Effect of Freezing on the Release Rate of Gentamicin Palmitate and Gentamicin Sulfate from Bone Tissue

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Received 16 December 2013; accepted 30 January 2014
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22602

ABSTRACT: In this study we evaluated gentamicin palmitate salt and gentamicin sulfate salt mixed with bone chips after storage at −80°C. Different concentration rates of gentamicin sulfate and gentamicin palmitate were mixed with human bone chips and stored for 1–6 months at −80°C. Nonstored samples were used as control. The release of the antibiotics from the bone was carried out in phosphate-buffered saline. Antibiotic concentrations in the elutions were determined with microbiological agar diffusion assay using Bacillus subtilis. Susceptibility tests were carried out using Staphylococci strains. The rate of gentamicin base (GB) released from bone was similar for all gentamicin salts and all storage conditions. The elutions released were efficient on reducing S. aureus and S. epidermidis CFU during all storage time. In resume, the capacity of bone grafts to act as gentamicin carriers has been confirmed in this study. GS + GP showed equivalent efficacy against S. aureus and S. epidermidis compared with GS pure. The lower delivery rate of GS + GP, related to its affinity with fat tissue can be an advantage for longer release times, increasing the local protection against infections. Storage at −80°C does not interfere on the gentamicin salts activity used. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: bone allografts; storage; anti-infective; gentamicin palmitate; bacillus subtilis; staphylococci

In orthopedic surgery bone grafts are used for reconstructing bone defects caused by implant associated complications, trauma, and tumors.[1,2] While autografts can be used, donor site morbidity can be avoided using allografts.[3–5] Bone grafts can either be used as large samples of bone grafts from post-mortem donors or as bone chips donated by living patients undergoing hip arthroplasty. Such bone chips are used to fill defects that require biomechanical stability, which can be achieved by compressing the chips into the defect site.

Freezing is the most common method for storing bone samples used in skeletal reconstructions. Bone banks protocols suggest that bone must be kept at low temperatures for more than 90 days and can be stored up to 5 years.[6] Fresh frozen method (storage at −80°C immediately after retrieval from donor without further procedures) is preferred because it does not compromise the original osteoconductive and osteoinductive proteins once viable cells remain alive. In addition, fresh frozen grafts are mechanically stronger, and better incorporated than, for example, freeze-dried grafts.[7]

However, fresh frozen chips can add the risk of local contaminations.[8,9] Surgery with bone allografts is complex and time-consuming; therefore it is per se prone to a higher infection rate (2.0–2.5%).[10–12] Additionally, the impaction of the foreign bone tissue creates an avascular area where local circulation is disrupted. In the case of a site infection, systemically administered antibiotics cannot reach the infected bone graft.[13] As a known complication factor, biofilms can be formed on the surface of foreign materials thus increasing antibiotic resistance.[14] Staphylococcus epidermidis and Staphylococcus aureus are the germs which mostly colonize implant surfaces.[15]

Antibiotics delivered from an implanted biomaterial can be potentially used to prevent infections, providing high concentrations of antibiotics at the surgical site without local or systemic toxicity. In addition, these materials should be osteoconductive and osteoinductive, thus supporting bone healing without further surgery.[16] Morselized bone allografts can be used as carriers by impregnating them with antibiotic solutions[17,18] or by mixing them with antibiotic powders.[19,20]

Anti-infective coatings containing a combination of antibiotics and/or antiseptics and fatty acids have been developed for medical implants and sutures. Matl et al.[21,22] developed a novel anti-infective coating consisting of a lipid-based drug-delivery system combined with antiseptics to avoid post-operative infections. Antibiotic coatings methods allow continuous drug release and superior anti-infective characteristics.

Gentamicin sulfate (GS) salt is commonly used antibiotic for local application in orthopedic surgery, for example mixed with PMMA cements. Gentamicin base (GB) consists of a mixture of gentamicin C1, C1a, and C2a + b. Gentamicin sulfate is highly water soluble and does not adhere well to metal surfaces. This substance can be used as a coating material for biomaterials and tissues by turning the water-soluble GS into a low-soluble gentamicin fatty acid salt (converting gentamicin sulfate to gentamicin palmitate; GP).[23,24]

As the freezing processes were never investigated on the activity of gentamicin palmitate, in this study

Conflict of interest: None.
Grant sponsor: Heraeus Medical GmbH, Wehrheim, Germany.
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we evaluated gentamicin palmitate (GP) and gentamicin sulfate (GS) mixed with bone chips (BCh) after freezing. The efficacy of the coated BCh was measured by drug release tests and bacterial susceptibility using B. subtilis, S. epidermidis, and S. aureus.

METHODS

Preparation of Bone Chips

Femur heads were obtained from the bone bank of the Medical University Innsbruck, Austria. The femur heads were removed during femoral head osteotomy from patients who had undergone hip replacement surgery. Throughout the procedure, the bone was rinsed and cooled with sterile 0.9% saline to prevent damage. Cortical and cartilage tissues were removed from the femoral heads with a bone saw. BCh were prepared from the spongious tissue using a bone mill (Noviomagus Bone Mill, Spierings Medische Techniek BV, Nijmegen, The Netherlands). BCh were mixed to achieve homogenous bone quality. All patients gave their written consent that the removed tissue was allowed to be used for research purposes.

Antibiotics

Gentamicin sulfate (GS) and gentamicin palmitate (GP) were kindly donated by Heraeus Medical GmbH (Wehrheim, Germany).

BCh Mixing With Antibiotics

The BCh were defrosted and added to tubes. The two different antibiotics following the specified range and groups (Table 1) were added to the BCh. The BCh with antibiotic powders were mixed with a spatula and then vortexed for 1 min. The same concentration rate was repeated three times for the sub-groups: no storage; −80°C 1 month and −80°C 6 months. All samples were carried out in triplicate.

Gentamicin Base Release

The release of the antibiotics from the BCh was carried out by using phosphate-buffered saline (PBS) pH 7.4 (Sigma–Aldrich, Schnelldorf, Germany). To that, 3 ml of PBS were added into each tube containing BCh mixed with each antibiotic. The tubes were vortexed for 1 min and placed on a rocking table (Rocky® Biometra, Goettingen, Germany; 20 cycles per min) at 37°C. After 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days the elution medium was completely removed and fresh PBS was added. The elution was vortexed for 1 min and stored at −20°C for the microbiological tests.

Bacillus Subtilis Assay for Antibiotic Release Concentrations

Antibiotic concentrations in the elutions were determined with a conventional microbiological agar diffusion assay using Bacillus subtilis (Merck KGaA, Germany in Test Agar pH 8.0 Merck KGaA, Germany) as the indicator strain.[25] Using a 6-mm diameter metal punch, a hole was made at the center of each B. subtilis agar plate into which 100 μl of each elution or 100 μl of 10-fold dilutions (from 10.000 to 0.01 mg/L) of gentamicin sulfate (standard curve) were added. The plates containing the samples were incubated for 24 h at 37°C. After the incubation period, the diameter of the zones of inhibition in centimeter was measured for each plate with a ruler. The diameter was confirmed with a second measurement. The standard curve was obtained by logarithmic regression and used to predict the concentration of gentamicin in each elution. This assay was carried out in triplicate.

Table 1. Concentration Rate and Group Organization

Group	Concentration Rate
GS 1%	1 g BCh + 0.01 g GB(GS)
GS 3%	1 g BCh + 0.03 g GB(GS)
GS 5%	1 g BCh + 0.05 g GB(GS)
GS + GP 1%	1 g BCh + 0.01 g GB(GS) + 0.01 g GB(GP)
GS + GP 3%	1 g BCh + 0.03 g GB(GS) + 0.03 g GB(GP)
GS + GP 5%	1 g BCh + 0.05 g GB(GS) + 0.05 g GB(GP)

BCh, bone chips; GB, gentamicin base; GS, gentamicin sulfate; GP, gentamicin palmitate.

Susceptibility Tests

Staphylococcus aureus ATCC 29913 and Staphylococcus epidermidis ATCC 12228 suspensions were prepared (0.5 McFarland) and 10 μl were inoculated over Müller-Hinton agar plates. Using a 6-mm diameter metal punch, a hole was made at the center of each plate where 100 μl of each antibiotic elution sample were added. The plates were incubated for 24 h at 37°C. After 24 h, the zones of inhibition were measured on each plate with a ruler. These tests were carried out in triplicate.

Statistical Analysis

Analysis of variance (ANOVA) and Bonferroni post hoc test were carried out to detect the differences between the antibiotics delivery and storage procedures concerning the concentration using B. subtilis and after susceptibility tests with S. aureus and S. epidermidis. p values <0.05 were considered significant different. The software package SPSS (Version 20, IBM Corporation, Armonk, NY) was used for all statistical calculations. Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA) was used to create the graphs.

RESULTS

Estimation of Antibiotic Delivery Rate by Using Bacillus Subtilis Bioassay After Storage Conditions

The rate of gentamicin base (GB) released from BCh mixed with pure GS and GS + GP in combination was similar for all storage conditions. As the concentration of GB in GS + GP combination was higher than in pure GS, the release rate in the GS + GP group was significantly higher in comparison with the GS group. In this case, higher concentration of GB was detected by the 10th day in comparison with the GS group (Table 2; Fig. 1). Along the release days, some significant difference could be observed between the release of GB from GS 1%, GS 3%, and GS 5% as well as between the release of GB from GS + GP 1%, GS + GP 3%, and GS + GP 5% (Fig. 1).

Susceptibility Tests

The elution released of GB from BCh mixed with GS and GS + GP were efficient on reducing S. aureus and S. epidermidis CFU. However, S. epidermidis are more susceptible to GS + GP and GS than S. aureus (Figs. 2 and 3). The zones of inhibition measured on S. epidermidis and S. aureus plates showed differences

Table 2. Mean and Standard Deviation (SD) of Release Rate of GS and GS + GP After 1 and 10 Days for the Three Storage Condition Groups

No Storage	Initial Delivery Rates (mg/L) 1 Day; Mean (SD)	Final Delivery Rates (mg/L) 10 Days; Mean (SD)
GS 1%	4.650 (2.619)	0.20 (0.15)
GS 3%	10.997 (8.254)	0.11 (0.15)
GS 5%	19.522 (26.011)	0.63 (0.35)
GS + GP 1%	20.827 (54.783)	0.00 (0.00)
GS + GP 3%	144.099 (88.849)	0.04 (0.04)
GS + GP 5%	33.778 (144.099)	1.31 (0.19)
1 Month	**1 Day; Mean (SD)**	**10 Days; Mean (SD)**
GS 1%	2.619 (1.475)	0.26 (0.02)
GS 3%	4.650 (3.490)	0.84 (0.84)
GS 5%	19.522 (10.997)	0.84 (0.63)
GS + GP 1%	7.918 (4.882)	0.01 (0.49)
GS + GP 3%	144.099 (54.783)	0.07 (0.18)
GS + GP 5%	33.778 (54.783)	0.30 (0.07)
6 Months	**1 Day; Mean (SD)**	**10 Days; Mean (SD)**
GS 1%	1.475 (1.475)	1.13 (1.50)
GS 3%	4.650 (6.195)	6.32 (8.43)
GS 5%	10.997 (2.619)	8.43 (8.43)
GS + GP 1%	4.882 (3.010)	0.30 (0.30)
GS + GP 3%	144.099 (54.783)	5.60 (3.45)
GS + GP 5%	54.783 (20.827)	5.60 (9.08)

between GS and GS + GP groups in some intervals, which can be observed in the Figures 2 and 3. The susceptibility tests using S. aureus showed less resistance of the strain after 1 month of the elution storage. That resistance was not observed after 6 months of storage (Fig. 2).

DISCUSSION

The objective of this study was to evaluate the activity of the novel gentamicin palmitate (GP) salt mixed with gentamicin sulfate (GS) salt mixed with bone chips (BCh) after 1 and 6 months of storage at −80°C. The results showed that GP in combination with GS, as well as GS alone are suitable for mixing with bone tissue and freezing aiming local antibiotic application. The storage processes did not compromised the efficacy of the gentamicin salts mixed with BCh against S. aureus and S. epidermidis *in vitro*.

The BCh mixed with gentamicin salts in this study was carried out mixing the BCh with powder manually. This is a suitable procedure for an operation room during surgery. Some authors first dilute the antibiotic powder in a saline solution and then soak the bone grafts in this solution keeping it for weeks or months before use.[17,18] We believe that this is an efficient method for bone chips incorporation with antibiotics since the tissue would act as a sponge absorbing the solution. According to these authors, that could also be an alternative for long-term storage of the grafts with antibiotic solutions. However, according to Sorger et al., the preservation of the grafts for up to 100 h in an antibiotic solution might influence the mechanical stability of the bone.[26] Based on Parrish and Witsø and collaborators, mechanical testing of osteochondral and structural allografts impregnated with antibiotics in solutions should be performed before this option is taken into clinical use.[27]

Due to its hydrophobic profile, it is expected that the GP coat not only the bone tissue but also the fat around the BCh, which could increase the adsorption areas of the carrier. In this study, GS + GP in combination showed lower release rates compared to

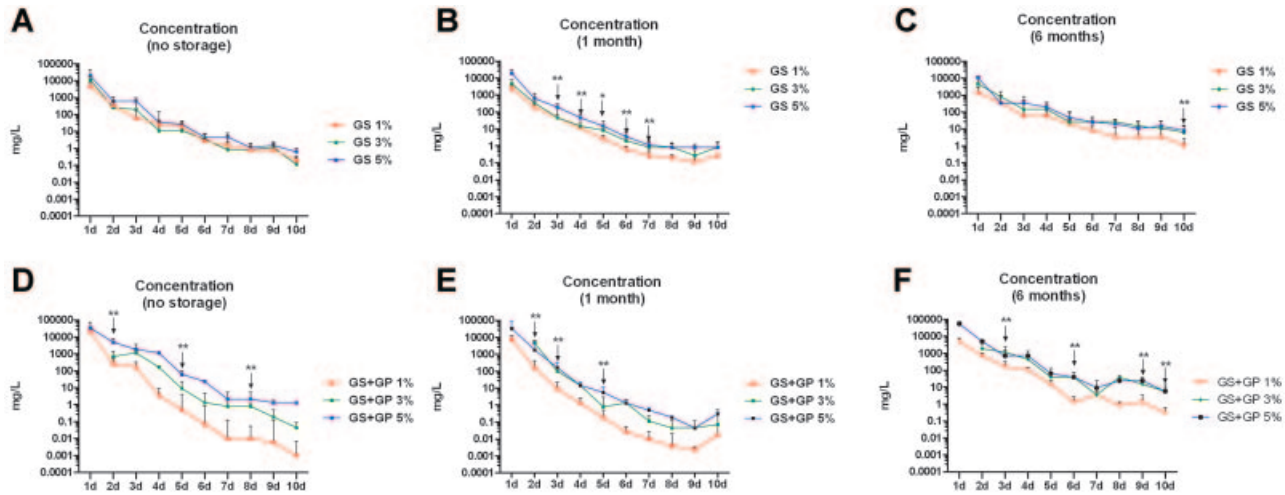

Figure 1. Release rate of gentamicin base (GB) from (A) GS 1%, GS 3%, and GS 5% without storage process; (B) GS 1%, GS 3% and GS 5% after 1 month at −80°C; (C) GS 1%, GS 3% and GS 5% after 6 months at −80°C; (D) GS + GP 1%, GS + GP 3% and GS + GP 5% without storage process; (E) GS + GP 1%, GS + GP 3% and GS + GP 5% after 1 month at −80°C; (F) GS + GP 1%, GS + GP 3% and GS + GP 5% after 6 months at −80°C. (*$p < 0.001$; **$p = 0.05$–0.001). Threshold 100.000 mg/L.

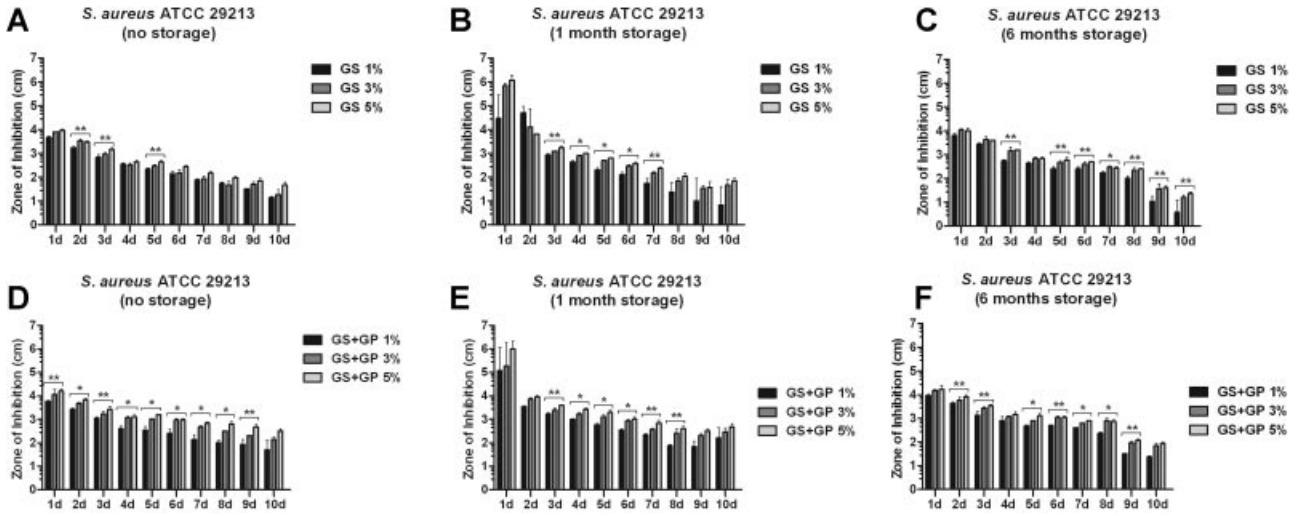

Figure 2. Susceptibility test using S. aureus ATCC 29213 strain after different storage conditions. (A) GS 1%, GS 3%, and GS 5% against S. aureus without storage process; (B) GS 1%, GS 3%, and GS 5% against S. aureus after 1 month at −80°C; (C) GS 1%, GS 3%, and GS 5% against S. aureus after 6 months at −80°C; (D) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. aureus without storage process; (E) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. aureus after 1 month at −80°C; (F) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. aureus after 6 months at −80°C. ($^{*}p < 0.001$; $^{**}p = 0.05$–0.001). Threshold = 7 cm.

GS pure in some periods. This could be due to its hydrophobic profile and affinity with the graft's fat tissue. Therefore, it could be an advantage of the combination of two the gentamicin salts (GS + GP), comparing with GS salt pure or other hydrophilic drugs that its concentrations are kept at homogeneous and constant rates. This could improve the protection of the bone grafts against infections for longer periods. In this study, drug concentrations were determined using a conventional microbiological agar well diffusion assay with Bacillus subtilis as indicator strain.[18,25] Because of the hydrophobic profile of GP which does not allow the obtainment of a homogeneous elution, we suggest this method for the concentration estimation instead of spectrometry techniques which could not show accurate results in these conditions.

A similar behavior of the GS + GP combination and GS pure against Staphylococcus aureus and Staphylococcus epidermidis was observed. Both strains, GS + GP in combination and GS pure were similarly effective. S. epidermidis are significantly more suscep-

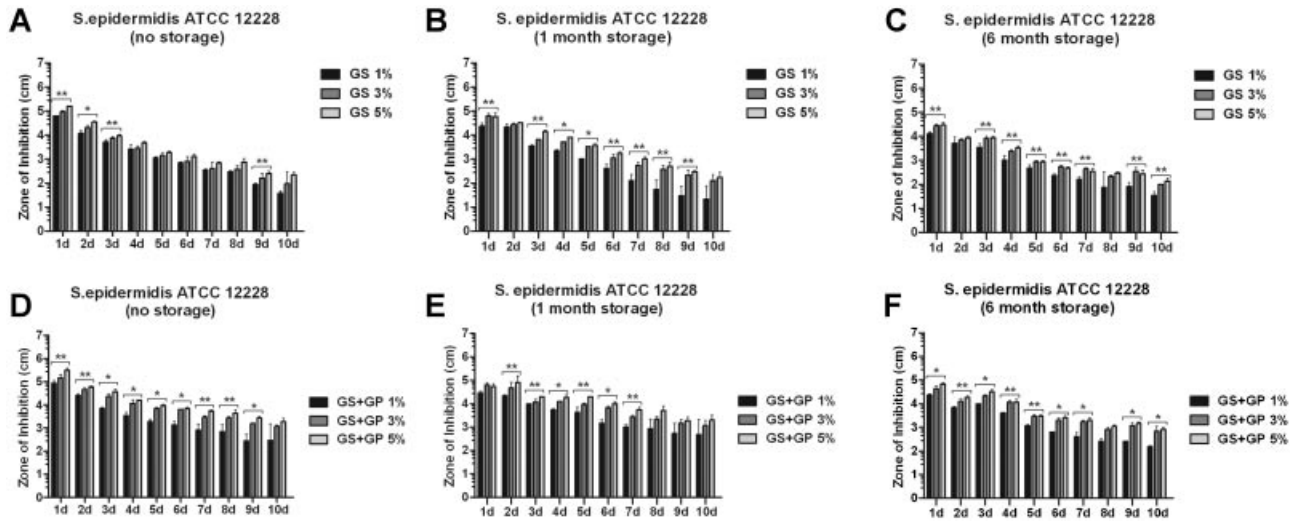

Figure 3. Susceptibility test using S. epidermidis ATCC 12228 strain after different storage conditions. (A) GS 1%, GS 3%, and GS 5% against S. epidermidis without storage process; (B) GS 1%, GS 3%, and GS 5% against S. epidermidis after 1 month at −80°C; (C) GS 1%, GS 3%, and GS 5% against S. epidermidis after 6 months at −80°C; (D) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. epidermidis without storage process; (E) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. epidermidis after 1 month at −80°C; (F) GS + GP 1%, GS + GP 3%, and GS + GP 5% against S. epidermidis after 6 months at −80°C. ($^{*}p < 0.001$; $^{**}p = 0.05$–0.001). Threshold = 7 cm.

tible to GS + GP in combination and GS pure than S. aureus. The efficacy of the antibiotics mixed with BCh against S. aureus and S. epidermidis *in vitro* was observed also after freezing the samples at −80°C for 1 and 6 months.

Antibiotic-coated cancellous bone is an alternative or supplement to non-biological material such as cement or metal in mega implants. Antibiotic-coated BCh can be used, for example, in revisions of aseptic and septic loosened hip and knee prostheses. In a clinical study of two-stage revision arthroplasties, the reinfection rate was lower using bone allografts impregnated with antibiotics than using grafts without antibiotics.[20] Antibiotic-containing allografts can also be used for non-healed fractures and, in particular, for infected pseudoarthroses.[18] The frequency of antibiotic resistance after using antibiotic-coated or impregnated bone allografts remains to be determined.

Freezing is the most common method for storing bone samples used in skeletal reconstructions. Bone banks protocols suggest that bone must be kept at low temperatures for more than 90 days and can be stored up to 5 years.[6] In this study we observed that the freezing processes, used in bone banks, do not compromise the antibiotic activity in the case the bone samples as mixed with it. Fresh frozen procedure for bone tissue helps keeping the osteogenic characteristics of the tissue. The mixing with antibiotics before the fresh frozen procedures could help decrease the risks of infection and also transform the bone sample in a carrier for local antibiotic treatment.

The susceptibility tests using S. aureus showed less resistance of the strain after 1 month of the elution storage. That resistance was not observed after 6 months of storage. Although some differences were observed on the S. aureus resistance after 1 and 6 months of freezing, that difference was not significant to tell that the storage interferes on the antibiotic activity.

In resume, the capacity of bone grafts to act as gentamicin carriers has been confirmed in this study. The combination of the two gentamicin salts (GS + GP) showed equivalent efficacy against S. aureus and S. epidermidis compared with GS pure. The lower delivery rate of the combination of GS + GP, related to its affinity with fat tissue can be an advantage for longer release times, increasing the local protection against infections. Storage at −80°C does not interfere on the antibiotic activity.

ACKNOWLEDGMENTS

We thank Dr. Sebastian Vogt from Heraeus Medical GmbH for their comments and improvement of this manuscript. Dr. Débora C. Coraça-Huber, Alexander Wurm, and Prof. Dr. Michael Nogler are paid employees of Medical University Innsbruck, Experimental Orthopedics. Dr. Manfred Fille and Dr. Hausdorfer are paid employees of Medical University Innsbruck, Division of Hygiene and Medical Microbiology. Prof. Dr. Klaus-Dieter Kühn is paid employee by Medical University Graz and Heraeus Medical GmbH.

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