Carboxymethyl chitosan–zinc coating for prevention of pin tract infection: An animal model

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Abstract

Background: Pin tract infection is a common problem in orthopedic and traumatology surgery. The aim of this study was to investigate the efficacy of an implant coated with carboxymethyl chitosan–zinc (CMC-Zn2+).

Materials and Methods: Twenty-four male New Zealand White rabbits were randomized into two equal groups (n = 12, uncoated and CMC-Zn2+). The implants were colonized with 1 × 10^6 colony forming units of Staphylococcus aureus and inserted into the lateral right proximal tibia in each rabbit. In each group, at 2 and 4 weeks post-surgery, five and seven rabbits were killed, respectively, to harvest the soft tissues around the implant as well as the hard tissue for histological analysis. The bone cross-sectional view, X-ray, and micro-computed tomography (μCT) were performed.

Results: The surgical sites in each animal were evaluated individually at both time points. No evident signs of infections were found in the CMC-Zn2+ group, while a high rate of infection was observed in the uncoated group where minor infections were 85.71% (n = 12) and major infections 14.29% (n = 12). The radiography, μCT, and histological analysis showed no evident signs of infection in both groups at 2 weeks post-surgery. However, at 4 weeks, signs of infection were found in all the animals in the uncoated group, whereas in the CMC-Zn2+ group, no infections were observed. The difference between the two groups was highly significant (p = 0.00).

Conclusions: Our study showed that CMC-Zn2+-coated implants were effective in preventing pin tract infection.

Keywords

animal models, antibacterial agent, carboxymethyl chitosan-zinc, coating implant, pin tract infection, Staphylococcus aureus

Date received: 5 April 2017; accepted: 24 July 2017

Introduction

Pin tract infection is the most common complication of external fixation devices in orthopedic and trauma surgery with an incidence rate from 10% to 100%, according to data from the literature. In the clinical setting, the early soft tissue infection occurred always from 1 week to 3 weeks after surgery, but from 3 to 10 weeks, signs of infections are obviously present in the soft tissue as well as in the bone tissue. These pins are susceptible to infection because the skin barrier has been disrupted. In order to reduce the incidence of such infections, numerous authors reported different protocols like dressing, cleansing, showering, coating antibacterial agent, and use of prophylactic antibiotics.

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antibiotic. However, there are several controversial research with regard to optimal care of pin tract infections.8–10

Carboxymethyl chitosan (CMC) is a derivative of chitosan, a product obtained by deacetylation of chitin. It has emerged as a promising candidate for different biomedical applications11 due to their better solubility in water, enhanced antibacterial property,12 biocompatibility,13 and safety for humans.13,14 Zinc ions have been reported as effective antimicrobial agents against Staphylococcus aureus (S. aureus).15 The purpose of this present study was to investigate the effect of CMC combined with zinc ion coated onto an implant in prevention of pin site infections.

Materials and methods

Bacteria growth

The S. aureus was prepared by an overnight culture in Luria-Bertani (LB) broth. A bacterial concentration was adjusted at an OD600 nm of around 0.5, corresponding to 10^6 CFU/mL by a microplate reader (SpectraMax M5, Molecular Devices, USA). The concentration of S. aureus in the inoculum and the OD suspension was confirmed by colony forming unit (CFU) quantification of serially diluted suspensions on agar. The bacterial suspension was then diluted 100-fold to 10^6 CFU/mL. After a concentration of 1/2×10^6 CFU/mL of bacterial solution was obtained, an implant was placed into a 15 mL centrifuge tube for incubation for 6 min before surgery.

Immobilization of carboxymethyl chitosan-zinc complex onto stainless steel pins

Stainless steel pins were polished using 1500 grit sandpaper. Subsequently, each of them was cleaned ultrasonically for 10 min in acetone, ethanol, and deionized water. The cleaned samples were activated by immersing them in a piranha solution (H_2SO_4:H_2O_2 = 3:1, v:v) for 15 min to produce a hydroxyl-enriched surface (SSP-OH) and then rinsed thoroughly with deionized water followed by argon blow-drying. The samples were further treated with a 2% (v/v) glutaraldehyde aqueous solution for 24 h at room temperature and then rinsed with deionized water to obtain an aldehyde-functionalized surface (SSP-CHO). To graft the CMC onto the surface, the SSP-CHO samples were immersed in a 2% (w/v) CMC aqueous solution for 24 h at room temperature and then rinsed with deionized water to obtain a CMC-grafted SSP surface (SSP-CMC). Finally, the SSP-CMC-Zn was prepared by immersing the SSP-CMC samples in a 0.1 mol/L ZnCl_2 solution for 10 h at 60°C, and then rinsed thoroughly with deionized water followed by vacuum drying. The process of immobilization of CMC-Zn onto stainless steel pins is shown in Figure 1.

Study design

Twenty-four adult male rabbits weighing 2.5–3 kg (4–8 months) were purchased from the laboratory animal centers of our institutions. Principles of laboratory animal care (NIH) were followed in this present study which had been approved by the ethical committee of laboratory of animals care. All rabbits were housed individually under constant conditions at 20 to 25°C, and relative humidity with a 12 h light–dark cycle and free access to water and food ad libitum.

Surgical procedure

The rabbits were randomly divided into two equal groups: carboxymethyl chitosan-zinc (CMC-Zn^{2+})-coated group (n = 12) and uncoated group (n = 12). The rabbits were anesthetized with an intramuscular injection of 3% pentobarbital sodium (1 mL/kg) and an intramuscular xylazine hydrochloride injection (0.1 mL/Kg). After anesthesia, the rabbit hair was carefully removed and the right posterior limb was shaved and disinfected with povidone iodine solution. Then, we made an incision on the lateral aspect of the proximal tibia of approximately 2.5–3 cm. After the bone was exposed, a power drill was used to perforate the tibiae from the lateral to medial cortex followed by an external saline solution irrigation. After the perforation, the implant was inserted using a manual screwdriver. Before insertion, all the implants were colonized with the bacteria by incubating them into a solution of 1×10^6 CFU/mL of S. aureus (ATCC 25923) for 6 min. Finally, the wound was closed layer by layer with vicryl 4–0. The bacteria strain dose and
inoculation time were selected in accordance with data from literature and the same protocol used in our previous study with the goal to create a persistent infection for over 4 weeks. In order to investigate and determine the possible source of the infection in rabbit models, a pilot study was carried out using the inoculation dose of $1 \times 10^6$ CFU/mL of *S. aureus* for contamination. After a 2-week observation, we found that all the pin sites in the CMC-Zn²⁺ group appeared clean with no clinical signs of infection compared to the uncoated group in which all pin sites were infected. This result also demonstrated the typical feature of infection as in a clinical setting stating *S. aureus* as the main cause of acute infection.

The postoperative care was carried out with an intramuscular injection of benzylpenicillin. The powder injection of benzylpenicillin (0.48 g) was diluted with 2 mL of saline solution; 1 mL was used during surgery and then 1 mL daily for two days after surgery.

The gross morphological appearance was recorded at 2 and 4 weeks after surgery. At both time points, the rabbits were killed using an overdose of pentobarbital sodium solution; 1 mL was used during surgery and then 1 mL daily for two days after surgery.

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### Classification of pin tract infection and cross-section of bone by visual inspection

In this study, pin tract infections were evaluated according to the Checketts–Otterburn classification. Minor infection: grade 1 represents slight redness and little discharge; grade 2 represents redness of the skin, discharge, pain, and tenderness in the soft tissue; and grade 3 represents persistent grade 2 symptoms even after treatment with oral antibiotics. Major Infection: grade 4 represents severe soft tissue infection involving several pins, sometimes with associated loosening of the pin; grade 5 represents the same grade 4 symptoms but with radiographic changes; and grade 6 represents infection after fixator removal. Radiographs showed new bone formation and sometimes sequestrums. The evaluation of pin tract infection was carried out by a physician who was blind to the group identification.

### Radiography and μCT

Radiography analysis is an indispensable investigation in orthopedic surgery. After the rabbits were sedated with an intramuscular injection of 3% pentobarbital sodium (0.5 mL/kg) and an intramuscular xylazine hydrochloride injection (0.1 mL/kg), a standard X-ray unit was used. The radiographs of the right tibia were obtained and assessed using the classification of radiological changes of implant. The radiography parameters were scored from 0 to 2 with score 0 corresponding to the absence of sequestral bone formation, periosteal new bone formation, soft tissue calcification, soft tissue swelling, and no destruction of bone, screw loosening, and peri-implant reaction; score 0.5 corresponding to equivocal periosteal new bone formation, soft tissue calcification, soft tissue swelling, and mild destruction of bone, screw loosening, and peri-implant reaction with only one area involved; score 1 corresponding to the presence of sequestral bone, periosteal new bone, soft tissue calcification, soft tissue swelling, and moderate destruction of bone, screw loosening, and peri-implant reaction with only one area involved; and score 2 corresponding to severe destruction of bone, screw loosening, and peri-implant reaction with multiple areas involved. The scores for each rabbit were given by two blinded radiologists.

In order to investigate the infection on pin tract and implant hole, the specimens were examined using a μCT system (SKYSCAN 1176, Belgium) with a tube voltage of 80 kV, tube current of 313 μA, and voxel size of 17.40 × 17.40 × 18 μm³ under an isometric resolution of 18 μm. The cross-sectional images of bone at the right proximal tibia are shown in Figure 5.

### Histological evaluation

The histological analysis was assessed under aseptic condition. First, after a soft tissue around the implant was removed, we extracted carefully the implant by a screwdriver followed by complete removal of the right tibia. Specimens were fixed separately in 4% buffered formaldehyde solution for 48 h. The bone tissue samples were cut longitudinally, washed under a slowly running tap water for 30 min, and then placed into a 50 mL centrifuge tube containing formic acid solution and distilled water after a preparation of 40 mL 8% formic acid stock solution and 460 mL distilled water. The complete decalcification was obtained after 2 weeks by changing the fresh solution three times in the week. The physical test was performed by inserting a 1 mL syringe needle into the bone tissue. After checked for rigidity, the specimens were washed thoroughly prior to processing. The samples were dehydrated and embedded in paraffin. Sections of 4-μm thickness in the cross-sectional plane were prepared and stained with hematoxylin and eosin. All sections were examined by light microscopy (Olympus DP72, Canada). All sections were observed and scored by a blinded pathologist in accordance with the histological soft and hard tissue inflammation scores ranging from grade 0 to grade 2.

### Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). All data were analyzed using nonparametric test (Mann–Whitney test), with $p < 0.05$ considered significant.
Results

Classification of pin tract infection and cross-section of bone by visual inspection

According to the Checketts–Otterburn classification, we found that 100% of the infections in the uncoated group (Figure 2(a) and (b)) were minor infections at 2 weeks post-surgery: grade 1 \( (n = 5; 60\%) \) and grade 2 \( (n = 5; 40\%) \). At 4 weeks, we found grade 1 \( (n = 7; 42.86\%) \), grade 2 \( (n = 7; 42.86\%) \), and grade 5 \( (n = 7; 14.29\%) \) infections in the uncoated group, while no signs of infection were observed in the CMC-Zn\(^{2+}\) group (Figure 2(c) and (d)). All rabbits from the experimental group did not show signs of infection at 2 and 4 weeks post-surgery, while all rabbits (100%) from the uncoated group showed clinical signs of infection (see Table 1) at both time points after surgery and five rabbits showed radiographic signs of infection after 4 weeks. There were significant differences between the two groups at two time points \( (Z = -4.504, p < 0.001) \).

In the medullary cavity, no signs of infection were observed at 1 and 2 weeks post-surgery in both groups, but at 4 weeks, a yellow formation of pus was found in the uncoated group (Figure 3(a)), while the experiment group was clean without any signs of infection (Figure 3(b)).

| Implants group | Animals receiving implants | Animals for evaluation | Animals with clinical signs of infections | Animals with radiographic signs of infections |
|----------------|---------------------------|------------------------|------------------------------------------|---------------------------------------------|
| Uncoated 2W    | 5                         | 5                      | 5                                        | 0                                           |
| CMC-Zn\(^{2+}\) 2W | 5                         | 5                      | 0                                        | 0                                           |
| Uncoated 4W    | 7                         | 7                      | 7                                        | 5                                           |
| CMC-Zn\(^{2+}\) 4W | 7                         | 7                      | 0                                        | 0                                           |

CMC-Zn\(^{2+}\): carboxymethyl chitosan-zinc; 2W: 2 weeks post-surgery; 4W: 4 weeks post-surgery.

Radiography and \( \mu \)CT

Radiographic images of the right tibia in both groups were taken (Figure 4). Three parameters, destruction of bone, peri-implant reaction, and soft tissue swelling, were observed in this study. In the uncoated group, the radiographs of the right tibia showed mild destruction of the bone in five/seven rabbits, mild peri-implant reactions in five/seven rabbits, and the presence of soft tissue swelling.
in seven/seven rabbits with a mean score of $1.71 \pm 0.49$, while in the CMC-Zn$^{2+}$ group, no signs of infection were observed in all rabbits. The difference was highly significant ($Z = -3.435, p < 0.001$).

The right tibiae were harvested and evaluated using a μCT (SKYSCAN 1176, In vivo X-ray Microtomograph, Belgium) under an isometric resolution of 18 μm. Considerable destruction of bone and soft tissue was observed in the uncoated group (Figure 5(a) and (b)).

**Histological analysis**

Histological changes in the rabbit’s tibia were analyzed using a light microscopy (Figure 6). Two weeks after
surgery, the histological inflammation of the soft tissues in the uncoated group was 0% (grade 0), 100% (grade 1), and 0% (grade 2), whereas the percentages of grade 0, grade 1, and grade 2 inflammation in the CMC-Zn$^{2+}$ group were 100, 0, and 0%, respectively. At 4 weeks, the percentages of soft tissue inflammation were 0% (grade 0), 14.29% (grade 1), and 85.71% (grade 2) in the uncoated group, while those in the CMC-Zn$^{2+}$ group were 100% (grade 0), 0% (grade 1), and 0% (grade 2). The histological scores at 2 weeks after surgery showed that bone inflammation was 0% (grade 0), 100% (grade 1), and 0% (grade 2) in the uncoated group, whereas the percentages of bone inflammation in the CMC-Zn$^{2+}$ group were 100% (grade 0), 0% (grade 1), and 0% (grade 2). As for the histological inflammation of the osseous tissue in the uncoated group after 4 weeks, we observed a destruction of cortical bone compared with the CMC-Zn$^{2+}$ group (Figure 7(a)). The percentages in all rabbits in the uncoated group were as follows: 0% (grade 0), 28.57% (grade 1), and 71.43% (grade 2). However, the CMC-Zn$^{2+}$ group did not present obvious deterioration with the percentages of 100% (grade 0), 0% (grade 1), and 0% (grade 2). There were significant differences between the two groups at two time points ($Z = -4.522$, $p < 0.001$).

**Discussion**

The most common problem expected with external fixation management is pin site infection. As we know, an implant or wire passage through the bone cortex allows an entrance of bacteria around the skin to the deeper soft tissues and to the bone. Earlier care or prevention by coating implants with antibacterial agents is a unique way to avoid post-surgery complication when external fixation device is used. Implants coated with antibiotic loading, apatite, titanium alloy, prophylactic antibiotics$^{2,23}$, and HA have been

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**Figure 5.** Overview of the μCT and 3-D reconstruction analysis of rabbit tibia at 4 weeks post-surgery. The μCT obtained from the rabbits with CMC-Zn$^{2+}$-coated implant ((c) and (d)) did not showed obvious signs of cortical bone destruction and periosteal reaction, whereas the uncoated group ((a) and (b)) of rabbits exhibited a cortical bone destruction and periosteal reaction on the cross-sectional view (b). μCT: micro-computed tomography; CMC-Zn$^{2+}$: carboxymethyl chitosan-zinc.
designed by researchers to minimize the rate of pin tract infection.

Oka et al.²⁴ have assessed the potential of TiO₂ photocatalyst in the inhibition of the bacteria colonization on percutaneous implants. They inoculated 1 mL of $10^8$ CFU/mL of MRSA around the femoral pin site in each rabbit after implantation. Three days post-surgery, their pin tracts were first cleaned with sterile saline and then

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**Figure 6.** The microscopic sections of soft tissue around implants at 4 weeks post-surgery were stained by H&E, the presence of intense inflammatory cell infiltration can be seen in the uncoated group (a). H&E: hematoxylin and eosin.

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**Figure 7.** (a) Representative histological images obtained from the decalcified cross-sectional sections of rabbits’ tibia at 4 weeks post-surgery ((a), magnification ×100). The evidence of inflammation of the medullary cavity can be seen in the uncoated group, even the magnified views of implants’ entry point (black box, cortical bone) showed a severe destruction of cortical bone. (b) Representative histological images obtained from the decalcified cross-sectional sections of rabbits’ tibia at 4 weeks post-surgery ((c), magnification ×100). No evident sign of histological inflammation of bone tissues were observed on the cross-sectional sections view (c) as well as on the magnified views of implants’ entry point ((d) black box, cortical bone).
illuminated with a 40-W BLB (UVA) for 60 min per day for 5 days. One week after surgery, pins were removed and examined by sonication. The authors conclude that TiO_2 photocatalyst has a bactericidal effect against MRSA and can inhibit the bacterial colonization on percutaneous implants. However, this method might probably have a disadvantage of possible technical errors related to bacterial detachment and culturing.

Another finding has proposed nitric oxide (NO)-releasing, xerogel-coated titanium implants to decrease the bacterial colonization on external fixation pins which were surgically implanted into the rat’s 3rd, 4th, and 5th tail vertebrae. Three days before surgery, each animal received 250 mg/kg of acetaminophen elixir in its drinking water. On day 48, the animals were killed and microbiological analysis was performed. The authors found that pin tract bacterial colony counts in the NO releasing group (170 K ± 181 K) were significantly lower than in both the xerogel-coated group (677 K ± 675 K) and the control group (1181 K ± 2717 K) 48 days postoperatively (p < 0.05). However, their results suggested that the high NO flux at early time points was bactericidal and could reduce initial adhesion of bacteria to implant surfaces. Recently, Kose et al. reported that silver ion-doped calcium phosphate–based ceramic nano powder coating to orthopedic implants might prevent bacterial colonization and infection in open fractures compared with those for implants without any coating. The authors selected 50 µL solution containing 10^6 CFU/mL of MRSA and injected into the intramedullary canal of femur in rabbits before implantation of nails. Ten weeks after surgery, animals were killed and rods were extracted for assessment. Their results proved that adding a silver ion-doped nano size calcium phosphate–based powder coating to orthopedic implants might prevent infection as compared with uncoated implants without significant cytotoxicity. However, silver has been revealed to be toxic and not likely to be used as an antibacterial material due to its toxicity and low biocompatibility. Additionally, other authors reported that the inoculation dosage of bacteria strain was critical to successful creation of infection. It is noted that an excessive dose of inoculation dosage can lead to certain complications, while an insufficient dosage may present false results. However, the optimal dose of bacteria strain inoculated before or after insertion of the coating materials might be a new point of discussion. Another point is that the inoculation of bacteria around the pin tract is still a critical topic of debate, since it is difficult to confirm the depth of bacteria strain injected into a large number of animals for entire experiment. It is probable that the bacteria are inoculated directly to the bone in certain cases, while in others, the bacteria are inoculated superficially or deeply to the soft tissue without reaching the bone.

In this present study, our method using the CMC-Zn^{2+} as an antibacterial agent has advantages of water solubility, antibacterial activity, absorbability, antioxidation, hemostatic effect, bleeding analgesic, and safety. Although it is known that CMC has antibacterial activity, biocompatibility character, and ability to accelerate wound healing and reduce scarring, zinc ion can induce platelet aggregation and play a certain role in thrombosis and hemostasis. Zinc ions have been reported to possess capabilities of both inhibiting bacterial activity and promoting bone growth, and thus are effective antimicrobial agents against S. aureus as well. In this perspective, CMC was combined with zinc ions for prevention of pin tract infection. According to our project design, animals were divided into two groups equally (uncoated, n = 12 and CMC-Zn^{2+}, n = 12), and a comparison was made. At 2 and 4 weeks after surgery, five rabbits in each group and seven rabbits in each group, respectively, were killed to investigate histological and radiography examination. We found clinical signs of infections at 2 weeks post-surgery in 12 rabbits in the uncoated group, but no signs of infections were observed in the CMC-Zn^{2+} group. S. aureus strain was used in our study has been reported to be one of the bacteria most frequently associated with manifestation of pin tract infection. Based on the visual inspection, the signs of infections were determined by the presence of redness with tenderness and yellow or white drainage around the implant area. Additionally, in our study, the destruction of the bone, peri-implant reaction, soft tissue swelling, cortical bone, and periosteal reaction have confirmed the complications of pin tract infection, which when left untreated can lead to severe osteomyelitis. Based on the present study, we believe that the use of CMC-Zn^{2+} coating around an orthopedic implant might be an effective way to prevent pin tract infection. The enhanced antibacterial property makes this chitosan derivative particularly suitable for applications in wound healing management, tissue engineering, and drugs delivery.

In conclusion, pin tract infection is a considerable problem in orthopedic and trauma surgery. Despite the solutions provided by researchers to reduce the incidence of infection in animal models or in vitro studies, until now, no promising solutions have been shown in clinical practice. Our animal model proves effective in the prevention of pin tract infection by S. aureus, but further research is needed to confirm the efficiency of CMC-Zn^{2+} in a clinical setting before our finding can be used to meet the challenge in the management of pin tract infection.

Authors’ note
Vidmi Taolam Martin and Wang Lei contributed equally to this work.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (grant no. 2016B090913004/201508020035).

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