

Establishment of an animal model of chronic osteomyelitis with *Staphylococcus aureus* by ligating the femoral artery of rats

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Abstract

Background

Osteomyelitis caused by *Staphylococcus aureus* (*S. aureus*) is an important post-operation complication, especially after fracture internal fixation and artificial joint replacement. Animal models play an indispensable role in exploring the pathogenesis of osteomyelitis. Most models use internal fixation, bacterial suspension and vascular sclerosing agent to destroy blood vessels. Vascular sclerosing agents not only damage blood vessels but also lead to local inflammatory immune disorders, which is different from simple vascular disease and osteomyelitis caused by ischemia in clinical practice.

Methods

The experimental animals were randomly divided into three groups: femoral artery ligation group, vascular sclerosing agent group and non-infection aseptic operation group. In the femoral artery ligation group, the femoral artery was ligated to reduce the blood flow of the affected limb to simulate the clinical ischemic state and increase the susceptibility, then the Kirschner needle with *S. aureus* biofilm was implanted into the tibia of rats, and the bone defect was sealed with aseptic paraffin. The non-infection aseptic operation group and the infection model group caused by vascular sclerosing agent have been used as the blank and positive control group. After operation, survival rate, body temperature and incision healing were monitored. Four weeks later, radiological and pathological changes of all animals were evaluated, and the secretions from osteomyelitis experienced etiological separation and cultivation.

Results

The chronic osteomyelitis model was established successfully by ligating femoral artery and implanting Kirschner needle covered with *S. aureus* biofilm. Signs of chronic osteomyelitis were observed in all rats of femoral artery ligation infection group and positive control group. No signs of infection and chronic osteomyelitis were found in the non-infection aseptic operation control group.

Conclusion

The method of ligating the femoral artery and implanting Kirschner needle with *S. aureus* biofilm into the tibia of rats can effectively establish a stable and reproducible chronic osteomyelitis model which is closer to the clinical pathogenesis and natural route of infection. This model could be useful for the study of pathogenesis and therapeutics of chronic osteomyelitis with *S. aureus*.

Background

Chronic osteomyelitis is a kind of infection and destruction of bone, which can be caused by aerobic or anaerobic bacteria, mycobacteria and fungi. The most common pathogen of Chronic osteomyelitis is *S. aureus*^[1]. Chronic osteomyelitis most often occurs in the long bones. It is common for adults who developed post-traumatic fracture infection in the feet and who are diabetic patients with poor blood supply, penetrating bone injury caused by trauma or operation. Hematogenous osteomyelitis is uncommon in adults but often occur in children. It is one of the most frequent invasive bacterial infection in the long bones with good blood supply, such as the tibia or the epiphysis of the femur^[2,3]. In clinic, it often occurs repeatedly and does not heal for a long time, which seriously affects physical, mental health and labor ability^[4]. Acute osteomyelitis begins with high fever and local pain, and when it turns to chronic osteomyelitis, there will be rupture, pus, dead bone or cavity formation. Severe patients are often in danger of life, which have to take emergency measures of amputation, resulting in lifelong physical disability^[5]. Thorough debridement, opening cancellous bone graft and repeated irrigation are the most commonly method of clinical treatment of osteomyelitis so far^[6]. In addition, puncture and aspiration, windowing and drainage, extraction of dead bone, filling of pedicled muscle flap, amputation, resection of massive diseased bone are also be used, but the therapeutic effect are not ideal, often requires multiple operations and prolonged antibiotic administration, and the recurrence rate are still very high^[7,8]. The treatment of osteomyelitis is still a difficult problem in orthopedic surgery at present. A good animal model must effectively and accurately reproduce the clinical osteomyelitis, which is the basis and key to study the new treatment of osteomyelitis. Existing animal models of osteomyelitis are mainly established by injecting sclerosing agents into sclerosing blood vessels to reduce local blood flow and implanting internal fixation to simulate clinical osteomyelitis^[9], which are different from the clinical causes of osteomyelitis. Clinically, most of osteomyelitis are caused by implanting with post-traumatic internal fixation which can bring local vascular diseases and result in local aseptic ischemia and osteomyelitis which formed after bacterial invasion^[10]. Compared with the previous animal models of osteomyelitis, using rabbits as experimental subjects will lead to more expensive and inconvenient management; using mice as experimental subjects is inconvenient for users to operate because the bones of mice are too small; the simulation of clinical ischemia is through the injection of vascular sclerosing agent, which is not close enough to the pathogenesis of clinical osteomyelitis^[11]. In this paper, purchasing and raising costs, the balance of convenience, size requirements, especially the clinical comparability have been considered. The purpose of this study was to develop a rat model that mimics chronic osteomyelitis with *S. aureus* by ligating the femoral artery of rats to simulate ischemia and implanting the Kirschner needle with bacterial biofilm into the tibia of rats to simulate the infection after clinical internal fixation, which were more similar to the clinical pathogenic factors.

Materials And Methods

Experiment animals

The 8 weeks old female SD rats, SPF, weighting 250~270g were purchased from the Changsha Tianqin Biological Company. Rats were raised by the Animal Center of Zunyi Medical University (3 rats per cage), provided with standard food, and had free access to bottled drinking water. In addition, the temperature and humidity of the facility environment can be controlled uniformly. Animals were acclimatized for a week prior to the initiation of this study. The weight and temperature of all rats were measured for 3 days before the experiment and 7 days after operation. After operation, phenobarbital sodium (3%) was used as the postoperative analgesic. All rats were executed by cervical vertebrae dislocation after phenobarbital anesthesia on the 28th day after operation.

Experiment grouping

Eighteen rats were randomly divided into 3 groups. Group A: femoral artery ligation + Kirschner needle with *S. aureus* biofilm internal fixation; Group B (positive control, vascular sclerosing agent group^[12]): vascular sclerosing agent + Kirschner needle with *S. aureus* biofilm internal fixation; Group C (blank control): non-infected aseptic operation group and sterile Kirschner needle internal fixation.

Preparation of bacterial strains and bacteria-carrying Kirschner needle

The standard *S. aureus* strain (ATCC25923) was inoculated on blood agar plate and incubated at 37°C for 24 hours. The pathogens were grown to 0.6 of OD_{600nm} in MHB medium at 37°C with shaking at 250 rpm, diluted to approximate 1×10⁶ CFU/ml with distilled medium^[13]. Then, the sterile Kirschner needle with diameter of 1mm and length of 5mm were inoculated in bacterial suspension and incubated for 18 hours at 37°C with agitation. Crystal violet staining confirmed the existence of biofilm on the Kirschner needle head.

Establishment of osteomyelitis model

Rats were anesthetized by intraperitoneal injection with 3% pentobarbital sodium (30mg/kg) before operation. The limbs of the rats were fixed, the right lower abdomen and right lower extremities were accepted pre-operative skin preparation, sterilization by iodine, then covered with disposable aseptic treatment towels. In group A, a 2cm longitudinal incision was made from the right groin to the subcutaneous, hemostatic clamp was used to separate the subcutaneous soft tissue, the inguinal ligament was used as the sign to find the femoral artery, then the femoral artery was separated and ligated, and then the subcutaneous soft tissue and skin were sutured. After that, skin incision of 2cm was made along the medial side of the anterior tibial crest under the knee joint and reached the periosteum on the medial side of the anterior tibial muscle to expose the anterolateral tibial crest of the upper tibia; drilled a bone hole by a hand drill with diameter of 1.5mm, explored the cancellous bone with a small curette. As for group A and group B, a Kirschner needle with *S. aureus* biofilm (sterile Kirschner needle in the blank group) was implanted into the hole. In addition, the local blood flow was reduced by ligating the femoral artery of the right lower limb in group A and injecting vascular sclerosing agent in group B, respectively. Finally, the bone window was sealed with bone wax and sutured layer by layer. Each rat was fed in a single cage and observed for 4 weeks after operation.

Detection and evaluation

The physiological status, death, induration, edema, purpura or dehiscence of the model incision of the right lower limb, pus and sinus formation were observed every day. Radiological examination was performed at the 4th week of modeling. Then all rats were killed by cervical vertebra dislocation after anesthesia. The secretions and tissue samples from the disease area were collected under aseptic conditions and cultured to confirm the presence of *S. aureus*, and the bone tissue was taken for HE pathological examination to confirm the presence of chronic osteomyelitis.

Clinical signs of infection

Animals were examined for clinical signs of infection (swelling and reddening of the right hind leg, loss of passive motion in knee and ankle joints. Moreover, the body temperature was measured with a digital thermometer on days -3 to 7 and the weight was also be determined.

Radiographic evaluation

Radiographs were taken in posterior–anterior and lateral views on days 28 after operation. For the X-rays, the digital films and X-ray unit were used to assess development and progression of bone infection. Each radiograph was evaluated by two independent observers in a blinded manner to look for evidence of chronic osteomyelitis based on the presence of periosteal reaction, osteolysis, soft-tissue swelling, deformity, sequestrum formation and spontaneous fracture.

Microbiological evaluation

The contents of any abscess cavities at the end point of this study were cultured using a sterile inoculation ring directly plated onto agar plates, which used to confirm the presence of *S. aureus*. The plates were evaluated by two different microbiologists at 24 hours for colony growth.

Gross view of bone tissue

The soft tissues of the affected limbs of the rats were completely removed, and the tibia was separated for comparison. The healing of the bone tissue in the holes of the modeled bricks was observed based on the presence of sinus and pus, dead bone formation, deformity and fracture.

Histopathological examination

Histopathological examination was performed in all rats, and the infection process of bone and surrounding soft tissue was also be observed. The tibia of each rat was removed aseptically and immediately fixed in 10% formaldehyde in EDTA decalcified solution. They were then embedded in paraffin, sectioned

and 4 mm sections were placed on glass slides. Slides underwent deparaffinization and staining by hematoxylin and eosin (H&E). For morphometric analyses, images of H&E stained were observed and recorded under light microscope.

Results

Animals infected with *S. aureus* (ATCC 25923)

All rats survived in the surgery and establishment of chronic osteomyelitis. On the 5th to 7th day after operation, the rats in group A and group B had abscess formation of the right lower extremity and purulent secretion in the incision. There were fistulas in the incised limbs deep to the marrow cavity. There were no signs of suppurative infection observed in control group C, and the incision healed completely around the 7th day after operation.

Body temperature and weight

Temperature monitoring of rats inoculated with bacterial biological kirschner needle in Group A and Group B indicated that the temperature began to rise rapidly to more than 38°C on the first day after the operation and maintained for a week. The body temperature monitoring in the Control group C increased on the first day after the operation, with the maximum temperature reaching 38.8°C, and the body temperature returned to the same level as before the operation on the second day after the operation (Figure 3). Compared with group C, the growth rate of body weight decreased in Group A and Group B.

Gait signs

Limping of the right lower extremity occurred for 4 weeks in Group A and Group B implanted with *S. aureus* biofilm Kirschner needle (Figure 4). No claudication was observed in Group C implanted with sterile Kirschner needle, and they gradually returned to normal on the 5th day after operation.

Radiographic examination

Radiographic examination of the surgical area indicated that there were soft tissue swelling and periosteum reaction at the right tibial modeling site of rats in groups A and B, as well as nonunion of bone cortex, enlargement of local bone marrow cavity, formation of dead bone and new cladding. In group C, X-ray results showed there was no swelling shadow in the soft tissue of the surgical area, no enlargement in the bone marrow cavity, no dead bones or new envelopment formation (Figure 5). The X-ray analysis in this present rat model of *S. aureus* chronic osteomyelitis are very similar to those observed in human patients.

Bacterial culture and bone healing

After skin incision, the tibial brick hole of rats in Group A and Group B was still present with a large number of suppurative secretions, while the tibial brick hole 7). Classical *S. aureus* colony was identified in all rats of group A and group B, while no bacteria was observed in group C (Figure 6). For comparison with the group C (blank control), *S. aureus* was cultured from bone secretions, which confirmed the diagnosis of chronic osteomyelitis.

Histological examination

The development of chronic osteomyelitis in infected bones of this study was further confirmed by histological examination. The group A and B were characterized by suppurative inflammation with foci of intense bacterial multiplication and necrosis which was similar to the signs of human patients. There are a great many of inflammatory cells infiltrating the bone marrow cavity in Group A and Group B, and significantly fewer inflammatory cells in Group C than in Group A and Group B (Figure 8).

Discussion

Chronic osteomyelitis can occur in any bone in the human body. It is a recalcitrant condition in which symptoms have been present for longer than 3 months and a source of disability in humans^[14, 15]. At present, it is still a difficult problem for orthopedic doctors. And there is no better treatment plan^[16]. The incidence of osteomyelitis also decreases with the increase of traffic accidents and patients with traumatic fracture. Epidemiological investigation shows that the incidence of adult osteomyelitis in developing countries is 24.4/100000, and the incidence of osteomyelitis in males who are the main labor force is higher than that in females^[17]. Among these patients with osteomyelitis, osteomyelitis caused by *S. aureus* plays a major role^[19]. The main cause of recurrent chronic osteomyelitis is the formation of bacterial biofilm. When the bone is infected with bacteria for more than 24 hours, the bacteria begin to form biofilm locally^[20]. Bacteria biofilm blocks the drug and routine surgical cleaning treatment, which is effective for enhancing bacterial survival in hostile environments and prevention of bacterial infection^[21, 22]. As we all know, blood circulation not only plays a critical role in providing nutrition to tissues and organs, but also plays an important role in the transportation of anti-inflammatory factors. Turkey et al found that there was a positive correlation between the incidence of osteomyelitis and the arterial ischemic disease. Incidence of amputation among patients with diabetes who also have peripheral arterial disease and osteomyelitis increased significantly^[23, 24]. In previous animal osteomyelitis models, local injection of vascular sclerosing agent was used to simulate clinical ischemic symptoms. However, there are few patients with osteomyelitis caused by local injection of vascular sclerosing agent into bone tissue^[12]. Therefore, there is a gap between the osteomyelitis model made by sclerosing agent and the actual incidence of osteomyelitis, which may lead to changes in the inflammatory pathological mechanism of the bone infection model, which is not conducive to the later study of the treatment of osteomyelitis. In clinic, most patients have arterial blood flow disorders and the insufficient supply of branch vessels, which lead to the deterioration of local immune function and bone tissue infection^[26]. In the previous osteomyelitis model with internal fixation, a certain amount of bacterial suspension was inoculated on the basis of bone defect or injected directly into bone tissue. Despite this method successfully improved the success rate of osteomyelitis model, it is still insufficient in simulating the pathogenesis of osteomyelitis in clinic^[27]. The Kirschner needle with biofilm is closer to the clinical pathogenic factors. In the selection of

animal model of osteomyelitis, the rabbit as the experimental object in the early stage, but it is more difficult to raise and more expensive than rats^[28]. Mice also be used as the experimental object of osteomyelitis model. However, the small bones of mice are not convenient for the surgical operation, which increase the difficulty of internal fixation and cannot well simulate the incidence of clinical osteomyelitis^[29]. Therefore, in this study, we report a novel model of chronic osteomyelitis in the rat which is based on many advantages over other animal models. Firstly, the rat is large enough to simulate the clinical, radiographic, and histologic characteristics of human. In contrast to most published models in which *S. aureus* are directly placed into the bones, in this model (model A) the Kirschner needle with *S. aureus* biofilm was implanted to facilitate *S. aureus* infection, which mimics the natural route of infection in hematogenous osteomyelitis and can facilitate the identification of bacterial factors involved in bone tropism. More importantly, an additional advantage of our model, the local blood flow was reduced by ligating the femoral artery of the right lower limb, which is more similar to the clinical pathogenesis^[30, 31]. It also has its advantages in the balance of convenience, purchasing and raising costs.

The most important requirements of a reliable animal model of chronic osteomyelitis are: high infection rate, inability to heal, low mortality in the course of the experiment, and little difference in symptoms among infected animals in the same group. The rat model of chronic osteomyelitis in this experiment reliably mimics the natural route and clinical features of chronic osteomyelitis. The first phase of infection is highly symptomatic and characterized by the significantly increased body temperature (Fig. 3) and decreased growth rate of body weight. Typical inflammatory secretion which shows a single bacterial colony and hemolytic ring on the agar blood culture can be seen in group A and group B (Figs. 6 and 7). The changes in the bone structure were already examined by x-ray. The infected bones undergo the enlargement of local bone marrow cavity, the formation of new bone and cladding, the thickened and uplifted bone tissue, and the swollen soft tissue (Fig. 4). The formation of sequestrum were detected in chronic osteomyelitis model, group A and B (Fig. 5 and Fig. 7). Histopathological evaluation of group A and B revealed a large quantity of inflammatory cells during the chronic phase of osteomyelitis (Fig. 8).

According to the above results and comparing with positive control group B which has been reported as chronic osteomyelitis model, the rat model developed in this study can serve as animal model to characterize the chronic osteomyelitis caused by *S. aureus* successfully. In addition, the most important advantage of this rat model is that the local blood flow can be reduced by ligating the femoral artery, which mimics the natural and clinical pathogenesis. As a disease with a high recurrence rate^[32], it is very necessary to make a stable animal model which closer to the current clinic to develop the new therapeutic strategies for patients with chronic osteomyelitis.

Conclusion

This present model is a novel animal model of chronic osteomyelitis by ligating the femoral artery and implanting the Kirschner needle with *S. aureus* biofilm, which closer to the clinical pathogenesis and natural route of infection. This model has a high success rate and a good balance of the survival rate, clinical characteristics, convenient, cost and size requirements, providing an important platform for studying the pathogenic mechanism and the new therapeutic strategies of chronic osteomyelitis.

Declarations

Acknowledgements

Part of the content of this research was the model making in the genetic laboratory of Zunyi Medical University, X-ray examination and pathological analysis in the affiliated hospital of Zunyi Medical University.

Authors' contributions

Feng Jian-Bo was responsible for the study design, most of the laboratory work and writing the article. Peng Jia-chen and Wang Xiao-Min were responsible for SEM and revision of the manuscript. Yang Li-dan, Wang Yu-qi and others assisted in most of the laboratory work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the affiliated hospital of Zunyi medical university council for animal experimentation.

Consent for publication

Not applicable

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Conflict of interest

Authors declares that there is no conflict of interest.

Ethical approval

All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Zunyi Medical University.

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Figures

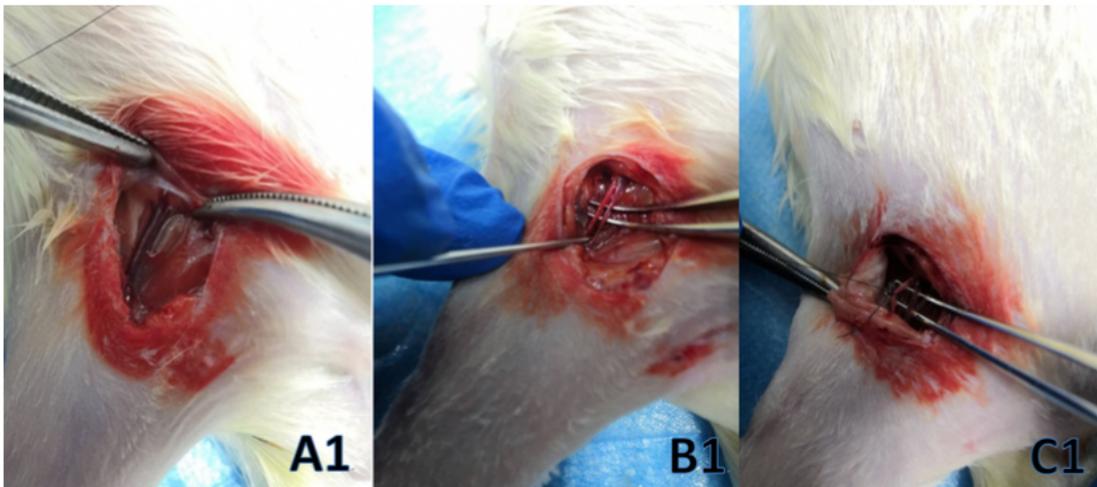


Figure 1

The progress of femoral artery ligation. The femoral artery was separated (A1) and ligated (B1 and C1).

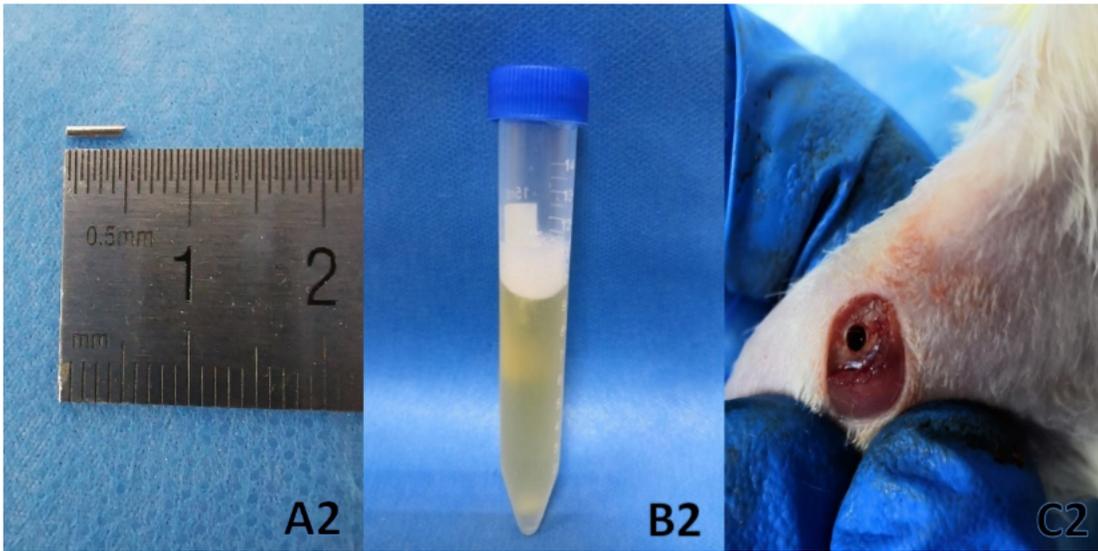


Figure 2
 The diagram of the bone hole and the materials of the Kirschner needle with *S. aureus* biofilm. Figure A2 showed a sterile implanted Kirschner needle with length of 5mm and a diameter of 1mm. The culture of *S. aureus* was shown in figure B2. A bone hole, 2mm diameter, located on the right tibial spine, was made for implanting a Kirschner needle (figure C2).

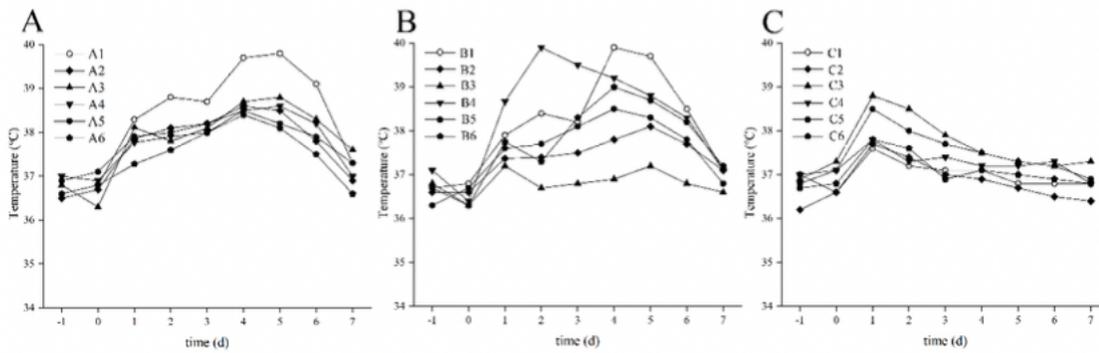


Figure 3
 The average rectal temperature of all rats from the 1 day before surgery to 7 days after operation. (A) A group; (B) B group; (C) Control group.

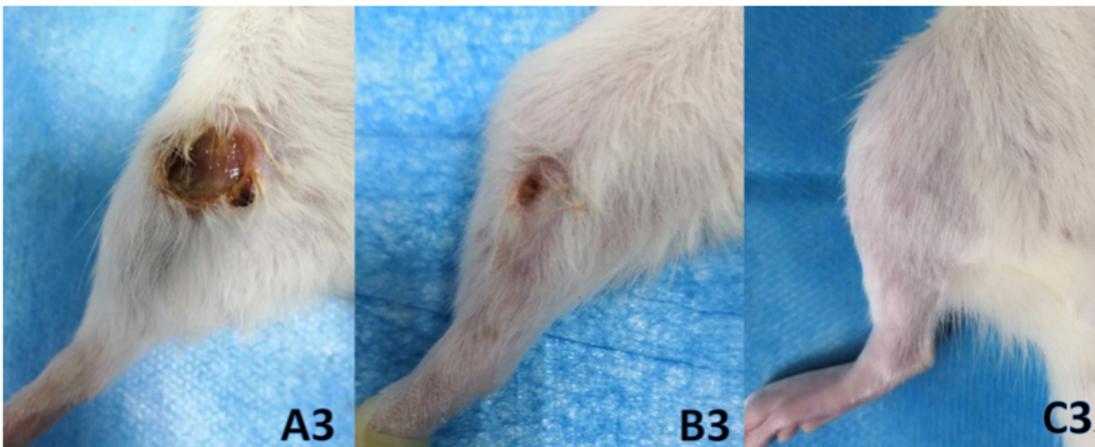


Figure 4
 General observation of rats at 4 weeks after operation in each group. Compared with Control group C (C3), group A (A3) and group B (B3) were characterized with the local swelling and purulent secretion.

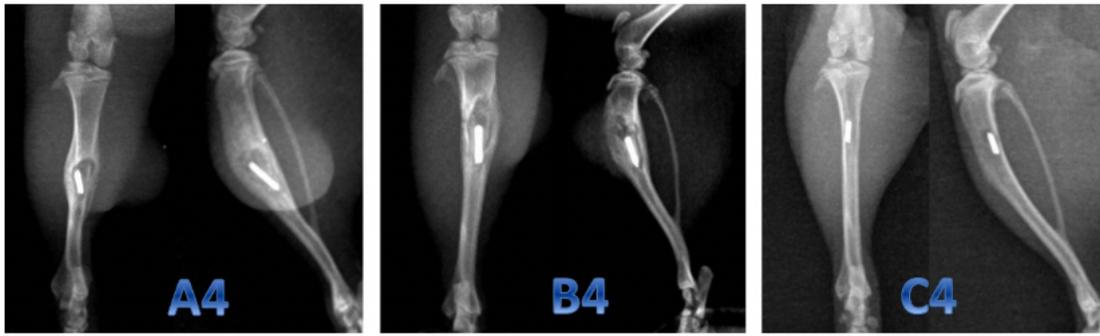


Figure 5

Representative radiographs from a rat in each group at 4 weeks after surgery. The group A (A5) and group B (B5) were characterized by the enlargement of local bone marrow cavity, the formation of new bone and cladding, the thickened and uplifted bone tissue and the swollen soft tissue.



Figure 6

The culture results of secretion collected from the disease area at 4 weeks after surgery.

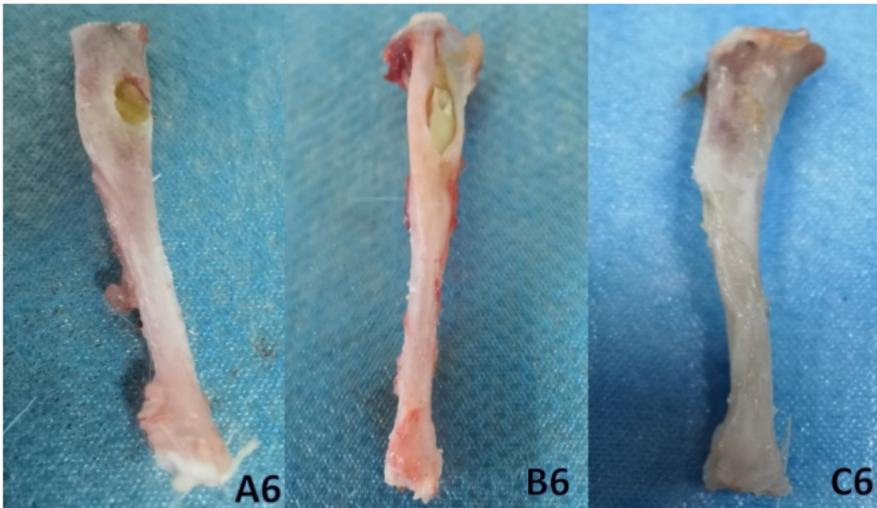


Figure 7

S. aureus contribute to the occurrence of chronic osteomyelitis. Obvious purulent secretion in the bone marrow cavity, the formation of new bone and dead bone were found in group A and B.



Figure 8

Bone histopathology from different experimental groups. (A7) A group; (B7) B group; (C7) Control group. A large number of inflammatory cell infiltration could be seen in group A and B, but no inflammatory cell infiltration was observed in group C.