Bone 59 (2014) 162–172

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Analysis of parameters influencing the release of antibiotics mixed with bone grafting material using a reliable mixing procedure

N. Bormann [a,b,1], P. Schwabe [c,1], M.D. Smith [d], B. Wildemann [a,b,c,*]

[a] Julius Wolff Institute, Charité — Universitätsmedizin Berlin, Germany
[b] Berlin-Brandenburg Center for Regenerative Therapies, Charité — Universitätsmedizin Berlin, Germany
[c] Center for Musculoskeletal Surgery, Charité — Universitätsmedizin Berlin, Germany
[d] German Institute for Cell and Tissue Replacement, Berlin, Germany

ARTICLE INFO

Article history:
Received 14 June 2013
Revised 1 November 2013
Accepted 5 November 2013
Available online 12 November 2013

Edited by: Thomas Einhorn

Keywords:
Antibiotics
Bone graft
Release kinetics
Infection prevention
Perioperative mixing

ABSTRACT

Local infections arising from fracture fixation, defect reconstruction or joint replacement can cause extreme pain and impaired healing, lead to revision operations, prolong hospital stay and increase costs. Treatment options including prophylaxis are afforded by the use of grafts and biomaterials loaded with antibiotics. These can produce local therapeutic concentrations with a reduced systemic concentration and reduced systemic side-effects. Patient-specific loading of osteogenic graft materials with antibiotic could be an important option for orthopaedic surgeons. A local therapeutic concentration must be available for the desired duration and cytotoxic effects must be kept within an acceptable range. The present study investigates a simple and reliable mixing procedure that could be used for the perioperative combination of antibiotic powders and solutions with bone grafting materials. The potential influence of concentration and sampling regime on the release kinetics of gentamicin, tobramycin and vancomycin was studied over a period of 56 days and potency and cytotoxicity were evaluated. In all treatment groups, gentamicin and tobramycin were completely released within 3 days whilst vancomycin was released over a period of 14 days. The results clearly show that the main parameter influencing release is the molecular weight of the drug. Growth of *Staphylococcus aureus* was inhibited in all 3 treatment groups for at least 3 days. Cell viability and alkaline phosphatase activity of primary osteoblast-like cells were not significantly affected by the antibiotic concentrations obtained from the elution experiments.
Bone grafting is an established component of surgery for bone defect filling and for biological stimulation of healing. Patient-specific enhancement of such procedures by incorporation of antibiotics for infection prevention or by addition of cytokines for promotion of impaired healing or for treatment of critical size defects will be a relevant issue in the future.

Introduction

Infections in soft tissue and bone can occur as severe complications in orthopaedic surgery. The incidence of infection is a function of fracture type (closed fracture 1–2%, open fracture up to 30%), nutrition, malignancy, lifestyle (smoking, alcohol, drug and glucosteroid consumption), diabetes mellitus, immune diseases and vasculitis [1,2]. Medical devices used for fracture stabilization or as endoprostheses increase the risk of infection. In revision surgery the infection risk can reach 40% [3,4]. When biofilms develop on the surfaces of medical devices, anti-infective treatment becomes difficult [5] and expensive. Rubin et al. calculated the direct medical costs for treatment of a patient with a bony osteomyelitis to be $35,000 [6]. Increasing life expectancy and increases in mobility and sporting activity in the elderly population cause the incidence of fractures to raise annually [7]. The likelihood that such an infection will involve multiresistant bacteria is also steadily increasing (National Nosocomial Infections Surveillance System 2004). Infection prophylaxis and treatment are therefore an important challenge in orthopaedic and trauma surgery.

The use of antibiotic-loaded bone cements is common in endoprosthetic surgery. Several products are available that ensure easy and reliable mixing of the cement with a specific antibiotic, sometimes however with a clinically relevant decrease in the strength of the cement [8]. Studies investigating the in vitro release kinetics of such mixtures report potency for up to one week [9–17]. One clinical study reports the in vivo release of antibiotics for up to 4 months after implantation with highly therapeutic levels of tobramycin but not of vancomycin [18]. Elson et al. anecdotally describe an antibacterial activity of antibiotic-loaded cement 2.25 years after implantation [19]. In a case study, antibiotic-loaded beads were explanted after 5 years from one patient and gentamicin-release was still detected in vitro. Bacteria

* Corresponding author at: Julius Wolff Institute, Berlin-Brandenburg Center for Regenerative Therapies, Charité — Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. Fax: +49 30 450 559938.
E-mail address: britt.wildemann@charite.de (B. Wildemann).
[1] These authors contributed equally to this work as first authors.

8756-3282/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bone.2013.11.005

isolated from the surface of the beads were gentamicin-resistant coagulase-negative Staphylococci [20]. These observations suggest that inappropriate antibiotic release kinetics may lead to selection of resistant bacteria [21,22].

In addition to these drawbacks, non-degradable cement cannot be used for biological defect regeneration that is generally preferred in bone loss situations. Larger bone defects will usually not heal without the support of a grafting material. In situations with a high infection risk (e.g. open fracture, compromised patient) prophylaxis may be indicated, whereas a post-infection treatment after debridement of the infection might be necessary [23]. Amongst the bone growth supporting graft materials available to surgeons are allogeneic tissue transplants in the form of cancellous, cortical or demineralized bone (DBM), xenogeneic allografts and assorted alloplastic materials usually based on calcium phosphate [24]. Autologous grafting with material from the iliac crest is still considered by many to be the gold standard. There remains a lack of level 1 evidence in this area and as a consequence the choice of materials is largely based on local experience and material availability. There is some evidence from published studies that allografts are adequate alternatives to autologous grafting in terms of clinical outcome and may reduce morbidity [25,26]. A perioperative approach to combining bone graft materials with antibiotics would provide the means for a target-oriented antibiotic therapy against specific pathogens that are considered to pose a threat to the patient in question. Simple and reliable mixing and defined elution kinetics are prerequisites for such an approach.

The goal of the present study was to investigate such a mixing system. For the first time, mixing properties and release kinetics of different antibiotics mixed with demineralized bone matrix (DBM) in a sodium hyaluronate carrier were investigated using different experimental setups. *DBM pastös* is clinically used as bone grafting material and was mixed with gentamicin (powder or solution), tobramycin or vancomycin. An initial concentration comparable to the concentration in commercially available/clinically used antibiotic-loaded bone cements was chosen [27], and also higher and lower concentrations. In order to gather initial information about how local consumption, removal, or accumulation of released antibiotics might influence the release kinetics, three sampling regimes were investigated. Release was determined over a period of 56 days. The antibiotic activity was evaluated by a zone of inhibition assay and the effects of the elution samples on cell viability and osteogenic activity were also investigated.

Material and methods

Drug-delivery material/grafting material

Demineralized bone matrix *pastös* (DBMp) was supplied by the German Institute for Cell and Tissue Replacement (DIZG, Berlin, Germany). DBMp is a ready-to-use allogeneic tissue transplant paste consisting of sterilised and freeze-dried DBM from screened human donors and sterile sodium hyaluronate. DBMp is approved for use in Germany as a medicinal product.

Antibiotics (Abx)

Gentamicin sulphate powder (~60% pure gentamicin; Fujian Fukang Pharmaceutical Co., Ltd., China); gentamicin solution (40 mg/ml; Merckle GmbH, Germany); tobramycin solution (40 mg/ml; cell pharm GmbH, Germany) and vancomycin hydrochloride powder (pure grade; LEK Pharmaceutical, Germany) were used.

Mixing

DBMp and antibiotic were combined in mixing devices supplied by Medmix Systems AG (Switzerland). These consist of a cylindrical polyamide barrel and a custom plunger with an integrated mixing propeller that can be rotated about the radial axis of the barrel and also moved along its length. A removable threaded cap with a luer-lock fitting permits filling and extrusion via the luer connector or if required with the cap removed. The materials were pharmaceutical grade (USP 6 compliant) and the syringes were gamma-sterilised (25 kGy) prior to use. 50 mg of antibiotic (Abx) was mixed with 1 g bone graft (Bg). This concentration was chosen in accordance with commercially available/clinically used antibiotic loaded bone cements [27].

Release experiment

Cell culture inserts with a polycarbonate-membrane (0.4 μm nominal pore size; Nunc, Germany) were filled with the different Abx/DBMp mixture ensuring a constant and comparable amount of the antibiotics and then placed into the wells of a 12-well plate pre-filled with 5 ml elution medium MEM Earl's/HAM's F-12 1:1 (Biochrom, Germany) supplemented with 10% (v/v) foetal bovine serum (Biochrom, Germany), 0.05 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, Germany) and 0.05 mM β-glycerol phosphate (Sigma-Aldrich, Germany). Medium was chosen for the elution experiments because cytotoxicity tests with primary human osteoblasts were done with the elution samples. The plates were incubated at 37 °C in an atmosphere with 5% CO_2 and 95% relative humidity for 56 days. Samples were taken and medium was replaced at different time points as described in the "Sampling and mixture variants" section. All elution samples and the inserts with the Abx/DBMp mixture (after 56 days) were stored at −20 °C.

Quantification of antibiotics was carried out by a certified analytic lab "Labor Berlin" (Charité Vivantes GmbH; Berlin, Germany). Gentamicin was quantified using kinetic interaction of microparticles in a solution (KIMS; Roche Diagnostics, Germany). Tobramycin and vancomycin were measured with a homogeneous enzyme immune assay using the Roche/Hitachi Cobas® c system (Roche Diagnostics GmbH, Germany). All assays had a defined measurement range (Genta: 0.4–10.0 μg/ml; Tobra: 0.33–10 μg/ml; Vanco: 1.7–80.0 μg/ml) and samples where diluted, if necessary, before analysis with phosphate buffered saline (Biochrom, Germany). Calibration measurements were conducted with Abx/medium mixtures. These mixtures were also used to evaluate the stability of the antibiotic solutions (37 °C, 56 days).

Cumulative release was calculated according to the following formula:

$$M_{t_n} = V\left(C_{t_n} + \sum_{t_o}^{t_{n-1}} C_t \cdot f_t\right)$$

where c = measured concentration (μg/ml); n = sample number; V = volume (ml), and f = dilution factor arising from sampling.

Sampling and mixture variants

The homogeneity of the mixtures was investigated by quantifying the release of gentamicin from different zones of the mixture mass (first, middle and final third of the material cylinder in the mixing device). DBMp and gentamicin solution or powder (25 mg/g Abx/DBM) were filled into the mixing device and mixed with 2, 10 or 20 complete strokes of the cylinder of the mixing propeller (Fig. 1a). Afterwards, each mixture was weighed in three inserts and incubated as described under 'Release experiment' section. This test was run in triplicate and repeated once. Furthermore, mixing of small quantities (1/3 of the device filled with DBMp) was analysed in six independent experiments each mixed with 20 strokes to prove that the mixing procedure results in the same quantity of eluted samples. Data are provided for samples taken after 1 h, 4 h, and 1 day.

Sampling regimes

Three antibiotics (gentamicin solution, tobramycin solution and vancomycin powder) were used to generate 50 mg antibiotic per