ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.2018.291

Reactions of bone, liver and kidney tissues to orthopaedic implants with silver nanoparticle doped hydroxyapatite coatings: microscopic examination in a rabbit model

Łukasz Łapaj[1], Waldemar Woźniak[1], Paweł Chodór[1], Jan Zabrzyński[2], Łukasz Paczesny[3]

[1] Department of General Orthopaedics, Musculoskeletal Oncology and Trauma Surgery, Poznan University of Medical Sciences, Poland
[2] Department of Orthopedic Surgery, Multidisciplinary Hospital, Inowrocław, Poland
[3] Department of Orthopedic Surgery, Orvit Clinic, Toruń, Poland

ABSTRACT

Introduction. Periprosthetic joint infections are severe complications of arthroplasty, difficult to manage due to biofilm formation on the components. Recently, the use of silver nanoparticles (SNs) has emerged as a method of preventing biofilm formation on orthopaedic implants, however little is known about the systematic toxicity of SNs.
Aim. This study used a rabbit model to examine the tissue response of bone, liver and kidney to prototype components with SN doped hydroxyapatite (HA) coatings.
Material and Methods. Twelve prototype implants (six with HA, six with SN doped HA coatings) were implanted into the femora of twelve New Zealand Rabbits. After 6 weeks, the animals were euthanised, their femora and samples of livers and kidneys harvested to prepare microscopic slides. The slides were examined for the presence inflammatory or toxic reactions to SNs, implants were examined using scanning electron microscopy (SEM) to determine structural changes related to implantation and verify retention of SNs *in vivo*.
Results. SEM demonstrated that SNs formed submicron conglomerates, which were retained after 6 weeks *in vivo* and did not interfere with osseointegration. Histologic studies of bone fragments demonstrated no signs of acute toxicity and inflammation. No inflammatory reaction was observed in kidneys, although in some samples signs of acute renal failure related to euthanasia were found. No severe toxic reaction was found in liver samples, however fatty degeneration of liver was found in some animals.
Conclusions. This study documented good osteointegration of implants with SN doped HA coatings with low systematic toxicity of SNs.

Keywords: osseointegration, silver nanoparticles, hydroxyapatite coatings.

Introduction

Total joint arthroplasty is perhaps the most successful orthopaedic procedure, performed in cases of end-stage osteoarthritis of the hip, knee and other joints [1, 2]. The number of such surgeries performed in developed countries is increasing due to ageing populations and it is estimated that approximately 50 thousand arthroplasties are performed annually in Poland. Arthroplasty is often performed in young, more active patients due to encouraging long-term clinical outcomes. In such cases, long-term function of the implants

relies on biologic fixation with host bone by means of osseointegration, ingrowth of bony tissue into various coatings of the components, predominantly hydroxyapatite (HA) [1, 2].

Unfortunately, as the number of joint replacements performed annually is increasing worldwide, the financial burden related to complications of these procedures is becoming more severe [1]. Perhaps the most devastating complication of arthroplasty is periprosthetic joint infection (PJI). This is because infections involving orthopaedic implants are associated with formation of biofilms, complex polymeric conglomerates of proteins, polysaccharides and DNA [3]. Biofilms encapsulate bacterial cells present on the surface of implants and protect them from antibiotics or the host immune response [3]. To date, no biofilm-disrupting drugs are available, consequently surgical exchange of components and prolonged antibiotic therapy are a necessity in the management of most patients with PJIs [1]. Unfortunately, this type of treatment is associated with unacceptably low success rates and suboptimal clinical results.

For this reason, development of methods allowing prevention of PJIs has become a research priority. In recent years, application of silver nanoparticles (SNs) on the surface of orthopaedic components has been suggested [2–4] based on data from laboratory studies, which indicated that SNs have antimicrobial properties and can prevent biofilm formation. This is also an attractive alternative for the industry, as such modification could be easily used in existing implant designs with long-term clinical record. However, there is a lack of data regarding the safe application of SNs on orthopaedic coatings and their possible local and systematic toxicity, especially in kidneys and liver.

Aim

This study used a rabbit model to examine tissue reactions of bone, liver and kidney to implantation of prototype components with SN doped hydroxyapatite coatings.

Material and Methods

This experimental study based on a New Zealand White Rabbit model compared the response of bone, liver and kidneys to implantations or prototype orthopaedic implants with conventional HA and SN doped HA coatings. First, prototype implants (cylinders with a diameter of 4 mm and length of 22 mm) were manufactured from TiAl6V4 alloy, plasma sprayed with a 50 µm thick HA coating (2PS, Montbazens, France). Coatings of randomly selected implants were then doped with SNs with a mean diameter of 66 nm (Particular GmBH, Hannover, Germany). Briefly, implants were immersed in a suspension of SNs with a silver concentration of 106 mg/l and stirred for 14 hours at room temperature. The components were then rinsed with physiological saline, dried and sterilised. Next, characterisation of the coating from both types of implants was performed using scanning electron microscopy (SEM): implants were rinsed in acetone to remove any fatty residues, vacuum dried, glued to aluminium stubs using a conductive carbon tape and coated with a 10-nm layer of gold using a JEOL JFC 1200 sputter coater. Samples were then examined using SEM (JEOL JSM-6400) with an accelerating voltage of 20–30 KV. The chemical composition of osseointegrative layers was verified using Energy-Dispersive X-ray Spectroscopy (EDS, Oxford Instruments Inca); analyses were performed in randomly selected, square regions (10 x 10 µm) at an accelerating voltage of 25 KV, with a bandwidth of 10 KeV and noise peak cut-off at 0.3 KeV. Analyses of larger regions were performed to minimise differences in silver content related to random distribution of SNs on the surface of the implants.

Six components with SNs (study group) and six implants with conventional HA coatings (controls) were implanted into the femora of New Zealand White rabbits (a total of twelve animals), according to approval granted by the local bioethical committee. Briefly, animals were fist anaesthetised (ketamine + medetomidine) and distal epiphyses of their left femora were exposed using a lateral approach. A 4-mm hole was then drilled through the bone, following by implantation of a prototype component and wound closure. All rabbits survived the procedures with no major complications, and were kept in cages receiving food and water *ad libitum* for 6 weeks. At that time, animals were euthanised (intracardiac phenobarbital injection) and their femora containing the implants, livers and kidneys were imme-

diately harvested. Fragments of bone adjacent to the implants were removed using a 10-mm chisel and used for further studies. All obtained tissue samples were fixed in 10% buffered formalin for 72 hours.

Liver and kidney samples were used to prepare routine pathological slides (H+E staining). Since toxic effects of silver may result in fatty degeneration of liver, these samples were evaluated according to the NAS scoring system developed by Kleiner et al. [5]. The scale comprised 14 histological features, 4 of which are evaluated semi-quantitatively: steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2), and fibrosis (0–4). In this system scores higher than five are required to diagnose steatohepatitis.

Bony fragments underwent electrochemical decalcification in Romeis fluid (solution of hydrochloric and formic acid) using a current of 100 mA and were then stained using conventional H+E and McManus PAS techniques. The healing response of bone around implants was then evaluated using a modified Solchaga score [6]. The system comprised the presence of new bone (0–3), vascularisation of the bone (0–1), presence of osteoblasts/osteocytes/osteoclasts (0–3 each), presence of immature bone (0–3), trabecular bone (0–3) and Haversian channels (0–3), inflammation (0–2) and granulation tissue (0–2). The scale yields 0–26 points and was used to quantify bone healing within a mantle of 4 mm around the implant.

Finally, all retrieved prototype implants were vacuum dried, prepared for electron microscopy using the same protocol as for characterisation of newly manufactured coatings and examined using SEM to verify bone ingrowth into the surface of components and retention of SNs using EDS.

Results

Coating characterisation

A uniform HA layer was obtained on all implants fabricated for this study. SEM analysis demonstrated that the coatings were composed of small irregular splats and grains of hydroxyapatite, which formed a complex three-dimensional structure (**Figure 1a**). The deposition of nanoparticles had no effect on the morphology of the HA coating, which was identical to the control samples (**Figure 1b**). SEM examination under high magnifications showed a tendency of SNs to form larger conglomerates (approx. 100 nm – 1 µm in diameter; **Figure 1c**). EDS analysis of the coatings confirmed the presence of calcium and phosphors in all samples; a well-defined peak

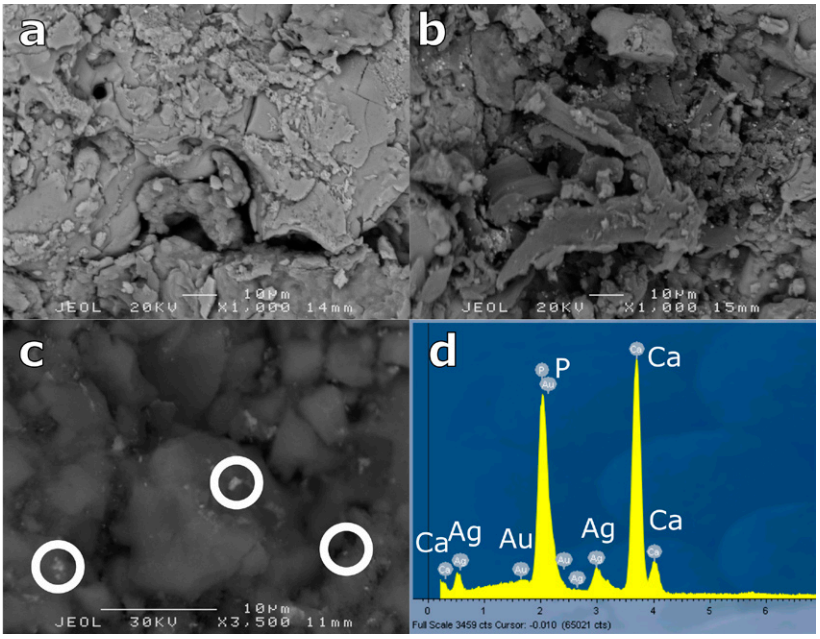

Figure 1. SEM studies of HA coatings used in the study: a) microstructure of the HA coating in control group; b) microstructure of the HA coating doped with SNs; c) high magnification image of SN doped HA coating demonstrating conglomerates formed by the nanoparticles (in circles); d) EDS analysis of SN doped HA coating confirming presence of silver

confirming presence of silver was observed in all SN doped implants (**Figure 1d**). Dependent on the region, the quantitative EDS analysis indicated a mean silver content of 3.3% (range 2.7–4.2%).

Characterisation of bone tissue reactions

There were no qualitative differences between bone samples around the implants in both groups. In all cases, the periosteum was properly structured from the connective tissue, and osteoblasts were only observed in regions adjacent to the implant border, where the periosteum was disrupted during implantation (**Figure 2**). The cortical bone was formed from significantly mineralised tissue with typical Haversian canals in all samples, while the trabecular bone exhibited a typical structure, with an increasing gradient of trabeculae towards the epiphyses. Areas around the implants had a mixed pattern of various features including formation of fibrous tissue (fibroplasia), small regions of cartilaginous tissue (chondroplasia) and new bone elements (osteoplasia) with features of lamellar and trabecular bone (**Figure 2**). There were no cases of cellular infiltration around the implants, indicating a lack of inflammation. The presence of implants did not influence the structure of bone marrow; intertrabecular sinuses contained yellow and red bone marrow with a typical morphology and occurrence of myelogenesis. A good healing response was observed in all cases, with comparable mean healing scores for HA (mean 18.3; range 15–23) and HA+SN (mean 17.8; range 16–22) samples.

Characterisation of kidney and liver tissues

Kidney samples from all animals retained a typical microscopic structure of nephrons (**Figure 3a**); the border between the cortical and medullar parts were clearly visible. Hyperaemia was observed in some cases within the glomerular (3 HA and 2 SN animals) and borderline (2 HA and 2 SN rabbits) renal layers, indicating acute renal failure, and are often described as "shock kidney".

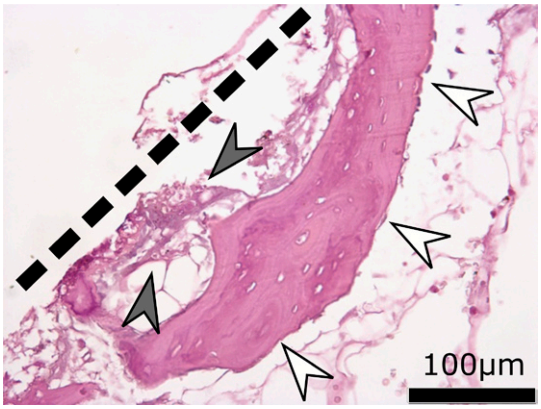

Figure 2. Bone formation around the implant. Dashed line indicates the location of implant, white arrows indicate trabecular bone, and grey arrows indicate localised chondroplasia

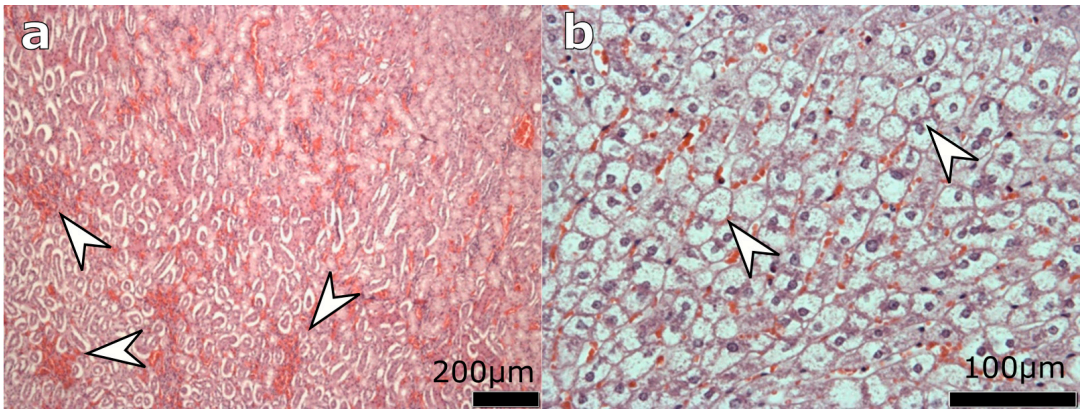

Figure 3. Microscopic images of kidney and liver samples: a) fragment of kidney tissue with localised hyperaemia indicated by arrows; b) liver sample from one animal with severe fatty degeneration (white arrows)

A normal morphology of liver with typical hepatic lobules was observed in most rabbits. However, a varying extent of hyperaemia in hepatic vessels (3 HA and 3 SN rabbits), localised lymphocytic infiltrations (1 HA and 3 SN rabbits) as well as fatty and vacuolar degeneration of hepatocytes (2 HA and 4 SN rabbits) were observed in some samples from both groups. In five cases where signs of fatty degeneration were present, the NAS score was 4 (borderline steatohepatosis), while pronounced damage with a score 6 was observed in one SN rabbit (**Figure 3b**).

Retrieval analysis

SEM examination of explanted components demonstrated ingrowth of trabecular bone into implants with conventional HA coatings and components with SNs (**Figure 4a**, **b**). In all implants the coating remained intact, except for localised damage caused by surgical tools used to extract bony fragments for histologic analysis. There were no qualitative differences between the newly formed bone on the surface of the components; in all cases formation of well attached trabeculae was confirmed. In all implants with SN doped coatings, conglomerates of silver nanoparticles were visible after six weeks *in vivo*, and EDS analysis confirmed their atomic composition (**Figure 4c**).

Discussion

In current clinical practice, the use of implants anchored by means of biologic fixation is becoming the gold standard in joint arthroplasty, including hip, knee, shoulder and ankle replacements [4, 7]. Such implants are being used in a growing number of patients, including young, active individuals. Many arthroplasties performed in developed countries are associated with an increasing number of complications following these procedures, and PJIs are perhaps the most severe [1]. Since the development of PJIs heavily depends on formation of biofilms on implants, it has been suggested that application of SNs to their surface could minimise the risk of infection [2–4]. Although antimicrobial properties of SNs are widely recognised, it is unclear whether their application in orthopaedic components is associated with localised or systematic toxicity [2, 3, 8, 9]. This study demonstrated that hydroxyapatite coatings doped with SNs are not associated with a localised toxic response of host bone, kidneys and liver.

There are several limitations to this study, predominantly due to the fact that it includes a small number of samples and animals. This was due to ethical reasons, which required the lowest possible number of animals to be used, and this applies

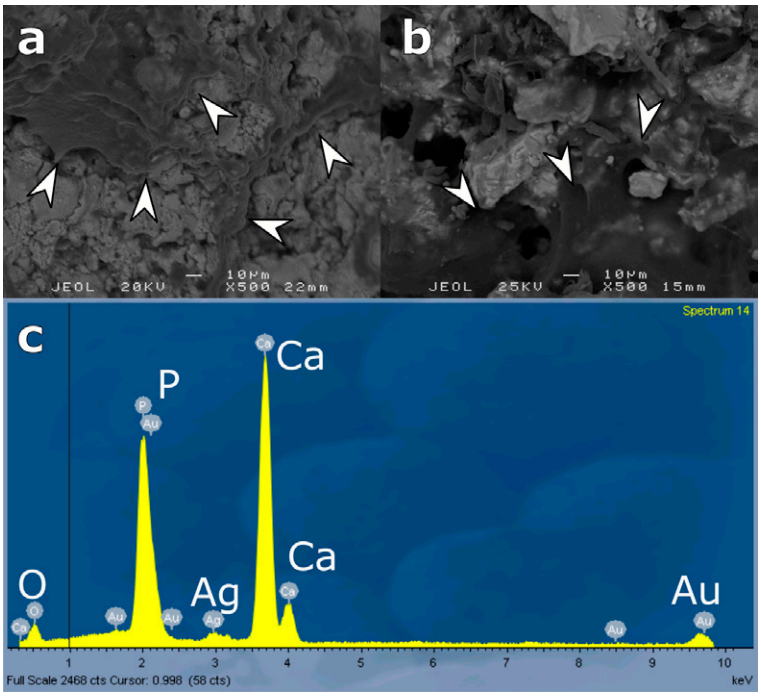

Figure 4. SEM analysis of retrieved samples: a) bony ingrowth (arrows) into conventional HA coating; b) ingrowth of bone (arrows) into SN doped HA coating; c) EDS analysis of fragment of SN doped coating not covered by bone confirming presence of silver nanoparticles

to other similar studies [3, 9]. Another limitation is that conventional, decalcified bone samples were included [10, 11]. It is possible that the decalcification procedure could potentially alter the morphology of bone tissue, however performing this procedure allowed the SEM examination of the retrievals. Lastly, the examination of the toxic effects related to implantation of components with SNs has limited precision since it is based on histologic examination [5, 8, 11].

Good osseointegration of SN doped HA coatings and retention of nanoparticles on the surface of the coatings is perhaps the most important finding from this study. Although results of EDS analyses suggest that a large portion of silver was retained after six weeks *in vivo*, it is not possible to precisely determine the extent of silver release from such coatings [9]. The HA layer has a complex porous structure, and EDS analysis allows the silver content to be determined only in areas directly exposed to the electron beam of the SEM. Unfortunately, the small size and weight of particles make it practically impossible to determine the amount of SNs retained in areas overgrown by trabecular bone. Theoretically focused ion beam milling could be used to gradually remove fragments of bone and expose the coating and SNs. However, even the use of such advanced techniques would yield very imprecise results due to irregularities in particle distribution and the complex three-dimensional coating structure [3]. Nonetheless, histological studies demonstrated that the presence of SNs does not affect bone ingrowth, with no inflammatory reactions associated with the presence of SNs.

The examination of kidney and liver tissues performed in this study aimed to determine the systematic cytotoxicity of SNs. Although some of samples examined in this study exhibited abnormal findings, they are most likely not associated with SNs. Kidney tissue samples exhibited findings typical of acute kidney failure, which are considered characteristic for hypovolemic shock rather than chronic damage, in agreement with data from other authors [11]. We believe that they were most likely formed when the animals were euthanised. However, some pathologies observed in the liver samples, predominantly fatty degeneration, developed over a relatively long time. It is essential to consider other etiological factors in these cases which may cause the hepatic lesions, such as the type of culture, nutrition, infections and stress-inducing conditions [2, 7–9]. Taking the above into consideration, it is not possible to confirm that lesions noted in experimental groups of rabbits are associated with the type of implant used.

Conclusions

This study demonstrated good osseointegrative properties of SN doped HA coatings, comparable to that of conventional hydroxyapatite layers. The presence of pathologic lesions in livers and kidneys of some animals can be attributed to the harvesting procedure (shock kidney) or have multifactorial origin (fatty degeneration in some livers). The findings suggest the antimicrobial potential of SN doped HA coatings to prevent biofilm formation *in vivo,* but this requires confirmation in further studies.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
This study was financed by 2011–2015 funds from the Polish Ministry of Science and Higher Education as part of an international project "Development of new nanocomposites using materials from mining industry" NANOMINING, EU grant number 2204/7.PR/2011/2.

References

1. Vanhegan I, Malik A, Jayakumar P, Islam SU, Haddad F. A financial analysis of revision hip arthroplasty: the economic burden in relation to the national tariff. J Bone Joint Surg Br. British Editorial Society of Bone and Joint Surgery. 2012;94(5):619–23.
2. Kose N, Otuzbir A, Pekşen C, Kiremitçi A, Doğan A. A silver ion-doped calcium phosphate-based ceramic nanopowder-coated prosthesis increased infection resistance. Clin Orthop Relat Res. 2013;471(8):2532–9.
3. Besinis A, Hadi SD, Le H, Tredwin C, Handy R. Antibacterial activity and biofilm inhibition by surface modified titanium alloy medical implants following application of silver, titanium dioxide and hydroxyapatite nanocoatings. Nanotoxicology. 2017;11(3):327–38.
4. Cheng Y, Gao B, Liu X, Zhao X, Sun W, Ren H, Wu J. In vivo evaluation of an antibacterial coating containing halogenated furanone compound-loaded poly (l-lactic acid) nanoparticles on microarc-oxidized titanium implants. International journal of nanomedicine. 2016;11:1337.
5. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313–21.

6. Lucaciu O, Băciuț M, Băciuț G, Câmpian R, Soriță O, Bran S, et al. Tissue engineered bone versus alloplastic commercial biomaterials in craniofacial reconstruction. Rom J Morphol Embryol. 2010;51(1):129–36.
7. Ciobanu CS, Iconaru SL, Chifiriuc MC, Costescu A, Le Coustumer P, Predoi D. Synthesis and antimicrobial activity of silver-doped hydroxyapatite nanoparticles. BioMed Research International. 2013;2013:916218.
8. Kim T-H, Kim M, Park H-S, Shin US, Gong M-S, Kim H-W. Size-dependent cellular toxicity of silver nanoparticles. J Biomed Mater Res A. 2012;100(4):1033–43.
9. Xie C-M, Lu X, Wang K-F, Meng F-Z, Jiang O, Zhang H-P et al. Silver nanoparticles and growth factors incorporated hydroxyapatite coatings on metallic implant surfaces for enhancement of osteoinductivity and antibacterial properties. ACS applied materials & interfaces; 2014;6(11):8580–9.
10. Punke C, Zehlicke T, Just T, Holzhüter G, Gerber T, Pau HW. Matrix change of bone grafting substitute after implantation into guinea pig bulla. Folia Morphol (Warsz). 2012;71(2):109–14.
11. Kose N, Çaylak R, Pekcsen C, Kiremitçi A, Burukoglu D, Koparal S et al. Silver ion doped ceramic nano-powder coated nails prevent infection in open fractures: in vivo study. Injury; 2016;47(2):320–4.

Acceptance for editing: 2018-06-30
Acceptance for publication: 2018-07-02

Correspondence address:
Łukasz Łapaj
Department of General Orthopaedics
Musculoskeletal Oncology and Trauma Surgery
Poznan University of Medical Sciences, Poland
email: llapaj@ump.edu.pl