ANTIBIOTIC TREATMENT

Antibiotic-loaded hydroxyapatite blocks in the treatment of experimental osteomyelitis in rats

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A novel drug delivery system for osteomyelitis was developed in which a porous hydroxyapatite block (HAB) is loaded with antibiotic by a centrifugation method. In this study, implantation of HABs loaded with the aminoglycoside antibiotic, arbekacin, were tested in established *Staphylococcus aureus* osteomyelitis in the proximal tibia of rats after debridement of the marrow cavity. The animals were observed for radiographic signs of infection and tissue was examined histologically. The infections were also evaluated by bone cultures. Bacterial counts were statistically lower in rats implanted with an antibiotic-loaded HAB than in those given a drug-free HAB. Radiographical and histological observations also showed beneficial effects of the antibiotic-loaded implant. The results suggest that the centrifugation method for loading HABs provides a simple drug delivery system. These antibiotic-loaded HABs may be useful for filling grafts in osteomyelitis.

Introduction

Despite various methods of therapy, chronic osteomyelitis still remains one of the most difficult diseases to treat. Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$; mol. wt 1004.63) has excellent biocompatibility and a porous structure. It can be used to fill the dead space after curettage and provides a drug delivery vehicle for antibiotics. We have reported a novel drug-delivery system for osteomyelitis in which a hydroxyapatite block (HAB) is loaded with antibiotics by a centrifugation method, and then gives a slow release of antibiotic activity *in vitro* [1].

The present *in-vivo* study assessed the efficacy of antibiotic-loaded HABs in a rat model of osteomyelitis caused by *Staphylococcus aureus*. Efficacy was evaluated by residual bacterial counts after treatment, radiographs and histological changes in experimental osteomyelitis in the tibia of rats.

Materials and methods

Preparation of antibiotic-loaded blocks

HABs (Boneceram-P, Sumitomo Pharmaceutical Co. Ltd, Tokyo) with the following properties were used:

mol. wt, 1004.63; porosity, 50%; pore size, 50–300 μm ; dimensions, $2 \times 2 \times 3$ mm. Fresh HABs were mixed with arbekacin (Meiji Seika Ltd, Tokyo, Japan) 400 mg dissolved in 2 ml of physiological saline solution, and centrifuged at 1500 rpm for 15 min. The antibiotic absorption rate by this method was 35%/10-mm³ volume, so that 0.84 mg of arbekacin was captured into one experimental block.

In-vivo study

Wistar male rats weighing 200–250 g, were used. The animals were anaesthetised with an intraperitoneal injection of pentobarbital sodium, 50 mg/kg. The medullary cavity of the proximal tibia was opened and an 8-mm long silk thread soaked in a bacterial suspension containing 1.0×10^8 cfu of *S. aureus* IM2-42 (Institute of Anaerobic Bacteriology, Gifu University School of Medicine) was inserted. The cavity was then closed with bone wax under sterile conditions. The rats were fed a standard antibiotic-free rat diet throughout. The MIC of arbekacin for the *S. aureus* strain used was 0.25 $\mu\text{g/l}$.

The HAB was implanted 4 weeks after the infection was induced. Each rat was re-anaesthetised and tibial X-rays were obtained. All rats showed gross pathological and radiographical signs of osteomyelitis at this time (purulent material, widening of the proximal tibia and disruption or pitting of bone architecture). The right proximal tibia was disinfected, and then prepared

for surgery. A 3×5 mm rectangular window was cut through into the medullary cavity with a dental burr. At this point the rats were divided into four groups. The first group consisted of three animals that received no treatment for 4 weeks after the inoculation of *S. aureus*. In experimental group I ($n = 21$) an arbekacin-loaded HAB was implanted into the marrow cavity of each rat after debridement and irrigation of the marrow cavity with 40 ml of normal saline. In control group II ($n = 21$) rats were similarly given implants of drug-free HABs. Animals in group III ($n = 21$) were treated with arbekacin 10 mg/kg daily for 1 week by subcutaneous injection after debridement and washing of the wound only.

Bacteriological assessment

The tibia was removed from soft tissues, crushed and homogenised with 2 ml of saline under sterile conditions. Serial dilutions of this suspension were inoculated onto three plates of Mueller-Hinton Agar (Nissui Co. Ltd, Tokyo, Japan). After incubation for 48 h, the viable count (cfu/ml) was calculated. Statistical significance was tested by Student's *t* test.

Radiological evaluation

Radiographs of tibias were obtained with Softex film (Fuji Co. Ltd, Tokyo, Japan) 1 and 7 weeks after implantation of the HABs.

Histological examination

Two animals from each group were killed 1, 3, 5 and 7 weeks after the implantation. Each tibia was fixed with formalin 4%, sliced into sections $3 \mu\text{m}$ thick, and stained with haematoxylin and eosin. For the non-decalcified sections saw cuts of *c.* $500 \mu\text{m}$ were made perpendicular to the long axis of the tibia. Tissues were milled down to *c.* $60 \mu\text{m}$ with a micromilling device (Maruto Co. Ltd, Tokyo, Japan) and stained with toluidine blue.

Pharmacokinetic studies

Samples of serum were obtained from three rats 30 min after implantation of an arbekacin-loaded HAB into the marrow cavity of the tibia and at random times thereafter. The specimens of serum were kept at -20°C until measurement of arbekacin concentrations by fluorescence polarisation immunoassay (Meijiseika Co. Ltd, Tokyo).

Results

Bacteriological assessment

Bacterial counts in untreated rats 4 weeks after infection with *S. aureus* averaged 2×10^6 SEM 2.7×10^6 cfu/ml of tibial homogenate. One week after

implantation of arbekacin-loaded HABs bacterial counts averaged 1.8×10^4 SEM 1.2×10^4 cfu/ml; at 3 weeks average counts remained at 1.7×10^4 SEM 0.9×10^4 cfu/ml. After 5 and 7 weeks, bacteria were no longer detectable in some rats and were present in counts of $(1.5 \times 10^3) - (9.5 \times 10^3)$ cfu/ml in others (Table 1). In rats given drug-free implants, bacterial counts 1 week after the implantation were on average 1.6×10^5 SEM 0.4×10^5 cfu/ml; counts remained above 3.8×10^6 SEM 3.1×10^6 cfu/ml for the 7-week period of observation. In rats treated with arbekacin by subcutaneous injection, bacterial counts after 1 week were 2.3×10^5 SEM 1.8×10^5 cfu/ml. Overall, total counts of bacteria in rats receiving arbekacin-loaded HAB implants (1.5×10^4 SEM 0.5×10^4 cfu/ml) were significantly lower than those observed in rats given drug-free implants (2.1×10^5 SEM 1.0×10^5 cfu/ml) or drug injections only (5.5×10^6 SEM 4.6×10^6 cfu/ml) ($p < 0.01$ and $p < 0.05$, respectively). No bacterial growth (limit of detection $< 1 \times 10^2$ cfu/ml) was observed in five rats given the arbekacin-loaded implant, in one rat treated with antibiotic alone, and was not observed in any of the animals given a drug-free implant (Table 1).

Radiological evaluation

X-ray evaluation of the treatment with arbekacin-loaded HAB showed that a radiolucent area surrounding the implant diminished gradually (Fig. 1). Almost all of the implants were united with bone and a radiolucent lesion with a periosteal reaction was observed in only one case. In rats given a drug-free implant, a radiolucent area persisted in the vicinity of the implant and osteosclerotic areas still existed after 7 weeks (Fig. 2). Osteolytic and extensive lesions were observed in all cases.

Table 1. Bacterial counts in tibial homogenates of rats experimentally infected with *S. aureus*

Period after treatment (weeks)	Viable count (cfu/ml of homogenate) in		
	Group I	Group II	Group III
1	5.9×10^3	2.2×10^5	5.8×10^5
	4.2×10^4	1.0×10^5	3.1×10^4
	5.9×10^3	1.6×10^5	7.5×10^4
3	4.0×10^3	1.3×10^6	4.5×10^6
	3.4×10^4	1.0×10^7	1.2×10^5
	1.4×10^4	8.0×10^4	5.6×10^7
5	$< 1 \times 10^2$	2.3×10^6	4.3×10^6
	$< 1 \times 10^2$	6.3×10^5	3.3×10^3
	1.5×10^3	1.2×10^6	1.4×10^3
7	9.5×10^3	3.6×10^5	1.4×10^5
	$< 1 \times 10^2$	1.3×10^5	5.0×10^2
	$< 1 \times 10^2$	1.4×10^6	$< 1 \times 10^2$
	$< 1 \times 10^2$	1.0×10^7	1.5×10^4
	$< 1 \times 10^2$		

Group I, rats with arbekacin-loaded HAB implant; group II, rats with antibiotic-free HAB implant; group III, rats treated with arbekacin alone (no implant). Difference between results in groups I and II statistically significant, $p < 0.01$.

Fig. 1. X-rays of rat tibias: **a**, 1 week; **b**, 3 weeks; **c**, 7 weeks after implantation of an arbekacin-loaded HAB. After 3 weeks the radiolucent zone was diminished; after 7 weeks it had almost vanished.

Fig. 2. X-rays of rat tibias: **a**, 1 week; **b**, 3 weeks; **c**, 7 weeks after implantation of an antibiotic-free HAB. After 3 weeks bone sclerosis surrounding the implant was visible; after 7 weeks osteosclerosis in the vicinity of the implant increased and a radiolucent zone clearly existed.

Histological findings

After implantation of arbekacin-loaded HABs, new bone formation was visible at the surface of the HAB and complete contact without fibrous tissue was evident at the interface between the bone and implant at 7 weeks (Fig. 3a,b). Few leucocytes were seen before 7 weeks without new bone formation. In animals given antibiotic-free HABs, leucocyte infiltration, proliferation of fibrous tissue and bone abscess formation were seen in the vicinity of the HAB (Fig. 3c).

Pharmacokinetic studies

The serum arbekacin concentration reached a peak of $8.7 \pm 1.4 \mu\text{g/ml}$, 1 h after implantation (Fig. 4). Antibiotic was undetectable by immunoassay after 3 h.

Discussion

Despite advances in antibiotic treatment, chronic osteomyelitis is still difficult to cure, as low concentra-

Fig. 3. Histopathological changes 7 weeks after implantation of HABs. Longitudinal section of bone with arbekacin-loaded HAB: **a**, $\times 9$; **b**, $\times 90$, showing block in contact with new bone (arrows) (non-decalcified toluidine blue staining); **c**; section of bone with drug-free HAB ($\times 9$) showing inflammatory cells and abscess formation (arrows).

Fig. 4. Changes in serum arbekacin concentration after the implantation of antibiotic-loaded HAB (0.84 mg arbekacin/block).

tions of antibiotics are achieved in the lesion, because of bone necrosis, sclerosis and decreased blood flow. The causative bacteria are known to produce a large amount of extracellular glyocalyx material [2] and necrotic bone provides a surface suitable for the development of a biofilm [3]. For these reasons, the surgical removal of necrotic tissues, with long-term delivery of high concentrations of drug at the site of the lesion are the primary methods of treatment of chronic osteomyelitis.

Currently, antibiotic-loaded cement beads [4,5] of polymethylmethacrylate are successfully used as a drug delivery system. However, the local insertion of this compound has the disadvantage of requiring subsequent surgery for replacement with an autograft. Moreover, drugs that are resistant to the heat necessary for the production of polymethylmethacrylate must be chosen.

In the search for suitable carrier systems, various biological substances have been tested: tricalcium phosphate [6], plaster of Paris [7], a composite of D,L-lactic acid oligomer [8] and fibrinogen [9] have been investigated as absorbable carriers, mainly in experiments with animals. However, unlike hydroxyapatite, these materials would not be able to withstand mechanical forces. Hydroxyapatite has been widely used as a bioactive ceramic material for bone defects because of its excellent biocompatibility and non-antigenicity [10,11]. In a preliminary study, Shinto *et al.* [12] used porous calcium hydroxyapatite in which an antibiotic powder was packed into a cylindrical cavity. Yu *et al.* [13] also reported the experimental use of a self-setting hydroxyapatite cement formed into cement pellets.

Itokazu *et al.* recently described a drug-delivery system in which HABs were loaded with antibiotics by a centrifugation method [1]. The slow release of the activity of the aminoglycoside antibiotic arbekacin was demonstrated. A concentration of 0.5 $\mu\text{g/L}$

(sufficient to inhibit most pathogens) was still maintained after 20 exchanges of phosphate-buffered saline over 40 days. In the present evaluation of the efficacy of arbekacin-loaded HABs in a rat model of osteomyelitis, bacterial counts in animals given implants of antibiotic-loaded blocks were lower than those in the control group throughout the experimental period. Moreover, complete cure was obtained in five of seven rats over a 5-week period of observation. Radiographical evaluation showed improvement in almost all animals with arbekacin-loaded implants. In contrast, deteriorating X-ray changes were seen in all animals given drug-free implants or subcutaneous arbekacin. Histological findings showed marked improvements in rats receiving antibiotic-loaded implants, confirming the benefit of the presence of antibiotics, although bacteria persisted in some lesions. Similar results have been reported in the treatment of experimental osteomyelitis of rabbits with gentamicin-loaded plaster of Paris [7]. In that study bacteria persisted in most animals but there was marked clinical improvement.

Monitoring of serum arbekacin concentrations after implantation of the antibiotic-loaded HABs showed that a peak concentration was achieved after 1 h. Antibiotic was undetectable 3 h after implantation. These results suggest that antibiotic-loaded HABs may be safe to use in combination with intravenous injections. Slow release of antibiotics from HABs produces high localised concentrations which cannot be attained by parenteral therapy because of the risk of side-effects. Antibiotic-loaded HABs prepared by centrifugation may offer a valuable new form of local antibiotic therapy primarily for strut graft and osteomyelitis. Such an approach may be particularly useful in treating bone and joints infected with methicillin-resistant *S. aureus*.

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