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Effect of two cleaning processes for bone allografts on gentamicin impregnation and in vitro antibiotic release

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ABSTRACT

Background: Bone allografts are a useful and sometimes indispensable tool for the surgeon to repair bone defects. Microbial contamination is a major reason for discarding allografts from bone banks. To improve the number of safe allografts, we suggest chemical cleaning of the grafts followed by antibiotic impregnation.

Objectives: Comparison of two chemical cleaning processes for bone allografts aiming for antibiotic impregnation and consequently delivery rates in vitro.

Materials and Methods: Bone chips of 5-10 mm were prepared from human femoral heads. Two cleaning methods (cleaning A and cleaning B) based on solutions containing hydrogen peroxide, paracetic acid, ethanol and biological detergent were carried out and compared. After the cleaning processes, the bone chips were impregnated with gentamicin. *Bacillus subtilis* bioassay was used to determine the gentamicin release after intervals of 1, 2, 3, 4, 5, 6 and 7 days. Differences were compared with non-parametric Mann-Whitney U-tests.

Results: The zones of inhibition obtained from the bone grafts cleaned with both cleaning processes were similar between the groups. The concentration of the released antibiotic was decreasing gradually over time, following a similar pattern for both groups.

Conclusions: The cleaning procedure A as well as the cleaning procedure B for bone allografts allowed the impregnation with gentamicin powder in the same concentrations in both groups. The delivery of gentamicin was similar for both groups. Both cleaning procedures were easy to be carried out, making them suitable for routine use at the bone banks.

KEYWORDS

Bone transplantation, allograft, gentamicin, sterilization, bone bank

INTRODUCTION

Bone allografts are a useful and sometimes indispensable tool for the orthopaedic surgeon to repair bone defects and to improve function. Bone grafting is indicated for joint reconstruction, repair of congenital defects of the skeleton or reconstructive procedures after trauma and diseases (Hinsenkamp et al. 2012; Putzer et al. 2011). Allografts have some advantages over autograft bone, e.g. avoidance of the donor site morbidity and availability (Barbour and King 2003; Butler et al. 2005; Haimi et al. 2008). By establishing tissue banks, the use of allografts has become widely accepted. Therefore, tissue banks must optimize procedures to increase the number of available safe allografts. Contamination is a major reason for discarding allografts (Deijkers et al. 1997; Schubert et al. 2012).

The need to ensure maximum safety has lead to a search for different sterilization methods for bone allografts. The most commonly used methods are chemical sterilization and beta and gamma irradiation (Vastel et al. 2004; Grieb et al. 2005). Chemical cleaning of bone allografts provides an additional level of safety but it is important not to jeopardize the graft by cleaning it. Commonly used chemical methods employ aqueous solutions of detergents, surfactants, peroxides, organic solvents, acids and alcohol. These methods can be used in combination with mechanical methods such as pressure or vacuum, ultrasonic baths, agitation or centrifugation to facilitate the cleaning (DePaula et al. 2005).

Surgery with bone allografts is more complex and time-consuming than, for example, primary hip surgery, which may contribute to a higher infection rate with percentages ranging from 2.0% to 2.5% (Parvizi et al. 2008; Parvizi et al. 2007; Blom et al. 2003). The impaction procedure for bone allografts creates an avascular area where local circulation is disrupted. If infections arise, this may prevent systemically administered antibiotics from reaching the infected bone (Isefuku et al. 2003; Mathijssen et al. 2010). In addition, when the bacteria, which colonized the implant surfaces, produce a biofilm they become more resistant against antibiotics (Coraça-Huber et al. 2012). Bone cements containing antibiotics were developed to solve this problem. These cements may serve as drug delivery system and prophylaxis against infections as they make it possible to achieve higher local drug concentrations. However, controversy exists on the efficacy of the antibiotic-loaded cements. Probably 90% 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). In addition, the formation of cracks in the cements reduces the stability of the cemented implant leading to prosthetic loosening and related complications (Choi et al. 2010).

An ideal antibiotic carrier for prevention and treatment of periprosthetic joint infections should be able to carry a substance with bactericidal activity against most pathogens. It should be able to provide and sustain high concentrations of the antibiotic at the surgical site without local or systemic toxicity. In addition, it should improve osteoconductivity and osteoinductivity, supporting the bone healing without further surgery (Saraf et al. 2010). Several studies have shown that morselized bone allografts can act as a carrier for antibiotics either by impregnating the bone grafts with antibiotic solutions (Witsø et al. 2005; Winkler et al. 2000) or by mixing them with antibiotic powders (Buttaro et al. 2005; Buttaro et al. 2003).

To increase the safety of bone allografts as well as to obtain an efficient antibiotic carrier, why not impregnate the bone allografts with an antibiotic after chemical cleaning procedures? Some studies have been carried out with morselized bone grafts without chemical sterilization which were impregnated with antibiotic substances for local delivery (Buttaro et al. 2005; Mathijssen et al. 2010). However, only few studies investigate the delivery rate of antibiotic from chemically sterilized bone grafts (Winkler et al. 2000).

In this study, we compared two chemical cleaning processes for human bone allografts followed by gentamicin impregnation. Our objective was to determine the cleaning process which allows higher levels of antibiotic impregnation and consequently higher delivery rate in vitro.

MATERIALS AND METHODS

Bone Allografts

Femoral heads were removed during total hip replacement surgery following femoral head osteotomy and sliced in the horizontal plane under sterile conditions. Throughout the procedure the bone was rinsed with sterile 0.9% saline to prevent damage by the high temperatures during drilling. For the experiments, cortical and cartilage tissues were removed from the femoral heads with a bone saw. Bone chips (BCh) with a size of 5-10 mm were prepared from the spongious tissue using a bone mill (Noviomagus Bone Mill, Spierings Medische Techniek BV, Nijmegen, The Netherlands). All morselized bone was carefully mixed to achieve homogenous bone quality and to minimize differences due to patient specific bone characteristics. The femoral heads were fresh frozen at -80°C. All patients approved previously the use of the specimens in this study. Ten grams of BCh were divided in two groups: 5 g divided in 5 tubes for cleaning process A and 5 g divided in 5 tubes for cleaning B.

Cleaning Process A

This procedure was based on the process used by Haimi and collaborators (Haimi et al. 2008). An amount of 1g BCh was placed in 15 ml Falcon tubes. Five tubes containing 1g of BCh each were used for this cleaning process. Cleaning process A consists of 12 successive steps **(Figure 1)**. For the first step, 3 ml of sterile distilled water was added in each tube after which the samples were sonicated in an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 15 min at 45-50°C. The samples were washed and agitated (Tube Rotator Stuart® SB3, Staffordshire, UK) for 30 min on 40 rpm at 37°C followed by centrifugation (1900g) for 15 min at room temperature (RT). The samples were washed and agitated (40 rpm) for 10 min at 37°C. After 10 min, the samples were centrifuged (1900g) for 15 min at RT, washed and agitated (40 rpm) for 10 min at 37°C, followed by centrifugation (1900g) for 15 min at RT. The washing, agitation and centrifugation steps were repeated once. The washing solution (distilled water) was replaced before each repetition. The next step after the last centrifugation was to remove the distilled water and add a 1:1 solution of 3% hydrogen peroxide (Sigma-Aldrich, Schnelldorf, Germany) with 0.02% paracetic acid (Sigma-Aldrich, Schnelldorf, Germany) after which the samples were sonicated for 10 min at RT. After 10 min the solution was removed and 70%

ethanol was added. The tubes were sonicated for 10 min at 45-50°C. The ethanol was removed and the samples were washed and agitated in distilled water for 20 min at 37°C. At the end of the process the washing solution was removed and the samples stored in a refrigerator at 3-4°C before the antibiotic impregnation process. These procedures were carried out in triplicate.

Cleaning Process B

This process was based on a procedure used by DePaula and collaborators (DePaula et al. 2005). An amount of 1g BCh was placed in 15 ml Falcon tubes. Five tubes containing 1g of BCh each were used for this cleaning process. The cleaning process B consists of 10 successive steps **(Figure 1)**. For the first step 3 ml of 1% Triton X-100 (Sigma-Aldrich, Schnelldorf, Germany) were added in each tube and the samples were sonicated for 30 min at 45-50°C. After 30 min the Triton X-100 solution was removed and 3 ml of sterile distilled water were added. The samples were sonicated for 5 min at 45-50°C. After 5 min the distilled water was replaced with sterile distilled water and the samples were sonicated for an additional 10 min at 40-45°C. For the next step, the solution was removed from the tubes and 14 ml of 3% hydrogen peroxide (Sigma-Aldrich, Schnelldorf, Germany) solution was added. The samples were sonicated for 60 min at RT. After 60 min, the hydrogen peroxide was removed and the sterile distilled water was added to the tubes. The samples were sonicated for additional 5 min at RT. The solution was renewed and the procedure was repeated for 5 more minutes. The solution was removed from the tubes and new sterile distilled water was added. At this time the samples were sonicated for 30 min at RT. After 30 min the solution was removed and 70% ethanol was added in each tube and the samples were sonicated for 60 min at RT. After 60 min the ethanol was removed and sterile distilled water was added in the tubes. The samples were sonicated for 10 min at RT and after renewing the distilled water, sonicated for an additional 30 min at RT. At the end of the process the washing solution was removed and the samples stored in a refrigerator at 3-4°C before the antibiotic impregnation process. These procedures were carried out in triplicate.

Antibiotic Impregnation

For the BCh impregnation, gentamicin was chosen as the antibiotic substance (Swieringa et al. 2008; Nadrah and Strle 2011). After cleaning, 8 g of gentamicin sulfate powder (equivalent 5 g of gentamicin basis), (SERVA GmbH, Heidelberg, Germany) was added in each

tube containing 1 g of bone chips and mechanically mixed with the help of a sterile spatula. The samples were stored at RT for 1 h prior the release assay.

Antibiotic Release Assay

The release of gentamicin from impregnated BCh was carried out by using phosphate-buffered saline (PBS) pH 7.4 (Sigma-Aldrich, Schnelldorf, Germany). After 1 h at RT, 3 ml of PBS was added into each tube. The tubes were vortexed for 1 min and placed on a rocking table (Rocky® Biometra, Goettingen, Germany) at 37°C to simulate the tissular conditions. After 1, 2, 3, 4, 5, 6 and 7 days the elution medium was completely removed and fresh PBS was added. The elution was vortexed for 1 min and stored in Falcon tubes at -20°C.

***Bacillus subtilis* Assay**

The antibiotic delivered was determined after each interval with a conventional microbiological agar diffusion assay with *Bacillus subtilis* as the indicator strain (Merck KGaA, Germany in Test Agar pH 8.0 Merck KGaA, Germany) (Stevens et al. 2005). For this purpose, a standard curve was created with known concentrations of the gentamicin sulfate (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10 mg/ml). Using a 6 mm diameter metal punch a hole was made at the center of each *B. subtilis* agar plates where 50 µl of each concentration were added. The plates containing the samples were incubated for 24 h at 37°C. After the incubation period, the zones of inhibition were measured on each plate with a ruler and a standard curve was obtained for future comparison with the measurements obtained from drug release tests.

After the intervals of 1 to 7 days, 50 µl of each elution medium were added to *B. subtilis* agar plates following the same procedures carried out for the standard curve obtainment. The plates containing the samples were incubated for 24 h at 37°C. After the incubation, the zones of inhibition were measured from each plate with a ruler. Logarithmic regressions of the standard concentration curve were calculated to predict the concentrations of the eluted medium after each delivery interval.

Statistical Analysis

Non-parametric Mann-Whitney U-tests were used to find statistically significant differences between the delivery rates of gentamicin after each interval, also taking into consideration both cleaning processes. SPSS 17.0 (IBM, Chicago, Illinois) was used for the statistical analysis.

RESULTS

Zone of inhibition obtained with *B. subtilis* bioassay

The zones of inhibition, obtained by carrying out the *B. subtilis* bioassay from the BCh cleaned by the process and A and by process B, were similar in both groups. Both groups had an average zone of inhibition of 4.2 cm at the first day which had been reduced to an average of 3.0 cm by the last day **(Figure 2)**.

Comparison of gentamicin release rate between cleaning processes

The gentamicin release from the BCh was similar for both groups. The concentration of gentamicin sulfate released at the first day was of 0.855 ± 0.130 mg/ml (mean ± SD) decreasing to 0.312 ± 0.101 mg/ml at the second day regardless of the cleaning process. The release concentration was decreasing gradually over the periods with a similar behaviour for both groups with a measured concentration of 0.020 ± 0.006 mg/ml. A higher concentration of gentamicin sulfate was detected from BCh after cleaning A in comparison with BCh after cleaning B but the difference was not statistically significant (p=0.08) **(Figure 3)**.

DISCUSSION AND CONCLUSIONS

The goal of this study was to compare two chemical cleaning processes for human bone allografts followed by gentamicin impregnation. Our objective was to determine a cleaning process which allows higher levels of antibiotic impregnation and consequently higher delivery rates in vitro.

During the processing of the bone allografts in this study, the femur heads were submitted to tissue debridement where muscle, fat and cartilage tissue was mechanically removed, followed by chemical cleaning. Two different processes for chemical cleaning were used, based on soaks with hydrogen peroxide, paracetic acid, ethanol and detergents. After the cleaning process, the bone allografts were impregnated with gentamicin sulfate. It is believed that cleaning the bone to remove fat, bone marrow and soft tissue makes it easier for the solution to penetrate through the canaliculi of the bone tissue, increasing the surface available for antibiotic adsorption (Winkler et al. 2000). However, is important to highlight that cleaning processes for bone allografts improve the safety of the samples but can also decrease the osteogenic properties of the same. The optimal bone allograft or bone substitute should provide structural strength, be osteoconductive (provide structure for the attachment and growth of cells), osteoinductive (contain active factors like hormones which stimulate cell migration and differentiation), osteogenic (contain cells from osteogenic linage) and biocompatible. Bormann and collaborators (2010) tested the osteoinductive potential of bone allografts after different processes and concluded that osteoinductive factors can be present even after chemical cleaning. Nevertheless, the authors identified the osteoinductive factors by ELISA which detects not only active but also inactive growth factors (Bormann et al. 2010). To ensure the osteoinductive potential of bone allografts after chemical cleaning processes we suggest further studies with cleaned allografts for the bioactivity of these factors.

The cleaning process A used in this study was based on a procedure used by Haimi and collaborators (2008). This process involves the use of distilled water, hydrogen peroxide, paracetic acid and ethanol solutions. The samples are cleaned with the help of ultrasonic baths, agitators and centrifuge. The bactericidal, fungicidal, virucidal and sporicidal effects of paracetic

acid are well known (Pruss et al. 1999). The addition of ethanol after the treatment with paracetic acid reduces the surface tension. Negative pressure removes the gas vesicles, which prevent complete tissue penetration of the sterilizing medium (Pruss et al. 2003). Hydrogen peroxide was also involved in the cleaning process B, as well as ethanol and biological detergent for fat removal. DePaula and collaborators also affirm that hydrogen peroxide has bactericidal effects; however, it can decrease the osteoinductivity of bone allografts after 5 h of treatment. In accordance with DePaula, we chose to treat the bone allografts in our study with hydrogen peroxide for 1 h, thus preventing the osteoinductivity loss of the grafts (DePaula et al. 2005). In addition, the two cleaning processes used in this study are easy to be carried out, making them suitable for routine use at the bone bank.

After the cleaning processes, the bone allografts were impregnated with gentamicin powder. The safety of the bone grafts is crucial for a successful procedure as well as the prevention of local infections during surgery. Antibiotic impregnation of allografts can reduce the infection rates in surgical procedures which employ them. The chemical cleaning of bone allografts improves the safety by eliminating contaminating factors from the samples. We believe that both processes in combination can help increasing the safety of bone allografts in general. We chose gentamicin for the impregnation of the samples since it usually is indicated for treatment of periprosthetic joint infections (Swieringa et al. 2008; Nadrah and Strle 2011). In addition, the use of antibiotic-impregnated cancellous bone from all origin, and not only femoral heads, might be an alternative or supplement to antibiotic-containing bone cements. It can be used in revisions of aseptic and septic loosened hip and knee prostheses. In a clinic study on two-stage revision of 30 total hip arthroplasties, the reinfection rate was 3% when using bone allografts supplemented with antibiotics (Buttaro et al. 2005). Besides revision prosthetic surgery, antibiotic-containing allografts could be used when grafting non-healed fractures, in particular infected pseudoarthroses (Witsø 2007). It is important to highlight that until now no studies describe the antibiotic resistance rates after using such impregnated bone allografts. To answer this question, further studies should be carried out.

We chose to manually mix the samples with the powder to impregnate the bone grafts with the antibiotic. This is a relatively easy procedure which could be suitable for an operating

room where the grafts could be mixed with antibiotic drugs during surgery. Some authors first dilute the antibiotic powder in a 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 bone chips impregnation since the tissue would act as a sponge absorbing the solution. According to these authors, this procedure could also be an alternative for long-therm storage of the grafts with antibiotic solutions. However, according to Sorger et al (2001), the preservation of the grafts for up to 100 hours in an antibiotic solution might influence the mechanical stability of the bone (Sorger et al. 2001). Based on Parrish (1973) and Witsø and collaborators (2005), mechanical testing of osteochondral and structural allografts impregnated with antibiotics in solutions should be performed before this option is taken into clinical use (Parrish 1973; Witsø et al. 2005).

After the impregnation, the delivery rate of the gentamicin was evaluated comparing process A and process B. The antibiotic delivered was determined after each interval by using a conventional microbiologic agar diffusion assay with *Bacillus subtilis* as the indicator strain (Stevens et al. 2005). The measurement of the zones of inhibition and the predicted concentration obtained after each interval showed a statistically similar delivery rate of gentamicin in both groups. Taking into consideration that a standard-length hip reconstruction typically requires about 100 g of bone chips (Steele RG 2010), once impregnated with gentamicin at the same rate used in this study, the locally deliver rate could reach 80 mg/mL after 24 h to 2 mg/mL after 7 days. This is not a high concentration for local antibiotic therapy. Other studies showed that higher concentrations can be achieved by using bone cements and gentamicin impregnated-beads (Springer et al. 2004). However, is important to remember that aminoglycoside nephrotoxicity occurs in 10%-15% of patients with serum concentrations of gentamicin in the range of 0.9-2 mg/l (detectable for example in patients which received around 200-400 gentamicin-PMMA beads). To clarify this comparison, each gentamicin-PMMA bead normally contains 7.5 mg of gentamicin sulfate (de Klaver et al. 2012; Matzke et al. 1983). As the aim of this study was the comparison of the delivery rate between bone grafts cleaned with different processes, we affirm that the concentrations used are not crucial but the delivery behaviour along the evaluated periods is. The gentamicin concentrations for bone graft impregnation can be increased in future studies when the objective is to achieve higher delivery rates.

In conclusion, we can affirm that both cleaning procedures for bone allografts allowed the impregnation of gentamicin powder in the same concentrations. The delivery of gentamicin was similar in both groups. Both cleaning processes were easy to be carried out making them suitable for routine use at bone banks. We suggest further studies with cleaned allografts for the bioactivity of osteoinductive factors and for impregnation and local delivery of higher antibiotic concentrations.

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Figure legends:

Fig. 1 - Flowchart of the cleaning process A and cleaning process B.

Fig. 2 – Zone of inhibition measured from *B. subtilis* bioassay for gentamicin concentration obtained from BCh after cleaning process A and cleaning process B.

Fig. 3 – In vitro release of gentamicin from cleaned BCh during the period of 1 to 7 days. Comparison between cleaning process A and cleaning process B. Mann-Whitney U-test ($P \leq 0.05$).

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