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The Influence of Biological Environment on the Silver-Coated Implants

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Abstract

The environment of the human body is very aggressive, containing among others bacteria, which contribute to the degradation of metal implants. Therefore sometimes implants are covered with nanometals to prevent development of aggressive bacteria. This paper deals with implants covered with nanosilver (15nm), which is antibacterial. The tested implants included: PE vein implant, an intramedullary implant made of stainless steel and brass implant for tracheotomy. The results showed an appearance of implants covered with silver as dependent on the type of bacteria: although silver significantly protected implants against some bacteria, a presence of some amounts of Staphylococcus aureus and Staphylococcus epidermidis was noticed after long term exposure in the human body. Only single bacteria could be observed on the surface of the tested materials. Such behavior is evidence, that silver coatings are effective for different form of materials in the presence of various bacteria, however, such behavior is related to form of bacteria.

Key words: implants; silver coatings; bacteria.

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1. Introduction

It is well-known, that the insertion of implants into human body carries the risk of bacterial infection, mainly as a result of biofilm formation at the implant surface. The first step to form the biofilm is bacteria adhesion. Biofilm is a surface-attached aggregates of microorganisms embedded in an extracellular polysaccharide matrix. Biofilm forming bacteria act as efficient barriers against antimicrobial agents and the host immune system, resulting in a persistent colonization and/or infection at the site of the biofilm formation. Bacteria living within the biofilm structure are more difficult to be destructed by immunity system, as well as are more resistant to antibiotics and may cause general body infection in case of weak immunity resistance.

The silver is known for a long time as a biologically active compound preventing biofilm formation [1]. Such efficiency was shown for stainless steels [2,3], Ti and its alloys [4], TiNi alloys [5] and polymers [6]. It was used for antibacterial protection for some medical devices [7-9].

It is uncertain, whether Ag nanoparticles or Ag^+ ions are responsible for the antibacterial action. Silver ions were more toxic to E. coli, than nanosilver [10]. Hatchett and White [11] proposed a synergistic toxic effect of the silver nanoparticles and the silver ions, which they produce. The ions moved into the cells and lead to the production of reactive oxygen species. The potency of Ag as an anti-bacterial coating was suggested to be dependent on its biologically active form, soluble Ag ions or Ag clusters, to interfere with the integrity of the bacterial cells [12] and to bind to the enzymes and proteins within the bacteria [13,14]. Ag film deposited by magnetron sputtering was reported to be in the form of Ag nanocrystalline clusters [15,16]. For the TiNiAg alloy immersed in a body fluid, the release of ionized Ag into the surrounding fluid was postulated [5]. The critical level for the antibacterial efficiency of Ag was very low and has been estimated at 0.1 ppb [17] or 0.5 ppb [18].

According to Navarro et al. [19] nanosilver can impart toxicity in both ion and particle forms, and silver ions were more toxic to E. coli, than nanosilver. Nanoparticle toxicity is size dependent with smaller size particles presenting higher toxicity [20-23]. The release rate and an effective killing time can be 15 days [4] and in case of silver inside the oxide nanotubes the bacterial adhesion is maintained without obvious decline for 30 days, which are normally long enough to prevent post-operation infection in the early and intermediate stages and perhaps even late infection around the implant [24]. In other work [25] the Ag effective concentration was at least 0.06 mM, and in 0.02 mM the antibacterial ratio to Staphylococcus aureus decreased to 63.30% [25].

AgNPs due to the wide spectrum of antimicrobial activity pay particular attention in contemporary orthopedics and bone and tissue engineering. However, still unexplained information about AgNPs-induced cytotoxicity in mammalian cells limited their use in tissue repair. It was demonstrated that exposure to AgNPs leads to an increase of oxidative stress, apoptosis and genotoxicity in cultured cells and animal tissues [26]. Interestingly, it was found that co-exposure of human gingival fibroblast cells to AgNPs and fluoride resulted in enhancing of cytotoxic damage [27]. Also, contradictory results are found in an available literature. Hackenberg *et al.*[28] observed a decrease in human mesenchymal stem cell after 1 h treatment with AgNPs <50 nm at concentration of 10 μg/mL. On the other hand, Samberg *et al.*[29] observed no toxicity for progenitor human

adipose-derived stem cells exposed to up to 100 μg/mL AgNPs with size 10 and 20 nm for 24 h. Therefore, in our study we decided to assess the impact of commercially available AgNPs with size 15 nm on human osteoblast cells viability.

The silver can be effective against different forms of bacteria for different materials. Even, if this role of silver is well known, less is known about selective biological activity and strength of silver in the presence of many different bacteria, which may together and in different contents appear in dangerous and frequent clinical inflammations. For the Ti-6Al-4V alloy such ability was observed against Staphylococcus epidermidis [4], against, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus with more than 90% antibacterial ratio [25], against the Staphylococcus epidermidis and Staphylococcus aureus [30].

The composition and structure of surface for Ag deposition is also important even, if this effect is moderate. Different solutions were postulated and investigated. The Ag deposited on PE showed almost 100% anti-bacterial ability [6]. Another tested form is the Ag particles dispersed within the titanium oxide obtained by anodic spark deposition in an aqueous electrolyte on the medical grade Ti-6Al-4V alloy, as small particles of sizes below 200 nm [4]. The next implementation technique for Ag ions based on a immersion of vacuum plasma sprayed titanium coatings in Ag^+ containing calcification solution [25]. TiO2-capped Ag nanorods can be also applied as a lasting and strong antibacterial material with controll-ability of the silver ion release through the mesoporous and aqueous cap layer. Mesoporous TiO2 cap layer is controlled the water and silver inter-diffusion [31]. Three kinds of antibacterial ingredients were loaded into the hydroxyapatite (HA) coating: antibiotic (Ampicillin sodium salt), silver ions and water soluble chitosan. The coatings with a porous structure showed improved antibacterial properties, likely due to better loading and more sustained release of the antibacterial ingredients, except for the highly water-soluble antibiotics [32]. Recently, nanosilver cement also proved to possessed high-antibacterial activity against multiresistant bacteria, including methicillin-resistant S. epidermidis(MRSE), and methicillin-resistant S. aureus (MRSA), and free of in vitro cytotoxicity [33]. The synthesized Ag–TiO2/(a)TiO2 nanocomposite thin film can be utilized as a promising and effective bactericidal material in the future [34]. The interesting research on four types of silver coatings with various surface energies prepared on stainless steel plates using $AgNO_3$ based on electroless plating solutions showed, that bacterial adhesion decreased with the total surface energy of the coatings decreasing, but also decreased with the electron donor component increasing [2]. Micro-electrochemical cells and galvanic effects between Ag nanoparticles and Ti matrix play an important role in the interactions with the attached cells supporting the direct deposition of silver inside the titanium [35]. Titania nanotubes (TiO2-NTs) incorporated with silver (Ag) nanoparticles can be fabricated on Ti implants to achieve this purpose. The Ag nanoparticles adhere tightly to the wall of the TiO2-NTs prepared by immersion in a silver nitrate solution followed by ultraviolet light radiation. The amount of Ag introduced to the NTs can be varied by changing processing parameters such as the $AgNO_3$ concentration and immersion time. In the last years the increasing interest in loading of nanosilver into the oxide nanotubes can be noticed with long term biological efficiency [24]. The silver can be also introduced into hydroxyapatite coatings by coprecipitation or plasma spraying. Both coatings can slowly release silver nanoparticles and Ag + ions in simulated body fluids [36]. Such solutions presumably made the coatings exhibit a good anti-bacterial effect. The bioactivity of the first solution is claimed to be higher, than the last one.

Even of the antimicrobial effect of silver ions has been studied extensively, the effects of silver on bacteria and the bactericidal mechanism are not fully understood. Bacteria affected by nanosilver cannot breathe, because the transfer of electrons in a cell is destroyed. Silver prevents metabolic reactions in a bacteria cell, because it reacts with the –SH enzyme groups [37]. The silver nanoparticles anchor to and penetrate the cell wall of Gram-negative bacteria [38,39]. It might be assumed, that the resultant structural change in the cell membrane could cause an increase in cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane and ultimately cell death. The antibacterial mechanism of silver nanoparticles may be also related to the formation of free radicals and subsequent free radical-induced membrane damage [40,41]. However, no damage by silver ions was postulated by Hwang et al. [42]. The silver nanoparticles may modulate the phosphor-tyrosine profile of putative bacterial peptides, that could affect cellular signaling and therefore inhibit the growth of bacteria [43]. Silver and copper can cause bacterial inactivation in vitro by binding to microbial DNA, preventing bacterial replication, and disrupting the sulfhydryl groups of metabolic enzymes in the bacterial electron transport chain [30,44-46].

Silver is a metal, that may in excess cause some detrimental effects. The toxic effects of silver substances are proportional to the rate of release of free silver ions from them [35]. The silver nanoparticles may interact with proteins and enzymes with thiol groups within mammalian cells. Then apoptogenic factors like cytochrome C are released and programmed cell death is a final result. Besides mitochondrial destruction, damage to cell membranes appears to be another part of nanosilver's mechanism of cytotoxicity, that precedes mitochondrial perturbation [47-50]. Silver ions released from nanosilver may react with the microbial membrane and inactivate cell functions, while small particles may enter the cells to disrupt microbial metabolism, making nanosilver highly toxic [21-23]. Therefore, the toxicity of nanosilver may be controlled by particle size (cell internal-ization), Ag release rate (particle stability), and surface characteristics (e.g., surface film formation). The first evidence for a cell-type-specific uptake of Ag-NP by peripheral blood mononuclear cells (PBMC) and the resultant cellular responses after exposure.

The research was aimed at verifying, whether a silver coat already developed effectively as antibacterial protection against different kinds of bacteria together, which may appear under hospital conditions. The objects of research included implants applied at hospital conditions: vein implant, steel implant and brass pipe for tracheotomia. The purpose for this experiment is an assumption, which if any antibiotic may be helpful to a single group of bacteria, silver ions or nanosilver clusters may be effective against a wide range of bacteria. Even, if usually the hospital inflammations occur, because of a presence of a single bacteria and can be prevented or cured with antibiotics, silver or nanosilver may substitute or accompany the traditional treatment. The second objective of this work is to contribute into explaining the exact mechanism of silver antibacterial action.

2. Materials and research methods

Three specific implants for investigations included: (i) polymer (polyethylene) vein implants covered with silver (the amount of silver ranged from 0.07 to 0.16 mg/cm^2), i.e. SILVER GRAFT (Figure 1); (ii) intramedullary nail for long bones made of stainless steel (17.57Cr, 14.29Ni, 5.66 Mo), covered with silver (average size of

nanoparticles of silver were 40 nm) by PVD method (Figure 2); (iii) brass pipe (64Cu, 34Zn, 0.8Pb, 0.05Al, 0.1Fe, 0.3Ni, 0.1Sn) for tracheotomy covered with silver on the outer side by PVD method (average size of nanoparticles of silver were 40 nm) (Figure 3). All implants were made by the Aesculap Chifa Ltd., Poland; the chemical composition is according to the supplier`s specification.

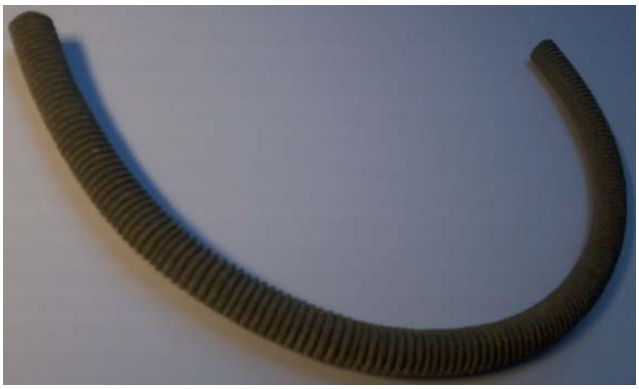

Figure 1: Antibacterial vein implant SILVER GRAFT covered with silver [51]

Figure 2: The intramedullary nail covered with silver [51]

Figure 3: The pipe used for tracheotomy [51]

All implants were cut for the tests into pieces of 10x15 mm. For the products the steam sterilization was performed in an autoclave, at a pressure of 0.2 MPa and temperature 134^0C for 10 min.

Simultaneously the research of cythotoxicity on human osteoblasts was carried out.

AgNPs (size 40 nm) water dispersion were purchased from MK Nano Company.

Human fetal osteoblast cell line (hFOB 1.19) was obtained from the American Type Culture Collection ([ATCC], Manassas, VA, USA) and maintained as a monolayer culture in T-75 cm^2 flasks. The cells were grown in a mixture of Dulbecco's Modified Eagle's Medium and Ham Nutried Mixture (Sigma-Aldrich)

containing sodium pyruvate (110 mg/L) and supplemented with 10% fetal bovine serum, 6 μg/mL penicillin-G, and 10 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% O2 and 5% CO2. hen confluent, cells were detached enzymatically with trypsin-EDTA and sub-cultured into a new cell culture flask. The medium was replaced every 2 days. These hFOB cells were indicated to be an excellent model system for the study of osteoblast biology *in vitro.*

Human fetal osteoblast cell line (hFOB 1.19) were treated with 40 nm AgNPs (1-80 μg/mL) for 24 hours. The dilutions of AgNPs were prepared just before adding to the cells in fetal bovine serum-free (SF) culture medium. AgNPs solutions were vortexed for 1 minute to prevent aggregation following the manufacturer's instructions. Control was cells untreated with AgNPs.

Cell viability was measured by MTS assay. The hFOB 1.19 cells were seeded triplicate at a density of 10^4 cells/100 μL of cell-culture medium into a 96-well plate. The following day, the hFOB 1.19 cells were treated with AgNPs as specified in section *Treatments*. Mitochondrial activity assay (MTS) evaluates mitochondrial activity (assesses cell growth and cell death) based on mitochondrial dehydrogenase enzyme's ability to convert the tetrazolium salt (MTS) to formazan - a colored reaction product. MTS assay was performed by adding a premixed optimized dye reagent to culture wells. Absorbance was read at 450 nm (reference: 630 nm) Absorbance values were also corrected with blank NPs. Treated-cell viability was calculated and expressed as a percentage (%) of the viability of control cells (100%) based on mean absorbance values at 450 nm.

The examinations of surfaces after exposure were made with the scanning electron microscope Philips XL 30 to qualitatively assess the bacteria presence and possible surface degradation.

3. Results

In the research, we analyzed the effect of AgNPs (size 40 nm) on the viability of hFOB 1.19 cells. It was found that 24 hours treatment of osteoblast cells with AgNPs at a concentration of 1, 5, 10, 20 μg/mL did not cause statistically significant changes in cells viability. This range of concentration AgNPs was not cytotoxic to hFOB 1.19. Nevertheless, under higher concentrations: 40-80 μg/mL AgNPs, a significant decrease of cells viability was observed (Figure 4). AgNPs in this concentration range exhibited a cytotoxic effect on hFOB 1.19 cells.

AgNPs (40 nm) induced death of hFOB 1.19 cells after 24 h exposure. Data are presented as the means ± SE for at least three independent results, analyzed by One-way ANOVA combined with Tukeys Multiple Comparison Test. *$p<0.05$; **$p<0.01$; ***$p<0.001$ AgNPs-treated cells versus control (untreated cells).

The major novel finding of this study is that AgNPs at low concentration (1-20 μg/mL) does not cause impairment of cell viability. Higher concentration of AgNPs (40-80 μg/mL) significantly decreases the viability of human fetal osteoblasts cells.

For the ***vein implant*** (Figure 5) only separate bacteria were observed - Staphylococcus aureus and Staphylococcus epidermidis (Figure6).

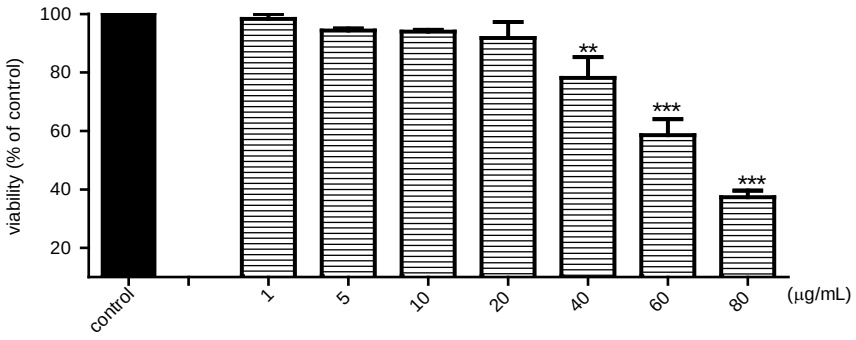

Figure 4: Induction of cell death of hFOB 1.19 cells treated with AgNPs (40 nm)

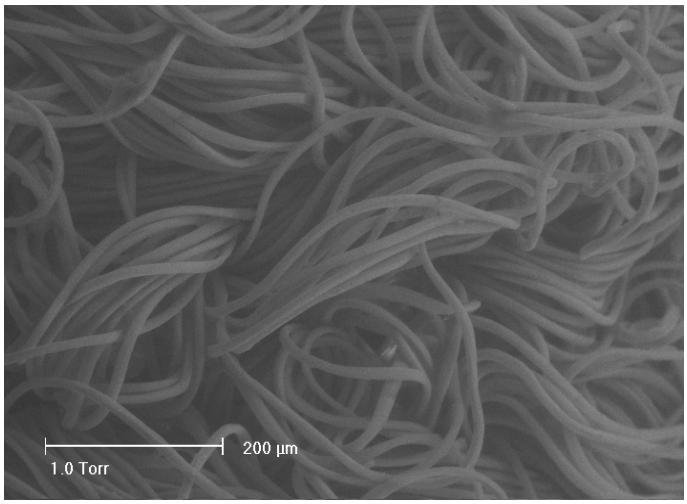

Figure 5: The surface of the vein implant

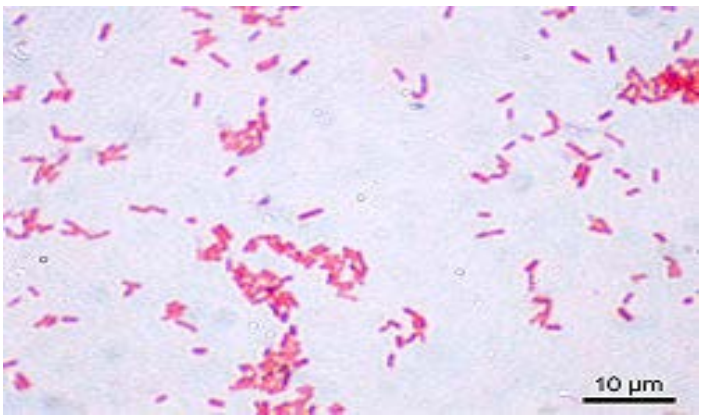

Figure 6: The separate bacteria among the fibers of the vein implant - Staphylococcus aureus and Staphylococcus epidermidis

The results of microscopic examinations of the ***intramedullary nail implant*** covered with silver are presented in Figure 7. which shows no noticeable changes in the area covered with silver appeared.

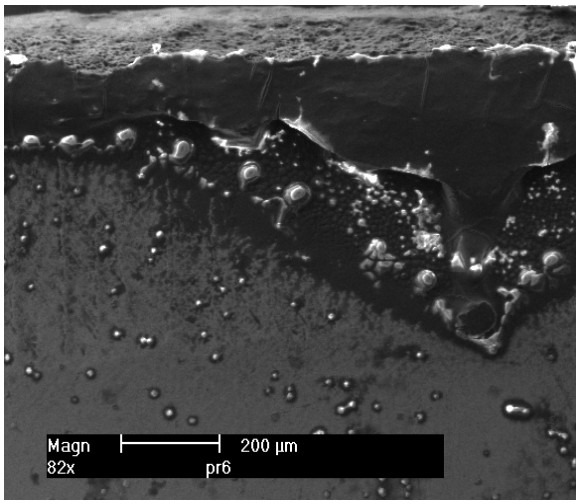

Figure 7: The surface of the intramedullary nail covered with silver

Figure 8 shows the brass pipe for tracheotomy. After cleaning the surface with Octanisept no degradation was noticed.

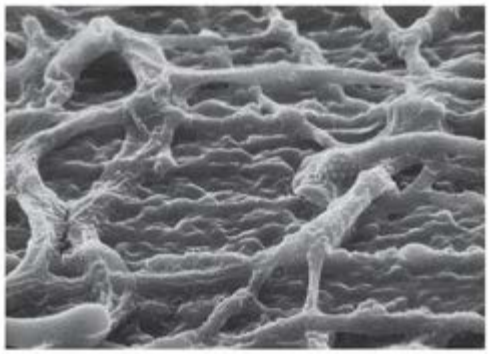

Figure 8: The surface of the pipe for tracheotomy

4. Discussion

The bacteria gather not only on the surface of body cells. Owing to their adhesive properties, they stuck to different kinds of materials. The great challenge for contemporary medicine is the formation of biofilms on biomaterials. Despite the efforts to obtain a smooth surface of biomaterials, the microscopic analysis shows, that the surface is rough, which makes it more accessible for bacteria adhesion. In a few seconds of contact with biological fluids, it covers the surface with proteins. The cells contained in body liquids or tissues immediately recognize the surface of the implants as a “strange body”. It results in the formation of biofilm.

The formation of the biofilm aims at the protection of microorganisms (creating biofilm) against degradation

activity of environmental factors, including antibiotics. The biofilm is responsible for long lasting diseases, especially infections caused by using drains and inserting implants. It is an undesirable problem in hospital infections. The complicated structure of the biofilm and various features of microorganisms, which constitute it, explain their high resistance to different kinds of fighting their factors, including antibiotics. A biofilm is not just a simple layer of slime but a complex multicellular community. Bacteria form biofilms to exploit their environment and protect themselves from their hosts.

This is likely the first attempt to examine the behavior of various bacteria altogether, which might appear on the surface of an implant during inflammation process. Such a case may always occur as a consequence of not fully aseptic implant and chirurgical instruments, improper conditions at a hospital and even in a surgery room, no knowledge of either of a patient or a medical team on the presence of some bacteria in a body. As it is then impossible to eliminate totally a risk of bacteria-related inflammation and then a danger of rejection of an implant, it would be useful to look for further prevention measures, rather than general and not specific activity. These results demonstrate, a little surprisingly, that silver itself, in amounts usually used and allowed, may be effective against some different bacteria. There are many reports, citied above, that silver may be valuable for killing or preventing Acinetobacter, Bacillus, Clostridium, Enterococcus, Escherichia coli, Klebsiella pneumonia, Listeria, Pseudomonas aeruginosa, P. gingivalis, Salmonella, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus and Vibrio, but not all of them together. That means, that this biometal is more effective, than antibiotics when placed on a surface of an implant.

The patients with silver coated implants do not need any additional antibiotic treatment. Such behavior may be evidence, that silver is highly harmful and kills almost all bacteria at applied amounts and released rates.

The mechanism of protection is not fully clear. For one side, it may be selective adhesion of nanoclusters or single atoms to the bacteria making their supply with oxygen impossible. On the other hand, almost equal effects on different forms of bacteria may suggest rather physical, than chemical explanation, i.e. by making an adhesion of bacteria to the surface impossible or very difficult; only bacteria present on the surface and not in the liquid volume were detected.

It seems, that the antibacterial property of silver is not directly related to size of silver particles. It is not then or at least it has been never proved, that silver nanoparticles themselves have a so outstanding surface, because of their small size and likely unsaturated chemical bonds. We think, that silver in both of micro- and nanometric size may be efficient, related to the release rate of silver ions, which are fully responsible for preventing effects. However, nanoparticles have a much more developed surface, so that they may produce significant amounts of silver ions and in such a way demonstrate a great potential. Such model explains, why both nanoparticles and silver ions possess an antibacterial efficiency and why the positive effect of decreasing particle size is observed. The model shows, that the future research should take into account the release rate of silver ions, both for nano- and microcoat, and time to reach the lowest critical concentration sufficient to be active in killing the bacteria, which are related to the possible form of attachment of silver to an implant – as a coating, a part of composite coating or of oxide nanotubes in Ti and its alloys, etc [31].

Similarly to our findings, Pauksch et al. [52] indicated, that therapeutical window for the use of AgNPs in orthopedic products exist. They demonstrated, that AgNPs induced impairment of osteoblast cell viability at higher concentrations. Albers, at al. [53] observed that AgNPs with size 50 nm exhibited strong cytotoxic effects on osteoblast. Importantly, they noticed, that antibacterial effects occurred at AgNPs concentrations, which were 2-4 times higher, than those exerting cytotoxic effects. It was also shown, that the adipogenic and osteogenic differentiation of human mesenchymal stem cells was impaired even at subtoxic concentrations of AgNPs. Importantly, Qin et al. [54] found, that 20 nm AgNPs are induced osteogenic differentiation of urine-derived stem cells advance osteogenetic differentiation at noncytotoxic concentrations after exposure for 24 hours. Samberg et al. [29] observed, that treatment with 10 and 20 nm AgNPs did not affect the differentiation of the human adipose-derived stem cells and at antimicrobial concentrations of AgNPs induced a minimal decrease in viability.

5. Conclusions

The silver coatings are almost equally effective against different forms of bacteria, whatever base for their deposition, stainless steel, brass or PE is used.

The mechanism of the antibacterial efficiency of silver against bacteria may involve either a direct killing of bacteria by stopping the delivery of oxygen or preventing the adhesion of bacteria on an implant surface.

The effect of Ag may be observed for both micro- and nanoparticles, with the latter more effective, because of the more developed geometric surface; whatever way, Ag ions are responsible for the antibacterial activity.

The AgNPs exert a cytotoxic effect on human osteoblast cell in a concentration-dependent manner a *concentrations* in the *range* of *1–20* μg/mL do not adversely affect the human fetal osteoblast cells viability and can be used as antimicrobial additives in implants. However, higher concentrations of AgNPs (40-80 μg/mL) should be thoroughly tested and optimized, because it may possess cytotoxic effect against hFOB 1.19 cells.

More research has to be performed to assess the effects of different AgNPs on bone cells and their usage in orthopaedic surgery and tissue engineering.

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