{g/ml}$ respectively. For chlorhexidine gluconate, mean MIC for S. mutans was $300 \pm 111.80 \,\mu\text{g/ml}$, S. oralis $-150 \pm 55.90 \,\mu\text{g/ml}$, L. acidophilus $-450 \pm 111.80 \,\mu\text{g/ml}$, L. fermentum $-450 \pm 111.80 \,\mu\text{g/ml}$ and Candida albicans $-150 \pm 55.90 \,\mu\text{g/ml}$. MIC and MBC values of AgNP were significantly lower than chlorhexidine gluconate and statistically significant (p < 0.05).

Conclusion: Silver nanoparticles exhibited better bacteriostatic and bactericidal effect with concentration less than five folds as compared to chlorhexidine. Silver nanoparticles when used in appropriate concentration as safe alternative to present chemically derived other antimicrobial agents.

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1. Introduction

Oral biofilm is a habitat for numerous species of bacteria causing various oral diseases. Dental caries, periodontal disease and other oral diseases are caused due to various bacteria present in pathologic biofilm that is also known as plaque. These biofilm associated diseases still pose a great degree of challenge to dental professionals (Miles et al., 1938). Various species of microorganisms have been identified in plaque which is acidogenic and aciduric nature and responsible initiation as well as progression of dental caries in humans. Microorganisms, especially streptococcus, serve as an essential etiologic agent in plaque associated oral diseases, due to its peculiar property of synthesizing extracellular polysaccharides and fermenting sugars (Sheiham, 2005). The primary etiologic agents of coronal caries and root caries are the Streptococcus mutans, in association with Streptococcus sobrinus for initiation and progression of disease (van Houte et al., 1994). Secondarily implicated bacteria responsible for caries are the Lactobacillus species and some non-mutans streptococci in coronal caries, particularly the acid-tolerant strains: Streptococcus sanguis, Streptococcus gordonii, and Streptococcus oralis (Liljemark and Bloomquist, 1996; van Houte et al., 1994). Targeting such micro-organisms to prevent plaque formation and maturation has been the first line of therapy (Holla et al., 2012). Due to inadequacy of chemically synthesized antimicrobial agents and emergence of multi drug resistant microorganisms, the quest to find out more reliable, biologically safe and naturally available agents to target such microorganisms continues. The use of various chemically synthesized antimicrobials have been found to be inadequate and insufficient (Sheiham, 2005).

Nano-sized metal particles show specific biochemical and physical features when used against pathogenic microorganisms (Miles et al., 1938). Silver ions (Ag+/Ag++) are generally recognized as the bioactive agent, supplied for various clinical applications in form of numerous silver containing formulations comprising silver salts, silver oxide, metallic silver, silver chelates, and silver particles (Holla et al., 2012). Silver based compounds have been used extensively in many bactericidal applications due to its strong toxicity to a wide range of microorganisms (Morones et al., 2005). Silver ions and / or nanoparticles modulate the phosphotyrosine profile of putative bacterial peptides affecting bacterial signal transduction and inhibiting the growth of the organism and

cell lysis (García-Contreras et al., 2011). Silver compounds like silver nitrate in solution or cream form are used in medical field to treat burns and variety of infections (Morones et al., 2005).

Considering these property of silver nanoparticles (AgNP), we aimed to conduct this in vitro experimental study to evaluate antimicrobial efficacy of silver nano-particles against five common oral pathogenic bacteria.

2. Materials and method

We carried out this in vitro experimental study at Department of Pediatric and Preventive Dentistry and Department of Microbiology after gaining clearance from institutional ethical committee, letter no. MIDSR/V/PG/5231/966/A/2016.

Materials used for the microbiological experiment

- Experimental group Commercially available chemically derived AgNP powder of size-30–50 nm (Nano-world Company, Calcutta, India).
- Positive Control group Commercially available Chlorhexidine Gluconate 2% v/w (Perioshield mouth wash, Sunstar GUM-Butler, USA).

2.1. Bacterial strains tested

All the bacterial strains were procured from IM-TECH, Chandigarh, India

- 1. Streptococcus mutans (MTCC 497)
- 2. Streptococcus oralis (MTCC 2696)
- 3. Candida albicans (MTCC 183)
- 4. Lactobacillus acidophilus (MTCC 10307)
- 5. Lactobacillus fermentum (MTCC 903)

2.2. Preparation of microbial inocula

A direct colony suspension of each bacterial isolate was prepared in brain heart infusion broth after revival of bacterial cells and turbidity of each aliquot was adjusted to 0.5 McFarland standards for all.

2.3. Preparation of stock solutions of antibacterial agents

We prepared the stock solutions of both antimicrobial agents as suggested by Miles et al. (1938). We added 1 mg of AgNP powder in 10 ml of normal saline and sonicated to obtain a homogeneous suspension of AgNPs at concentration of $1000 \mu g/10 \text{ ml}$ [stock solution equivalent to $100 \mu g/ml$], (Solomon et al., 2007). Similarly we prepared a stock solution from commercially available 2% chlorhexidine gluconate solution. We added 0.4 ml of chlorhexidine in 9.6 ml of sterile distilled water to achieve a concentration of $100 \mu g/ml$ (Qaiyumi, 2007).

2.4. Determination of minimum inhibitory concentration (MIC) of AgNP and chlorhexidine through serial dilution method

We determined the antibacterial activity of AgNP and chlorhexidine gluconate through serial dilution method in brain heart infusion broth. From each stock solution, we added 1 ml of AgNP suspension and chlorhexidine solution in test tubes and diluted it further at two fold serial dilution respectively. We added 5 μ l of bacterial aliquot to all the test tubes containing antimicrobial agents (Figs. 1 and 2). The test tubes were shaken properly and incubated at 370C for 24 h. We determined MIC of both antibacterial agents by visual inspection and confirmed through spectrophotometry (OD600:0.6–0.7). The procedure was repeated five times to minimize errors (Solomon et al., 2007).

2.5. Determination of minimum bactericidal concentration (MBC) using colony forming unit method

The minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antibiotic killing the majority (99.99%) of bacterial inoculums. Since MIC is the ability of inhibitory status, it is possible that if the antibiotic is removed, the micro-organism will begin to grow again (Ten Cate, 2012). As AgNP particles from stock solution were non diffusible on nutrient agar, we decided to conduct MBC procedure for both antimicrobial agents through inoculation of MIC broth on culture plates containing nutrient agar. The total number of colonies appearing on culture media determined MBC of the antimicrobial agents (Figs. 3 and 4). If the culture plates showed bacterial growth in form of colony forming units (CFU), less than 30, then it is considered as sensitive and MBC for that antimicrobial agent (Pérez-Díaz et al., 2015).

2.6. Statistical analysis

The data obtained was subjected to statistical analysis with SPSS version 22.0 statistical package for MS Window (SPSS Inc. Chicago, IL, USA). Intra group mean and standard deviation was analyzed using paired 't' test. Inter group mean and standard deviations were analyzed using One way ANOVA and statistical significance was analyzed using Tucky's Post Hoc HSD test (P < 0.05).



Fig. 1 MIC of AgNPs by broth dilution method.



Fig. 2 MIC of chlorhexidine by broth dilution method.



Fig. 3 MBC of AgNPs by CFU counts.

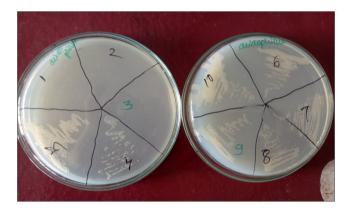


Fig. 4 MBC of chlorhexidine by CFU counts.

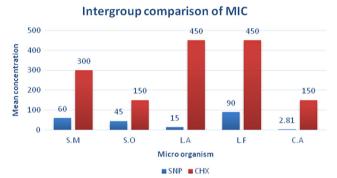
3. Results

Following tables show results of the antimicrobial agents used against bacteria in this study. Tables 1 and 2 shows that the mean MIC of AgNP particles lies between 2.82 \pm 0.68 and

 $90\pm22.36~\mu l/ml~(p<0.001-highly significant)$ as compared to mean MIC of Chlorhexidine gluconate was seen between $150\pm55.90~$ and $~450\pm111.8~\mu g/ml~$ (p <0.001~- highly significant).

It is evident that the amount of AgNP particles required to inhibit bacteria was five times lesser than chlorhexidine gluconate. Graph 1 show intergroup comparison of MIC between AgNP particle and Chlorhexidine gluconate. The number of bacteria inhibited by these antibacterial agents were variable, of which S. Mutans (p = 0.002) and S. Oralis (p = 0.003) were moderately inhibited. However L. Acidophilus (p < 0.001), L. Fermentum (p < 0.001) and C. Albicans (p < 0.001) were significantly inhibited by AgNP (see Table 3).

Tables 4 and 5 show that the mean MBC of AgNP particle was seen between 9 \pm 2.23 and 119.6 \pm 4.33 µl/ml (p < 0.001 – highly significant) as compared to mean MBC of Chlorhexidine gluconate lying between 11.80 \pm 0.27 and 18 \pm 1.06 µg/ml (p < 0.001 – highly significant). However to kill bacteria, the amount of AgNP particles required were approximately same as chlorhexidine gluconate except L. Fermentum which required very high amount of AgNP particles were required to kill bacteria (see Table 6).



Graph 1 Mean MIC of AgNP and Chlorhexidine against oral pathogenic bacteria.

Organism	Samples, $N =$	Minimum	Maximum	Mean	S. D.	P value
S. Mutans	5	50.00	100.00	60.00	22.36	< 0.001**
S. Oralis	5	25.00	50.00	45.00	11.18	
L. Acidophilus	5	12.50	25.00	15.00	5.59	
L. Fermentum	5	50.00	100.00	90.00	22.36	
C. Albicans	5	1.60	3.12	2.82	0.68	

Organism	Samples, N =	Minimum	Maximum	Mean	S. D.	P value
S. Mutans	5	250.00	500.00	300.00	111.80	< 0.001**
S. Oralis	5	125.00	250.00	150.00	55.90	
L. Acidophilus	5	250.00	500.00	450.00	111.80	
L. Fermentum	5	250.00	500.00	450.00	111.80	
C. Albicans	5	125.00	250.00	150.00	55.90	

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Table 3 Intergroup comparison of Mean MIC of AgNP particles and Chlorhexidine gluconate in ug/ml against oral pathogenic bacteria.

Organism	AgNP		CHX		Mean Difference	P value
	Mean	SD	Mean	SD		
S. Mutans	60	22.36	300	111.80	-240	0.002*
S. Oralis	45.0	11.18	150	55.90	-105.0	0.003*
L. Acidophilus	15	5.59	450	111.80	-435.0	< 0.001**
L. Fermentum	90	22.36	450.0	111.80	-360.0	< 0.001**
C. Albicans	2.81	0.61	150	55.90	-147.18	< 0.001**

^{*} Significant (p < 0.05).

Table 4 Mean MBC of AgNP particles in ug/ml against oral pathogenic bacteria. S. D. Minimum P value Organism Samples, N = Maximum Mean S. Mutans 5 18 19.5 18.5 < 0.001** 0.67 S. Oralis 5 10 13 12 1.14 L. Acidophilus 5 12 9 2.23 6 L. Fermentum 5 114.0 126.0 119.6 4.33 C. Albicans 5 42.0 56.0 48.0 5.47 Highly significant (p < 0.001).

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Table 5 Mean MBC of Chlorhexidine gluconate in ug/ml against oral pathogenic bacteria.							
Organism	Samples, N =	Minimum	Maximum	Mean	S. D.	P value	
S. Mutans	5	15.0	17.0	16.20	0.91	< 0.001**	
S. Oralis	5	11.5	12.0	11.80	0.27		
L. Acidophilus	5	17.5	19.0	18.00	0.61		
L. Fermentum	5	17.0	19.5	18.00	1.06		
C. Albicans	5	14.0	16.0	15.00	0.79		

^{**} Highly significant (p < 0.001).

Table 6 Intergroup comparison of Mean MBC of AgNP particles and Chlorhexidine gluconate in ug/ml against oral pathogenic bacteria.

Organism	AgNP		CHX		Mean Difference	P value
	Mean	S. D.	Mean	S. D.		
S. Mutans	18.5	0.65	16.20	0.91	2.30	< 0.001**
S. Oralis	12.0	1.14	11.80	0.27	0.20	0.514 (NS)
L. Acidophilus	9.0	2.23	18.00	0.61	-9.0	< 0.001**
L. Fermentum	119.6	4.33	18.00	1.06	101.6	< 0.001**
C. Albicans	48.0	5.47	15.00	0.79	33.00	< 0.001**

NS - Not Significant.

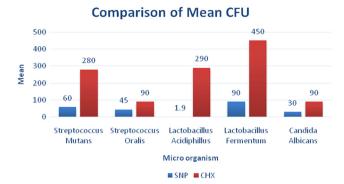
Graph 2 show intergroup comparison of MBC between AgNP particle and Chlorhexidine gluconate. It can be seen that S. Mutans (p < 0.001); L. Acidophilus (p < 0.001); L. Fermentum (p < 0.001); and C. Albicans (p < 0.001) were significantly killed by AgNP. However, S. Oralis have shown less activity against AgNP particles.

4. Discussion

In the past few decades, infections arising from antibioticresistant microorganisms have posed a great deal of threat to the field of medical science owing to increased tolerance of microorganisms against antimicrobial agents through various

^{**} Highly significant (p < 0.001).

^{**} Highly significant (p < 0.001).



Graph 2 Mean MBC of AgNP and Chlorhexidine against oral pathogenic bacteria.

mechanisms (Balappanavar et al., 2013). Many of such infectious agents grow in/on the biofilms (Sheiham, 2005). Dental plaque is an example of such pathogenic biofilm revealing a highly complex structure fashioned through bacterial activities. It is influenced by local environment, type of bacteria and oral hygiene measures (Holla et al., 2012). Likewise mechanical removal of plaque from tooth surfaces by means of brushing does not suffice sometimes due to unfavorable conditions, such as physically or medically compromised health, crowding of teeth, orthodontic treatment or periodontal diseases. Use of supplementary preventive and therapeutic measures employs antimicrobial agents, either chemically or biologically derived, to aid in plaque removal (Sheiham, 2005).

Conversely limited efficacy of antibacterial agents to penetrate the biofilm destroy the bacteria further engraves the problem (Miles et al., 1938). Thus the limited horizons of these agents have always inspired clinicians and researchers to seek biologically safer and effective agent to counteract associated problem. In the same attempt Chen and coworkers proposed the division of new anti-biofilm technologies into two groups: (i) treatments that specifically inhibit process of biofilm formation and (ii) modified biomaterials for use in medical devices to make them resistant to biofilm formation (Allaker, 2010). Several new antimicrobial agents have been developed and investigated for their efficacy to inactivate clinically important pathogenic microorganisms. Chlorhexidine gluconate is one such agent that has been studied thoroughly and proved to be effective against many oral plaque forming microorganisms (Balappanavar et al., 2013). Irrespective of its benefits, numerous untoward effects have been documented with its use (Gomes et al., 2013). Nanotechnological approaches to combat biofilm are based on the use of nano-particles on biomaterials. Surfaces of these biomaterials are functionalized through impregnating or embedding nano materials on/into the substrate (Allaker, 2010). Amongst metals, silver nano-particles (AgNP) have shown better antimicrobial activity at relatively low dose which is biologically safe for eukaryotic cells (Allaker, 2010). That's why we thought of investigating efficacy of AgNP in aqueous suspension against selected five oral pathogenic bacteria involved in plaque formation and maturation process.

Silver is known for its broad spectrum antimicrobial activity against bacteria, fungi, protozoa, and certain viruses, including antibiotic resistant strains (García-Contreras et al. 2011; Qaiyumi, 2007). These nanoparticles attack more than

one site in the bacteria like respiratory chain or cell division mechanism, reducing chance of bacteria to develop resistance (Hwan et al., 2011; Shrivastava et al., 2007). When it comes in contact with DNA and RNA of cell, interacts with soft bases of nucleic acids through interaction with sulfur and phosphorus group destroying the associated nucleic acids (García-Contreras et al. 2011). Chlorhexidine digluconate is the most thoroughly studied and effective antiplaque agent. It is widely used and considered the gold standard due to broad spectrum antimicrobial activity (Pérez-Díaz et al., 2015). It shows bacteriostatic action at low concentration and bactericidal at higher concentrations. CHX destabilizes the bacterial cell wall and interferes with osmosis (Cheung et al., 2012). Bacterial uptake of chlorhexidine is very fast which facilitates rupture of the cell wall and finally cytoplasmic membrane causing cell death (Markowska et al., 2013). However it exhibits very low toxicity due least systemic absorption and excreted in unchanged form through gut (Allaker, 2010).

In the present study, we aimed to evaluate antimicrobial efficacy of silver nanoparticles and chlorhexidine against five different oral pathogenic bacteria those are frequently found in multiple infectious oral and dental pathologic conditions like dental caries, endodontic infections, gingivitis and aphtous ulcers, etc (Allaker, 2010; Balappanavar et al., 2013; Gomes et al., 2013). In this study, we determined minimum bactericidal concentration (MIC) of both antibacterial agents through the serial dilution method. This method is more accurate compared with the disc diffusion test and is more easily interpreted (O'Leary et al., 1972). Most of the previous studies investigated the antibacterial properties of silver nanoparticles on fewer oral pathogenic bacteria and data available is also limited in terms of bactericidal properties of these nanoparticles, when compared against chlorhexidine. We haven't found any studies which have shown direct comparison of silver nanoparticles and Chlorhexidine as antibacterial agent against multiple oral pathogenic bacteria and ours may be the first one of its own kind. However there are few studies, those can be mentioned where both the antibacterial agents have been used.

Nagendrababu et al. (2017) compared antibacterial efficacy of silver nanoparticles with chlorhexidine against E. feacalis biofilm. They observed that silver nanoparticles showed better potential to eliminate bacteria as well as bacterial biofilm and possessed better disinfecting potential compared to chlorhexidine when used as an intracanal medicament. Wu et al. (2014) evaluated in-vitro antibacterial efficacy of silver nanoparticles as an intracanal irrigant (0.1% AgNP solution and 0.02% and 0.01% AgNP gel) or medicament against E. feacalis biofilms. They observed that use of 0.02%AgNP gel as medicament disrupted the biofilm structure and the proportion of viable bacteria in the biofilm significantly than irrigation with 0.1% AgNP solution and other agents, thus suggesting dependency of AgNP application mode responsible for its antibiofilm efficacy.

Besinis et al. (2012) investigated toxicity of silver and other nanoparticles with chlorhexidine against the oral pathogenic species of S. mutans. They assessed bacterial growth through MIC assay for growth, and observed that Ag NPs had the strongest antibacterial activity among all the nanoparticles tested, with equivalent bacterial inhibition at a concentration 25-fold lower than that of chlorhexidine. With AgNP concentration 0.1 mg/ml, the survival rate of bacteria was found to be only 2% compared to 60% with chlorhexidine, while the

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lactate concentration was 0.6 and 4.0 mM, respectively. Likewise silica and titanium dioxide nanoparticles had limited effects. Luckie et al. (2018) evaluated the antibacterial effect of biologicallysynthesised silver nanoparticles and chlorhexidine against S. mutans and L. casei. In this study, they observed that Ag-NPs inhibited both L. casei and S. mutans better than chlorhexidine. Ag-NPs at a concentration of 1 mg/ml showed statistically significant antibacterial effect compared to 20 mg/ml of chlorhexidine gluconate. Sadeghi et al. (2010) evaluated bactericidal effect of silver nanoparticles and chlorhexidine against S. mutans after 30 s of application as mouth wash, and they observed that silver nanoparticles exhibited statistically significant bactericidal effect as compared to chlorhexidine. Our study is in agreement to these studies.

Ahrari et al. (2015) evaluated antibacterial effects of colloidal solutions containing Silver (Ag), zinc oxide (ZnO), copper oxide (CuO), and titanium dioxide (TiO2) nanoparticles on S. mutans and S. sanguis and compared the results against chlorhexidine and sodium fluoride mouth rinses. In this study, they observed the nanoTiO₂-containing solution resulted in less number of S. sanguis after 1 min of exposure compared to other nanoparticle-containing solutions and its antibacterial effect was comparable to that of chlorhexidine. The solutions containing nanoCuO, nano ZnO and nanoTiO2 resulted in less number of S. mutans colonies after 1 and 5 min of bacterial exposure in comparison to the solution including silver nanoparticles. The antibacterial effects of all the nanoparticle groups were significantly lower than that of the 0.2% chlorhexidine mouth rinse against S. mutans. The probable reason for reduced activity of all nanoparticles in this study might be related to agglomerates of nanoparticles formed in the suspension with the larger diameter that reduces adherence and penetration of these nanoparticles inside bacterial cells to kill them. Another reason that can be mentioned about better antimicrobial efficacy of silver nanoparticles as compared to chlorhexidine is due to acidogenic and aciduric nature of the bacteria.

Most of the non haemolytic streptococci and lactobacilli can produce abundant amount of acid as metabolites in the microenvironment around them that favors oxidation of AgNP to release Ag+ and Ag++ ions (Tian et al., 2018). The antibacterial activity of AgNPs is dependent on the concomitant release of Ag+ and Ag++ ions that cause irreversible aggregation of thiol- or amine bearing molecules known as oligodynamic effect (Le Ouay and Stellacci, 2015; Xiu et al., 2012). As reported previously, the oxidation of AgNPs proceeds through complex mechanisms and is modulated by several factors. AgNPs oxidized in aqueous solutions results in the release of Ag+ at low pH environment exhibiting its antibacterial effects (Plessas et al., 2008).

Chlorhexidine is a synthetic cationic bis-guanide base, stable as a salt. In most of the oral preparations, chlorhexidine gluconate is water soluble and at physiologic pH, it readily dissociates and releases the positively charged chlorhexidine component. Conversely the quantity of chlorhexidine ions starts getting depleted due to dilution in oral cavity with time. Secondly tenacious nature of plaque does not allow chlorhexidine to penetrate to the deeper layers and eliminate microorganisms there even though it has good antimicrobial activity (Mohammadi and Abbott, 2009). The same is not true with silver nanoparticles. Being metal nano particle, it gets mechanically entangled in plaque and remains less diluted in aqueous environment. Silver nano particles being smaller in size can

penetrate into the deeper layers of biofilm and ultimately leads to the destruction of microbial cells (Tian et al., 2018).

However it should be noted that that, the study was conducted as in-vitro experiment. And it is not possible to simulate all the oral cavity conditions in the laboratory setup. Furthermore, the antibacterial agents are kept in constant contact with microorganisms in the culture media or test tubes, but the contents of antibacterial agents are diluted and neutralized in the oral cavity in very short span of time. In addition to that, we would like to advocate detailed in-vivo investigations in the same area to elucidate the antimicrobial effects of nanoparticle solutions when used as an antiplaque agent and find out side-effects of these agents on oral microflora.

5. Conclusion

In this experimental study, solution containing silver nanoparticles showed significantly higher bacteriostatic and bactericidal effect against five different oral pathogenic bacteria compared to chlorhexidine. These findings suggest that silver nanoparticles in solution or suspension form can be used as an effective antiplaque agent at very safe biological concentration.

Acknowledgement

For his valuable time to time guidance during this microbiological experiment we are thankful to, Dr. Kishor Bhat, Professor & Head, Department of Microbiology, Director, Department of molecular Biology and Immunology, Maratha Mandal's NGH Institute of Dental science & research Centre, Belgum, Karnataka, India. Pin code – 590010.

Conflicts of interest

Declared none.

Source of support

Nil

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