Effect of storage temperature on gentamicin release from antibiotic-coated bone chips

D. C. Coraça-Huber, J. Hausdorfer, M. Fille & M. Nogler
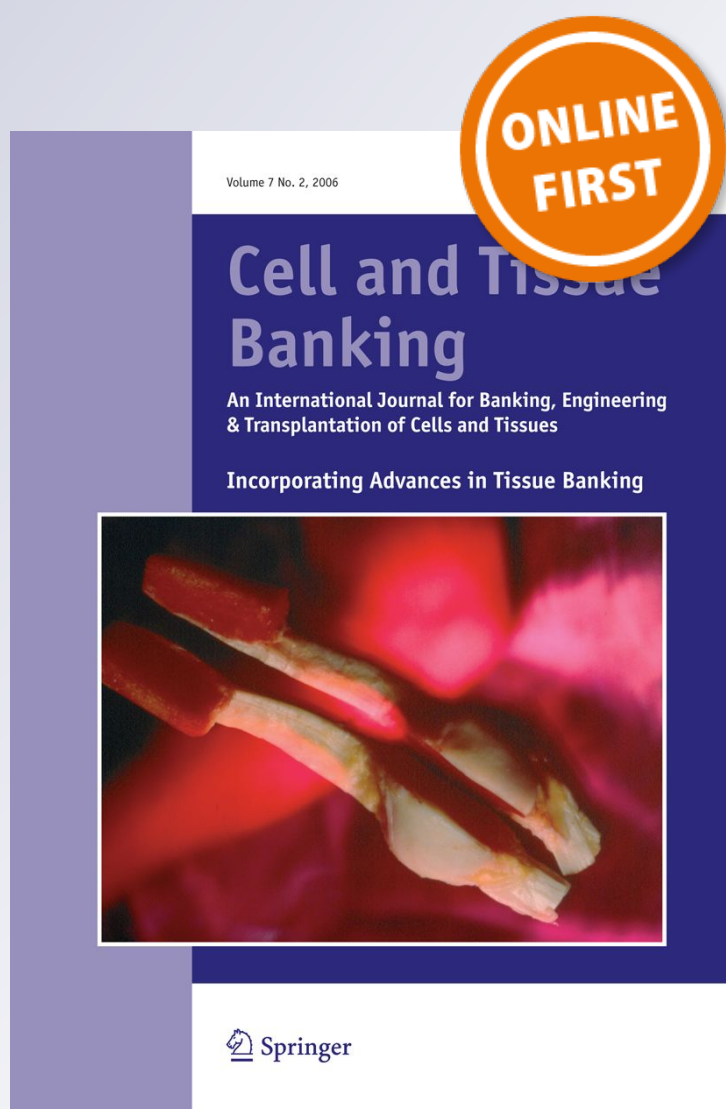
ONLINE FIRST

Springer

Springer

ORIGINAL PAPER

Effect of storage temperature on gentamicin release from antibiotic-coated bone chips

**D. C. Coraça-Huber · J. Hausdorfer ·
M. Fille · M. Nogler**

Abstract Freezing is the most common method for storing bones until use in skeletal reconstruction. However, the effect of freezing on antibiotic delivery from antibiotic-coated bone has not been evaluated. In this study, we compared antibiotic delivery in vitro from gentamicin-coated human bone stored at different temperatures. Bone chips obtained from human femur heads were chemically cleaned and mixed with gentamicin sulfate. Samples were stored for 4 months at −20 °C, 4 months at −80 °C, or evaluated immediately without freezing. Antibiotic release from the bone chips was measured using *Bacillus subtilis* as an indicator strain. Zones of inhibition and rates of gentamicin release were similar in all three groups. Storage at −20 and −80 °C for bone allografts has no effect on gentamicin release from chemically cleaned bone chips.

Keywords Bone transplantation · Allograft · Gentamicin · Storage · Bone bank

D. C. Coraça-Huber (✉) · M. Nogler
Experimental Orthopaedics, Department of Orthopaedic Surgery, Medical University Innsbruck, Salurnerstrasse 15, 6020, Innsbruck, Austria
e-mail: debora.coraca-huber@i-med.ac.at

J. Hausdorfer · M. Fille
Division of Hygiene and Medical Microbiology, Medical University Innsbruck, Innsbruck, Austria

Introduction

Bone grafting is indicated for joint reconstruction, repair of congenital skeletal defects and reconstruction of bone tissue after trauma and disease (Hinsenkamp et al. 2012; Putzer et al. 2011). Bone grafting can be carried out using autografts (tissue from the same patient) or allografts (tissue from the same species). Bone allografts avoid problems of autografts, including donor site morbidity and availability (Barbour and King 2003; Butler et al. 2005; Haimi et al. 2008). However, allografts are immunogenic (Stevenson and Horowitz 1992) and can transmit infectious diseases (Chapman and Villar 1992). To eliminate these problems and make human bone tissue suitable for transplantation, bone banks routinely decontaminate bone samples by mechanical and chemical cleaning and by freeze-drying (Lomas et al. 2000; Scarborough 1992; Holzmann et al. 2010). Also, infections can occur at the surgical site of contaminated bone grafts, leading to biofilm formation (Coraça-Hubér et al. 2012). Such biofilms can be difficult to treat after surgery involving impaction because the impaction creates an avascular area where local circulation is disrupted, limiting the ability of systemically administered antibiotics to reach the infected bone (Isefuku et al. 2003).

Bone cements containing antibiotics were developed to prevent and treat implant-related infections. However, the efficacy of the antibiotic-loaded cements is uncertain. Most of the antibiotics contained in the

cements are never released. Only when cracks are formed in the cement layer will a small, sub-inhibitory amount of antibiotic be released into the surrounding tissue. This release can continue for years, potentially inducing bacterial resistance (van de Belt et al. 2001; Winkler et al. 2006). Also, infections can be prevented by coating or impregnating morselized human bone with an antibiotic solution (Witsø et al. 2005; Winkler et al. 2000) or by combining it with antibiotic powders (Buttaro et al. 2003, 2005). Clinical studies using antibiotic-coated bone grafts have been performed with positive results (Winkler 2009; Borkhuu et al. 2008). Winkler et al. (2008) reported the use of allografts mixed with vancomycin and tobramycin in 37 one-stage revisions of infected total hip replacements. Nearly all (92 %) of the operated hips remained free from infection and stable at a mean follow-up of 4.4 years (Winkler et al. 2008).

Freezing is the most common method for storing bone to be used in skeletal reconstructions. Bone banks protocols suggest that bone must be kept at low temperatures for more than 90 days and that it can be stored up to five years (Farrington et al. 1998). However, the effect of cryopreservation on gentamicin release is unknown. In this study, we quantitatively compared the release of gentamicin from coated human bone chips (BCh) after different storage temperatures.

Materials and methods

Preparation of BCh

Femur heads were obtained from the bone bank of the Medical University Innsbruck, Austria. The femur heads were obtained during femoral head osteotomy from patients who had undergone hip replacement surgery at the Medical University of Innsbruck. 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 (5–10 mm diameter) 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 previously approved the use of the specimens for research purposes.

Chemical cleaning, storage, and gentamicin coating of samples

BCh were cleaned by chemical method based on a procedure described by DePaula and collaborators (DePaula et al. 2005). BCh (1 g) were placed in polypropylene centrifuge tubes and sonicated using an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) in the following series of solutions (Fig. 1): (i) 3 ml of 1 % Triton X-100 (Sigma-Aldrich, Schnelldorf, Germany) for 30 min at 45–50 °C, (ii) 3 ml of sterile distilled water for 5 min at 45–50 °C, (iii) 3 ml sterile distilled water for 10 min at 40–45 °C, (iv) 14 ml of 3 % hydrogen peroxide (Sigma-Aldrich, Schnelldorf, Germany) for 60 min at room temperature, (v) 3 ml sterile distilled water for 5 min at room temperature, (v) 3 ml sterile distilled water for 5 min at room temperature, (vi) 3 ml sterile distilled water for 30 min at room temperature, (vii) 3 ml of 70 % ethanol for 60 min at room temperature, (viii) 3 ml sterile distilled water for 10 min at room temperature, and (ix) 3 ml sterile distilled water for 30 min at room temperature. Finally, the water was removed and the samples stored in a refrigerator at 3–4 °C. Each 1-g sample of BCh was mechanically mixed with 8 mg of gentamicin sulfate powder (equivalent to 5 mg of gentamicin basis; SERVA GmbH, Heidelberg, Germany) using a sterile spatula. Samples were stored for 4 months at −20 °C (n = 5), 4 months at −80 °C (n = 5), or examined immediately (control; n = 5).

Measurement of gentamicin release from BCh

Frozen samples were thawed at room temperature for 1 h and then mixed with 3 ml of phosphate-buffered saline (PBS) pH 7.4 (Sigma-Aldrich, Schnelldorf, Germany). Samples were vortexed for 1 min and placed on a rocking table (Rocky® Biometra, Goettingen, Germany) at 37 °C. Every day for 1 week, the solutions were transferred to a centrifuge tube and replaced with 3 ml fresh PBS. Collected elutions were vortexed and frozen at −20 °C.

After 1 week, 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 (Stevens et al.
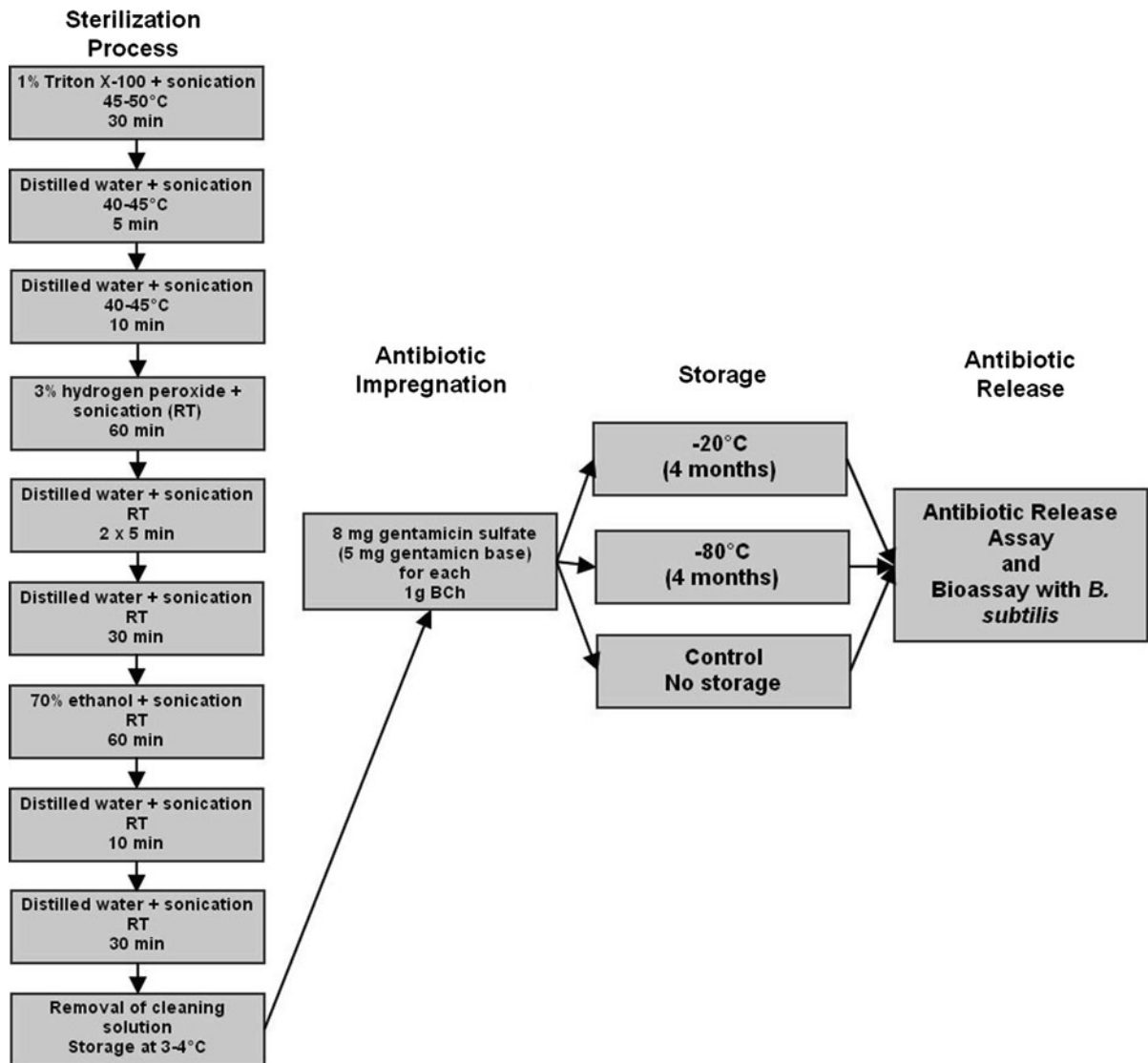
Sterilization Process

1% Triton X-100 + sonication 45–50°C 30 min

↓

Distilled water + sonication 40–45°C 5 min

↓

Distilled water + sonication 40–45°C 10 min

↓

3% hydrogen peroxide + sonication (RT) 60 min

↓

Distilled water + sonication RT 2 × 5 min

↓

Distilled water + sonication RT 30 min

↓

70% ethanol + sonication RT 60 min

↓

Distilled water + sonication RT 10 min

↓

Distilled water + sonication RT 30 min

↓

Removal of cleaning solution Storage at 3–4°C

Antibiotic Impregnation

8 mg gentamicin sulfate (5 mg gentamicin base) for each 1g BCh

Storage

-20°C (4 months)

-80°C (4 months)

Control No storage

Antibiotic Release

Antibiotic Release Assay and Bioassay with *B. subtilis*

Fig. 1 Flowchart showing the experimental procedure. *RT* room temperature

2005). Using a 6-mm diameter metal punch, a hole was made at the centre of each *B. subtilis* agar plate into which 50 μl of each elution or 50 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 cm was measured from each plate using a ruler. The diameter was confirmed with a second measurement. This method was carried out in triplicate. The standard curve was obtained by logarithmic regression and used to predict the concentration of gentamicin in each elution.

Statistical analysis

One-way analysis of variance was used to analyse the differences between the release rates of gentamicin. Sheffe and Games-Howel post hoc analysis were used to confirm differences in all cases. P values ≤ 0.05 were considered statistically significant. SPSS 17.0 (IBM, Chicago, Illinois) was used for the statistical analysis.

Results

Zone of inhibition measured with *B. subtilis* bioassay

Zones of inhibition measured from the *B. subtilis* bioassay showing similar diameter for all storage conditions (−20 °C, −80 °C and control; Fig. 2). Overall, the mean zone of inhibition was 4.1 cm on day 1 and decreased to mean of 3.0 cm on day 7.

Effect of storage conditions on gentamicin release rate

The concentration of gentamicin sulfate released from samples stored at −20 °C was 823 ± 266 mg/l (mean ± SD) on day 1 and 270 ± 71 mg/l on day 2 (Fig. 3). Release of gentamicin from samples stored at −80 °C was 1,214 ± 708 mg/l day 1 and decreased to 210 ± 45 mg/l on day 2. Release from the control group was similar for the samples stored at −20 °C. On day 7, the release from the samples stored at −20 and −80 °C were similar, with a mean of 19 ± 7 mg/l. Overall, the release of gentamicin decreased gradually over time at a similar rate in all groups. The rate of gentamicin delivery did not differ between the groups ($P = 0.49$ by one-way analysis of variance).
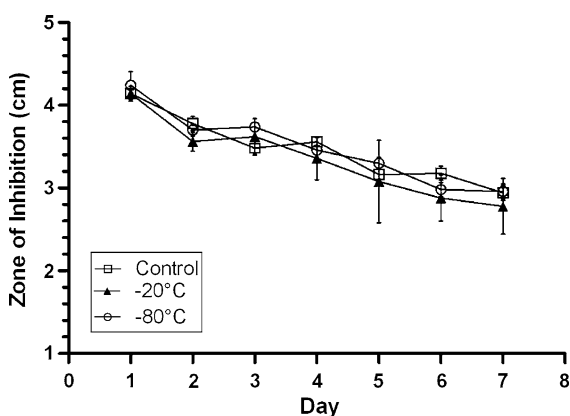

Fig. 3 In vitro release of gentamicin from gentamicin-coated BCh. Comparison between cleaning process storage at −20 °C, storage at −80 °C and control. Gentamicin release was not significantly different between groups (One-way analysis of variance; $P = 0.49$)

Fig. 2 Zone of inhibition. BCh were treated as described in Fig. 1. Gentamicin released from the BCh was measured by using a *B. subtilis* bioassay. *Symbols* indicate the mean diameter of the zone of inhibition. *Error bars* represent the standard deviation
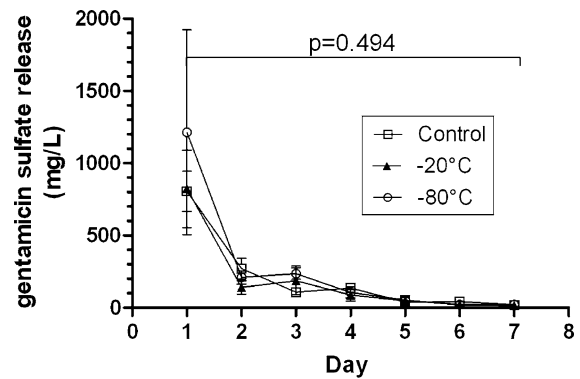
Discussion and conclusions

This study quantitatively compared antibiotic release from gentamicin-coated BCh after storage at −20, and −80 °C, and non-stored samples. We found that the release of antibiotic from gentamicin-coated BCh was similar between the groups.

In this study, the BCh were cleaned with hydrogen peroxide followed by ethanol and biological detergent baths for fat removal. However, because infection remains a concern, coating bone grafts with antibiotic powder or its impregnating in antibiotic solution has been considered (Winkler et al. 2000, 2006). In this study, to coat the bone grafts, we manually mix them with the gentamicin powder. This is a relatively easy procedure that could be suitable for an operating room. Also, antibiotic coating can be carried out prior the cryopreservation in bone banks. Some authors first dilute the antibiotic powder in saline solution and then soak the bone grafts in this solution, storing them for weeks or months before use (Witsø et al. 2005; Winkler et al. 2000). We believe that this is an efficient method for BCh impregnation. However, Sorger et al. (2001) suggested that preserving the grafts for up to 100 h in an antibiotic solution might compromise the mechanical stability of the bone. Based on Parrish (1973) and Witsø et al. (2005), allografts impregnated with antibiotics in solution should be tested for mechanical and structural characteristics before clinical use.

Antibiotic-impregnated or coated cancellous bone might be an alternative or supplement to bone cements.

Antibiotic-coated BCh can be used 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 (Buttaro et al. 2005). Antibiotic-containing allografts can also be used for non-healed fractures and, in particular, for infected pseudoarthroses (Witsø et al. 2005). The frequency of antibiotic resistance after using antibiotic-impregnated bone allografts remains to be determined. Also, the deleterious effect of gentamicin on osteogenesis in antibiotic-coated or impregnated cancellous bone needs to be clarified. According to Isefuku (2003), gentamicin, at high concentrations, as achieved following topical application, inhibits cell proliferation in vitro and, therefore, may be detrimental to the repair process in vivo.

In conclusion, we found that storage at -20 and -80 °C has no effect on gentamicin release from chemically cleaned BCh. Therefore, our results suggest that BCh can be coated with antibiotics prior to short-term cryopreservation of human bone allografts. Further studies on long-term storage are necessary to recommend this practice to bone banks.

References

Barbour SA, King W (2003) Basic science update. The safe and effective use of allograft tissue—an update. Am J Sp Med 31(5):791–797

Borkhuu B, Borowski A, Shah SA, Littleton AG, Dabney KW, Miller F (2008) Antibiotic-loaded allograft decreases the rate of acute deep wound infection after spinal fusion in cerebral palsy. Spine (Phila Pa 1976) 33(21):2300–2304. doi:10.1097/BRS.0b013e31818786ff

Butler AM, Morgan DA, Verheul R, Walsh WR (2005) Mechanical properties of gamma irradiated morselized bone during compaction. Biomaterials 26(30):6009–6013. doi:10.1016/j.biomaterials.2005.03.007

Buttaro MA, Gonzalez Della Valle AM, Pineiro L, Mocetti E, Morandi AA, Piccaluga F (2003) Incorporation of vancomycin-supplemented bone incorporation of vancomycin-supplemented bone allografts: radiographical, histopathological and immunohistochemical study in pigs. Acta Orthop Scand 74(5):505–513. doi:10.1080/00016470310017884

Buttaro MA, Pusso R, Piccaluga F (2005) Vancomycin-supplemented impacted bone allografts in infected hip

arthroplasty. Two-stage revision results. J Bone Joint Surg Br 87(3):314–319

Chapman P, Villar R (1992) The bacteriology of bone allografts. J Bone Joint Surg Br 74-B(3):398–399

Coraça-Hubér DC, Fille M, Hausdorfer J, Pfaller K, Nogler M (2012) Evaluation of MBEC^{TM}-HTP biofilm model for studies of implant associated infections. J Orthop Res n/a-n/a. doi:10.1002/jor.22065

DePaula CA, Truncale KG, Gertzman AA, Sunwoo MH, Dunn MG (2005) Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties. Cell Tissue Bank 6(4):287–298. doi:10.1007/s10561-005-3148-2

Farrington M, Matthews I, Foreman J, Richardson KM, Caffrey E (1998) Microbiological monitoring of bone grafts: two years' experience at a tissue bank. J Hosp Infect 38(4):261–271

Haimi S, Vienonen A, Hirn M, Pelto M, Virtanen V, Suuronen R (2008) The effect of chemical cleansing procedures combined with peracetic acid-ethanol sterilization on biomechanical properties of cortical bone. Biologicals 36(2):99–104. doi:10.1016/j.biologicals.2007.06.001

Hinsenkamp M, Muylle L, Eastlund T, Fehily D, Noel L, Strong DM (2012) Adverse reactions and events related to musculoskeletal allografts: reviewed by the World Health Organisation Project NOTIFY. Int Orthop 36(3):633–641. doi:10.1007/s00264-011-1391-7

Holzmann P, Niculescu-Morzsa E, Zwickl H, Halbwirth F, Pichler M, Matzner M, Gottsauner-Wolf F, Nehrer S (2010) Investigation of bone allografts representing different steps of the bone bank procedure using the CAM-model. ALTEX 27(2):97–103

Isefuku S, Joyner CJ, Simpson AH (2003) Gentamicin may have an adverse effect on osteogenesis. J Orthop Trauma 17(3):212–216

Lomas R, Drummond O, Kearney JN (2000) Processing of whole femoral head allografts: a method for improving clinical efficacy and safety. Cell Tissue Bank 1(3):193–200. doi:10.1023/a:1026512312385

Parrish FF (1973) Allograft replacement of all or part of the end of a long bone following excision of a tumor. J Bone Joint Surg Am 55(1):1–22

Putzer D, Mayr E, Haid C, Reinthaler A, Nogler M (2011) Impaction bone grafting: a laboratory comparison of two methods. J Bone Joint Surg Br 93(8):1049–1053. doi:10.1302/0301-620X.93B8.26819

Scarborough NL (1992) Current procedures for banking allograft human bone. Orthopedics 15(10):1161–1167

Stevens CM, Tetsworth KD, Calhoun JH, Mader JT (2005) An articulated antibiotic spacer used for infected total knee arthroplasty: a comparative in vitro elution study of Simplex$^®$ and Palacos$^®$ bone cements. J Orthop Res 23(1):27–33. doi:10.1016/j.orthres.2004.03.003

Stevenson S, Horowitz M (1992) The response to bone allografts. J Bone Joint Surg Am 74(6):939–950

van de Belt H, Neut D, Schenk W, van Horn JR, van der Mei HC, Busscher HJ (2001) Infection of orthopedic implants and the use of antibiotic-loaded bone cements. A review. Acta Orthop Scand 72(6):557–571. doi:10.1080/000164701317268978

Winkler H (2009) Rationale for one stage exchange of infected hip replacement using uncemented implants and antibiotic impregnated bone graft. Int J Med Sci 6(5):247–252

Winkler H, Janata O, Berger C, Wein W, Georgopoulos A (2000) In vitro release of vancomycin and tobramycin from impregnated human and bovine bone grafts. J Antimicrob Chemother 46(3):423–428. doi:10.1093/jac/46.3.423

Winkler H, Kaudela K, Stoiber A, Menschik F (2006) Bone grafts impregnated with antibiotics as a tool for treating infected implants in orthopedic surgery—one stage revision results. Cell Tissue Bank 7(4):319–323. doi:10.1007/s10561-006-9010-3

Winkler H, Stoiber A, Kaudela K, Winter F, Menschik F (2008) One stage uncemented revision of infected total hip replacement using cancellous allograft bone impregnated with antibiotics. J Bone Joint Surg Br 90-B(12):1580–1584. doi:10.1302/0301-620x.90b12.20742

Witsø E, Persen L, Benum P, Bergh K (2005) Cortical allograft as a vehicle for antibiotic delivery. Acta Orthopaedica 76(4):481–486. doi:10.1080/17453670510041457