Hanan Ali Hameed et al /J. Pharm. Sci. & Res. Vol. 10(5), 2018, 1157-1160

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Evaluation of Antibacterial Properties of Copper Nanoparticles Surface Coating on Titanium Dental Implant

Hanan Ali Hameed[1] , Azirrawani Ariffin[2] ,Norhayati Luddin[3] , Adam Husein[4]

[1.] *PhD student, Prosthodontic Department, School of Dental Science, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia. And Asst. Lecturer, Prosthdontic Department, Babylon University College of Dentistry, Iraq.*
[2.] *Senior Lecturer, Prosthodontic Unit, School of Dental Science, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia.*
[3.] *Assoc Prof, Prosthodontic Unit, School of Dental Science, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia.*
[4.] *Professor, Prosthodontic Unit, School of Dental Science, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia.*

Abstract

Prevention of implant associated infection has been one of the main challenges in oral implantology. The emergence of antibiotic resistance crisis due to the prescribed antibiotics to treat the infection further complicates the challenge. The objective of this study was to compare the antibacterial activities of Ti, Ti Cu, Ti HA and Ti Cu/HA against *Porphyromonas gingivalis* which is considered as one of the microorganisms that most commonly related to the dental implant failure. Both qualitative and quantitative tests clearly showed that copper nanoparticles have antibacterial effect. The results suggest that the surface modification of Ti-6Al-7Nb alloy with nCu/HA may be good for use as an attractive coating for local control of infection around dental implant.

INTRODUCTION

Dental implant has become one of the therapeutic alternatives for the rehabilitation of totally or partially edentulous patients. Commercially pure Titanium (Cp-Ti) and Ti-6Al-4V alloy are widely used materials in dental implants. Nevertheless, Cp-Ti is unfavourable on its own as it displays poor mechanical properties. Hence, the use of Ti-6Al-4V alloy has been advocated to improve the mechanical properties (Semiatin *et al.*, 1997). However, some studies have discovered that Ti-6Al-4V alloy induces some inflammatory responses associated with the release of vanadium which has been reported to be toxic and affects the proliferation of peri implant cells (Venkataraman and Sudha, 2005; Ngwa *et al.*, 2009; Manivasagam *et al.*, 2010). As a result, Ti-6Al-7Nb alloy has been recommended as an alternative to Cp-Ti and Ti-6Al-4V alloy (Geetha *et al.*, 2009).

Problems of the osseous healing of implants seem to be resolved by surface modification with bioactive materials such as hydroxyapatite (HA), which is considered as a promising modification due to the similarity of its chemical composition with an inorganic component of human hard tissues like bone and teeth (Djošić *et al.*, 2012). Despite the improvement in osseointegration with HA modification, the subsequent accumulation of bacteria on implant surfaces is still the main catalyst for the initiation of inflammatory processes. The quality and quantity of plaque that accumulates on the implant abutment surface that come in contact with gingival tissue can lead to progressive loss of alveolar bone surrounding the implant (Ong *et al.*, 1992). Peri-implantitis, the pathological inflammatory change that takes place surrounding the load bearing implant tissues is considered the most common complication in orofacial implantology (Mombelli and Lang, 1998; Göthberg *et al.*, 2003). The pathogenesis of this infection has been related to the biomaterial surfaces largely affected by the initial adhesion and the colonization of bacteria.

The microorganisms that most commonly related to the failure of an implant are the Gram-negative anaerobes, like *Prevotella intermedia, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacterioides forsythus, Treponema denticola, Prevotella nigrescens, Peptostreptococcus micros and Fusobacterium nucleatum*. Some of these microorganisms look like those found around natural teeth in patients with severe chronic periodontitis, especially *Porphyromonas gingivalis* (Lee *et al.*, 1999; Heydenrijk *et al.*, 2002; Shibli *et al.*, 2003; Pye *et al.*, 2009; Henry *et al.*, 2012).

The incorporation of antibacterial agents in metal ions consisting of copper (Cu^{2+}), silver (Ag^{+}), and zinc (Zn^{2+}) in HA have been proposed to resolve the problem of implant related infections that have been associated with deficiency of antibacterial capacity in HA (Borkow *et al.*, 2010; Grass *et al.*, 2011). Several studies reported that the above metal ions in the implant coatings play a key role in minimizing or preventing initial bacterial adhesion (Kim *et al.*, 1998; Yang *et al.*, 2009; Stanić *et al.*, 2011). Among these ions, Cu^{2+} is a necessary trace element for mammals and plants because it stimulates several enzymes activities and performs a function in crosslinking of collagen and elastin of bones (Habibovic and Barralet, 2011). Cu^{2+} has demonstrated high antibacterial ability whilst maintaining a low cytotoxicity (Habibovic and Barralet, 2011; Radovanović *et al.*, 2014; Shanmugam and Gopal, 2014). To our knowledge, this is the first study that has been conducted to investigate the antibacterial properties of copper ion doped hydroxyapatite as a coating on Ti-6Al-7Nb against *P. gingivalis*.

The primary aim of the current study was to evaluate the antibacterial activity of Ti-6Al-7Nb alloy coated with copper (nCu), hydroxyapatite (nHA) and copper ion doped hydroxyapatite (nCu/HA) nanoparticles by electrophoretic deposition technique against *Prophyromonas gingivalis* bacteria. The evaluation of antibacterial agents was performed using agar diffusion and broth culture tests.

MATERIALS AND METHODS

Sample preparation

The Ti-6Al-7Nb alloy rod (American Elements, USA) were cut into disks (10 mm in diameter, 2 mm in thickness) using a lathe machine. The samples serially ground by 320, 400, 600, 800, 1000, 1200, 1500 and 2000 grits silicon carbide abrasive papers and lastly polished with 1µm diamond paste to remove any scratches. The samples were connectionally cleaned with acetone and then distilled water after each step of grinding or polishing to remove the abrasive debris. The Ti-6Al-7Nb samples (Ti) were then coated with nCu (Ti Cu), nHA (Ti HA) and nCu/HA (Ti Cu/HA) by electrophoretic deposition following process from previous studies (Razak and Sharin, 2007; Alzubaydi *et al.*, 2009).

Investigations of antibacterial effects

Agar diffusion method

In this study, the antibacterial properties of the Ti (control), Ti Cu, Ti HA and Ti Cu/HA samples were investigated against *P. gingivalis* strain (ATCC33277). A 0.5 McFarland suspension (containing approximately 1.5×10^8 CFU/ml) of standard *P. gingivalis* strain was prepared and an aliquot was spread on Colombia Sheep Blood Agar plate (OXOID Ltd., England). The coated and uncoated samples were posed on the bacteria covered

plate. Finally, all plates were incubated in an anaerobic chamber in an atmosphere of 85 % N_2, 10 % CO_2 and 5 % H_2 at 37 °C for day 1, day 2 and day 3 (Hamza *et al.*, 2015). After the incubation periods, the antibacterial activity of the samples was manifested by the formation of clear zone around each sample. The diameter of each zone was measured in milliliter (mm) using an electronic digital caliper (Libera kingdom, China). Three readings of the inhibition zone diameter surrounding each sample were taken and the average results were noted.

Broth culture test

According to international standards (ISO/EN 10993-5, 2009; ISO/EN 10993-10, 2009), the extract of Ti (control), Ti Cu, Ti HA and Ti Cu/HA were sterilized by autoclaving at 121 ◦C for 20 min and then were immersed in brain heart infusion broth with a surface area-to-volume ratio of 1.25 cm^2/ml under a condition of 37 ◦C and 5% CO_2 for 72 h to prepare the extract (Holm *et al.*, 2015).

The concentration of the bacterial solution was adjusted to 1.5×10^8 cfu ml^{-1}. Then, 1 ml of the suspension of bacteria was inoculated with each test tube containing 1 ml of extract solution. After that, the test tubes together with the control tube (growth control) were incubated in an anaerobic chamber in an atmosphere of 85 % N_2, 10 % CO_2 and 5 % H_2 at 37 °C for 24 hours. The evaluation of growth was observed by getting the optical density (OD) measurements, a primary reading was taken at zero hour in densitometer and afterward readings were taken after each two-hour up to 24 hours. The inhibition percentage of bacteria was calculated using the formula:

Inhibition percentage = $(C_0 - C)/C_0 \times 100$ (1)

where C_0 is the number of bacteria on the control tube and C is the number of bacteria on the tube with extract of samples. The statistical analysis of the antibacterial activity test was determined by repeated measures ANOVA. The results were considered significant at $p < 0.05$ level.

RESULTS

Disk diffusion test

The inhibition zone displays the degree of susceptibility of the coated sample against the bacteria. Bacteria that were sensitive to antibacterial agents showed larger inhibition zone, whereas those resistant exhibit smaller or no inhibition zone.

Figure 1A, B and C show the antibacterial activities of Ti, Ti Cu, Ti HA and Ti Cu/HA against *P. gingivalis* at day 1, day 2 and day 3 on Colombia Sheep Blood Agar. The results indicated a complete growth of bacteria that was incubated with Ti and Ti HA. However, no bacterial activity was detected around Ti Cu and Ti Cu/HA coated disks representing a remarkable antibacterial activity around the disks

The mean differences of diameter of the inhibition zones around Ti Cu and Ti Cu/HA by incubation period and by treatment group were compared and represented in Table 1 and Table 2 respectively. Table 1 shows a significant decrease in the diameter of inhibition zones in both groups from day 1 to day 3 while Table 2 reveals that Ti Cu significantly exhibited a larger zone of inhibition than Ti Cu/HA coated disk.

Broth culture test

Table 3 displays the percentage of inhibition among the groups against *P. gingivalis.* The results showed that Ti Cu and Ti Cu/HA have higher antibacterial effect on *P. gingivalis* when compared with Ti and Ti HA coated extracts. *P. gingivalis* treated with Ti and Ti HA exhibited rapid growth after 4–24 hours of incubation compared to Ti Cu and Ti Cu/HA as shown in Figure 2. The absorbance in initial stage and final stages are similar in Ti Cu and Ti Cu/HA indicating that *P. gingivalis* was highly susceptible to Ti Cu and Ti Cu/HA. Figure 3 represents the differences in the turbidity between four treatment and control (broth and bacteria) groups. The turbidity for control, Ti and Ti HA were higher as compared to Ti Cu and Ti Cu/HA groups.

Table 1: Comparison of inhibition zone formed against *P. gingivalis* among treatment groups by day

Comparison	Ti Cu n= (9)	Ti Cu/HA n= (9)
	MD(95%CI) P-value	MD(95%CI) P-value
Day 1 - Day 2	0.69< **0.001***** (0.40, 0.99)	0.69 < **0.001***** (0.38, 1)
Day 1 - Day 3	1.27 < **0.001***** (0.86, 1.67)	1.34 < **0.001***** (1.02, 1.65)
Day 2 - Day 3	0.57 < **0.001***** (0.34, 0.81)	0.65 < **0.001***** (0.54, 0.76)

Repeated measures ANOVA within group analysis was applied followed by pairwise comparison with confidence interval adjustment

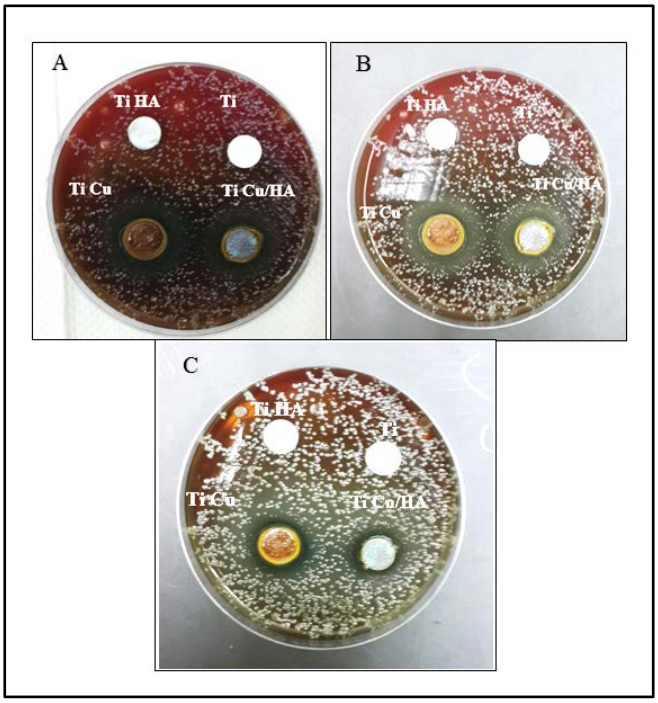

Figure 1 Zone of inhibition formed around Ti, Ti Cu, Ti HA and Ti Cu/HA at: A- day 1, B- day 2 and C- day 3

Table 2: Mean differences of the inhibition zones between Ti Cu and Ti Cu/HA against *P. gingivalis*

Mean(SD)		MD	95% CI	p-value
Ti Cu	Ti Cu/HA			
9.40(0.39)	6.37(0.35)	3.02	2.66, 3.37	< **0.001***

F-stat (df) = 3124.226(3), P-value=0.000
Repeated measures ANOVA between groups analysis was applied followed by post-hoc multiple comparisons
Level of significance at 0.05

Table 3: The percentage of inhibition among groups against *P. gingivalis*

Groups	*P. gingivalis* (n=9)
	Mean (SD)
Ti	28.57(1.96)
Ti Cu	75.46(0.87)
Ti HA	56.57(1.16)
Ti Cu/HA	87.77(0.85)

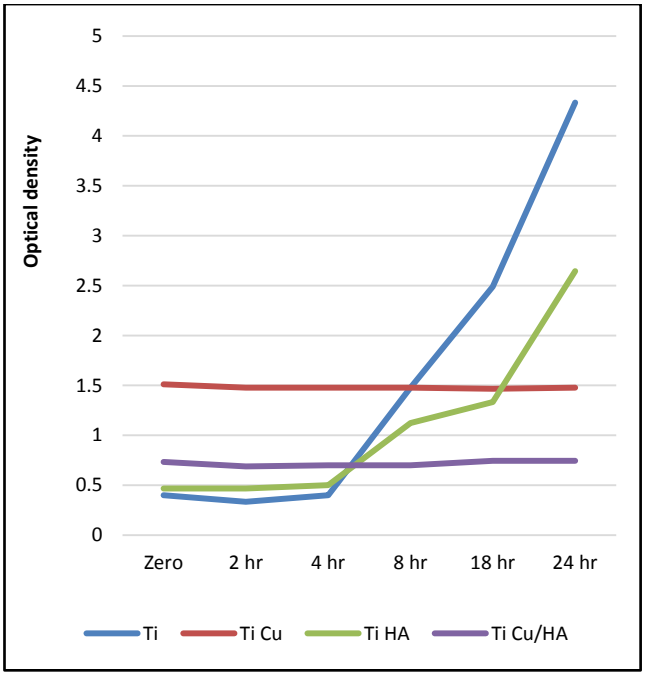

Figure 2: The antibacterial activity of Ti, Ti Cu, Ti HA and Ti Cu/HA

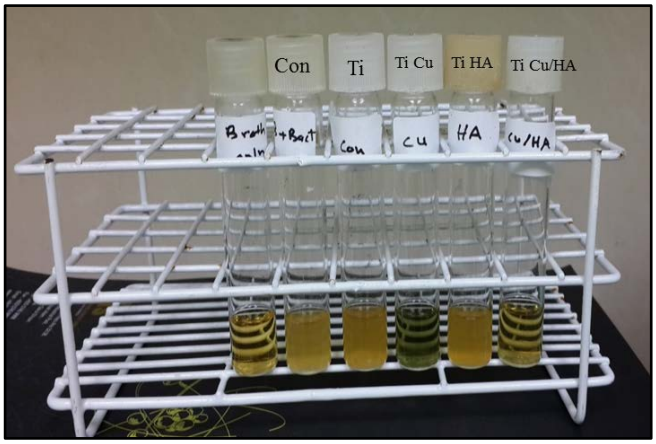

Figure 3: Turbidity evaluation for broth culture formed for Ti, Ti Cu, Ti HA and Ti Cu/HA

DISCUSSION

The main objective of our work was to study the antibacterial activity of Cu and Cu/HA nanoparticles using agar diffusion and broth culture tests. The growing interest in this field is related to the bacterial infection around dental implant which remains as a significant complication (Kalaivani *et al.*, 2014). Therefore, developing dental material with high antibacterial property helps to decrease or limit the growth of any bacteria, even after treatment in infected defects. The agglomeration of nanoparticles inside the agar medium was considered the main disadvantage of using agar diffusion test. To avoid any false negative results which present in agar diffusion tests, a broth culture analysis is required to double-check the results.

Our results revealed that Cu^{2+} released from Ti Cu and Ti Cu/HA exerted a strong antibacterial effect against *P.gingivalis* suggesting that Cu^{2+} showed good antibacterial activity against the microorganism. This finding was in accordance to the work done by Liu *et al.* who compared the antibacterial efficiency of Cu-bearing titanium alloy (Ti-Cu) in comparison to pure Ti against the same microorganism by real-time polymerase chain reaction method. The antibacterial property improves greatly when using Cu^{2+} particles in nanosize as demonstrated by (Medina *et al.*, 2007; Grass *et al.*, 2011) Likewise, in the present study, the Cu^{2+} and HA particles were synthesized in nanoparticles to improve their bactericidal effect by increasing the contact surface area against *P. gingivalis.* Until now, the exact mechanism of bacterial cell death by Cu nanoparticles is not entirely understood. Metal ion incorporation results in progressive release of membrane proteins and lipopolysaccharide molecules which can in turn cause changes in membrane permeability and deformation of the outer membrane (Madigan and Martinko, 2005). The bacterial cell death may be due to possible attachment of Cu nanoparticles to negatively charged bacterial cell wall because of the negatively charged lipopolysaccharides (Lin *et al.*, 1996). Copper ion nanoparticle surfaces interact directly with the bacterial outer membrane, causing the membrane to rupture and kill the bacteria (Chatterjee *et al.*, 2012).

In this study, the antibacterial effect of Cu nanoparticles alone was found to be highly effective than Cu/HA. The reduction in the latter group may be due to the absence of antibacterial activity observed in HA coated Ti against *P. gingivalis*, therefore reducing the diameter of inhibition when compared with samples coated with Cu nanoparticles only. The existing results are in accordance with a study by Stanić *et al.* (2010) who found the Cu/HA samples clearly confirmed an antibacterial effect on *Escherichia coli* and *Candida albicans* in disk diffusion test when compared with HA and Zn/HA that did not show antibacterial results. However, the zone of inhibitions produced on day 3 were determined considerably smaller as compared to those produced on day 1 and day 2 for Cu and Cu/HA nanoparticle groups. This decline in antibacterial activity might be retracted to the associated decline in the available concentrations of Cu nanoparticles. The decline in antibacterial activity over extended periods of time has been invariably reported by the previous researchers as well (Turkun *et al.*, 2008; Yesilyurt *et al.*, 2009; Prabhakar *et al.*, 2014). In the case of nanoparticles, these results cannot be considered conclusive due to diffusion complications.

From our study, broth culture analysis is a better index to study the antibacterial effect as the nanoparticles are always in direct contact with the bacteria. The results demonstrated that Cu and Cu/HA nanoparticles showed reduction of *P. gingivalis* in broth culture method comparable to the findings from disk diffusion test. However, Ti and Ti HA without Cu nanoparticles showed contradictory results in broth culture analysis when compared to the disk diffusion test as some bacterial reduction was observed. These findings proposed that the adhesion of bacterial cells to the particles of Ti and Ti HA may contribute to the overall reduction of the bacterial number in a liquid medium. The adhesion may facilitate contact of Cu^{2+} with cells walls and thus enable their destructive effect on the cells. However, further investigation is required to validate the proposed theory. Our findings were comparably similar to the antibacterial tests results in liquid medium from Stanić *et al.* (2010) which revealed that all metal-doped HA samples (HA, Cu/HA and Zn/HA) displayed viable cells reduction of all microorganism species.

CONCLUSION

In this work, Ti Cu and Ti Cu/HA samples clearly demonstrated an antibacterial effect on *P. gingivalis* in disk diffusion test and the results were corroborated by broth culture analysis. The antibacterial efficiency of Cu nanoparticles was dependent on the quantities of Cu^{2+}. According to this study, copper ion doped hydroxyapatite (Cu/HA) in nanoparticles is considered highly effective as an antibacterial agent and Titanium surface modification with this material can be recommended as an attractive coating for local control of infection around dental implant.

REFERENCES

[1] Alzubaydi, T. L., AlAmeer, S. S., Ismaeel, T., AlHijazi, A. Y. & Geetha, M. (2009). In vivo studies of the ceramic coated titanium alloy for enhanced osseointegration in dental applications. *Journal of Materials Science: Materials in Medicine*, **20(1)**, 35-42.

[2] Borkow, G., Okon-Levy, N. & Gabbay, J. (2010). Copper oxide impregnated wound dressing: biocidal and safety studies. *Wounds: a Compendium of Clinical Research and Practice*, **22(12)**, 301-310.

[3] Chatterjee, A. K., Sarkar, R. K., Chattopadhyay, A. P., Aich, P., Chakraborty, R. & Basu, T. (2012). A simple robust method for synthesis of metallic copper nanoparticles of high antibacterial potency against E. coli. *Nanotechnology*, **23(8)**, 085103-085114.

[4] Djošić, M., Janić, V., Stojanović, J., Mitrić, M. & Mišković-Stanković, V. (2012). The effect of applied current density on the surface morphology of deposited calcium phosphate coatings on titanium. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **400**, 36-43.

[5] Geetha, M., Singh, A., Asokamani, R. & Gogia, A. (2009). Ti based biomaterials, the ultimate choice for orthopaedic implants–a review. *Progress in Materials Science*, **54(3)**, 397-425.

[6] Göthberg, C., Bergendal, T. & Magnusson, T. (2003). Complications after treatment with implant-supported fixed prostheses: a retrospective study. *International Journal of Prosthodontics*, **16(2)**.

[7] Grass, G., Rensing, C. & Solioz, M. (2011). Metallic copper as an antimicrobial surface. *Applied and Environmental Microbiology*, **77(5)**, 1541-1547.

[8] Habibovic, P. & Barralet, J. (2011). Bioinorganics and biomaterials: bone repair. *Acta Biomaterialia*, **7(8)**, 3013-3026.

[9] Hamza, A. S., Aliyu, A. & Ibrahim, F. M. (2015). Evaluation of Antibacterial Activity of Sudanese Bee honey Against Four Species of Bacteria. *American Journal of Research Communication*, **3(4)**, 132-142.

[10] Henry, L. G., McKenzie, R. M., Robles, A. & Fletcher, H. M. (2012). Oxidative stress resistance in Porphyromonas gingivalis. *Future microbiology*, **7(4)**, 497-512.

[11] Heydenrijk, K., Meijer, H. J., van der Reijden, W. A., Raghoebar, G. M., Vissink, A. & Stegenga, B. (2002). Microbiota around root-form endosseous implants: a review of the literature. *International Journal of Oral & Maxillofacial Implants*, **17(6)**, 829-839.

[12] Holm, C., Morisbak, E., Kalfoss, T. & Dahl, J. E. (2015). In vitro element release and biological aspects of base–metal alloys for metal-ceramic applications. *Acta Biomaterialia Odontologica Scandinavica*, **1(2-4)**, 70-75.

[13] ISO/EN 10993-5. (2009). *Describes test methods to assess the in vitro cytotoxicity of medical devices. Test on extracts*.

[14] ISO/EN 10993-10. (2009). *Different methods and approaches for in vitro assessment of cytotoxicity - Part 10: Biological evaluation of medical devices*.

[15] Kalaivani, S., Singh, R. K., Ganesan, V. & Kannan, S. (2014). Effect of copper (Cu 2+) inclusion on the bioactivity and antibacterial behavior of calcium silicate coatings on titanium metal. *Journal of Materials Chemistry B*, **2(7)**, 846-858.

[16] Kim, T., Feng, Q. L., Kim, J., Wu, J., Wang, H., Chen, G. & Cui, F. (1998). Antimicrobial effects of metal ions (Ag+, Cu2+, Zn2+) in hydroxyapatite. *Journal of materials science: Materials in Medicine*, **9(3)**, 129-134.

[17] Lee, K., Maiden, M., Tanner, A. & Weber, H. (1999). Microbiota of successful osseointegrated dental implants. *Journal of Periodontology*, **70(2)**, 131-138.

[18] Lin, Y.-S. E., Vidic, R. D., Stout, J. E. & Victor, L. Y. (1996). Individual and combined effects of copper and silver ions on inactivation of Legionella pneumophila. *Water Research*, **30(8)**, 1905-1913.

[19] Madigan, M.-M. & Martinko, J. (2005) Prentice Hall. ISBN 0-13-144329-1.

[20] Manivasagam, G., Dhinasekaran, D. & Rajamanickam, A. (2010). Biomedical Implants: Corrosion and its Prevention-A Review. *Recent Patents on Corrosion Science*, **2**, 40-54.

[21] Medina, C., Santos-Martinez, M., Radomski, A., Corrigan, O. & Radomski, M. (2007). Nanoparticles: pharmacological and toxicological significance. *British Journal of Pharmacology*, **150(5)**, 552-558.

[22] Mombelli, A. & Lang, N. P. (1998). The diagnosis and treatment of peri-implantitis. *Periodontology 2000*, **17(1)**, 63-76.

[23] Ngwa, H. A., Kanthasamy, A., Anantharam, V., Song, C., Witte, T., Houk, R. & Kanthasamy, A. G. (2009). Vanadium induces dopaminergic neurotoxicity via protein kinase Cdelta dependent oxidative signaling mechanisms: relevance to etiopathogenesis of Parkinson's disease. *Toxicology and Applied Pharmacology*, **240(2)**, 273-285.

[24] Ong, E.-M., Newman, H., Wilson, M. & Bulman, J. (1992). The occurrence of periodontitis-related microorganisms in relation to titanium implants. *Journal of periodontology*, **63(3)**, 200-205.

[25] Prabhakar, A., Agarwal, S. & Basappa, N. (2014). Comparative evaluation of antibacterial effect and physical properties of conventional glass-ionomer cement containing 1% chlorhexidine and 1% xylitol. *International Journal of Oral Health Sciences*, **4(2)**, 63.

[26] Pye, A., Lockhart, D., Dawson, M., Murray, C. & Smith, A. (2009). A review of dental implants and infection. *Journal of Hospital Infection*, **72(2)**, 104-110.

[27] Radovanović, Ž., Jokić, B., Veljović, D., Dimitrijević, S., Kojić, V., Petrović, R. & Janaćković, D. (2014). Antimicrobial activity and biocompatibility of Ag+-and Cu 2+-doped biphasic hydroxyapatite/α-tricalcium phosphate obtained from hydrothermally synthesized Ag+-and Cu 2+-doped hydroxyapatite. *Applied Surface Science*, **307**, 513-519.

[28] Razak, A. & Sharin, M. F. (2007). *Electrophoretic deposition and characterization of copper selenide thin films*. (Dissertation). Universiti Putra Malaysia

[29] Semiatin, S., Seetharaman, V. & Weiss, I. (1997). The thermomechanical processing of alpha/beta titanium alloys. *Journal of the Minerals, Metals and Materials Society*, **49(6)**, 33-39.

[30] Shanmugam, S. & Gopal, B. (2014). Copper substituted hydroxyapatite and fluorapatite: synthesis, characterization and antimicrobial properties. *Ceramics International*, **40(10)**, 15655-15662.

[31] Shibli, J. A., Martins, M. C., Lotufo, R. F. M. & Marcantonio Jr, E. (2003). Microbiologic and radiographic analysis of ligature-induced peri-implantitis with different dental implant surfaces. *International Journal of Oral & Maxillofacial Implants*, **18(3)**.

[32] Stanić, V., Dimitrijević, S., Antić-Stanković, J., Mitrić, M., Jokić, B., Plećaš, I. B. & Raičević, S. (2010). Synthesis, characterization and antimicrobial activity of copper and zinc-doped hydroxyapatite nanopowders. *Applied Surface Science*, **256(20)**, 6083-6089.

[33] Stanić, V., Janaćković, D., Dimitrijević, S., Tanasković, S. B., Mitrić, M., Pavlović, M. S., Krstić, A., Jovanović, D. & Raičević, S. (2011). Synthesis of antimicrobial monophase silver-doped hydroxyapatite nanopowders for bone tissue engineering. *Applied Surface Science*, **257(9)**, 4510-4518.

[34] Turkun, L., Turkun, M., Ertugrul, F., Ateş, M. & Brugger, S. (2008). Long-term antibacterial effects and physical properties of restorative materials combined with chlorhexidine. *J Esthet Restor Dent*, **20**, 29-44.

[35] Venkataraman, B. & Sudha, S. (2005). Vanadium toxicity. *Asian J Exp Sci*, **19(2)**, 127-134.

[36] Yang, H., Zhang, L. & Xu, K.-W. (2009). Effect of storing on the microstructure of Ag/Cu/HA powder. *Ceramics International*, **35(4)**, 1595-1601.

[37] Yesilyurt, C., Er, K., Tasdemir, T., Buruk, K. & Celik, D. (2009). Antibacterial activity and physical properties of glass-ionomer cements containing antibiotics. *Operative Dentistry*, **34(1)**, 18-23.