ARTICLE

Antibacterial effect of Titanium alloys with Copper Nanoparticles: In vitro study.

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Abstract: Objective: To evaluate the in-vitro antimicrobial effect of Titanium alloys with Copper Nanoparticles (CuNP) against *Streptococcus mutans* and *Phorphyromonas gingivalis*. Materials and Methods: An in vitro study was carried out. The unit of analysis corresponded to 10 healing abutments. In 5 abutments Copper nanoparticles (CuNP) electrodeposition was applied. The remaining 5 abutments corresponded to control. The healing abutments were then immersed in culture medium for *S. mutans* and *P. gingivalis* for 14 days. Results: The agar plates with CuNP-coated abutments showed a lower growth, statistically significant for both bacterial strains. Conclusion: There is a statistically significant lower growth of *S. mutans* and *P. gingivalis* in healing abutments with CuNP.

Keywords: Metal Nanoparticles; Copper; dental implant; Anti-Bacterial Agents; Dental materials.

INTRODUCTION

Caries and periodontal disease are two of the most prevalent diseases worldwide *(Marcenes et al., 2013)*. In Chile, these diseases have a prevalence of around 98% in the population between 35-44 years old *(Urzua et al., 2012)*. Among the preventive treatments for these diseases, the application of Copper nanoparticles *(CuNP)* on dental materials has been recently described *(Hajipour et al., 2012; Zhang et al., 2013)*.

Cu appears as a material with high antibacterial levels and low toxicity, with biocidal properties and a role in the regeneration of endothelial and bone tissues *(Zhang et al., 2013; Kruk et al., 2015; Zhang et al., 2015)*. Some studies have revealed that adding Cu to a Titanium alloy results in strong antibacterial activity, without reducing mechanical properties nor corrosion resistance *(Vargas-Reus, 2012)*.

The antibacterial, antiviral and antifungal effect might derive from the size and elevated weight/volume proportion of the particles. In theory, such features should allow them to interact closely with the bacterial surface and its intracellular components *(Kruk et al., 2015; Zhang et al., 2015; Vargas-Reus, 2012; Allaker, 2010; Ren et al., 2009)*. The particles over 10nm would accumulate in the cellular membrane altering permeability and transport. On the other hand, the particles under 10nm would penetrate the membrane, accumulate intracellularly and have an effect on the nucleic acids *(Allaker, 2010; Ren et al., 2009)*.

The combination of Cu with Titanium or other medical and dental alloys has showed a great antibacterial effect on agar plates colonies count against *Staphylococcus aureus* and *Escherichia coli (Zhang et al., 2013; Kruk et al., 2015; Allaker, 2010)*. This is relevant since bacterial infections following

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implant placement are a significant source of complications *(Canullo, 2015; Belibasakis, 2015)*. Therefore, bacterial infections associated with medical and dental metal alloys still pose a great challenge in the quality of healthcare.

In spite of this, there isn't enough information regarding the antibacterial potential of CuNP in dental implants abutments against oral bacteria. The aim of this study is to evaluate the in vitro antimicrobial effect of Ti-CuNP alloys against *S. mutans* and *P. gingivalis*.

MATERIALS AND METHODS

An in vitro pilot study was carried out. The unit of analysis was 10 Alphabio-tec® healing abutments made up of a Titanium, Aluminum, Vanadium alloy *(Ti-6Al-4V)*. Electrodeposition of CuNP was applied to 5 abutments and the remaining 5 abutments were used as control.

Nanoparticle application protocol: At the Department of Materials Engineering of the Universidad de Concepción, 10 Alphabio-tec® healing abutments made up of a Titanium, Aluminum, Vanadium alloy *(Ti-6Al-4V)* were coated with CuNP. These CuNP were regular, with a size between 30 and 500 nm, and were distributed homogenously across the healing abutment. The particles were obtained from metallic Copper, reproducing the chemical features of HG Copper *(99,9% pure grade A cathode)*. An independent laboratory of the Faculty of Sciences of the Universidad de Concepción was in charge of corroborating the correct application of CuNP.

Electrodeposition of Cu on abutments was carried out using a 3-electrode setup, where a Platinum *(Pt)* plate was used as counter electrode and a standard Saturated Calomel Electrode *(SCE)* as reference electrode. Aluminum Vanadium *(Ti-6Al-4V)* dental abutments with an exposed area of 5.0mm2 were used as working electrode. Prior to the experiment, these pieces were cleansed with alcohol and distilled water. The electrolyte bath contains 120 mm of CuSO4-5H20 *(Merck 99.9%)* and 1.8 M of H2SO4. The process was performed by galvanostatic electrodeposition applying a constant current of -13,5 mA during 90 seconds. Immediately after electrodeposition, the samples were removed from the solution, washed with distilled water in order to remove non-adhered material and dried at room temperature.

Microbiological protocol: Freeze-dried strains reconstituted and cultivated at the Health Sciences laboratory of the Universidad del Desarrollo in Concepción were used.

S. mutans strain *(ATTC® 25175TM)* was spread over mitis salivarus agar 20% sacarose and then put into an anaerobiosis jar with a candle in its interior to create a microaerophilic environment for 48 hours at 37ºC in order to promote bacterial growth.

P. gingivalis strain *(ATTC® 33277TM)* was spread over Columbia Blood agar and then put into an anaerobiosis jar, which contains gas generating envelopes for the production of an anaerobic atmosphere *(GasPak)* at 37ºC during 14 days.

In order to cultivate each mentioned strain from broth to plates, 0.5% McFarland solution was prepared for McFarland visual scale. The purpose of this was to obtain a standard inoculum for each culture of *S. mutans* and *P. gingivalis* in physiological serum. This allowed the spreading of a standardized amount of bacteria *(>108 UFC)* over each agar. For each strain, three replicas were made, obtaining a total of nine Petri dishes.

Two controls in two different plates were used in order to validate the used methodology. 0.12% Clorhexidine *(CHX)* was used as positive control and sterile distilled water as negative control.

The prepared plates were marked and placed under bell jar with sterile cotton swab in order to obtain a bacteria covered plate.

The Titanium healing abutments, with and without CuNP, were sterilized and inserted by pressing them into each one of the specific agar plates prepared from each studied bacterium and incubated during 48 hrs. in anaerobiosis jars at 37ºC and 90% humidity.

After that, the antibacterial effect was observed through the presence of inhibition halo in the plates with *S. mutans* and *P. gingivalis*, according to inhibition zone width around the abutment measured in three points using a caliper. The width of the inhibition zone was calculated with the following formula.

$$\text{Inhibition zone width} = \frac{\text{(inhibition width – abutment width)}}{2}$$

In sterile test tube, 5 ml mitis salivarus broth with 200 µl bacterium *(S.mutans)* and 5 ml Columbia broth with 200 µl bacterium *(P.gingivalis)* were inoculated. These mixtures were incubated at 37ºC for 48 hrs. in culture oven. For each test tube, three replicas of each bacterial strain were made.

1 ml of 100% mixture was extracted and put over a Neubauer chamber, the sample was observed with CX 21 Olympus® microscope at 100x magnification. Three grids of the Neubauer chamber counting area were randomly selected and the bacteria present in each one of the *S. mutans* and *P. gingivalis* strains were directly counted.

A random count of the selected three areas was performed, obtaining an average, which was then multiplied by 103 in order to convert the values according to the chamber used.

Statistical analysis: The data obtained were analyzed using Stata 14.0 software *(StataCorp)*, with 95% confidence interval and 5% significance level. For descriptive analysis, the position, central tendency and variability statistics were used, in which maximum and minimum values, median, mean and standard deviation were considered. For inferential analysis, the normality was evaluated through Shapiro Wilk test, showing non-parametric distribution; therefore, Wilcoxon hypothesis tests were used.

RESULTS

Table 1 shows bacterial growth of *S. mutans* and *P. gingivalis* in abutments with and without CuNP *($p<0.05$)*.

DISCUSSION

This study revealed an inhibition of bacterial growth of *S. mutans* and *P. gingivalis* in abutments with CuNP. These findings agree with those reported by Amiri et al. *(2017)*, where an inhibition of bacterial growth of *S. mutans* in solutions of Copper oxide NP was observed, and by Vargas-Reus et al. *(2012)*, which also showed inhibition of growth in bacteria associated with peri-implantitis, such as *P. gingivalis*.

Currently, there is a tendency to strengthen biomaterials by incorporating components that reduce the risks of infection and foster tissue regeneration. Among these components, Cu, and particularly CuNP, have gained recognition as a complementary material to polymers and alloys already used in the health area, such as polypropylene, polystyrene and Ti alloys, among others. Actually, it is estimated that these polymers and alloys could benefit from the features of Cu, which include: bone regeneration, angiogenic ability, anti-inflammatory characteristics and antimicrobial features, among others *(Zhang, 2013; Allaker & Memarzadeh, 2014; Finney et al., 2009; Gérard et al., 2010; Barralet et al., 2009)*. The advantage of using CuNP, instead of proper Cu, is that it allows using less mineral amount, reducing the cost of its potential applications. Additionally, it would allow reducing the presence of potential resistance, corrosion, galvanism and cytotoxicity on cells. To sum up, reducing the concentration of the Copper mineral should have a positive impact on its biocompatibility.

Even though the biocompatibility of Copper has

TABLE 1. Bacterial count of *S. mutans* and *P. gingivalis* in abutments with and without nanoparticles.

Bacteria	Abutment	Minimum	Maximum	Median	Mean ± SD*	*p* - value
S. mutans	Without CuNP	3×10^7	3.32×10^7	3.12×10^7	$3.15 \times 10^7 \pm 1.62 \times 10^6$	0.0495
	With CuNP	0	2.4×10^6	1.6×10^6	$1.33 \times 10^6 \pm 1.22 \times 106$	
P. gingivalis	Without CuNP	2.56×10^7	2.92×10^7	2.6×10^7	$2.69 \times 10^7 \pm 1.97 \times 10^6$	0.0495
	With CuNP	0	1.2×10^6	0.4×10^6	$0.53 \times 10^6 \pm 0.61 \times 10^6$	

**Standard Deviation*

been proven, with less cytotoxicity than Silver and higher resistance to corrosion, adding Silver Nanoparticles on Ti surfaces as antimicrobial agent has been widely reported and few studies have considered adding CuNP for odontological use *(Zhang et al., 2015; Shirai et al., 2009; Ferraris & Spriano, 2016)*.

Our results show an antibacterial effect against oral pathogens, confirming what has been previously reported on fixed orthodontic appliances and Clorhexidine-based mouthwash *(Ramazanzadeh et al., 2015; Ahrari et al., 2015)*. However, the antibacterial effect of CuNP on TI alloy used for dental implants has been scarcely studied. The only findings in this field are against oral bacteria that are not classically linked to caries and periodontal/peri-implant disease, such as *E. coli* and *S. aureus (Zhang et al., 2013)*. This study is, therefore, one of the first that analyzes its effect against *S. mutans* and *P. gingivalis*.

The addition of CuNP appears as an innovative alternative, although up to date, all reported findings regarding dental use of CuNP have been developed on in vitro studies. Furthermore, regarding this and other surface treatments to improve antimicrobial properties on dental implants, it is worth noticing that one of the issues concerning this strategy is that it offers a high initial release of the agent, decreasing rapidly in the following days. Also, while with higher concentrations there is a greater effect, it must be noticed that with high concentrations there might be cytotoxical behavior *(Ferraris & Spriano, 2016)*. Nevertheless, gynecology has been using copper-based intrauterine contraceptive devices since the middle of the last century, with a high margin of safety.

Some limitations of this study are the reduced sample size and its in vitro nature. It is, thus, necessary to carry out further studies with a bigger sample size, using a substrate that is more similar to oral biofilm and recreating the conditions of the healing abutments in the oral cavity. After that, in vivo studies could be carried out.

CONCLUSION

The results showed that bacterial growth of *S. mutans* and *P. gingivalis* was statistically lower on Titanium abutments with added CuNP.

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