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Allograft/DBM Use in Infected Bone Regeneration When Combined with Silver Nanoparticles and BMP2

A thesis submitted in partial satisfaction

of the requirements for the degree

Master of Science in Oral Biology

by

Mona Farrahi Adams

2017

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ABSTRACT OF THESIS

Allograft/DBM Use in Infected Bone Regeneration When Combined with Silver Nanoparticles and BMP2

by

Mona Farrahi Adams

Master of Science in Oral Biology
University of California, Los Angeles, 2017
Professor Kang Ting, Chair

Background: Complete regeneration of contaminated/infected bone defects is a difficult clinical situation. Prevalence of multiantibiotic resistant organisms has renewed interest in the use of antiseptic silver as an effective, but less toxic antimicrobial with decreased potential for bacterial resistance. This project will systematically integrate a nanosilver (Ag^{NANO})-based antimicrobial and sustained-release bone morphogenetic protein 2 (BMP2) in a demineralized bone matrix (DBX®) carrier to create a “one-step” bone graft material that can be implanted into contaminated/infected bony defects at the time of initial debridement surgery. Our previous studies demonstrate that nanosilver (Ag^{NANO}) exhibit excellent antimicrobial activity against methicillin and vancomycin-*resistant S. aureus* Mu50 and *P. aeruginosa*, pathogens frequently isolated from bone and joint infections, without osteoblast toxicity. BMP2 is a Food and Drug Administration (FDA)-

approved osteoinductive factor in wide clinical use. Its release can be modified by apatite-coated β-tricalcium phosphate (aTCP) microcarrier which is a biocompatible ceramic material that is bioresorbable and can be replaced by bone tissue *in vivo.*

Methods: Methicillin and vancomycin-resistant *S. aureus* Mu50 and *P. aeruginosa*, were used in a bacterial infection model. Cell viability was estimated by MTT metabolism. Alkaline phosphatase (ALP) activity was assessed by using a colorimetric ALP assay and degree of mineralization was quantified in MC3T3-E1 cell culture supernatants by Alizarin Red S staining.

Results: In this study, we demonstrated that metallic nanosilver particles (with a size of 20-40 nm), DBX® and poly(lactic-*co*-glycolic acid) (PLGA) composite grafts have strong antibacterial properties. In addition, the composite grafts did not inhibit adherence, proliferation, alkaline phosphatase activity, or mineralization of ongrowth MC3T3-E1 pre-osteoblasts compared to DBX®- PLGA controls. Furthermore, nanosilver particles did not affect the osteoinductivity of BMP2.

Conclusion: This study demonstrated that 5.0% nanosilver in a bone graft device composed of biocompatible and biodegradable demineralized bone matrix and PLGA has bactericidal effect against both gram-positive *S. aureus*, and gram negative *P. aeruginosa* for 10^9 CFU/ml which far exceeds the typical 10^5 CFU/ml criteria for invasive tissue infection. Furthermore, there were no adverse effect on proliferation and osteogenic activity on mouse pre-osteoblastic cell line.

The thesis of Mona Farrahi Adams is approved.

Min Lee

Shen Hu

Kang Ting, Committee Chair

University of California, Los Angeles

2017

This thesis is dedicated to

All the mentors who have provided academic guidance,

my PI Dr. Ting, Dr. Zheng and Dr. Zhang,

the entire lab members who have contributed to this project,

and my family and friends – there are no words to describe their sacrifices–

who have supported me throughout my life

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Allograft/DBM Use in Infected Bone Regeneration When Combined with Silver Nanoparticles and BMP2

INTRODUCTION

For complete regeneration of contaminated or infected bone defects the body's endogenous tissues have to compete with infectious organisms that seek to contaminate, colonize and ultimately infect the implanted device. Despite a recent focus on aseptic surgical and procedural techniques, surgical implant-associated infections account for nearly half of the 2 million cases of nosocomial infections in the United States per year, with an average cost of combined medical and surgical treatment of US $15000[1,2], representing a significant healthcare and economic burden

Management of an implant-associated infection typically requires device removal, multiple debridement surgeries, and long-term systemic antibiotic therapy, despite the associated side effects and complications[3,4]. However, these additional surgical procedures and medical therapies not only increase the healthcare costs, but also result in an increased rate of recurrence, particularly because it is difficult to clear the infection from devascularized bone and other necrotic tissues[1]. Soon after introduction of an implant, a conditioning layer composed of host-derived adhesins (including fibrinogen, fibronectin, collagen, etc.) covers the surface of the implant[1]. This layer promotes adherence of free-floating (planktonic) bacteria, which subsequently form a three dimensional, extracellular polysaccharide biofilm[1]. Once a biofilm forms, it makes treating these infections extremely difficult because the biofilm blocks the penetration of both host

immune cells (such as macrophages) and systemic antibiotics, promoting further bacterial survival[5,6]. Given the difficulties in treating implant-associated infections, strategies aimed at preventing the infection and biofilm formation during surgery and in the immediate postoperative period may serve as a more effective alternative that can prevent these infections.

Prior studies have covalently linked antibiotics onto prosthetic materials to prevent bacterial infection during surgical implantation[7,8]. However, efficacy of local therapies is limited by the sensitivity of a given bacterial species to a specific antibiotic used[9]. An implant-related infection can be caused by a wide spectrum of bacteria, including Gram-positive *Staphlococcus. aureus*, *S. epidermidis*, and Streptococci species, as well as Gram-negative *Pseudomonas* and *Enterobacter* species[10,11]. The use of narrow-spectrum antibiotics may inadequately cover these infecting bacterial species, while the use of broad-spectrum antibiotics can contribute to the development of antibiotic resistance, which is especially relevant as there is an increasing number of infections caused by methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE) strains[9,12]. Furthermore, in most cases high doses of antibiotics are used to reach an effective therapeutic concentration in bone which can cause systemic toxicity as well as increase in multidrug-resistant bacteria[13,14].

In this study, gram positive *S. aureus* Mu50 and gram negative *P. aeruginosa* PAO-1 were chosen to be the source of infection. *S. aureus* Mu50 is the leading pathogen responsible for ~80% of all human osteomyelitis cases and a bacterial burden typical of invasive tissue infection[15–18], while P. aeruginosa PAO-1 is a biofilm-forming bacterial strain[17,19].

An ideal compound to control infection after definitive debridement would have broad-spectrum bactericidal activity, be minimally toxic, be easy to apply, and be less likely to incur resistance. Due to their relatively less toxic effects and lower likelihood of promoting bacterial resistance, antimicrobials in the form of nano-sized silver have evoked renewed interest. They have rapid and broad-cellular activities which require several mutations in bacterial systems to develop resistance against[20,21]. When delivered locally, they can be administered in higher antimicrobial concentrations at the site of infection for prolonged duration without systemic toxicity since only doses well above the antimicrobial levels are toxic to humans[22]. There are numerous *in vitro* studies that have shown bactericidal nanoscale silver without any discernible toxicity to osteoblasts[23–25]. *In vivo* silver in any form is not thought to be toxic to the immune, cardiovascular, nervous or reproductive systems and it is not considered to be carcinogenic, the prevailing view is that except for a permanent grey or blue grey discoloration of the skin (argyria), primarily in sun exposed regions and eye (argyrosis), silver is relatively non-toxic[26].

Mechanistically, all silver-based materials are thought to release silver ions Ag^+ in aqueous solution, which account for their antibacterial properties by attaching to specific thiol (–SH) groups containing sulfur and hydrogen found in a variety of structural and functional bacterial proteins[20,27]. Nanoparticles, which are defined as particulate dispersions or solid particles with a size in the range of 10-100 nm, exhibit remarkable physical, chemical, and biological properties. Silver nanoparticles (Ag^{NANO}), made up of 20–15,000 silver atoms have greater active surface, higher solubility, and chemical reactivity due to the greater surface-to-mass ratio[27,28]. Although the reservoir form for Ag^{NANO} is also elemental silver (Ag^0), Ag^{NANO} have higher release of oxidative Ag^+ and/or

partially oxidized Ag^{NANO} with chemisorbed (surface-bound) Ag^{+} and thus have higher antibacterial activity compared with conventional silver preparations [29].

Nanoscale silver preparations are commonly synthesized through chemical reduction of a silver salt such as silver nitrate ($AgNO_3$) by reducing agents. During chemical reduction, silver ion (Ag^{+}) receives an electron from the reducing agent and reverts to its metallic form (Ag^{0}) eventually clustering to form Ag^{NANO} particles. Generally, a capping agent [e.g., poly(N-vinyl-2pyrrolidone) (PVP)] is used during chemical reduction to stabilize the nanoparticles and prevent them from aggregating. However, reducing agents and organic solvents used in chemical reduction, such as N,N-dimethylformamide, hydration hydrazine, and sodium borohydride, are highly reactive and pose potential environmental and biological risks[27]. Moreover, the inability to control the size of the Ag^{NANO} obtained by chemical reduction could result in a wide distribution of sizes of Ag^{NANO} obtained. Photoreduction of silver salt in the presence of PVP or citrate by ultraviolet (UV) light is another simple strategy to produce Ag^{NANO}. However, most photo reduction methods still use organic solvents, such as PVP, and are unable to control the size of obtained Ag^{NANO}[27,29]

In order to overcome these challenges, in collaboration with QuantumSphere, Inc., non-chemically based Ag^{NANO} (QSI-Nano® Silver, QuantumSphere, Inc., Santa Ana, CA) with highly purified (> 99.9% pure), 20-40 nm Ag^{NANO} was used in this study. The active surface of QSI-Nano® Silver (15-25 m^2/g) is much greater than that of commercial non-nanoscale silver powder (1-2 m^2/g) and previously *in vitro* studied chemically synthesized nanoparticles (4 m^2/g)[30,31]. This highly purified, more homogenous Ag^{NANO} with greater active surface is more effective than other nanoscale silver preparations. Our lab has

previously demonstrated that up to 2% wt [weight percentage nanosilver with respect to poly(DL-lactic-*co*-glycolic acid (PLGA)], Ag^{NANO} are non-toxic, effectively bactericidal *in vitro*, and that silver nanoparticle-based bone grafts, using PLGA, combined with bone morphogenetic 2 (BMP2) successfully regenerate bone in vivo in a rat FSD infected with vancomycin-resistant *S. aureus* Mu50[9].

PLGA has been most widely used for biomedical applications, in particular, tissue engineering scaffolds and drug delivery systems due to excellent physicochemical properties, biocompatibility and biodegradability. The degradation period of PLGA can be controlled from weeks to over a year by varying the composition ratio and processing conditions. However, its applications have been hampered by immunologic responses. PLGA degrades into its original monomers, lactic acid and glycolic acid, which decreases the pH in the surrounding tissues, leading to local inflammatory reaction and potential delayed tissue regeneration[32].

The most favorable microenvironment for bone repair would require a resorbable, biocompatible bone graft device that provides structure, promote bone regeneration and has antimicrobial properties. While, autogenous bone is the “gold standard” of bone graft material in orthopedic surgery[33], its major disadvantage is the donor site pain and morbidity after harvesting[34]. Moreover, in procedures requiring large amounts of graft, there may not be adequate quantities of autogenous bone available[34].

The next best available graft alternative is an allograft which is any tissue harvested from one individual and implanted into another of the same species[34]. A review article of spine fusion treatments from 2014 reported that demineralized bone matrix

(DBM) shows similar fusion rates with autologous bone graft in lumbar spine fusion, when used as a graft extender with local autologous bone of autologous iliac crest bone graft[32]. DBM is a derivative of allograft bone which has long been recognized as a powerful inducer of new bone growth. After a demineralization process - developed by Urist et al in 1965[35] - an acid-insoluble matrix of collagen and growth factors is left behind. In the bone defect site, as well as in non-skeletal areas, DBM induces osteogenesis without a fibrous reaction[32].

As a carrier, DBM is superior to synthetic scaffolds since in addition to being osteoconductive it exerts osteoinductive effects, potentially lowering dose requirements of BMP2, and avoids potential detrimental inflammatory responses elicited by synthetic scaffolds. To improve and standardize bone regeneration, BMP2 can be added to bone graft extenders such as DBM to increase osteoinductive effects.

Musculoskeletal Transplant Foundation (MTF) has developed a DBM derived, moldable putty called DBX® which was recently approved by the Food and Drug Administration (FDA) for clinical use. DBM particles from cortical bone (bone content by weight, 31%) between 212 and 850 µm in diameter are processed with sodium hyaluronate (by weight in solution, 4%) in order to provide a structural framework for bone growth. DBX® is nonhemolytic and models the PH of human blood which ensures compatibility with the surrounding autogenous blood cells. Furthermore, the combination of demineralized bone and sodium hyaluronate results in a greater textured surface area that makes it attractive to cells.

The osteoinductivity and regeneration capacity of DBX® can be exceeded by BMP2 and extended with apatite coated β-tricalcium phosphate (aTCP) as a bioresorbable microcarrier[36–42]. TCP is a biocompatible ceramic material that is bioresorbable and can be replaced by bone tissue *in vivo*[41] . It has been widely used as a platform for osteogenic growth factor delivery and bone regeneration due to its attractive osteoconductive properties[38]. Our group has successfully shown that we can localize the delivery of bioactive growth factors using TCP in a rat spinal fusion model [36]. In addition, by adding apatite coating to the TCP microcarrier, we can achieve further sustained release of bioactive molecules. Since rapid BMP2 release (or burst effect) may increase local BMP2 concentration and consequent adverse effects[43], the use of surface-modified TCP microcarriers should maximize the extended release of BMP2.

This project integrated a nanosilver-based antimicrobial and sustained-release BMP2 in a demineralized bone matrix framework in order to study its cytotoxic and antimicrobial properties with the ultimate goal of creating a “one-step” bone graft material that can be implanted into contaminated/infected bony defects at the time of initial debridement surgery. This material significantly decrease the morbidity of multiple surgeries and prolonged non-unions.

MATERIAL AND METHODS

2.1.Nanosilver

Nanosilver used in this study is in 20-40 nm silver particle form (QSI-Nano® Silver) obtained from QuantumSphere, Inc. (Santa Ana, CA).

2.2. Preparation of surface-modified TCP microcarrier

β-TCP granules 1.4–2.8 mm in diameter (Synthes Spine) were ground and sieved into particles 200–300 μm in diameter. β-TCP granules were coated with apatite in order to make aTCP via the following method: stimulated body fluid (SBF) solution was prepared via sequentially dissolving CaCl2, MgCl2·6H2O, NaHCO3, and K2HPO4·3H2O in ddH2O; solution pH was lowered to 6 by adding 1M hydrochloric acid to increase solubility; Na2SO4, KCl, and NaCl were added and the final pH was adjusted to 6.5 (SBF 1); Mg2+ and HCO3− free SBF (SBF 2) was prepared by adding CaCl2 and K2HPO4·3H2O in ddH2O and pH was lowered to 6; KCl and NaCl were added and the final pH was adjusted to 6.8; all solutions were sterile filtered through a 0.22 μm polyethersulfone membrane[44]. The granules were then incubated in SBF 1 for 12 h and changed to Mg2+ and HCO3− free SBF 2 for another 12 h at 37°C under gentle stirring. Coated granules were washed with ddH2O to remove excess ions and lyophilized before further studies.

2.3. Fabrication of antimicrobial bone graft device

The bone graft device were manufactured using a combination of our preliminary data[9,44]. The desired amount of nanosilver was mixed thoroughly with 17.5% (w/v) PLGA [85:15 poly(DL-lactic-*co*-glycolic acid), inherent viscosity: 0.64 dl/g in dichloromethane anhydrous >99.8% (50-150 ppm amylene as stabilizer); Sigma Aldrich]-dichloromethane solution. The concentration of silver refers to the weight ratio of nanosilver with DBX®. The samples were then placed in a chemical hood for 24 h, lyophilized for 24 h and stored in -20 degrees C. The surface morphology of the samples was evaluated using variable pressure scanning electron microscopy (NOVA 230 Nano SEM). Based on previous *in-vitro* studies, where a BMP2 dosage range of 50-500 ng/ml reported, we used the relatively low dosage 300ng/ml for the cytotoxicity assay[45,46]. BMP2 was coupled with aTCP and lyophilized for 24 h. Mixing all together, a DBX® /Ag^{NANO} /PLGA/$BMP2^{aTCP}$ bone graft device was fabricated.

2.4. In vitro antimicrobial activity

Methicillin and vancomycin-resistant *S. aureus* Mu50 and *P. aeruginosa*, were used in a bacterial infection model. The Gram-positive vancomycin-intermediate *S. aureus* (VISA/MRSA) strain Mu50 (ATCC 700699) was cultured in brain heart infusion broth (BHIB; BD, Sparks, MD) at 37°C; while biofilm-forming, Gram-negative opportunistic pathogen P. *aeruginosa* PAO-1 (ATCC 15692)[47] was cultured in Luria Bertani broth (LB; Fisher Scientific, Hampton, NH) at 37°C. 10^9 colony forming units (CFU) of bacteria were suspended in 1 ml culture broth and incubated with the DBX®/ Ag^{NANO} /PLGA bone graft

device at 225rpm on a shaker for 18 h. At the end of the incubation, 100 µl *S. aureus* Mu50 and *P. aeruginosa* PAO-1 bacteria were plated onto 10-cm BHIB or LB culture medium plates overnight, respectively. After 18 h incubation, the number of colonies on each plate was quantitated following protocols set forth by the U.S. Food and Drug Administration (FDA) in their Bacteriological Analytical Manual Aerobic Plate Count Method. If resultant colonies per plate were within 25-250, the undiluted colony numbers were utilized for quantitation. If there were over 250 colonies per plate, the bacterial solution was diluted by factors of 10 (e.g.,1:10, 1:100, 1:1000 dilutions) until resultant colonies per plate were again within 25-250, and colony numbers were then calculated accordingly.

2.5. In vitro cytotoxicity testing

Passage 18 mouse pre-osteoblastic MC3T3-E1 cell line (subclone 4, ATCC CRL-2593) was employed for *in vitro* cytotoxicity evaluation of DBX®/BMP2^{aTCP} / Ag^{NANO} /PLGA composite grafts devices. MC3T3-E1 cells were maintained in a-minimal essential medium(a- MEM) supplied with 10% fetal bovine serum (FBS), 1% HT supplement, 100 units/ml penicillin and 100 mg/ml streptomycin (maintenance medium) at 37°C with 5% CO^2. Two thousand five hundred cells were seeded on the bone graft devices for testing. All media for cell culture were purchased from Gibco (Invitrogen, Carlsbad, CA). Cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) metabolism using commercially available Vybrand® MTT Cell Proliferation Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA) with Tecan Infinite f200 microplate

reader. With MTT test, yellow MTT is reduced to purple formazan crystals by dehydrogenases in cells.

Alkaline phosphatase (ALP) activity was assessed after cultivation in osteoblastic differentiation medium (maintenance medium supplied with 50 mg/ml ascorbic acid and 10 mM b-glycerophosphate) by using colorimetric ALP assay kit (abcam ab83369).

Alizarin Red S staining (Sciencell 8678) was used to quantify the degree of mineralization in MC3T3-E1 cell culture supernatants[48,49].

2.6. Statistical analysis

The results were graphically depicted as the mean ± the standard deviation (SD). One-tailed t-test were performed (SPSS 13.0 for Windows, SPSS, Chicago, IL) to detect statistically significant differences. P value < 0.05 was considered statistically significant

3. RESULTS

3.1. Nanosilver coupled on DBX®/PLGA bone composite graft devices

The microstructure of DBX® - (demineralized bone matrix) - silver (metal) - PLGA (polymer) composite graft devices was analyzed by SEM. The different components of the graft device were integrated (Fig. 1 a-c). Aggregates or particles sintered together were not present in the DBX®/Ag^{NANO} /PLGA composite graft devices containing up to 5.0% silver (Fig. 2a-c). On the other hand, nanosilver particles aggregated in the composite devices at higher silver concentrations (Fig. 2d). Because the bioactivity of silver is mostly based on generation and/or release of oxidative silver[21], asymmetric distribution of nanosilver particles will result in uneven local concentration of oxidative silver followed by variable local antibacterial activity and cytotoxicity. Therefore, with this result the ceiling concentration of nanosilver in this study was established as 5.0%.

Visualization of the DBX®/Ag^{NANO} /PLGA composite graft device could not be obtained due to the complete opacity of the nanosilver layer on the graft material.

Fig. 1. Nanosilver, DBX®, aTCP components of the graft device



Fig. 1. SEM image of QSI-Nano® silver, particle sizes ranging from 20-40 nanometers (a). DBX demineralized bone matrix (b). SEM image of aTCP particles at a magnification of x400 (c).

Fig. 2. Scanning electron microscopy of bone graft device

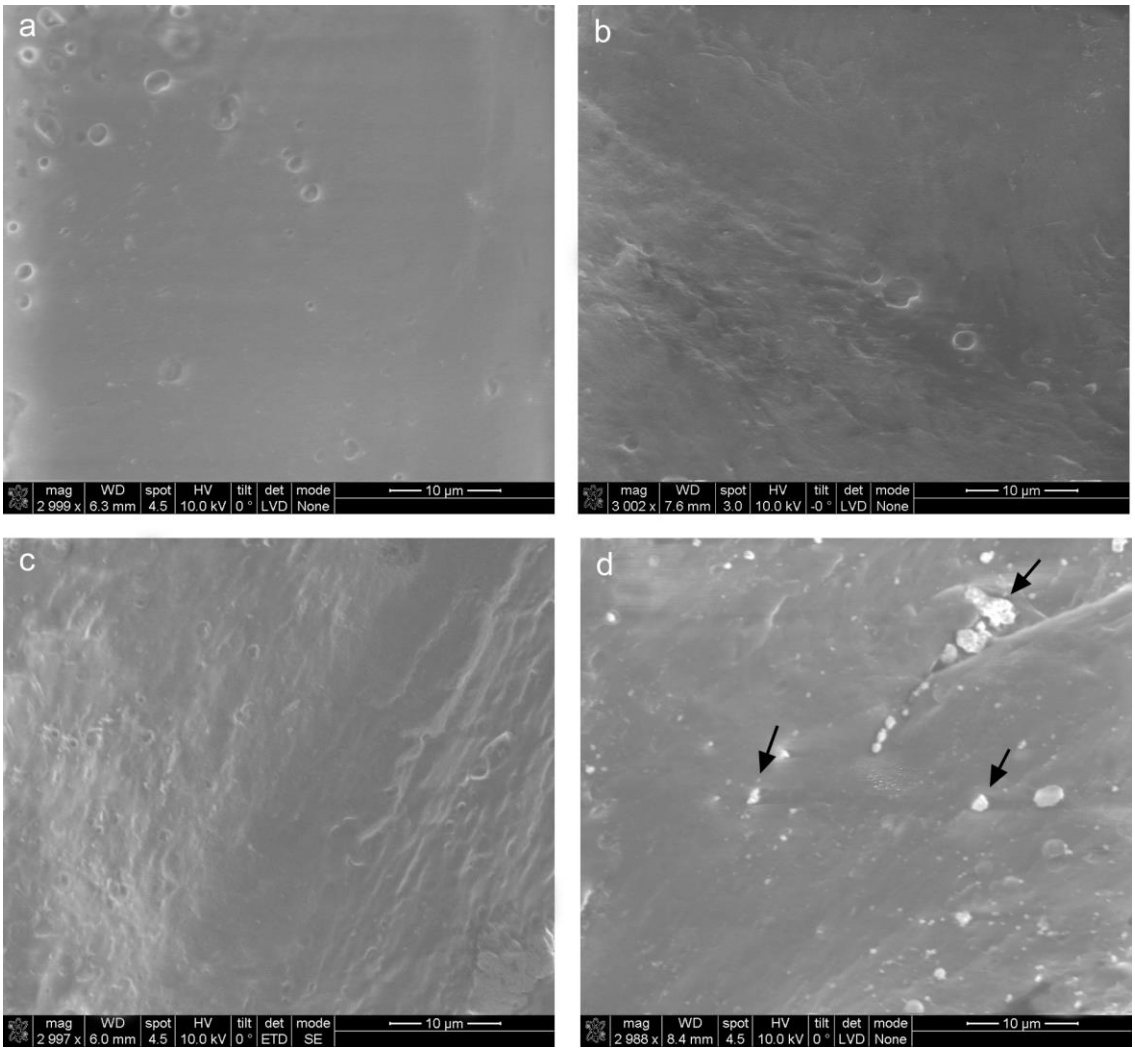


Fig. 2. Scanning electron microscopy of bone graft composite devices at magnification of x3000. Compared to control DBX®/PLGA composites (a), no discernible difference was found in DBX®/Ag^{NANO} /PLGA composite grafts with 2.0% (b) and 5.0% (c) nanosilver particles. However, particles aggregated (arrows) in the composite grafts with 10% nanosilver (d).

3.2. Antibacterial activity of nanosilver

Control DBX®/PLGA graft devices with no nanosilver did not inhibit proliferation *of S. aureus* Mu50 (Fig. 3a) nor *P. aeruginosa* (Fig. 3b) *in vitro* by 24 h, while at the new established ceiling concentration of 5.0% nanosilver, bacterial proliferation of 10^9 CFU was completely inhibited *in vitro.* This finding demonstrates the efficacy of Ag^{NANO} in decreasing localized bacterial bioburden in challenging *in vitro* environment contaminated with up to 10^9 CFU/ml *S. aureus Mu50* as well as *P. aeruginosa*.10^9 CFU/ml of bacterial culture was used since it far exceeds the typical 10^5 CFU/ml criteria for invasive tissue infection[50]*.* As a consequence, for future *in-vivo* studies a bacterial inoculum above 10^5 CFU/ml and up to 10^9 CFU/ml can be used.

Fig. 3. In vitro antibacterial activity of composite graft devices

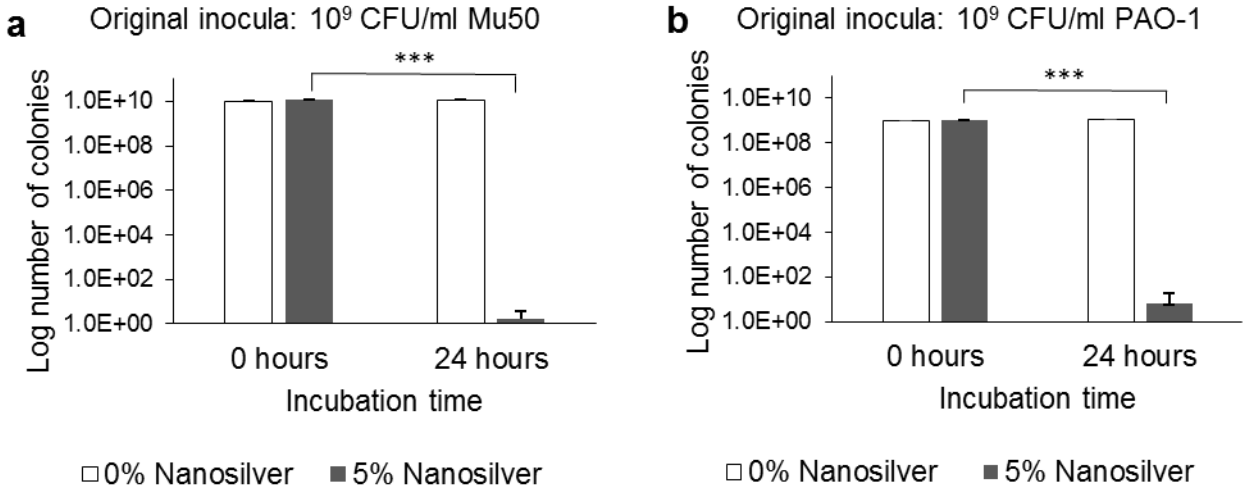


Fig. 3. *In vitro* antibacterial activity of DBX®/Ag^{NANO} /PLGA composite graft devices. 10^9 CFU/ml Mu50 (a) and 10^9 CFU/ml PAO-1 (b). *** P<0.001, N=6 for each test.

3.3. Cytotoxicity

Cell growth activity measured by MTT assay showed that neither 0.3 microgram/ml BMP2 nor up to 5.0% nanosilver affected the viability of MC3T3-E1 cells. Mouse pre-osteoblastic MC3T3-E1 cells grew into the DBX®/Ag^{NANO} /PLGA graft devices in osteoblastic maintenance medium with no significant difference in cell viability between the tested samples measured on day 1, 3 and 6 with and without BMP2 (Fig. 4a-b)

Fig 4. *In vitro cytotoxicity of DBX®/Ag^{NANO} /PLGA composite grafts devices*

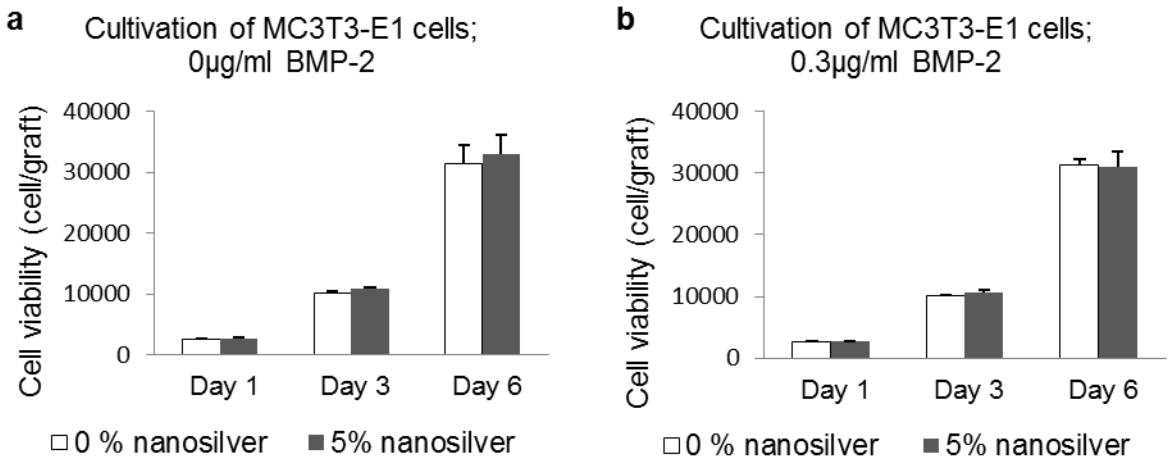


Fig. 4. *In vitro* cytotoxicity of DBX®/Ag^{NANO} /PLGA composite graft devices. In maintenance medium, 5.0% nanosilver did not affect MC3T3-E1 viability with 0 (a) or 0.3 µg/ml (b) BMP. No significant difference in cell viability was found between the tested grafts. $P < 0.05$; N=6 for each test.

3.4. Osteogenic activity of DBX®/Ag^{NANO} /PLGA graft devices

To assay osteoblastic cell function, ALP activity in MC3T3-E1 cells was measured after 9 and 15 days in osteoblastic differentiation medium (Fig 5a). 5.0% nanosilver samples had no adverse effect on ALP activity measured on culture day 9 and 15.

To qualitatively assess cell-mediated calcium accretion, mineralization was measured using Alizarin Red S on days 21 and 28 (Fig. 5b). Interestingly when mineralization was measured on culture day 21 the 5.0% nanosilver group had significantly higher amount of calcium deposition measured by Alizarin red (Fig 5b). Otherwise, no significance was detected between the tested graft devices on day 28.

Fig 5. ***In vitro osteoinductive activity of DBX®/Ag^{NANO} /PLGA composite graft devices***

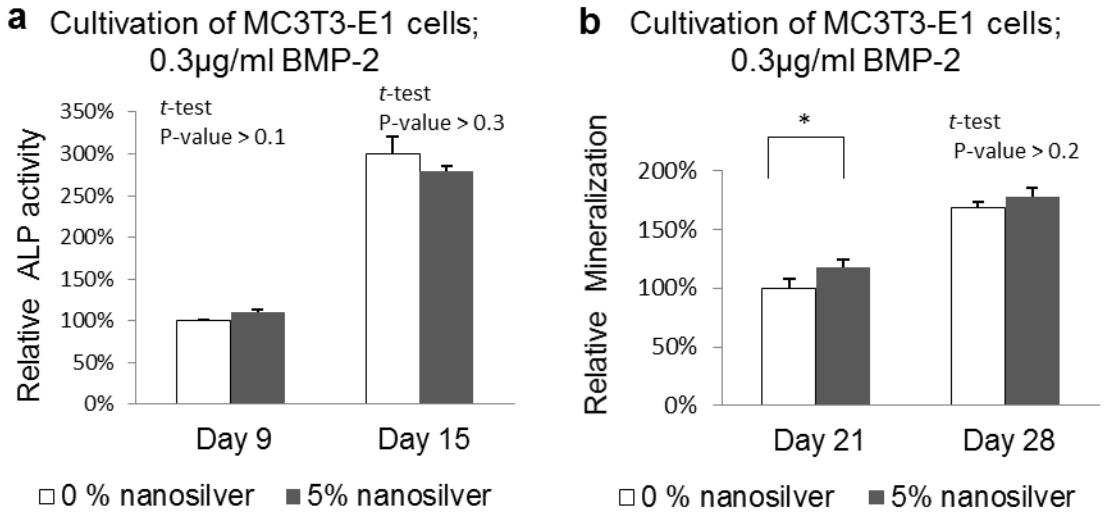


Fig. 5. *In vitro* osteoinductive activity of DBX®/Ag^{NANO} /PLGA composite *grafts*. There were no significant difference in ALP activity in differentiation medium on day 9 and 15 between 0% and 5.0% nanosilver containing grafts (a). The grafts with 5.0% nanosilver promoted significant mineralization on day 21 (b). N=6; *, $P < 0.05$.

DISCUSSION

The treatment of infected segmental bone defects continues to be a troublesome problem in orthopedics. Microorganisms, such as *S. aureus* and reported *P. aeruginosa*[51], infect bone through one or more of three basic methods: (a) hematogenous spread from other sources of infection, (b) from the skin or other external contaminants through open fracture or trauma, or (c) following placement of internal fixation devices[2]. Once the bone is infected, pus spreads affecting both the medullary and periosteal blood supplies, thereby impairing blood flow. Decreased vascular function likely decreases the amount of oxygen available to tissues at and around the fracture site, impairing the exchange of nutrients, and potentially leads to problems during recruitment of cells to the injury site[2]. These events, alone or in combination, lead to the formation of sequestra or necrotic bone, which is considered the hallmark of chronic disease and may contribute to delayed healing of any segmental defect. Once diagnosed via gold standard diagnostic modality for osteomyelitis – bone biopsy, conventional treatment includes drainage, extensive debridement of all necrotic tissue, obliteration of dead spaces, adequate soft tissue coverage, and restoration of an effective blood supply. This treatment is coupled with high doses of systemic administration of antibiotics for at least several weeks which can cause systemic toxicity as well as increase of multidrug-resistant bacteria[13,14]. Furthermore, in addition to the limited vascular supply which precludes obtaining therapeutic levels of antibiotic in the infected bone, the biofilm protects the bacteria from the host immune responses and antibiotics[5,6,52–54]

An alternative to systemic antibiotic therapy is in situ implantation of a local antibiotic delivery system, which initially works to obliterate bacteria in the area and

together with bone graft substitutes such as allogeneic demineralized bone matrix reduces the dead space.

The major advantages of allograft bone harvested from cadavers are its ready availability in various shapes and sizes, avoidance of the need to sacrifice host structures, and no problems of donor-site morbidity. DBM does not evoke any appreciable local foreign-body immunogenic reaction, as the antigenic surface structure of the bone is destroyed during demineralization[35] The biological activity of DBM is presumably attributable to proteins and various growth factors present in the extracellular matrix and made available to the host environment by the demineralization process. Prepared by acid extraction of allografts in addition to proteins and other growth factors, it also retains collagen. DBM can also augment and expand autologous cancellous bone graft when the supply of autogenous bone is limited or the defect is very large.

This study used DBX® from Musculoskeletal Transplant Foundation which is DBM with sodium hyaluronate to form a moldable putty and as such it can be packed into any defect size or shape. DBM is a derivative of allograft bone which has since long time been proven to be inducer of bone development. It is an inert biocompatible carrier, pH-balanced and nonhemolytic. DBM is superior to synthetic scaffolds since in addition to being osteoconductive it exerts osteoinductive effects, potentially lowering dose requirements of BMP2. However, BMP2 can be added if due to processing and storage requirement the osteoinductive potential is diminished. Another advantage is that sterilization, donor screening and sterile harvesting keeps the host immune response to a minimum. Furthermore, the new bone composition comprises a higher surface area which is attractive to cells and can induce significantly more and faster bone formation.

Biodegradable synthetic polymer, PLGA was added to the graft for even distribution and sustain release of the antibacterial nanosilver [9]. PLGA has been used for decades in clinical applications, including prosthetic devices, implants, and microspheres for drug delivery[55,56]

Silver, because of its antimicrobial properties, has been extensively used in water recycling and sanitization and for treatment of wound infections[9]. Currently, silver is gaining renewed attention as a medical antimicrobial agent due to its broad antibacterial spectrum and the difficulty of developing bacterial resistance to silver[9]. For instance, silver is used to reduce bacterial colonization in a variety of pharmaceutical devices including vascular and urinary catheters, endotracheal tubes, and implantable prostheses[9]. Mechanistically, silver prevents cell division and transcription by binding to and disrupting multiple components of bacterial structure and metabolism, including cellular transport, essential enzyme systems such as the respiratory cytochromes, and synthesis of cell wall components, DNA and RNA; nevertheless, the reservoir form of the active silver form may be diverse[9]. Previously, ionic reservoir forms of silver such as silver nitrate ($AgNO_3$) and silver sulfate (Ag_2SO_4) have been used to provide protection against bacterial infections[9]. However, despite their effective short-term antibacterial activity, these ionic salts have high solubility, inadequate local retention and hence, undesirable for continually preventing bacterial colonization[9].

Recent reports have shown that that 20-25 nm silver nanoparticles effectively inhibit microorganisms without causing significant cytotoxicity[9]. *In vivo* 10-20 nm silver nanoparticles are nontoxic in mice and guinea pigs when administered by the oral, ocular and dermal routes[57]. In a recent oral toxicity study of 60 nm silver nanoparticles in rats

there was a dose-dependent accumulation of silver content in a broad range of tissues including blood, liver, lungs, kidneys, stomach and brain [58]. In humans, the finding of argyria after dermal exposure of burned skin with nanosilver containing wound dressings (Acticoat) indicates that ingested colloidal silver is absorbed by the gastrointestinal tract, distributed by the blood to organs and eventually accumulates (partially) in the skin[59]. If nanoparticles are absorbed by the gastrointestinal tract, they will be transported directly to the liver via the portal vein. In general, the liver is able to actively remove compounds from the blood and transform them to chemical forms that can easily be excreted. However, no evidence exists for metabolism of nanosilver by enzymes in the liver and the rest of the body. Most probably, nanosilver is able to bind specifically to metallothioneins. Metallothionein proteins that are present in all living cells have a unique structure, depending on their ability to bind metals like zinc and silver. They regulate the cellular metal homeostasis[59]. The typical excretion route for nanosilver is via the kidneys since it has been reported that treatment of burn wounds with nanosilver containing wound dressings (acticoat) leads to detectable levels of silver in urine of patients[59].

Collectively, these findings suggest silver nanoparticles of the size evaluated in the present study are appropriate for therapeutic application from a safety standpoint. In order to prevent aggregation and stabilize nanoparticles several polymers have been used in previous studies, including poly(vinylpyrrolidone)[60] and chitosan[61]. The nucleophilic character of these polymers, albeit minor, is sufficient for them to bind to the metal particles by donating electrons[62]. The FDA-approved, biodegradable and biocompatible polymer PLGA was, however, used in this study because its hydrolyzable ester bonds are subject to nucleophilic interactions with the incorporated components such as silver

particles[63]. In addition, PLGA degradation is based on hydrolytic splitting of the polymer backbone into oligomers and release of lactic acid and glycolic acid, two byproducts of various metabolic pathways in the body under normal physiological conditions.

Thus, a local delivery system that incorporates silver nanoparticles into the polymer coating ensures not only high local concentrations around the implant for long periods but also reduced risks and side effects for the host organism compared to systemic drug application. Moreover, in order to combine the effect of infection-control with simultaneous and subsequent bone regeneration in infected segmental bone defects, a controlled delivery of BMP2 via an apatite-coated β-tricalcium phosphate microcarrier was utilized. BMP2 is a proven strong osteoinductive factor, and used for the treatment of many bone fractures and bone defects[55,64,65] . Therefore, the combination of a BMP2 with a PLGA-DBX® - bone graft has potential to be an ideal clinically applicable bone scaffold.

Taken together, this composite graft device - DBX® /Ag^{NANO} /PLGA/$BMP2^{aTCP}$ - represents a novel therapeutic approach of using a biomaterial with the dual function of infection control and bone regeneration. Collectively, this study showed that 5.0% nanosilver in a bone graft device composed of biocompatible and biodegradable demineralized bone matrix and PLGA has bactericidal effect against both gram-positive *S. aureus*, and gram negative *P. aeruginosa* for 10^9 CFU/ml which is significantly more than 10^5 CFU/ml count for invasive tissue infection[50].

Furthermore, there were no adverse effect on proliferation and osteogenic activity on mouse pre-osteoblastic cell line.

CONCLUSION

In this study, the efficacy of a nanosilver-releasing graft devices was evaluated *in vitro*. We found that a nanosilver concentration of 5% in a DBX (demineralized bone matrix)-silver (metal)-PLGA (polymer) composite graft device allowed for enough silver ion release to inhibit the growth of methicillin-resistant *S. aureus* and *P. aeruginosa* without causing cytotoxicity to mouse pre-osteoblastic cell line. In the creation of DBX® /Ag^{NANO} /PLGA/$BMP2^{aTCP}$ composite graft devices, the effect of nanosilver particle on BMP2 was also considered, as nanosilver particles could possibly interfere with essential cellular elements relating to BMP2 osteoinductivity when it binds to thiol groups. Fortunately, 5.0% concentration of nanosilver particles did not interrupt bone regeneration induced by BMP2 *in vitro* as shown in this study. Collectively, our findings support the application of DBM/silver nanoparticle/ PLGA composite for localized prophylaxis of implant associated infections.

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