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ORIGINAL ARTICLE

Antibiofilm and Antimicrobial Effectiveness of Chlorhexidine Hexametaphosphate Nanoparticles as a Coating for Orthodontic Miniscrews

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ABSTRACT

Introduction: Inflammation around miniscrews can be responsible for about 30% of failures of these devices that associated with miniscrews insertion transgingivally, which straightly accessible to all forms of microorganisms existing in the oral cavity. Chlorhexidine hexametaphosphate has been described as a material that offers persistent, slow release of active chlorhexidine over time when nacked to an aqueous conditions.

Aims of study: To test the antibiofilm and antimicrobial effects of OMS coated with antimicrobial nanoparticle (Chlorhexidine hexametaphosphate).

Materials and Methods: The sanitized orthodontic minisecrews coated by nanoparticles, to establish the effectiveness of nanoparticles against formation of biofilm for S. mutans, A. actinomycetemcomitans and C. albicans. The agar method was used to evaluate the antimicrobial validity of the nanoparticles coated OMS. Gram-positive bacteria including S. mutans and S. gordonii gram negative like A. actinomycetemcomitans, p. gingivalis and Candida were used to test the antimicrobial activity of the titanium and stainless steel coated orthodontic miniscrews. After incubation for 24 hours at 37℃, the agar plates were distant from the incubator and the zone of inhibition was examined for each OMS in mm using a ruler around each disc. The zone of inhibition was measured in terms of length (mm), breadth (mm), and area (mm^2) due to linear shape of samples. Area of the ellipse was calculated as π (l/2 × b/2).

Results: The inhibition areas around stainless steel coated orthodontic miniscrews were larger than those around the titanium ones for all types of microorganisms. It were statistically significant for S. mutans and A. Actinomycetumcomitance but not significance for S. Gordonii, P. gingivalis and candida. The inhibition areas for all bacteria were comparable but notably larger than that for candida. Chlorohexidine hexametaphosphate nanoparticles exhibited powerful antibiofilm activity against the selected S. mutans, A. Actinomycetumcomitance and candida.

Conclusion: This study revealed that OMS coated by CXH-HMP nanoparticles as antimicrobial agent can provide significant antimicrobial activity that inhibit bacterial growth and aid in biofilm reduction.

Keywords: Orthodontic miniscrew, CHX-HMP nanoparticle, Antimicrobial and antibiofilm.

INTRODUCTION

The use of orthodontic miniscrews are to be responsible for anchorage for force submission to be simplified and enhanced the effectiveness of various orthodontic treatments (Casaña-ruiz et al., 2020). The colonization of pathogenic bacteria on miniscrew surfaces has been mentioned as one of the causative factors for the failure of these means, which result in implant mobility and loss (Andrucioli et al., 2018; Garcez et al., 2020). These inflammations are accompanying with a 30% of the failure rate of these devices (Miyawaki et al., 2003; Wiechmann et al., 2007; Crismani et al., 2010).

The oral environs are a host to widespread range of micro-organisms which form a complicated system, from interacting of different species of microorganisms with each other along with the hard and soft tissues of the mouth. Whereas the incidence of microbes and biofilm is unavoidable, therefore managing the overall bacterial load along with the prevalence of specific pathogens is of great importance to ensure good oral health (Quirynen and de Soete, 2002; Garner et al., 2021). Miniscrews are inserted transgingivally and are therefore straightly accessible to all types of microorganisms present in oral cavity, mainly bacteria associated with periodontitis and peri implantitis. These bacteria can invade with the miniscrew, initiating infection of soft tissues and adjacent areas, especially in poor oral hygiene patients (Apel et al., 2009; Andrucioli et al., 2018).

The antibiotics uses to prevent postoperative infection associated with implant is questionable due to its ability to increase the antimicrobial resistance, alternate methods should be investigated (Park et al., 2018). Promising recent strategies in avoidance of biofilm creation on the implant surfaces are grounded on three lines. At first, changing the surface geography aid to avoid bacterial adhesion, releasing of antimicrobial agents from surfaces for a definite time for the adhesion inhibition as well as bacteria killing in the surrounding area, and in the surfaces treated with antimicrobial agents that bonded permanently to these surfaces that will avoid elongated attachment of bacteria to surfaces (Hickok and Shapiro, 2018).

Chlorhexidine (CHX) is widely used in general dentistry and not usually used as a part of orthodontic treatment. It has broad-spectrum antimicrobial activity, is a cationic bisbiguanide which is active against a wide variety of bacteria and yeasts. By binding to the inner membrane of bacteria and the permeability of the cell wall will enhanced, which lead to loss of cell components, cytoplasm precipitation, and cell death (Kamarudin et al., 2020).The solubility of CHX salt means that it will rapidly release when contacting with fluid, which result in a great early concentration without sustained release. Lately, compressed CHX phosphate salts have been described. CHX hexametaphosphate (CHX-HMP) is a cautiously soluble CHX which, when get a contact with aqueous condition, a sluggish release of active, soluble CHX over an long period of time under aqueous conditions (Duckworth et al., 2020).

Chlorhexidine hexametaphosphate (CHX-HMP NPs) were used to coat the dental implants, coated surfaces exhibited antimicrobial effect against S. gordonii, which is primary colonizing bacterium within 8 hours (Al-obaidy and Greenway, 2021). Recently were used to coat the orthodontic elastomeric ligature and power chain which also exhibited effective release of CHX to act as active antimicrobial agent (Kamarudin et al., 2020; Subramani et al., 2020)

Therefore, the main aims of this study were to estimate the antibiofilm and antibacterial activity of chlorhexidine hexametaphosphate of coated orthodontic miniscrew.

MATERIALS AND METHODS

Orthodontic Miniscrews: The orthodontic miniscrew (OMS) were divided into 4 groups: 2 control groups (one of stainless steel and one titanium). The other two experimental groups were stainless

steel and titanium OMS coated with the CHX-HMP nanoparticles (NP).

Miniscrews preparation and coating: Each miniscrew was supplied from the manufacturer in a small pouch. The miniscrews were sterilized by autoclave steam sterilization at 121ºC for twenty minutes, according to the manufacturer instructions. To coat the sample with nanoparticles, each sterilized miniscrew was carefully removed from the set to prevent any contamination and inserted directly a test tube containing 10ml of the previously prepared nanoparticle colloidal suspension for 30 seconds being stirred on a rapid stirrer device. Then it was picked up with a pair of tweezers, inserted in deionized water for 10 seconds, before being blotted to let dried in air and to remove excess water. Finally, it was inserted in a sealed tube with a clear label till the test day(Wood et al., 2015).

Assessment the antimicrobial effects of the coated miniscrews: The agar method included the subsequent steps: A standard inoculum of the isolated microorganisms was first obtained, since the bacterial number in a liquid medium can be detected by comparing the turbidity of the liquid medium visually to a standard the characterizes an identified number of bacteria in the suspension. Thus, standard inoculums were organized for each bacterial that fit to 0.5 McFarland Nephelometer Standard (Kim and Shine, 2014; Gholam, 2018; Fadhil, 2020).

Gram-positive bacteria including S. mutans and S. gordonii gram negative like A. actinomycetemcomitans, p. gingivalis and C. albicans were used to test the antimicrobial activity of the titanium and stainless steel coated orthodontic miniscrews.

Culturing: One hundred microliters from each type of bacterial suspension were taken individually using a micropipette, and then added to the petri dishes having Muller-Hinton agar then splashed using a sterile cotton swap with a gentle touch in all direction of the petri dish. The coated and uncoated OMS from stainless steel and titanium were shown on the Muller-Hinton agar directly by sterile tweezers using and it well modified on the surfaces agar, only two miniscrews from each type for every petri dish. Then, the petri dish lids were closed and incubated in an aerobic environment at 37 C^{o} within anaerobic jar with gas packs.

Measurements of the bacterial inhibition zones: After incubation for 24 hours at 37℃, the agar plates were tacked from the incubator and the zone of inhibition was examined for each OMS in mm using a ruler around each sample. Due to linear samples, the zone of inhibition (ZOI) was measured in terms of length (mm), breadth (mm), and area (mm^2) (Figure 1). The ellipse area was calculated as π (l/2 × b/2) (Venugopala et al., 2017; Hameed et al., 2018). The inhibition zones were measured by two microbiologists independently and for each zone, a mean for their readings was considered to be used for statistical analysis (Garg and Garg, 2010).

Measuring antibiofilm formation of the CHX-HMP nanoparticle: To define the effectiveness of the nanoparticles against biofilm formation, S. mutans, A. actinomycetemcomitans and C. albicans were used. Ninety-six well micro-titer plate method was practical (Kalishwaralal et al., 2010). Individual wells of the sterile microtiter plate were filled with 100 µL of MH broth and 100 µL of CHX-HMP nanoparticles were added from the stock of colloidal suspension. To the Mixture, 10 µL from bacteria added. The micro-titer plates were transferred to the incubator for 24 h at 37 °C. Then, the content of each well was removed carefully and washed with 0.2 mL of phosphate buffer saline for three times, in order to remove free-floating bacteria. Biofilms formed by microorganisms' adherence in wall of plate would fix with sodium acetate and then stained with crystal violet dye. Rinsing of the excess stain by washing with sterilized Millipore water and kept for drying. After that, 200 µL of 95% ethanol was added to the wells. By ELISA reader, the absorbance was measured at 620 nm, and the values achieved were reflected as an index of bacterial adherence to the walls of wells of developing biofilms. The inhibition percent was measured by using this equation:

% biofilm inhibition = {1 – (O.D. 620 of cells treated with CHX-HMP NPs /O.D. 620 of non-treated control) ×100} (Barapatre et al., 2016).

RESULTS

Antibacterial activity

Inhibition zones against all types of microorganism: The results of descriptive statistics included the min., max., means and standard deviation values of area inhibition for all types of microorganisms including S. mutans, S. gordonii, P. gingivalis, A. Actinomycetum comitance and C. albicans**.** All the data were regarded as normally distributed as Shapiro test values were greater than 0.05 for all types of microorganisms.

Figure (1) shows the inhibition zone around the coated OMS. The ZOI around all control stainless steel and titanium OMS were zero showing no microorganism growth inhibition. The inhibition areas around stainless steel coated orthodontic miniscrews were larger than those around the titanium ones for all types of microorganisms. Independent sample t-test revealed that these differences are statistically significant for S. mutans and A. Actinomycetumcomitance but not significance for S. Gordonii, P. gingivalis and candida. The inhibition areas for all bacteria were comparable but notably larger than that for candida (Table 1).

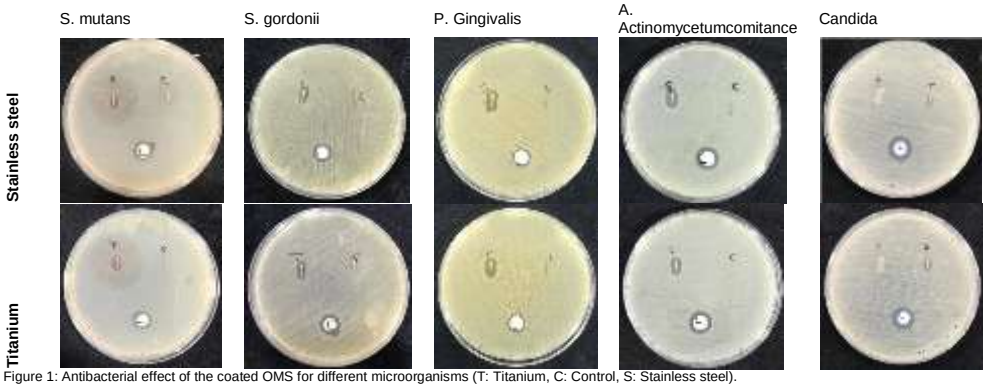

Figure 1: Antibacterial effect of the coated OMS for different microorganisms (T: Titanium, C: Control, S: Stainless steel).

Table 1: The inhibition areas of coated miniscrews for different microorganisms with independent sample t-test between the Stainless steel and Titanium miniscrews.

Microorganisms	Miniscrew type	Mean	Std. Deviation	T-test
S. mutans	S.S	36.76	0.961	p=0.011 S
	Ti	33.16	2.649	
S. gordonii	S.S	38.58	3.359	p=0.524 NS
	Ti	37.29	3.405	
A. Actinomycetumcomitance	S.S	37.53	1.400	p=0.018 S
	Ti	35.04	1.635	
P. Gingivalis	S.S	37.76	2.707	p=0.852 NS
	Ti	37.43	3.210	
Candida	S.S	27.95	1.525	p=0.064 NS
	Ti	26.25	1.290	

Antibiofilm formation: Chlorohexidine hexametaphosphate nanoparticles exhibited potent antibiofilm activity against the selected S. mutans and A. Actinomycetumcomitance and C. albicans. The prepared concentration of the nanoparticles (5mM) was greatly inhibit biofilm formation by all studied microorganisms. After different serial dilutions, the nanoparticles displays different antibiofilm activity against the tested microorganisms, regarding S. mutance the following calculated concentrations (5 mM, 2.5 mM, 1.25 mM, 0.650 mM) showed 100% of biofilm inhibition while for the next dilutions the percent of inhibition start to decrease gradually with the decrease in the concentration of CHX-HMP Nanoparticle Figure 2.a. While the antibiofilm activity of nanoparticles against A. Actinomycetumcomitance result in 100% of biofilm inhibition for the following calculated concentrations (5 mM, 2.5 mM) and the percent decreased gradually with the decrease in nanoparticles concentrations Figure 2.b.

Again, the antibiofilm activity was tested against C.albicans and the results shows that the percent of biofilm inhibition were 100% at 5mM only and the percent decreased gradually with the decrease in nanoparticles concentrations figure 2.c**.**

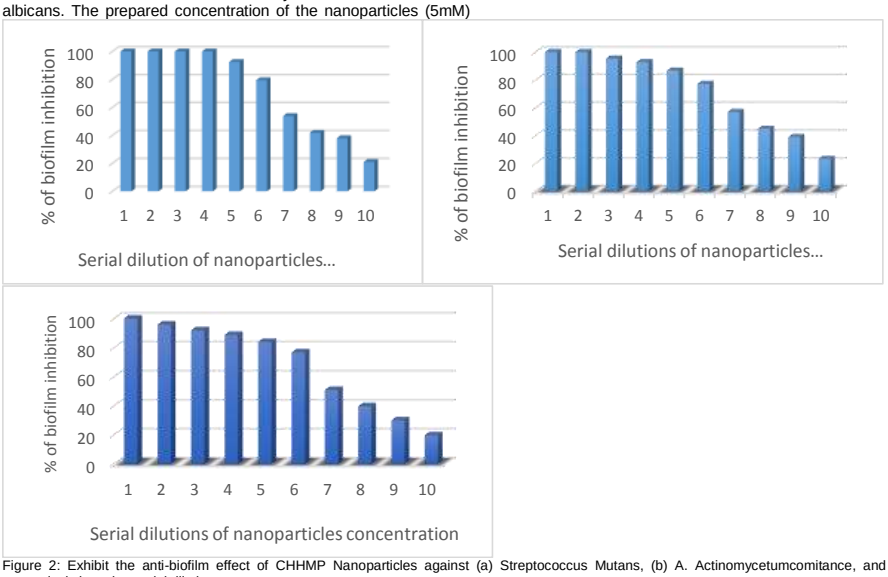

Figure 2: Exhibit the anti-biofilm effect of CHHMP Nanoparticles against (a) Streptococcus Mutans, (b) A. Actinomycetumcomitance, and (c)C. albicans respectively by using serial dilutions.

DISCUSSION

The microbiological results confirmed that sufficient and active CHX was released from OMS coated with CHX-HMP nanoparticle to be active against all studied microorganisms. This was in agreement with Subramani et al. (2020) who stated that the microbiological estimation established that sufficient and active CHX is released of CHX-HMP nanoparticle coated orthodontic elastic chain to be active against S. mutans and L. rhamnosus, where both of them are gram-positive bacteria. The results exhibit the formation of inhibition areas for all types of microorganisms. It demonstrated that the inhibition areas around stainless steel coated OMS were larger than those of titanium OMS for all types of microorganisms. This may be attributed to the larger release of CHX from stainless steel coated OMS in comparison to titanium coated OMS, as was shown in the results of the CHX elusion part of this study. Both types of coated OMS showed larger inhibition areas for S. gordonii followed by P. gingivalis, A. actinomycetumcomitance, S. mutans and the smallest was for C. albicans. These results are in agreement with a previous study by Garner et al. (2015)which stated that the metabolic activity of C. albicans was inhibited by existence of CHX-HMP-NPs in suspension due to CHX release from NP.

Sreenivasagan et al. (2020) found that around uncoated mini-implants, there were no inhibition of bacterial growth seen and maximum inhibition zone was observed for Staphylococcus aureus and the minimum for C. albicans. However, in this study the antimicrobial effect against C.albicans albicans were after 24 hours only while for the Sreenivasagan study it measured after 48 hours.

The data revealed that there are significant differences in the inhibition area around coated OMS between titanium and stainless steel on both S. mutans and A. actinomycetumcomitance, while for other types of microorganism there are no significance difference present between titanium and stainless-steel OMS. This may due to the difference in sensitivity of these microorganisms for the

selected type of OMS and antimicrobial nanoparticle or may be due to differences in the composition of bacterial cell wall.

The antimicrobial efficacy established here can be anticipated to be continued as long as the CHX–HMP NPs remain provide soluble CHX. As the releasing mechanism depend on dissolution that primarily related to the maximum coverage by NP that allow active osteoblast formation and maturation and bone production. In vivo the release of CHX from minisecrew site is generally to be slower than in vitro studies where the OMSs were submerge in water and strongly agitated. The microbial colonization on the implant surface had primary risk period during or as soon as after implantation procedure (Subramani et al., 2009). Effective treatment provided by chlorohexidine for peri-implant mucositis, so coating with CHX released NP can deliver CHX for months or weeks after surgery may be of value even if the effect is not undefined (De Siena et al., 2013; Wood et al., 2015).

The presence of biofilm may be considered as a source of dental diseases such as caries, periodontal or perimplant inflammation (Miyawaki et al., 2003). During formation of biofilm, Gram-positive bacteria such as Actinomyces and Streptococcus species are pioneer species because in general they cosider the first microorganisms that stick to the dental pellicle. Therefore, these species accomplish a crucial role in generating and sustaining circumstances that help other microorganisms to colonies and succeed on materials and dental surfaces. Their respiration route generates hypoxic conditions that render it favorable for the growth of several anaerobic species (Rimondini et al., 2015; Ramburrun et al.,, 2021). This effect may related to Glucans produced by S. mutans that provide attachment sites for the latecomer bacteria, that loss the ability to attach to the oral surface. After maturation of biofilm, the percentage of S. mutans decreases and the microbiota changes to late colonizer bacterial collections (Koo et al., 2010; Laosuwan et al., 2018).

The early losses of OMS are related to the primary bacterial biofilm formation and consequent release of metabolic byproducts (Sridhar et al., 2015). A recent review by Dhaliwal et al. (2021) found that the frequently used approaches for microbial biofilm decontamination on dental implant surfaces concentrated on the adding of chemical treatment and surface coating.

This study investigated the antimicrobial effect of CHX-HMP nanoparticles on the growth of biofilms and found that these nanoparticles exhibit a potent antibiofilm activity against S. mutans, A. actinomycetumcomitance and C. albicans.

After different serial dilutions, the nanoparticles shows different antibiofilm activity against the tested microorganisms. Antimicrobial resistance and biofilm formation related to many factors, including structure and nature, poor penetration of antibiotic, bacterial cells metabolic state, oxygen and availability of nutrient, and resistance to antimicrobial agent developed by mutation and gene transfer (Arciola et al., 2018).

Regarding S. mutans, the prepared NPs (5 mM CHX-HMP) showed 100% of biofilm inhibition and the percent of inhibition start to decrease gradually after the fourth serial dilution (0.625 mM CHX-HMP) with the decrease in the concentration of CHX-HMP nanoparticle. However, the antibiofilm activity of CHX-HMP nanoparticles on A. actinomycetumcomitance resulted in 100% of biofilm inhibition for the first two concentrations (5 and 2.5 mM) and the percent decreased gradually with the decrease in nanoparticles concentrations. This was in agreement with a previous research by Seneviratne et al. (2014) who studied the antibiofilm activity of Nano-CHX against S. mutans and A. actinomycetumcomitance and other bacterial species and concluded that Nano-CHX have potent antibacterial effects on oral biofilms.

For C. albicans, the antibiofilm activity results shows that the percent of biofilm inhibition were 100% at 5 mM only and the percent gradually decreased with the decrease in the concentrations of nanoparticles. A previous study by Alvendal et al. (2020), tested the antibiofilm effect of CHX digluconate for C. albicans, and they found that in already established biofilm, CHX digluconate discrete the biofilm and it was more effective in eliminating C. albicans compared to fluconazole. However, they used in-vitro established biofilm, which differs from that developed in the mouth that is affected by surface configuration, nutrients and mixed bacterial species provide a more favorable environment encouraging biofilm formation (Bevilacqua et al., 2018).

These results were in agreement with Garner et al. (2021) who found that the CHX released from the CHX-HMP coatings has ability to prevent formation of varies bacterial biofilm related to implant infection especially Streptococcus species, P. gingivalis and A. actinomycetumcomitance.

In conclusion, CHX-HMP nanoparticles exhibit actual antimicrobial activity against some Gram-positive and Gram-negative bacteria including Streptococcus species, P. gingivalis and A. actinomycetumcomitance in addition to C.albicans. It also shows potent antibiofilm activity regarding Streptococcus mutans, A. actinomycetumcomitance and least against C.albicans.

Commented [AA1]: There is no conclusion

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