Antibiotic-loaded bone void filler accelerates healing in a femoral condylar rat model

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Aims
Deminerlised bone matrix (DBM) is rarely used for the local delivery of prophylactic antibiotics. Our aim, in this study, was to show that a graft with a bioactive glass and DBM combination, which is currently available for clinical use, can be loaded with tobramycin and release levels of antibiotic greater than the minimum inhibitory concentration for Staphylococcus aureus without interfering with the bone healing properties of the graft, thus protecting the graft and surrounding tissues from infection.

Materials and Methods
Antibiotic was loaded into a graft and subsequently evaluated for drug elution kinetics and the inhibition of bacterial growth. A rat femoral condylar plug model was used to determine the effect of the graft, loaded with antibiotic, on bone healing.

Results
We found that tobramycin loaded into a graft composed of bioglass and DBM eluted antibiotic above the minimum inhibitory concentration for three days in vitro. It was also found that the antibiotic loaded into the graft produced no adverse effects on the bone healing properties of the DBM at a lower level of antibiotic.

Conclusion
This antibiotic-loaded bone void filler may represent a promising option for the delivery of local antibiotics in orthopaedic surgery.

The internal fixation of fractures may involve the use of metalwork and graft materials, and may be complicated by infection. Contributing risk factors, either specific to the patient or the procedure, can further increase the risk of infection. The duration of the operation, the length of hospital stay and co-morbidities such as obesity, diabetes and rheumatoid arthritis increase the risks of acquiring a surgical site infection (SSI). Infection can lead to the devitalisation of bone and soft tissues and subsequent nonunion. The management of an infection can include debridement of soft tissues and bone, removal of the metalwork and aggressive antibiotic treatment. SSIs are responsible for an estimated 12% to 16% of infections, with Staphylococcus aureus being a major contributor. Systemic antibiotics penetrate ischaemic and necrotic tissue poorly, and may have toxic side effects. Ideally, a locally administered prophylactic antibiotic is preferred.

The local delivery of antibiotics can be used for both prophylactic and therapeutic purposes. They may be administered for the treatment of orthopaedic infections through vehicles such as polymethylmethacrylate (PMMA) or calcium sulphate. Although the use of antibiotic loaded PMMA improves the outcome of periprosthetic infections, this form of delivery is not suitable for use when treating fractures. First, antibiotic-loaded PMMA cannot be used as a definitive procedure. It may be used to treat established infections as well as maintain overall bone length where there is significant bone loss. It is removed in subsequent procedures, being replaced by bone graft and fixation devices. In addition, the amount of antibiotic that is eluted and the rate of elution varies considerably depending on the physical environment and the shape and size of the defect. When the amount of antibiotic which is released falls below the minimum inhibitory concentration (MIC), the remaining PMMA can become a nidus for infection, leading to further complications. Finally, the curing reaction of PMMA is exothermic preventing the use of many antibiotics. Materials with a base of calcium
sulphate also has advantages and disadvantages. First, they do not require removal and with only a slight increase in temperature during setting, allow the use of a much broader range of antibiotics. They also allow a continual release of antibiotics, the rate of which is determined by the dissolution and resorptive properties of the calcium sulphate. As a filler of bone voids, however, their bone regeneration activity is limited with minimal osteoinduction and faster degradation than bone ingrowth which may lead to increased wound drainage.

An ideal antibiotic delivery device for use in orthopaedic surgery would be a bone graft with drug eluting properties that are biocompatible, degrade in concert with bony replacement, and possess osteoinductive and osteoconductive properties. The use of allograft bone is well established in many orthopaedic settings, decreasing the need for autograft, of which major complications include donor site morbidity and additional surgical sites. These materials are useful in augmenting the healing of bony defects caused by trauma, the resection of tumours, abnormal skeletal development, the removal of cysts and loosening of prostheses. Demineralised bone matrix (DBM) has also been shown to stimulate osteoinduction allowing improved bone growth and fusion. Allogenic DBM and bioactive glass possess osteoinductive properties and may serve as a drug delivery device in the prophylactic treatment of SSI in a variety of anatomical locations. The use of this type of bone void filler would allow the release of the entire quantity of antibiotic as the material is being resorbed and remediated. The concept of allograft impregnated with antibiotic is not new, having been initially described in the 1940s and further developed in the mid 1980s. Most studies, however, involve the use of cancellous bone allograft rather than a DBM based material. In addition, there are few reports of the prophylactic use of antimicrobial allografts. Here we describe an injectable gel comprised of DBM and bioactive glass that has been reconstituted with tobramycin present. Tobramycin is one of several antibiotics that are currently being used in prophylaxis and in the treatment of osteomyelitis due to its activity against gram-negative bacteria as well as Staphylococcus aureus, a leading cause of infection. The aim of this study was to evaluate the antimicrobial properties of tobramycin loaded on a DBM based gel using in vitro assays and to evaluate the impact tobramycin would have on bone healing. The hypothesis was that tobramycin can elute from the graft material and does not interfere with the healing properties of the graft.

**Materials and Methods**

The DBM-based gel (NanoFUSE, Nanotherapeutics, Alachua, Florida) was formulated essentially as previously described using a patented process which encapsulates the DBM and bioactive glass in porcine gelatin, and is sterilised using ionising radiation. The DBM used for these studies was harvested from the long bones of generously donated tissue and supplied by Allosource Inc. (Denver, Colorado). The demineralisation process was similar to that described by Urist and Dowell. The final size of the particles varied between 125 μm and 710 μm. Bioactive glass, 4555 (Mo-Si Health Care, LLC; Rolla, Montana), with a composition of 43% to 47% SiO₂, 22.5% to 26.5% CaO; 5% to 7% P₂O₅; and 22.5% to 26.5% Na₂O was used with a particle size between 90 μm and 710 μm (≥ 90%). The DBM-based gel was reconstituted with either sterile water (DBM) or a tobramycin/water solution to prepare an antibiotic-loaded graft (DBMT).

In order to prepare tobramycin loaded DBM-based gels, the DBM-based gel (1 g) was reconstituted with 2 mL of a tobramycin/water solution. The final compositions of the hydrated gels were 4% wt and 8% wt tobramycin (DBMT4 or DBMT8, respectively). DBMT was then placed in 5 mL of phosphate buffered saline (PBS) and incubated at 37°C. At designated time points, the PBS eluant was removed and replaced with fresh PBS. The recovered eluant was saved at 4°C until measured for tobramycin concentration of for bacterial inhibition. This process was performed in triplicate and repeated over 14 days. Tobramycin was quantified in each eluant using the Dimension Xpand Plus Clinical Chemistry System (Siemens, Erlange, Germany).

Then, 6 mm paper discs (Remel Microbiology Products, Lenexa, Kansas) were loaded with a 20 μl sample from the release kinetics study and 10 μg tobramycin was loaded onto blank paper discs for positive controls and PBS wetted blank paper discs were used as negative controls. Staphylococcus aureus UAMS-1 (ATCC 49230, Blacksburg, Virginia), at a concentration of 10⁷ colony forming units per millilitre (CFU/mL), was plated onto 100 mm diameter Mueller–Hinton agar plates (Remel). Each agar plate received three sample discs, one positive control disc and one negative control disc. The diameters of the zones of inhibition were measured following a 24 hour incubation at 37°C. Each sample of eluant was measured in triplicate.

All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations and the principles of the Guide for the Care and Use of Laboratory Animals. A total of 61 athymic nude rats (13.6 weeks, SD 0.7; Charles River, Wilmington, North Carolina) received a 3 mm diameter × 5 mm deep defect in the left femoral condyle. Athymic nude rats were chosen in order to limit any immune response to the human DBM within the NanoFUSE. Briefly, prior to surgery, the animals were given a slow release buprenorphine and were anaesthetised with 1% to 3% isoflurane. The surgical region was aseptically prepared with 70% ethanol and betadine. An incision was made over the medial portion of the distal femur to reveal the condyle. Using a 3 mm drill bit, a 5 mm deep defect was created while cooling generously with saline. The defect was subsequently filled with DBM, DBMT4, DBMT8, or left empty as the negative control (Table I). The tissues were closed in layers with 4-0 suture (Table I). The tissues were closed in layers with 4-0
vicryl sutures. The animals were recovered and allowed food and water ad libitum. Blood from the tail vein was taken at six and 24 hours, and seven days to measure systemic tobramycin levels. At six weeks post-operatively, 16 animals, four from each group, were killed. The remaining animals were killed 12 weeks post-operatively. The limbs were harvested, the soft tissue was removed, and the remaining bone structures were fixed in 10% neutral buffered formalin for Micro Computed Tomography (microCT) and methacrylate embedded for histology.

Immediately following surgery, at six weeks and 12 weeks, the limbs were scanned using microCT to quantitate the three-dimensional volume of bone within the femoral condylar defect. A VivaCT40 (Scanco Medical, Bassersdorf, Switzerland) was used to scan each femur with settings of 70 kV and 114 μA, 21 μm voxel size, and an integration time of 200 ms. The scans were re-orientated with DataViewer software (Bruker-microCT, Kontich, Belgium) to align the cylindrical defect along the z-axis. A cylindrical region of interest was designated with a diameter of 3002 μm, spanning 132 slices starting at the medial aspect of the defect. Using CT Analyser Software (Bruker-microCT), bone was separated from the surrounding tissue using a global threshold of 73 via the Otsu method. A ratio of bone volume-to-tissue volume (BV/TV) was determined. Due to the radiopacity of the DBM-based graft material, implanted material was not segmented from new bone growth.

The undecalcified fixed tissues were dehydrated through serial alcohol solutions and embedded in PMMA. Longitudinal sections (5 μm thick) were made along the sagittal plane of the bone. The sections were deplasticised with 2-methoxyethyl acetate, rehydrated and stained with Goldner’s Trichrome to evaluate for bone growth.

**Statistical analysis.** A two-way analysis of variance (ANOVA) was used where appropriate followed by a Bonferroni t-test. A t-test was used to differentiate the tobramycin release and ring of inhibition at each time point. A p-value < 0.05 was used to indicate significance.

**Results**

Tobramycin was released from the DBMT in a logarithmic order (Fig. 1). Both the 4% and 8% had a burst of tobramycin within the first four hours followed by a rapid release of tobramycin over the next three days.

Blood plasma mean concentrations of tobramycin peaked at six hours with 0.32 ug/ml SD 0.08 and 0.34 ug/ml SD 0.08 for DBMT4 and DBMT8, respectively. The levels of tobramycin decreased by 24 hours with mean minimally measurable quantities (0.1 μg/ml and 0.12 μg/ml for DBMT4 and DBMT8). By seven days, the levels of tobramycin within the plasma had fallen below detectable limits.

Although bone was observed in all samples, including the empty defect, there were significant differences (Fig. 3a). First, there was a significant overall increase in bone volume compared with the empty control in both the DBM and DBMT4 groups (two-way ANOVA with Bonferroni post-test; p = 0.006, p < 0.001, respectively). The addition of 4% tobramycin did not hinder the regenerative properties of the graft. There was no difference in bone volume

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<th>Time points (wks)</th>
<th>Number of animals</th>
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<tr>
<td>Empty defect</td>
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<td>4</td>
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<tr>
<td>DBM-based Gel (DBM)</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>DBMT+4% tobramycin</td>
<td>6</td>
<td>4</td>
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<td>DBMT+8% tobramycin</td>
<td>12</td>
<td>12</td>
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<tr>
<td>DBMT+4% tobramycin</td>
<td>6</td>
<td>4</td>
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<tr>
<td>DBMT+8% tobramycin</td>
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DBM, demineralised bone matrix based gel; DBMT4, DBM reconstituted with a tobramycin solution resulting in a 4% wt/wt tobramycin gel; DBMT8, DBM reconstituted with a tobramycin solution resulting in a 8% wt/wt tobramycin gel

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**Table I. The in vivo experimental design**

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**Fig. 1**

Mean release of tobramycin from demineralised bone matrix (DBM)-based biomaterial reconstituted with 4% (DBMT4) and 8% (DBMT8) tobramycin (weight-to-weight). DBMT8 released significantly more tobramycin on days 1 and 2 compared to DBMT4 (*, p = 0.013 and #, p = 0.048, respectively). Error bars represent standard error of the mean.
between the 8% tobramycin group and all other groups. Although bone per volume had subtle changes, the evidence of decreased bone regeneration was more apparent in the microCT slices and histology. All groups showed cortical bone formation, an expected outcome from a non-critical size defect. The DBM and DBMT4 also showed signs of bone formation within the metaphysis (Fig. 3b), a feature which was absent from the empty and DBMT8 groups. This was corroborated histologically (Fig. 4). There was evidence of bone in the DBM at 12 weeks and in the DBMT4 at both six and 12 weeks. There was an observational difference between the sections of 8% tobramycin and 4% tobramycin, namely the appearance of bone within the metaphysis with the 4% tobramycin group.

Discussion
A leading complication associated with open reduction is infection. Factors that increase the risk for infection include the duration of the operation, the general health of the patient and the extent of the injury. Although risk factors which are associated with the procedure can be mitigated, those related to the patient cannot be changed. High local concentrations of antibiotics are especially beneficial in the treatment of relatively avascular areas and in the presence of organisms resistant to the levels of antibiotics obtained via parenteral administration, including organisms in biofilms. It has recently been suggested that vancomycin powder introduced during spinal surgery reduces the incidence of infection. This does not contain properties to aid bone healing. The ideal materials for local antibiotic delivery to be used in orthopaedic surgery should be biocompatible, contain osteoconductive and osteoinductive properties as well as absorb and release active antibiotics at clinically relevant levels. Lewis et al have shown that a human DBM-based bone void filler loaded with gentamicin has no adverse effects on new bone growth in rat models and the
human DBM did not adversely affect the antibiotic characteristics of the gentamicin. In this study, we investigated a DBM/bioactive glass blended material that is reconstituted with tobramycin, with appropriate release kinetics, bacterial susceptibility and bone growth. The gel was reconstituted with two different concentrations of tobramycin. Most of the tobramycin was released within the first four hours with subsequent continuing release for three days. The released tobramycin eluted from DBMT was capable of inhibiting bacterial growth for three days, confirming that the form of tobramycin which was released was active. This release of tobramycin within the first days is crucial to mitigate the onset of infection induced by pathogens introduced at the time of surgery. The prophylactic use of local vancomycin powder has been shown to decrease the incidence SSIs of the spine from 2.6% to 0.2%,26,27

Previous authors have shown that this particular gel is biocompatible and possesses both osteoconductive and osteoinductive properties.17 When assessed for its use as a graft for bone healing, a lower concentration of tobramycin did not affect the regenerative properties of the graft. To our knowledge, this study is the first indication that higher growth volume compared to the unloaded bone graft. Lindsey et al28 showed that autologous bone graft with 3% tobramycin did not interfere with healing.

We chose to use tobramycin in this study because there is a long history of the local administration of this antibiotic. It provides broad spectrum cover for the bacteria that commonly cause SSI, and is relatively non-toxic to osteoblasts compared with other antibiotics.29 However, as with any antibiotic, there is limited activity against a broad range of bacteria. Additionally, although the material may be used to fill voids in bone, it has limited capacity for maintaining the space. Our investigation limited its use to a non-critical condylar defect without local infection. Further investigation is required to determine its capacity to reduce infection in vivo.

The release kinetics, histological and radiographic data presented here show that a DBM-based gel reconstituted with lower concentrations of tobramycin maintains its ability to accelerate bone healing while eluting effective amounts of tobramycin. Thus it is reasonable to anticipate that reconstitution of this material with tobramycin would allow the surgeon to treat an orthopaedic surgical site prophylactically with a resorbable and osteoinductive biological graft. The local treatment of fractures with a DBM-based biomaterial reconstituted with tobramycin should reduce the incidence of infection, allow increased bone formation and provide an improved local environment for healing, as well as potentially decreasing the number of additional operations performed on patients at risk.

**Fig. 4**

Representative histological results from non-critical defect in rat femoral condyle model. Sections stained with Goldner’s Trichrome stain (blue: connective tissue; red/pink, cytoplasm). The top of each image represents the medial border of the femoral condyle. Demineralised bone matrix (DBM)-based biomaterial reconstituted with 4% (DBMT4) and 8% (DBMT8) tobramycin.)

**Take home message:**

By loading a clinically available bone void filler with an antibiotic, there can be rapid translation of this delivery mechanism to aid in reducing surgical site infections.

**Author contributions:**

S. M. Shiels: Data collection, Data analysis, Primary manuscript writer.

R. R. Cobb: Study conception, Manuscript drafting.

K. M. Bedigrew: Study design, Surgery, Data collection.


J. F. Kirk: Study conception, Manuscript drafting.

A. Kimbler: Study conception.

I. Finger Baker: Study conception.

J. C. Wenke: Study design, Writing the manuscript.

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**References**


