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Journal of Orthopaedic Research 22 (2004) 1351–1357

Journal of Orthopaedic Research

www.elsevier.com/locate/orthres

Prevention and treatment of experimental osteomyelitis in dogs with ciprofloxacin-loaded crosslinked high amylose starch implants

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Received 9 April 2004; accepted 12 April 2004

Abstract

Crosslinked high amylose starch (CLHAS) matrix was used as a biodegradable drug delivery implant for the prevention and treatment of osteomyelitis. Thirty-two dogs underwent the femoral insertion of a screw inoculated with *Staphylococcus aureus* and were then randomly assigned to four groups: (A) prevention with ciprofloxacin-CLHAS implants, (B) surgical debridement (positive control), (C) surgical debridement and oral ciprofloxacin treatment and (D) surgical debridement and treatment with ciprofloxacin-CLHAS implants. At week 4 the osteomyelitis was confirmed, the infected site debrided and respective treatments initiated for groups B, C and D. Radiographs, macroscopic evaluations, bacterial cultures and histopathological examinations were used to evaluate the femora at week 10. Femora from preventive group A were almost normal. Dogs of both ciprofloxacin treatment groups C and D showed better bone healing, less periosteal reaction and less screw mobility than dogs from group B. Eradication of infection was observed at proximal/distal sites in B: 25%/12%, C: 37%/62% and D: 62%/75%. Both ciprofloxacin treated groups improved radiographically from week 4 to week 10. Periosteal and marrow neutrophilic and lymphoplasmocytic infiltrations were less severe in groups C and D versus group B. These data suggest that biodegradable ciprofloxacin-CLHAS implants are a safe and efficient modality for the prevention and treatment of osteomyelitis.

Keywords: Osteomyelitis; Local delivery system; Biodegradable implants; Starch; Ciprofloxacin

Introduction

Musculoskeletal infections can be difficult to treat with systemic antimicrobials because of their limited penetration into the devascularized tissues and biofilms that may be present at the infection site [5,8,15,16,19,28,38,40]. The current regimen of therapy for such infections includes an aggressive surgical debridement, soft tissue coverage, long term systemic antibiotic therapy and the removal or change of fixation devices without impairing bone stability [2,6,14,25,29,42]. The success rate with this approach varies between 80% and 90% [8,16,39–41]. To improve this treatment response, antimicrobial drug delivery systems (DDS) have been developed, allowing sustained high local drug concentrations with minimal potential of systemic toxicity. Furthermore, the preventive use of such systems has decreased infection rates in total hip arthroplasties and open fractures [4,31,41]. In past years, many efforts have been oriented towards the evaluation of various biodegradable DDS such as plaster of Paris, poly (D,L-lactide) and/or polyglycolide, calcium hydroxyapatite, calcium sulfate, collagen, chitosan and others [2,5,6,8,14,18,25,27,29,37,38]. Their major advantage over polymethylmethacrylate (PMMA) is to avoid the drawbacks of a second surgery for removal. However, one limitation of these newly developed DDS is the fact that their cost/benefit ratio is often too high and precludes their use on a regular basis [7,27].

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doi:10.1016/j.orthres.2004.04.007

Crosslinked high amylose starch (CLHAS) was first developed as a controlled release solid oral dosage form with a quasi-zero order drug release over 12–24 h [20,21]. It has been recently demonstrated that CLHAS is a biodegradable material characterized by an excellent biocompatibility and controlled local delivery properties after subcutaneous and intramuscular implantations [10,11]. Following perifemoral placement of ciprofloxacin-loaded CLHAS implants in rabbits, local muscle and bone ciprofloxacin concentrations were measured over 100 times in excess of the minimum inhibitory concentration (MIC) for a strain of *Staphylococcus aureus*, over at least 28 days [12]. Moreover, CLHAS implants are easily manufactured, which would be an advantage over most other degradable DDS [9]. Fluoroquinolones such as ciprofloxacin can penetrate bone at sufficient concentrations to inhibit most members of the family of Enterobacteriaceae, and a large percentage of *Pseudomonas* spp. and *Staphylococcus* spp. strains [23,34]. Although the performance of ciprofloxacin in treating experimental infections has been questioned [24,27,30], it has been reported that this agent is very active against the biofilm of *S. aureus* and *P. aeruginosa* [33,36], and that it may be a judicious option for antibiotic therapy of osteomyelitis [23,34]. Consequently, CLHAS implants containing ciprofloxacin may have a potential as a controlled DDS for prevention and treatment of musculoskeletal infections.

The objective of this study was to evaluate the preventive and curative efficacies of CLHAS implants loaded with ciprofloxacin in a canine experimental model of chronic femoral osteomyelitis. Our hypotheses were that the use of ciprofloxacin-CLHAS implants (1) prevents the development of osteomyelitis and (2) is equivalent to oral ciprofloxacin for the treatment of osteomyelitis in this canine experimental model.

Materials and methods

Bacterial isolate

The strain of *S. aureus* used in this experimental model, identified as American type culture collection (ATCC) 29213, was shown to be sensitive to ciprofloxacin and methicillin in vitro. The MIC of ciprofloxacin for this strain was 0.5 μg/ml. A suspension of $3–4\times10^8$ colony forming units (cfu) per ml was prepared for the purpose of the study.

Crosslinked high amylose starch implants

Crosslinked high amylose starch implants (Contramid®; Labopharm Inc., Laval, QC, Canada) of 200 mg, each containing 40 mg (20%) of ciprofloxacin hydrochloride (Betachem Inc., Upper Saddle River, NJ, USA), were prepared by direct compression as previously described [12]. This formulation provided the best delivery profile in vivo [12]. The implants were 7.1 mm in diameter and 4.8 mm thick.

Canine osteomyelitis model

Thirty-two 1–2 year old Beagle dogs (16 males and 16 females, 8.5–14.5 kg) were used for the creation of a chronic osteomyelitis model. Dogs were randomly assigned to four treatment groups ($n = 8$) (A, B, C and D). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Montreal. At week 0, each dog was premedicated with an intramuscular injection of hydromorphone (Sabex Inc., Boucherville, QC, Canada), induced under general anesthesia with intravenous thiopental (Penthotal®, Abbott Laboratories Ltd, Montreal, QC, Canada) and maintained with isoflurane (AErrane®, Baxter Corporation, Toronto, Ont., Canada). A morphine (Sabex Inc., Boucherville, QC, Canada) epidural was performed prior to every surgical procedure. The left hind limb was prepared for aseptic surgery. After lateral femoral exposure, a total dose of $0.6–0.8\times10^6$ cfu of the inoculum (0.02 ml) was directly dropped on a 2.0 mm cortical screw (Synthes Canada Ltd, Mississauga, Ont., Canada) using a sterile micropipette. The screw was inserted in a hole drilled through the cis-cortex at mid-diaphysis and its end rested in the medullary cavity. The fascia and subcutaneous tissues were sutured with 3-0 polyglyconate (Maxon®, Tyco Inc., Ville St-Laurent, QC, Canada) and skin with 3-0 nylon (Monosof®, Tyco Inc., Ville St-Laurent, QC, Canada). Post-operative hydromorphone and ketoprofen (Anafen®, Merial Inc., Baie d'Urfé, QC, Canada) were administered intramuscular to all animals. Dogs from group A simultaneously received eight ciprofloxacin-CLHAS implants directly on the lateral aspect of their femur before closure. This group has served after 10 weeks to evaluate the efficacy of preventive implantation.

Four weeks after inoculation, dogs from groups B, C and D were re-operated. The screw was removed to allow bacterial sampling centered over the empty hole, using a 5 mm Michele trephine (Instrumentarium, Terrebonne, QC, Canada) (Fig. 1A). The site was thoroughly debrided and a new 2.0 mm cortical screw was inserted 1.5 cm distally to maintain a foreign body within the infected site as would be encountered in the clinical setting (Fig. 1A). The same surgeon performed all debridements and was blinded to the treatment groups. Afterwards, the treatment plan was adopted depending on which group the dog was previously assigned to: dogs from group B did not receive any further treatment until the end the project and served as a positive control; dogs from group C were treated for 28 consecutive days with ciprofloxacin (10 mg/kg, PO, q12 h) (Cipro®, Bayer Inc., Etobicoke, Ont., Canada) and allowed the evaluation of oral antibiotic curative efficacy at week 10 and dogs from group D were treated with eight ciprofloxacin-CLHAS implants and allowed the evaluation of ciprofloxacin-CLHAS implant curative efficacy at week 10.

Ten weeks after inoculation, all dogs were euthanatized and prepared for sterile harvesting. After removal of the distal screw, two bone samples were taken adjacent to the proximal and distal sites for bac-

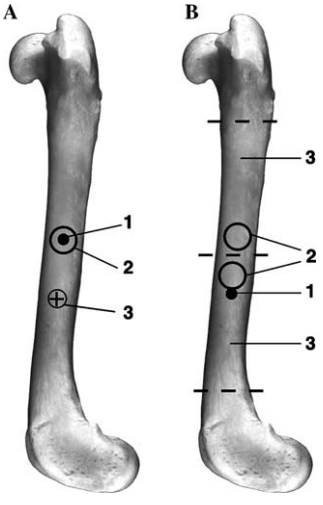

Fig. 1. (A) Manipulations at week 4 were sequenced: (1) first screw removal, (2) bacterial sampling and (3) second screw insertion. (B) Manipulations at week 10 were sequenced: (1) distal screw removal, (2) bacterial sampling and (3) sections of the diaphysis for histopathological examination.

terial evaluations (Fig. 1B). Since no distal screw was placed in group A at week 4, a sample was taken only beside the original hole. Sections of the diaphysis were then made to obtain cylindrical bone samples for histopathological examination (Fig. 1B).

Evaluation methods

(1) Radiographs: all dogs were radiographed at weeks 0, 4 and 10 using digitalized images (ADC Solo, AGFA-Gaevaert N.V., Mortsel, Belgium). Radiographic criteria of periosteal reaction, osteolysis, medullary sclerosis and proximal defect healing were graded as absent, mild, moderate or severe. All radiographs were reviewed by the same investigator who was blinded to the treatment groups.

(2) Macroscopic evaluations: after femoral exposure, a semi-quantitative scoring system was used to evaluate the severity of periosteal reaction, screw mobility, soft tissue adhesions and the importance of bone healing when appropriate. All surgical procedures and macroscopic evaluations were performed by the same surgeon who was blinded to the treatment groups.

(3) Bacterial cultures: all bone samples were crushed into pieces, weighed and homogenized (Vortex, Scientific Industries Inc., Bohemia, NY, USA) in a sterile saline solution. Serial dilutions of this suspension were inoculated on 5% blood agar supplemented with 5% sheep blood. After incubation at 35 °C for 24 h, the counts were performed. Bacterial growth was expressed as cfu/g of bone tissue, and the limit of detection of cfu/g was calculated at 1×10^4. Enrichment in a BHI broth was always performed to make sure that positive cultures with lower counts were not missed.

(4) Histopathological examinations: The distal samples were decalcified in formic acid, embedded in paraffin, cut and stained with hematoxylin and eosin. The proximal samples were not decalcified, embedded in methylmethacrylate, cut with a motorized microtome into 5 μm sections and stained with Goldner's trichrome. All slides were examined semi-quantitatively by the same pathologist who was blinded to the treatment groups for periosteal proliferation, cortex remodeling, endosteal proliferation, periosteal neutrophilic inflammation, periosteal lymphoplasmocytic inflammation, marrow lymphoplasmocytic inflammation, sequestrum and bacteria. The presence of vacuolated macrophages was also recorded and graded for group A and D specimens.

Statistical analysis

Comparisons of scores between treatment groups at week 4 and at week 10 were made with a G-test for independence followed by multiple comparisons between treatments. When the effect of a treatment was evaluated within a group from week 4 to week 10, a Cochran–Mantel–Haenszel test for repeated measures was performed. Differences in log-transformed bacterial counts at the proximal site between the two time periods were tested with a repeated analysis of variance. The Kruskal–Wallis test was used to test for differences among treatments in bacterial counts at each time period. When significant differences occurred, multiple comparisons relied on differences between mean ranks. The level of statistical significance was set at $p < 0.05$.

Results

Week 4

(1) Radiographs: all animals from groups B, C and D demonstrated mild to moderate signs of osteomyelitis. Periosteal reaction and bone lysis were lower ($p < 0.05$ and $p < 0.01$, respectively) for dogs in the preventive group A compared to animals of other groups. No significant differences were observed in the severity of lesions between groups B, C and D.

(2) Macroscopic evaluations: mild to moderate signs of osteomyelitis were observed in all dogs from groups B, C and D. Adhesions of the *vastus lateralis* were mild in every case, with occasional purulent material accumulation adjacent to bone. Periosteal reaction was scored as mild to moderate in all dogs, extending up to three cm away from the screw insertion site. Approximately 80% (20/24) of the screws were mobile when tested, with four of them that could be extracted with a minimal linear extraction force. Adhesions, periosteal reaction and screw mobility were not statistically different between the groups.

(3) Bacterial cultures: all sampled animals had a positive culture. Bacterial counts were not significantly different between groups B, C and D. These counts averaged in B: 166, C: 126 and D: 7983×10^5 (SEM 2×10^5) cfu/g of bone.

Week 10

(1) Radiographs: The absence of periosteal reaction and bone lysis was outlined in group A, with the only finding being mild medullary sclerosis that was significantly less ($p < 0.01$) than in the other groups. Bone lysis was less severe ($p < 0.01$), and cortical remodeling of the proximal defect was greater ($p < 0.01$) in both ciprofloxacin treated groups C and D versus group B (Fig. 2). Periosteal reaction was significantly lower ($p < 0.05$) in group D versus group B, but no differences could be found between groups C and B. A reduction in the severity of bone lysis was observed ($p < 0.05$) between week 4 and week 10 in groups C and D.

(2) Macroscopic evaluations: bone healing of the proximal defect created at week 4 by bacterial sampling was greater ($p < 0.01$) in both groups C and D versus group B. Periosteal reaction was significantly different ($p < 0.01$) between groups, with increasing severity of $A < C = D < B$, being absent in the preventive group A. All screws were mobile in group B whereas they were all firmly fixated in group A. The screw mobility was different ($p < 0.01$) between groups, with an increasing order of $A < C = D < B$. Soft tissue adhesions were higher ($p < 0.01$) in dogs of group B compared to all other three groups at week 10. In all CLHAS-implanted dogs, a complete resorption of the implants was observed.

(3) Bacterial cultures (Fig. 3): Bone cultures of proximal/distal sites were negative in A: 87%/-%, B: 25%/12%, C: 37%/62% and D: 62%/75%. The percentage of negative culture/total was significantly higher ($p < 0.01$) in group D versus group B at the distal site. Bacterial counts averaged in positive dogs A: 0.3, B: 25, C: 3 and D: 5×10^5 (SEM 2×10^5) cfu/g of bone. Counts in groups A and C (distal site) were significantly lower ($p < 0.05$) than in group B. Bacterial counts decreased ($p < 0.01$) from week 4 to week 10 in all three groups.

(4) Histopathological examination: No suggestive lesions of osteomyelitis were seen in any animals of group

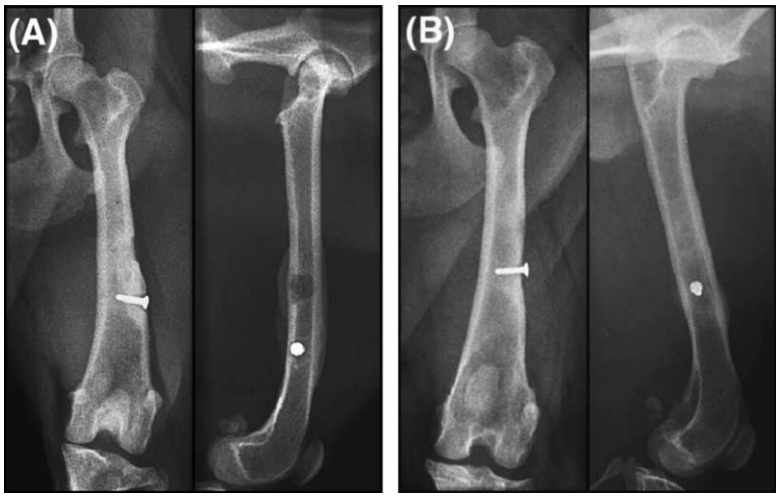

Fig. 2. (A) Group B: bone lysis and periosteal proliferation were scored as grade 2 and the proximal defect created at week 4 is still present. (B) Group D: bone lysis and periosteal proliferation were scored as grade 1 and the proximal defect is filled with remodeling new bone.

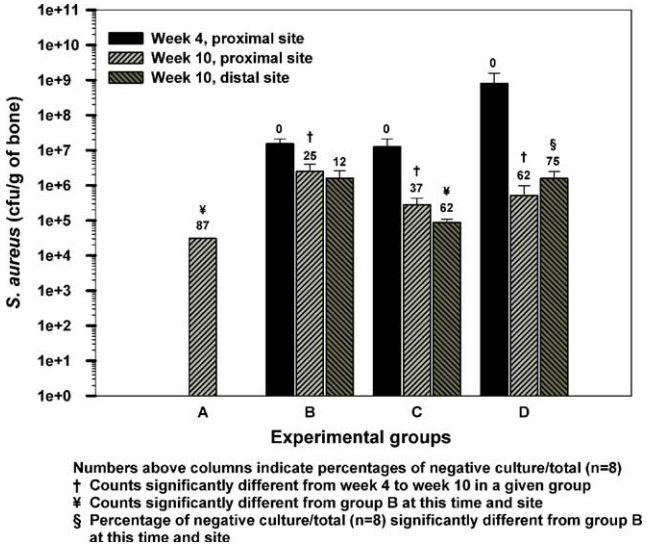

Fig. 3. Means (+SE) cfu of *S. aureus* in dogs with positive cultures and percentages of negative culture/total.

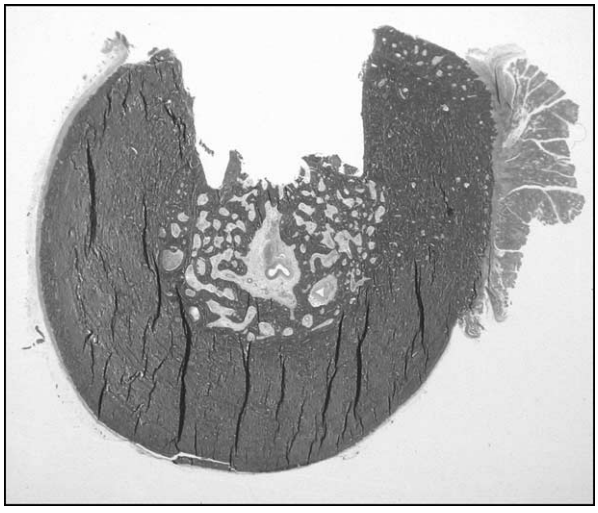

Fig. 4. Undecalcified histopathological transverse femur section of a dog treated with ciprofloxacin-CLHAS implants. Note the endocortical bone formation with the presence of trabecular bone and osteoid substance in the medullary cavity, the mild cortical remodeling with Havers channels enlargement and the absence of inflammatory infiltrate (Goldner's trichrome; original magnification ×1).

A. The mild bone reaction noted in this group was considered compatible with normal bone healing after implantation of a metallic screw in the diaphysis of a long bone. A moderate osteomyelitis was noted in all group B dogs. Both curative ciprofloxacin treatments had a reduced incidence of periosteal neutrophilic inflammation and lymphoplasmocytic infiltration versus group B. Similarly, more endosteal bone proliferation was noted in groups C and D dogs (Fig. 4). At the distal site, periosteal proliferation was milder in the implant versus the oral treated dogs. Vacuolated macrophages accumulation was noted in 3/8 and 9/16 specimens from groups A and D, respectively.

Discussion

This canine experimental model of chronic femoral osteomyelitis was elaborated to maintain a foreign body within the infected site at all times, as it is often necessary clinically to stabilize bone fragments. The model produced macroscopic, radiographic and bacteriologic evidences of osteomyelitis in all animals of groups B, C and D at week 4. The similarity of lesions between the groups indicates a good reproducibility of the model and a good reliability for the comparison of curative treatment protocols. Moreover, the reliability of the

positive control at week 4 and week 10 was helpful to assess the preventive efficacy of the starch implants. However, this model may not approximate clinical situations with compromised vascularization or extensive soft tissue damage.

In this study, the preventive efficacy of the ciprofloxacin-CLHAS implants was clearly demonstrated with the absence of any radiographic, macroscopic or histological sign of inflammatory reaction of bone and surrounding soft tissues. Surprisingly, one dog from the preventive group A was found positive on bacterial culture at week 10. However, the bacterial count was low and no corresponding signs of osteomyelitis were observed on microscopic examination. A small number of bacteria may have been present at the bone/screw interface, and osteomyelitis may have developed if given enough time. Similar results have been observed with preventive implantation in another study about gentamicin-impregnated PMMA, where 9 out of 10 tibiae exposed to *S. aureus* in presence of a foreign body were free of osteomyelitis at the end of the project [13]. In series of clinical cases, prophylaxis with antibiotic-loaded PMMA used in various surgical interventions has led to different infection rates. A ten-year prospective multicenter controlled study of 1688 cases of hip arthroplasty reported no difference in deep infection rates (1.6% versus 1.1%) or aseptic prosthetic loosening (55% versus 50%) when gentamicin-impregnated PMMA was used for prophylaxis versus systemic penicillins or cephalosporins [17]. In another study reviewing 1085 consecutive cases of open compound limb fractures, the overall infection rate was significantly lower in the group receiving gentamicin beads versus systemic antibiotics only (3.7% versus 12%), and the differences were greatest (6.5% versus 20.6%) among patients with initial severe soft tissue damage or impaired vascularity [32]. Given these different results, it is reasonable to assume that prophylaxis with antibiotic-loaded implants is superior to prophylaxis with systemic antibiotics only, but randomised prospective studies would be necessary to confirm this assumption.

The present study also demonstrated that in the presence of established osteomyelitis, oral ciprofloxacin and ciprofloxacin-CLHAS implants had an equivalent curative efficacy. Similar results in the treatment of *S. aureus* osteomyelitis were reported with calcium hydroxyapatite and D,L-lactide:glycolide [29,35]. In the latter study, a significant difference was not found between the vancomycin-loaded calcium hydroxyapatite and the systemic vancomycin treated groups. In contrast, efficacy between systemic and antibiotic DDS treatments was significantly different in several experimental models [5,25,27,37]. Further studies would be needed to evaluate antibiotic-CLHAS implants and systemic antibiotics in presence of a more severe osteomyelitis where their efficacies may differ because of increased devascularized tissues. Since the severity of the osteomyelitis induced in this study was moderate, it is effectively unclear whether a significant avascular local tissue component was present or not.

An excellent biocompatibility of CLHAS has been shown in rodents [10,11]. On day three after subcutaneous implantation in mice, the inflammatory response was reported to be less severe around the implants than around the skin incision [11]. In the present study, the difference in periosteal proliferation between the implant and the oral treatment groups outlines a good biocompatibility of these implants in dogs. Also, the ciprofloxacin-CLHAS implants allowed bone healing in a similar manner than the oral use of ciprofloxacin. Other advantages of this new excipient include biodegradability, readily accessible industrial manufacturing technology, high active ingredient loading and very good cost-effectiveness [11,21]. In order to target the clinical strain responsible for an infection, elution properties of other antibiotics from the CLHAS matrix could also be evaluated. Increasing antibiotic loading in these implants results in a steady delivery over time characterized by a plateau serum profile, minimizing the initial burst and/or the bimodal delivery profile as reported in many systems [7,12,38,41]. The rapid gelification of CLHAS is believed to be a key parameter in controlling the initial burst [9]. Neovascularization and fibrous septae eventually subdivide CLHAS implants which are then progressively phagocytosed by macrophages [11]. It is known that macrophages are a key component of the response to implanted CLHAS [10–12], and that starch has no effect on macrophage nonspecific defense response such as cytotoxic activity [3]. The vacuolated macrophages detected in groups A and D are consistent with phagocytosis of the starch implants.

The reduction of bacterial counts and number of positive sites from week 4 to week 10 for the positive control can be primarily explained by an effective surgical debridement. This finding is in agreement with the theory that the quality of surgical debridement remains the most critical factor in the successful management of chronic orthopaedic infections [35,39,40]. Adequate debridement can lower the number of bacteria and facilitate clearance by the immune system [35,39,40]. In concert with the results and recommendations of many other authors, our results support once more the importance of aggressive surgical debridement in the treatment of chronic osteomyelitis [15,16,19,25,27,28,35,39,40].

After oral administration of ciprofloxacin at 10 mg/kg q12h in dogs, the C_{max} is reported to be 1.55 mg/l [1]. This regimen in dogs corresponds to a conventional oral dose of 500 mg q12h (C_{max} = 1.5–2.8 mg/l) used in humans [22]. With a similar efficacy of ciprofloxacin-CLHAS implants and oral ciprofloxacin in dogs, it can

be assumed that CLHAS implants would be a viable DDS in humans. The site-specific antibiotic delivery allows the elimination of peaks and troughs of drug concentrations associated with conventional multiple high dose systemic administrations [4,18,19,26,38]. Thereby, not only the possible toxicity and side effects are minimized by lowering the systemic drug levels, but the efficacy of treatment may be enhanced due to higher sustained concentrations at the surgery site. Also, patients can not be noncompliant to medical treatment and miss doses, as it frequently happens when on prolonged oral therapy. Moreover, hospitalization time and costs related to the treatment may be reduced [2,4,8,10,14,18,19,27,35,37].

In the present study, *S. aureus* was chosen to produce a reliable and reproducible model of osteomyelitis because it has been previously used as a standard [13–15,27–29,33,35,37]. Nevertheless, quinolone resistance has become a concern among clinical isolates of *S. aureus* [24,27,30]. The strain used in our study was purposely selected to be sensitive to ciprofloxacin, in order to allow a reliable evaluation of CLHAS matrix implant efficacy for the prevention and treatment of osteomyelitis. As in conventional systemic antibiotic therapy, the development of resistance with drug delivery implants can be suspected. However, it seems more likely that resistance could develop in distant floras following implantation of an antimicrobial DDS because resulting systemic antibiotic concentrations remain below MIC over an extended period of time. Unfortunately, studies on bacterial sensitivity evolution with antimicrobial implant usage are lacking. Accordingly it would be very helpful to further investigate bacterial sensitivity patterns from a pathogen agent in bone and from distant floras (e.g. nasal, oral, rectal and cutaneous) during long term implant versus oral antibiotic therapy, in order to evaluate the risk/benefit ratio of antimicrobial DDS usage in clinical situations.

The use of ciprofloxacin-CLHAS implants has been determined as a safe and efficient modality for the prevention and treatment of the osteomyelitis developed in this experimental model. The judicious use of these implants in infection prone situations such as open fractures may help to reduce infection rates. Also, the treatment of established infections could be achieved with implantation performed at the time of surgical debridement, without the need of a second surgery for antibiotic implant removal.

Acknowledgements

This study was funded by Labopharm Inc., Laval, QC, Canada. The authors thank Dr. Serge Messier, Mr. Guy Beauchamp and Mr. Maxim Moreau for their assistance in this study and in manuscript preparation.

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