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The Effect of Changing the Concentration of Silver Nanoparticles on the Antibacterial Properties of Silver-PVP Composite-Coated Implants

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Abstract

Background: The body's ability to fight the infection in the vicinity of implants decreases, resulting in an increased risk of local infection around the implants. According to importance of this issue, this study investigates the antibacterial properties of implants coated *via* silver-PVP nanocomposite.

Methods: In this research, silver nanoparticles have been synthesized by green synthesis method and using the coffee powder. The X-ray Diffraction (XRD) pattern confirms the formation of metallic structure of silver nanoparticles. Particle size has been studied by Transmission Electron Microscopy (TEM) and Digital Light Scattering (DLS) analysis, showing that the average particle size is about 17 *nm*. The implants have been coated *via* silver-PVP nanocomposite using deep coating method at different concentrations of Ag nanoparticles, and antibacterial properties of these samples have been investigated. The obtained results demonstrate a very large non-growth halo in the presence of silver-PVP coating. Also, the repeatability of non-growth halo test, toxicology analysis and pull off adhesion of samples have been studied and analyzed.

Results: According to the MTT results, the appropriate concentration of silver-PVP nanocomposite without cell toxicity has been obtained equal to 118.6 *μg/ml*. At this concentration, the non-growth halo for both types of bacteria is clearly visible. By repeating the antibacterial test after 5 days, it was observed that the non-growth halo of implants is lasting.

Conclusion: Overall, it can be stated that this study presents a simple, operational, repeatable, and large-scale method for coating the implants and medical equipment that prevents the development of infection in the vicinity of implants.

Keywords: Bacteria, Implants, Infection, Silver Nanoparticles

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Introduction

Medical implants are widely used to replace a biological organ, support a damaged biological structure, or strengthen the structure in a part of the body. In general, the purpose of placement of implants is to integrate the implant with the surrounding tissue in order to restore the optimal function of the structure. However, implant surfaces provide an ideal environment for the growth of bacteria. In this case, the host cells must compete with the microorganisms that may be present for adhesion to the surface. If pathogens stick to the implant surface at the beginning of implant placement, there is a risk of microbial colonization formation on the surface of the biological material, resulting in the biofilm formation and causing infection. Although prophylactic measures and flawless surgical methods have effectively reduced the incidence of implant-related infections, the prevalence of these infections remains a significant problem. For example, the rate of implant-related infections in patients with orthopedic injuries is reported to be 5 to 10%. Implant-related infections can have the serious consequences, such as implant loss, the need for revision surgery, possible systemic infections, and rot, which can have the severe impacts on the health of patients and impose huge financial burdens on patients and the health care system. Current treatments rely on topical or systemic administration of antibiotics, but the microorganisms in the biofilms can react with the antibiotics and lead to the persistence or recurrence of infection. In addition, microbial resistance is a growing global challenge, reducing the effects of existing antibiotics and antifungals, and high doses of these drugs can be toxic to human tissues.

Given the increasing in the resistance of bacteria to common antibiotics, the antibacterial effects of nanomaterials such as silver nanoparticles, Ag nanocomposites or Ag nanoparticle-based have recently received much attention (1-9). The antibacterial activities of silver nanoparticle-based materials may help reduce the infection during burn treatment (10) and in addition, it prevents the bacterial colonization of implants, prostheses, and flexible tubes inserted into the body (11,12). Furthermore, polymers are able to load germicidal agents within their matrix. Polyvinyl Alcohol (PVA) and Polyvinyl pyrrolidone (PVP) are artificial polymers and are widely utilized in pharmaceutical needs owing to their biocompatibility (13). Regarding PVA, it is a multi-hydroxyl group (O–H) polymer; thus, it has been utilized for both electrical and medical purposes (14,15), owing to high tensile strength and elasticity, and degradability (16). On the other hand, the chemical composition of PVA offers a high hydrophilic feature, leading to its ability to interact with fluids in living cells directly, besides suppressing its calcification (17,18). This behavior reduces PVA bio-applicability. As a result, its combination with different polymers such as PVP offers a chance to use it in biomedical applications (19-21). Further, polymer blending offers new materials with characteristics based on its constituents. Additionally, nanoparticles doped polymers composites introduce an interesting line in biological investigations (22). Jegatheeswaran *et al* reported the synthesis of f-HAp/PVP/Ag nanocomposite under the ionic liquid medium. This report reveals that delivery of silver and fluorine ions from the fluorhydroxyapatite surface to the bacterial surface has been reducing the bacterial growth rate (23). Norbert Harrasser *et al* determined the antimicrobial potency of different surface treatments on a titanium alloy, which had been conversed to diamond-like carbon and doped with high (Ag: PVP = 1:2) and low (Ag: PVP = 1:10 and 1:20) concentrations of silver (24). The antibacterial activity of Ag^+-implanted titanium surface modified with poly vinyl pyrrolidone (Ag^+/PVP–Ti) against *Escherichia coli* (*E. coli*) colonies was evaluated *in vitro* by Shuge Peng and Yingchun Zhu. Their results exhibited that the sterilizing efficiency of Ag^+/PVP–Ti was approximately 100% against *E. coli* while keeping a low Ag^+ release amount which was far below the human cell toxicity concentration (25).

Previous studies by researchers show that silver nanoparticles at low concentrations (*mg/L*) kill the bacteria and do not show the acute toxic effects on human cells (2,26). Also, Ag nanopowder has not been shown to cause the bacterial resistance. Unlike antibiotics, this is probably due to the fact that Ag nanopowder does not exert the antibacterial effects in a specific location, but they exert the antibacterial effects in several surfaces, such as the bacterial wall, protein synthesis, and DNA. Silver can kill

a wide range of gram-negative and gram-positive bacteria, viruses, protozoans and fungi. However, its mechanism of action is not fully specified. Researches have shown that Ag releases the biologically-active ions from its surface. The released ions bind to a number of bacterial cell structures, including the cell wall of peptides, glycans, bacterial proteins, plasma membrane, and bacterial DNA. Binding of ions to the cell wall damages the outer layers of the cell, destroying the cell contents and causing the structural abnormalities. Since gram-positive bacteria have thicker cell walls, higher concentrations of Ag are needed to prevent the *bacterial growth* compared to gram-negative bacteria (27-30).

In order to counteract the growth of bacteria and prevent the infection, antimicrobial coatings containing silver nanoparticles can be used on a wide range of medical equipment such as catheters, implants and surgical instruments. Given the high prevalence of infections caused by medical equipment and the spread of antibiotic resistance, in the research, silver nanoparticles have been produced by green synthesis method in a very simple and low-cost way. The characterization of the synthesized nanoparticles has been thoroughly analyzed and investigated. Finally, silver nanoparticles are coated on the surface of medical equipment and their antibacterial effects and toxicity have been studied.

Materials and Methods

Green synthesis of silver nanoparticles

All chemicals used were of analytical grade and were used as received without any further purification and were obtained from Sigma-Aldrich. The method introduced in the reference (31) was utilized to synthesize silver nanoparticles. Brazilian coffee powder was used to synthesize the silver nanoparticles. 2 *g* of coffee powder was added to 200 *ml* of boiling water. It was kept in the boiling state for 10 *min*. Then, it was filtered using filter paper and its pulp was disposed.

0.3 *g* of silver nitrate was mixed with 20 *ml* of deionized water and stirred until it completely dissolved. The silver solution was mixed with 60 *ml* of filtered coffee and slightly stirred. The final solution was kept in darkness for 24 *hr*. The obtained nanoparticles were then filtered through filter paper and centrifuged 3 times with deionized water. The resultant products were collected and dried at 50°*C*. The obtained nano-powders were characterized by XRD, TEM, DLS, FTIR, and UV-Vis.

Implant coating

All implants used in this study were obtained from Osveh Medical Instrument Company, Iran. 1gr of medicinal PVP was gradually added to 100 *ml* of deionized water at 80-90°*C* under stirring to completely dissolve. The aqueous solution containing the silver nanoparticles, which was prepared in the section 2.1, was mixed with PVP solution under stirring for 60 minutes. In order to coat the implants and other medical equipment, they were immersed in the solution and kept in the solution for 30 seconds. Then the implants were removed and placed at 50°*C* for 30 *min* to slightly dry. This procedure was repeated 70- and 100- times. In the initial tests, immersion was tested 30- and 50- times, but the results did not show good antibacterial, so these samples were not examined further. For 70- and 100- time immersions, it was observed that with increasing the number of immersions, the adhesion of the layer decreases, thus higher immersions do not seem appropriate and the optimal sample with 70 immersions was selected to continue the work. Finally, the surfaces were dried in an oven at 100°*C* for 10 *hr*. The aqueous solution of silver nanoparticles at the concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 *M* was used to determine the effect of changing the concentration of Ag nanopowder on the antibacterial properties of coated implants.

Antibacterial assay

Antibacterial analysis by determining the diameter of the growth inhibition zone is a way to measure antibacterial effects. In this method, after isolating the bacteria, some of the bacterial colonies are removed and dissolved in the serum environment. After preparing the homogeneous solution, the solution is stirred with a swab and transferred to Müller-Hinton culture medium. The medium is then cultured with a swab, and after culture, antibiogram disks are transferred to the culture medium. After placing the discs, the plate is closed and the disks are kept in an incubator at 37°*C* for one day. After 24 *hr*, the plate is examined under light and the underdevelopment area

is measured. The larger the growth inhibition zone, the stronger the antibacterial effect.

Antibacterial properties of silver-PVP composite-coated implants at different concentrations were investigated by deep coating method. The *E. coli* ATTC 25922 (standard gram-negative bacteria) and *Staphylococcus aureus* (*S. aureus*) ATTC 25923 (standard gram-positive bacteria) were purchased from the Iran's Scientific-Industrial Research Center. Mueller Hinton Broth medium (Merck) was used. The test was performed by the non-growth halo method. The samples of gram-positive and gram-negative bacteria were cultured in Mueller Hinton Broth medium and incubated for 24 *hr* at 37°*C*. The results of experiments on *S. aureus* and *E. coli* bacteria were investigated for 24 *hr* on uncoated implants (reference sample) and silver-PVP composites-coated implants.

Cytotoxic assay

MTT test is performed to achieve viability under the influence of various drugs and materials. This is a simple method that has made it possible to quickly obtain the toxicity of the material and obtain the IC50. In this method, the cells are treated with different doses of drugs and the survival rate in each of them is obtained. Its importance in obtaining IC50 means the concentration at which half the cells die. The MTT test is run on multi-well plates and different doses of medication are treated in different wells, and no drug is treated in one well and is considered as a positive control. Finally, the number of cells in each well is measured relative to the positive cell and the survival rate is obtained. The term MTT belongs to a substance called 3- (4,5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide, which penetrates into the vicinity of living eukaryotic cells and in the mitochondria of cells, Formazan crystals form. Formazan is a chromophore compound that has light absorption in the wavelength range of 570 *nm* and this feature has caused that after treating living cells with MTT solution and forming Formazan crystals in mitochondria, by examining the light absorption of the samples, the amount of living cells based on the absorption was obtained. Thus, the higher absorption rate, the more living cells there are. Therefore, in MTT test, after treating the cells with drugs or material, MTT solution was added to them and after the required time, by examining the amount of light absorption of each sample, the survival percentage of each different dose is calculated and compared to the control sample. In this test, normal cell line was used to perform the MTT analysis.

Characterizations

The structure of silver nano-powder synthesized by green synthesis method was studied by X Ray diffraction (XRD) technique (model: D8 Advance Bruker YT) under CuKα radiation. Investigating the way of distributing the particles was performed by DLS device, Model SZ100, Horiba Co., Japan. The transmission electron microscopy (TEM), model CM120 made in the Netherlands with a maximum voltage of 100 *kV* was used to investigate the particle size. The chemical bonds of silver nano-powder were studied using Fourier transform infrared spectroscopy, Nicolet AVATAR370 device in the range of 500-4000 cm^{-1}. UV-Vis device, U3500 model was used for optical characterization of the samples in the wavelength range of 200-800 *nm*. Statistical analysis was not performed for the results obtained in this study.

Results

Characterization of silver nano-powder

Figure 1 shows the XRD pattern of crystallized Ag nanopowder prepared by green method. The

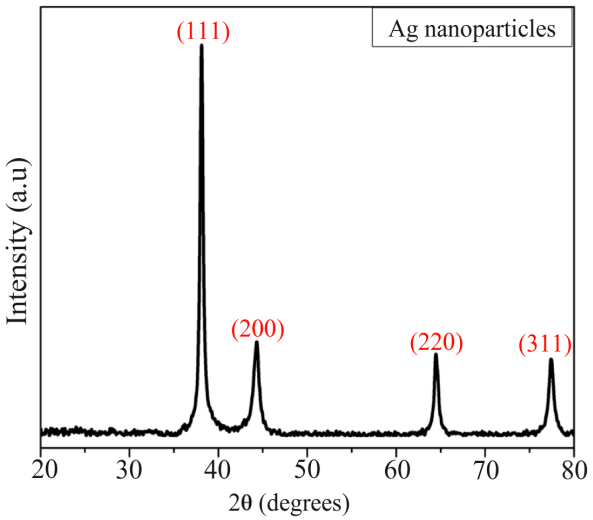

Figure 1. X-ray Diffraction pattern of crystalline silver nanoparticle.

diffraction pattern exhibits 4 sharp and well-defined peaks at angles of 38.15, 44.34, 64.5 and 77.46 degrees, which can be attributed to the reflecting plates (111), (200), (220) and (311), respectively, according to the face centered cubic (fcc) structure of metallic Ag (32). The lattice parameter calculated by XRD pattern is equal to a = b = c = 4.0862975Å. The diffraction pattern corresponds well with JCPDS card number: 04-0783 (32). High-intensity peaks, marked in the diffraction pattern of figure 1, confirm the excellent crystallinity of silver nanoparticles.

Dynamic Light Scattering (DLS) analysis is a physical method for determining the distribution of particles in suspensions and solutions. In the DLS analysis, after the laser light interacts with the particle, the scattering and the changes in light intensity are evaluated according to Brownian motion of the particles, and accordingly, the particle size distribution is measured (33).

The intensity of light scattering varies over time and at different angles due to the motion of particles and their interaction with the liquid environment. The particle sizes can be calculated from the correlation between these changes and colloidal parameters. In other words, the measurement of Brownian motion of the particles in DLS test is determined by calculating the number of fluctuations in the intensity of light rays scattered by small and large particles. This determination is based on changes in the point pattern that is in the form of dimming and brightening of dark and light points. Determining the intensity of scattering of light rays leads to the measurement of particles.

General information on the size distribution of silver nanoparticle (AgNPs) is obtained from the DLS results exhibited in figure 2. There are 2 visible peaks, demonstrating an average particle size of 12 and 17 *nm*, respectively. The peak observed at 17 *nm* is more intense, reaching 66.4% compared to the 12 *nm* peak at 33.6%. In order to describe the nature of the particles producing these 2 peaks, a Transmission Electron Microscopy (TEM) was used for having the more accurate structural information.

Figure 3 shows the image of transmission electron micrograph of silver nanoparticles. The average particle size is between 5-20 *nm*. As shown in TEM image, the particles are well separated from the

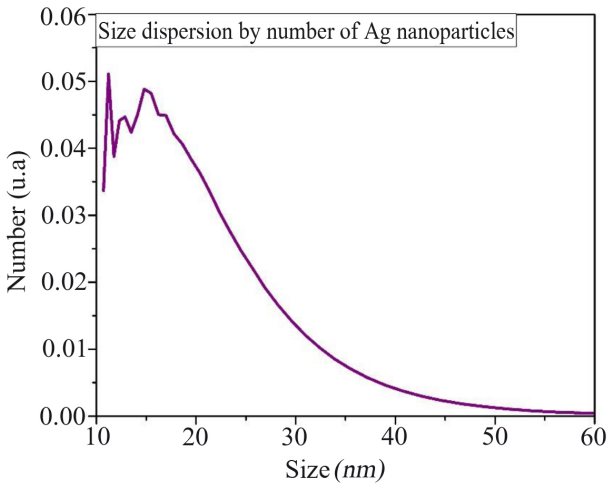

Figure 2. Particle size distribution of Ag nanoparticles obtained from DLS particle size.

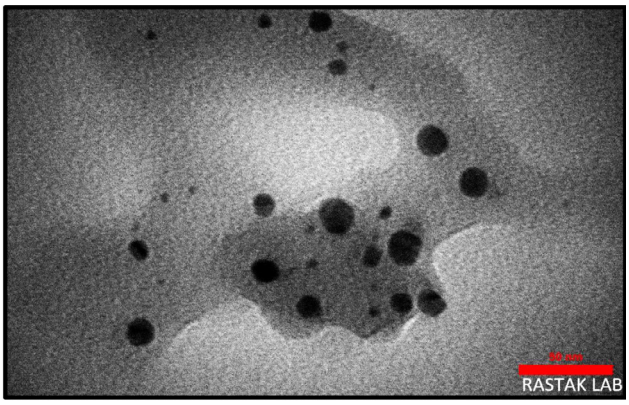

Figure 3. TEM analysis of crystalline silver particles. Scale bar corresponds to 50 *nm*.

adjacent nanoparticles. It is also clearly visible in the TEM image that the particles are cubic. In the images of FCC pattern, the silver particles shown are also in accordance with the observations made from the X-ray diffraction (XRD) pattern (34).

The FT-IR spectrum of silver nanoparticles synthesized by the green synthesis method is shown in figure 4. As shown in figure 4, the tensile peak at 3394 cm^{-1} is related to the OH vibration of the hydroxyl group. The two peaks identified in 2810 and 2925 cm^{-1} are attributed to C-H tensile bands. The tensile peak at 1731 cm^{-1} can be assigned to C=O elongations. The peak observed in 1655 cm^{-1} is assigned to the tensile vibrations of the C=O groups. The vibrating bands vI (C-C) vI / II (C-C) appear in 1561 and 1451 cm^{-1}, respectively (35).

Production of silver nanoparticles was followed by

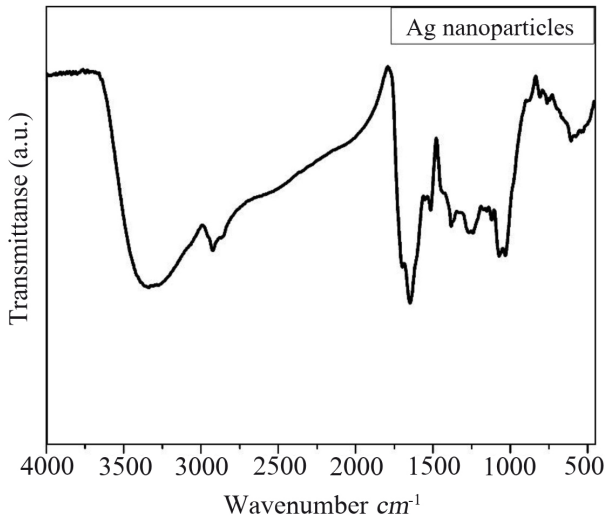

Figure 4. FT-IR spectrum of Ag nanoparticles.

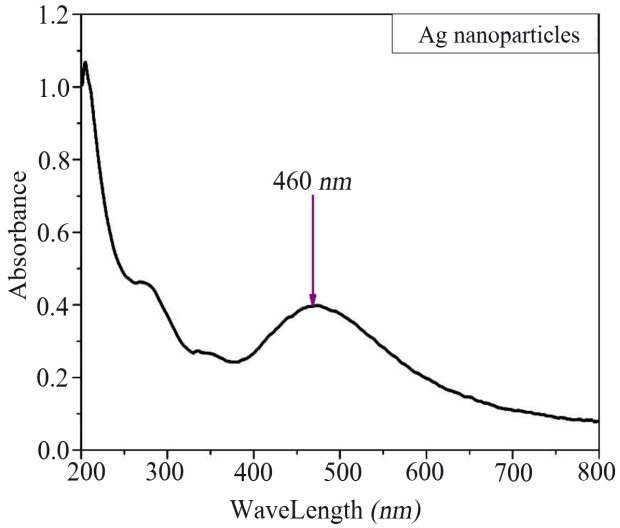

Figure 5. Absorption spectrum of Ag nanoparticles.

reduction of silver ions using green synthesis and UV-Vis spectroscopy. UV-Vis absorption spectrum of the synthesized silver nanoparticles is shown in figure 5. The peak in the silver nanoparticles solution was close to 460 *nm* during the observation period so that the nanoparticles were scattered in the environment (36).

Antibacterial and MTT toxicity

In the first step, the concentration of silver nanoparticles in silver-PVP composite was selected to be 0.02, 0.04, 0.06, 0.08, 0.1 *M* and the test was only performed on *E. coli*, which is a more resistant bacterium. The results of the non-growth halo test after 24 *hr* are shown in figure 6. The obtained results show the presence of antibacterial properties for all concentrations studied in the presence of *E. coli* bacterium, and a very large non-growth halo is clearly observed for all concentrations of silver in the figure 6.

According to the antibacterial results obtained in figure 6, the solution containing the lowest concentration of silver was selected and sent for toxicology test by MTT method. MTT toxicology test is one of the methods to study the amount of toxicity of materials on *in vitro* cells. In the method, the cells are treated with the studied materials after culture in the laboratory to check their amount of toxicity. The result is that the viability of the cells will be determined for each concentration of the material. The basis of MTT toxicology method is the formation of formazan dye due to the reduction of the combination of dimethyl thiazol-2 and 5-diphenyltetrazolium bromide (MTT) or other tetrazolium salts. The insoluble formazan crystals are formed by breaking the tetrazolium ring through mitochondrial enzymes in living cells, which are purple in color. The formation of these crystals shows the activity of respiratory chain enzymes and is a measure for cell viability. By calculating the amount of absorption by spectrophotometer at certain

Figure 6. Antibacterial properties of silver-PVP composite-coated implants at different concentrations by deep coating method in the presence of *E. coli* bacterium.

wavelengths, the percentage of surviving cells can be estimated. This percentage is calculated as follows:

100* Mean absorption of control samples/Mean absorption of treated samples = percentage of living cells

The results of the MTT toxicology test are plotted in figures 7 and 8. According to the results of MTT toxicology test, the appropriate concentration of silver-PVP composite without cell toxicity is 118.6 *µg/ml*.

After determining the appropriate dose of silver nanoparticles by MTT test, silver-PVP composite with the appropriate dose (118.6 *µg/ml*, according to the result obtained from MTT test, this is the concentration at which 50% of the cells survived. This is the safety concentration for nanoparticles) was synthesized and the resulting solution was coated on the surface of the implants according to the previous method. Antibacterial test was performed on two gram-positive and gram-negative reference

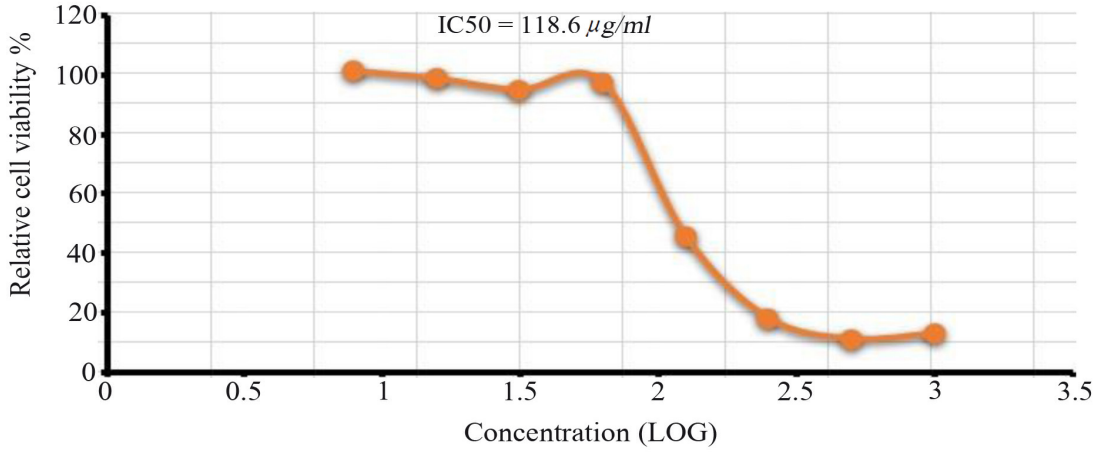

Figure 7. Diagram of changes in cell viability and IC 50 in terms of logarithm of the concentration.

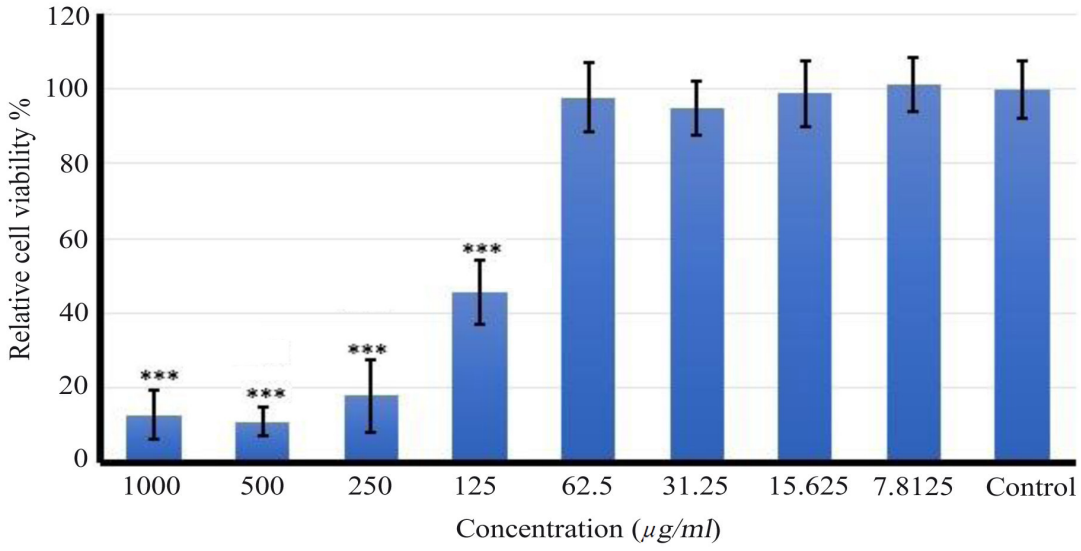

Figure 8. Diagram of changes in cell viability in terms of different concentrations of silver.

bacteria and the results are reported in figure 9A. According to the Figure, it can be observed that at this concentration, the non-growth halo for both types of bacteria compared to reference sample is clearly visible. In the next step, in order to test the durability of the applied coating, the coated implants in Figure 9A were removed from the culture medium and kept in the laboratory in normal air flow for 5 days. After 5 days, antibacterial test of the samples was performed again. The results are shown in figure 9B. Figure 9B shows that although the non-growth halo is slightly smaller than that of figure 9A, the halo is still clearly visible, indicating the very high antibacterial properties of the coated samples.

Pull off adhesion test

In general, the resistance of a coating to the removing and detaching from the surface on which it is applied is called adhesion. The adhesion of the coating to the bottom level (substrate) depends on many factors, including the type of coating, the substrate material, way of preparing the surface, and so on. This very complex phenomenon can be investigated in various ways, including the pull off test. The pull off test is used to investigate the tensile strength of the coating applied to a metal surface. In the method, the tensile stress applied to the surface reaches its maximum value compared to the shear stress, and in this regard, it is more accurate than other methods for determining the adhesion strength. In the pull off adhesion method, the dollies are glued to the surface of the coating using a specific epoxy adhesive, and after the epoxy adhesive is fully cured, the amount of force required to separate the dolly from the coating is reported as the adhesion of the coating. Pull off test was performed according to ASTM D4541-17 standard. Two samples of titanium metal with 70- and 100-times repetition of immersion process were selected to investigate the effect of immersion times. The results obtained from this analysis show that the mean pressure for two samples with 70- and 100-times repetition of immersion is 16.4 and 14.7 MPa, respectively. The adhesion amount of the coating to the substrate has decreased by increasing the number of immersion times and thereby, increasing the thickness of the applied coating.

Discussion

In implant surgery, postoperative infection is one of the most common events threatening the success of

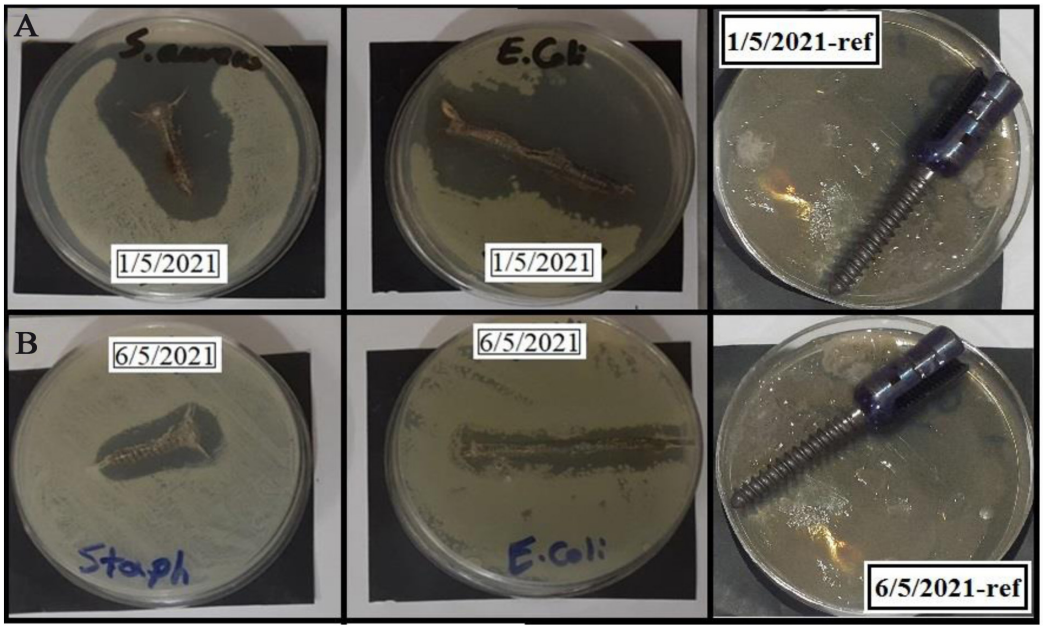

Figure 9. A) Antibacterial property test of silver-PVP composite-coated implants at the concentration of 118.6 *μg/ml* by deep coating method in the presence of two standard bacteria (*E. coli* and *S. aureus*) compared to reference sample, B) Repeatability test of antibacterial properties after 5 days.

surgery. Although modern surgical techniques, such as spinal surgery, have reduced the incidence of this complication, infections of spinal implants and other implants still occur significantly. This unwanted complication occurs due to the adhesion of bacteria and the formation of biofilms. The most common organism resulting from postoperative wound infections is Staphylococcus aureus. To overcome implant infection, many efforts have been made to improve implant technology and experimental implant models. Silver and their nanocomposites have been shown to have very high antimicrobial effects. The proposed mechanism to justify the antibacterial activity of silver was proposed by Chloe *et al* in 2000. According to their theory, silver ions enter into the cell and then immediately attach to DNA. As we know, the DNA molecule begins to replicate at rest, and when the DNA is compressed, it loses its replication properties. When silver ions enter into the bacterial cell, they lead to the bacterial DNA compression and lose the ability to replicate and eventually bacterial cell death (37,38).

The toxic effects of nanoparticles on living organisms can be explained by two main hypotheses (39-45). The first hypothesis states that the harmful activities of nanoparticles are caused by the release of metal ions. The second hypothesis states are due to the toxicity the formation of Reactive Oxygen Species (ROS). The resulting free radicals can damage any component of the cell and begin to produce the increasing number of reactive oxygen species. For example, the generated free radicals are able to oxidize the double bonds of fatty acids in the cell membrane, thereby increasing the permeability of the membranes and increasing the permeability involved in osmotic pressure. Reactive oxygen species may also inhibit the activity of enzymes by binding to enzymes and altering the DNA helix, which can lead to cell death. The formation of larger amounts of reactive oxygen species results from the higher levels of nanoparticles compared to their larger analogues. Nano particles may also damage the cell membranes, oxidize the proteins, be genotoxic, and interfere in energy conduction. Therefore, balancing the benefits and risks of nano-materials is essential to conducting the safe and responsible researches on their development.

Conclusion

The study is aimed to provide a simple, effective, repeatable and large-scale method to prevent the infection after implant placement. In the first part of the research, silver nanoparticles were produced by green synthesis method and using Brazilian coffee powder, and their structural, morphological and chemical properties were investigated. The results confirm the formation of metallic structure of silver nanoparticles with the average size of about 17 *nm*. In the second part, the surfaces of different implants are coated with silver-PVP nanocomposite at different concentrations by immersion method. Antibacterial and MTT results show a very large non-growth halo in the presence of silver-PVP nanocomposite coating with the appropriate dose of 118.6 $\mu g/ml$. By repeating the non-growth halo test after one week, the non-growth halo is still clearly visible. Overall, it can be concluded that the silver-PVP nanocomposite produced in the study can be used to coat the implants and medical equipment and prevent the development of infection in the vicinity of implants.

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