Journal of Visualized Experiments

Treatment with Vancomycin Loaded Calcium Sulphate and Autogenous Bone in an Improved Rabbit Model of Bone Infection

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57294R3
Full Title:	Treatment with Vancomycin Loaded Calcium Sulphate and Autogenous Bone in an Improved Rabbit Model of Bone Infection
Keywords:	Bone infection; rabbit model; Staphylococcus aureus; Tibia; vancomycin loaded calcium sulfate; autogenous bone
Corresponding Author:	Dan Shou Zhejiang Academy of Traditional Chinese Medicine Hangzhou, Zhejiang CHINA
Corresponding Author's Institution:	Zhejiang Academy of Traditional Chinese Medicine
Corresponding Author E-Mail:	shoudanok@163.com
Order of Authors:	Yang Zhang
	Lifeng Shen
	Ping'er Wang
	Weixing Xi
	Zhongming Yu
	Xiaowen Huang
	Xuping Wang
	Dan Shou
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	No. 132 Tianmushan Road, Hangzhou, Zhejiang, China

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;57294_R3.docx

TITLE:

Treatment with Vancomycin Loaded Calcium Sulphate and Autogenous Bone in an Improved Rabbit Model of Bone Infection

AUTHORS& AFFILIATIONS:

Yang Zhang[1]*, Lifeng Shen[2]*, Ping'er Wang[3], Weixing Xi[4], ZhongmingYu[1], Xiaowen Huang[1], Xuping Wang[1], Dan Shou[1†]

[1]Department of Medicine, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, ZJ, China
[2]Department of Orthopaedic Surgery, Tongde Hospital of Zhejiang Province, Hangzhou, ZJ, China
[3]Institute of Orthopaedics and Traumatology, the First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, ZJ, China
[4]Department of Clinical Laboratory, Tongde Hospital of Zhejiang Province, Hangzhou, ZJ, China

*The authors contributed equally.

Email Addresses of Co-authors:
Yang Zhang (zhangyang0310@163.com)
Lifeng Shen (hzshenlf@163.com)
Ping'er Wang (apple63209321@126.com)
Weixin Xi (877845932@qq.com)
Nani Wang (wnn8511@163.com)
ZhongmingYu(yzm5266@163.com)
Xiaowen Huang(bowen8883@126.com)
Xuping Wang (1050979506@qq.com)
Dan Shou (shoudanok@163.com)

[†]Corresponding Author:
Dan Shou

KEYWORDS:

Bone infection, rabbit model, *Staphylococcus aureus*, tibia, vancomycin loaded calcium sulphate, autogenous bone

SUMMARY:

This study presents an improved rabbit model infected with *Staphylococcus aureus* by blocking the same amount of bacteria in bone marrow. Vancomycin loaded calcium sulphate and autogenous bone are used for antibiotic and bone repair treatment. The protocol could be helpful for studying bone infection and regeneration.

ABSTRACT:

Bone infection results from bacterial invasion, which is extremely difficult to treat in clinical, orthopedic, and traumatic surgery. The bone infection may result in sustained inflammation, osteomyelitis, and eventual bone non-union. Establishment of a feasible, reproducible animal model is important to bone infection research and antibiotic treatment. As an in vivo model, the rabbit model is widely used in bone infection research. However, previous studies on rabbit bone infection models showed that the infection status was inconsistent, as the amount of bacteria was variable. This study presents an improved surgical method for inducing bone infection on a rabbit, by blocking the bacteria in the bone marrow. Then, multi-level evaluations can be carried out to verify the modelling method.

In general, debriding necrotic tissue and implantation of vancomycin-loaded calcium sulphate (VCS) are predominant in antibiotic treatment. Although calcium sulphate in VCS benefits osteocyte crawling and new bone growth, massive bone defects occur after debriding. Autogenous bone (AB) is an appealing strategy to overcome bone defects for the treatment of massive bone defects after debriding necrotic bone.

In this study, we used the tail bone as an autogenous bone implanted in the bone defect. Bone repair was measured using micro-computed-tomography (micro-CT) and histological analysis after animal sacrifice. As a result, in the VCS group, bone non-union was consistently obtained. In contrast, the bone defect areas in the VCS-AB group were decreased significantly. The present modeling method described a reproducible, feasible, stable method to prepare a bone infection model. The VCS-AB treatment resulted in lower bone non-union rates after antibiotic treatment. The improved bone infection model and the combination treatment of VCS and autogenous bone could be helpful in studying the underlying mechanisms in bone infection and bone regeneration pertinent to traumatology orthopedic applications.

INTRODUCTION:

Bone infection usually results from bacteria or other microorganism invasion after trauma, bone fracture, or other bone diseases[1]. Bone infection may induce a high level of inflammation and bone tissue destruction. In the clinic, *Staphylococcus aureus* (*S. aureus*) is the predominant causative agent of bone infection[2,3]. The bone infection is painful, debilitating, and often takes a chronic course that is extremely difficult to treat[4]. At present, debridement of necrotic tissue and implanting of vancomycin-loaded calcium (VCS) beads have been confirmed as an efficient strategy for controlling local infection[5,6]. However, 10% to 15% of patients experienced a prolonged bone repair process, delayed union, or non-union after anti-infection treatment[7]. The large segment of a bone defect is the most difficult issue for orthopedic surgeons. An autologous bone graft is considered the optimal bone replacement in bone non-union treatment[8,9].

To date, most of the studies on bone infection and autologous bone implantation have been conducted in various kinds of animal models, such as rats, rabbits, dogs, pigs and sheep[10,11]. Rabbit models are most commonly used for bone infection studies, as first performed by Norden and Kennedy in 1970[12,13]. In our previous study, we used rabbit models following Norden's method, and we found that the quantity of *S. aureus* injected into bone marrow could

not be quantified accurately, as the blood leaking out of bone marrow led to bacteria solution overflow.

This article presents an improved surgical method for inducing bone infection on rabbits. At the end of the procedure, a blood biochemistry test, a bacteriological examination, and a histopathologic examination were performed to verify the bone infection model. Then, VCS was implanted to inhibit infection, and autogenous bone was implanted to promote bone regeneration.

PROTOCOL:

The rabbits used in the present study were treated in accordance with the Guide for the Care and Use of Laboratory Animals. All the experimental procedures were followed by the rules of the Bioethics Committee of Zhejiang Academy of Traditional Chinese Medicine.

1. Preparation of the bacterial suspension

1.1. Dissolve 0.5 mg of *S. aureus* freeze-drying powder (ATCC 6538) with 0.3 mL of Luria-Bertani culture medium. Mix suspension completely.

1.2. Streak the bacteria suspension onto tryptic soy agar plates and incubate the bacterial colonies at 37 °C for 16 h.

1.3. Select a single bacterial colony forming unit (CFU) and culture in tryptic soy broth tubes for 24 h. Perform a subculture for approximately 24 h at 37 °C, and obtain mid-logarithmic growth phase bacteria after 16 to 18 h, when the optical density (OD) value is 0.6 at 600 nm[14].

1.4. Transfer 1 mL of bacteria suspension into a centrifuge tube. Centrifuge for 5 min at 825 x *g* and 4 °C, and discard the supernatant. Resuspend and wash the bacteria with 100 µL of phosphate buffered saline (PBS); repeat this step 3 times. Finally, resuspend bacteria with 3 mL of PBS.

1.5. Estimate the bacteria concentration using McFarland's turbidimetry[15].

1.5.1. Transfer 100 to 500 µL of bacteria suspension to a colorimetric tube until the turbidity is equivalent to a 0.5 McFarland standard.

1.5.2. Assess turbidity by visual comparison to the 0.5 McFarland, when the content of bacteria reaches approximately 10^8 CFU/mL.

NOTE: Make sure the volume of bacteria suspension is sufficient for the following protocols. For every rabbit, the volume of bacteria suspension is less than 1 mL.

1.6. Transfer 0.2 mL of bacterial suspension to an agar plate and apply it evenly. Incubate the plate at 37°C for 16 h. Count the bacteria colonies to verify the CFU of bacteria suspension.

2. Preparation of bone infection models

2.1. Keep male New Zealand white rabbits, aged 3 months, in individual cages, under air-controlled conditions (20 ± 1 °C) and 12 h/12 h light-dark illumination cycles. Offer routine diet and tap water daily.

2.2. Ensure that at the time of surgery that the rabbit weighs more than 3 kg.

2.3. Anaesthetize rabbits by intraperitoneal injection with pentobarbital sodium (3 mg per 100 g of body weight). Make sure rabbits are fully anesthetized by a failure to respond to a paw pinch. Fix rabbits on the operating table during operation procedure.

NOTE: Make sure that the modeling procedure duration is less than 1 h.

2.4. Shave the proximal tibia region using an electric shaver against the direction of hair growth. Disinfect the skin by applying a povidone-iodine solution.

2.5. Mark the upper end of the tibia and the drilling hole position for injection with *S. aureus* (the distance to the upper end of the tibia is 1.5 cm) with pen and ruler. Make sure the drilling hole positions are in the middle of the tibial plateau horizontally (**Figure 1A**).

2.6. Cut tibia skin using a No. 11 scalpel and make a 1 cm incision in the periosteum (**Figure 1B,C**). Punch a 2 mm diameter hole in the tibia using an electric bone drill unit (**Figure 1D**).

2.7. Press the 2 mm diameter holes in the tibial plateau with a cylinder of bone wax of 2 mm diameter and 2 mm height (**Figure 1E**). Remove the spare bone wax along the horizontal plane of the tibial plateau (**Figure 1F**). Check that the 2 mm hole is full of bone wax (**Figure 1G**).

NOTE: Ensure that the holes are full of bone wax by checking the hole with or without blood overflow.

2.8. Sew up the periosteum and skin with absorbable surgical suture in a vertical mattress suture to prevent the animal from chewing the sutures (**Figure 1H**).

2.9. Inject 1 x 10^8 CFU/mL of *S. aureus* solutions (30 µL per 100 g of body weight) with a 1 mL asepsis injector (**Figure 1I**). Make sure the needles penetrate the bone wax and inject the *S. aureus* solution into bone marrow slowly.

2.10. Keep the animal in warm and clean conditions to avoid heat loss after modelling. Monitor respiratory rate and heart rate. After waking up, house the rabbits in individual cages with free access to food and water.

3. Evaluation of bone infection model

3.1. At days 7, 14, 21 and 28 after infection, place rabbits into the rabbit fixer with the head and ear outside of the fixer.

3.2. Draw 2 mL of blood from the auricular veins into a dipotassium ethylenediaminetetraacetic acid (EDTA-K2) anticoagulant blood container. Draw 1 mL of blood from a blood vessel into a blood container. Centrifuge the serum for 10 min with a speed of 651 x *g* at room temperature.

3.2.1. Determine white blood cell count (WBC) in whole blood using a blood biochemical analyzer, and C-reactive protein (CRP) by an enzyme-linked immunosorbent assay (ELISA) method[16].

3.3. At days 7, 14, 21 and 28 after infection, anaesthetize one model rabbit with pentobarbital sodium at the dosage of 3 mg per 100 g body weight. Cut tibia skin using a No. 11 scalpel and make a 2 cm incision in the periosteum (**Figure 2A**).

3.4. Clean bone wax. Debride necrotic bone by punching two adjacent 4.8 mm diameter holes using an electric bone drill unit (**Figure 2B**). Debride necrotic bone marrow and granulation tissue using a bone spoon (**Figure 2C**).

NOTE: Clean bone tissue during the debridement to avoid bone tissue remaining in the bone marrow.

3.5. Scrape and clean the bone tissue between the two holes (**Figure 2D**).

3.6. Spread 1 mL of bone marrow onto sheep blood agar plates. Incubate plates overnight at 37 °C. Select plates of 30-300 colonies, and calculate the number of colonies.

3.7. At the end of day 28 after infection, extract tibia specimens along the edges of knee and ankle joints. Fix the tibia specimens in 4% paraformaldehyde for 24 h. Decalcify the tibia specimens in 10% EDTA for 8 weeks.

3.8. Dehydrate the tibia specimens in a graded series of ethanol dilutions, and then embed in paraffin wax. Cut 4 consecutive 5 µm sections from the coronal planes. Stain sections with a hematoxylin and eosin (H&E) staining kit.

3.9. Use a microscope to view the stained sections and record transmitted light images with standard software.

4. Preparation of VCS beads

4.1. Add 1 g of vancomycin hydrochloride powder to 9.5 g of medical grade calcium sulphate, and then add 3 mL of normal saline to the mixed power. Mix them thoroughly with a spatula for 30 to 45 s.

4.2. Place the mixed product into a flexible silica gel mold (cylinder of 4.8 mm diameter and 4.8 mm height), and dry at room temperature for 15 min. Remove the VCS beads by flexing the mold.

5. Antibiotic treatment and implantation of autogenous bone

5.1. Anaesthetize model rabbits with pentobarbital sodium at the dosage of 3 mg per 100 g body weight on the 28th day after infection. Shave the proximal tibia region using an electric shaver. Disinfect the skin by applying povidone-iodine solution.

NOTE: Make sure that the modelling procedure is less than 1 h.

5.2. Shave the tail region using an electric shaver and disinfect the tail by applying povidone-iodine solution.

5.3. Cut down the tail using surgical scissors. Cut tail skin using a No. 11 scalpel and reveal the tail bone. Remove any muscle, soft tissue and periosteum.

5.4. Detach the tail bone at each joint and transfer the bone fragment to a 100 mm plastic dish containing sterile saline.

5.5. Sew up the skin at the tail region with absorbable surgical sutures in a vertical mattress suture to prevent the animal from chewing the sutures.

5.6. Implant 4 pieces of VCS beads (cylinder of 4.8 mm diameter and 4.8 mm height, 1.25 mg vancomycin per piece of bead) into the marrow cavity using curved tweezers (**Figure 2E**).

5.7. Fill the bone defect with 8 pieces of autogenous bones (cylinder of 2 mm diameter and 4 mm height per each piece) using curved tweezers (**Figure 2E**).

5.8. Sew up the periosteum and skin with absorbable surgical sutures in a mattress suture manner (**Figure 2F**).

NOTE: Keep the temperature at 25 °C during the surgery.

5.9. Keep the animal in warm and clean conditions to avoid heat loss after surgery. Monitor respiratory rate and heart rate. After waking up, house the rabbits in individual cages with free access to food and water.

6. Assessments of antibiotic activity

6.1. Put rabbits into a rabbit fixer, and place the head and ear outside of the fixer at 2, 4, 6 and 8 weeks after treatment.

6.2. Draw blood from auricular veins with EDTA-K2 anticoagulant blood vessel. Draw 1 mL of blood from a blood vessel into a blood container. Centrifuge the serum for 10 min with a speed of 651 x *g* at room temperature.

6.3. Determine the white blood cell count (WBC) in whole blood by using blood biochemical analyzer, and C-reactive protein (CRP) by an ELISA method[16].

7. Assessments of bone regeneration

7.1. Euthanize rabbits by injecting with an over dosages of pentobarbital sodium, at the end of 8 or 12 weeks after treatment.

7.2. Extract tibia specimens, along the edges of knee and ankle joints. Debride muscles and fascial layers.

7.3. Analyze structure of tibia by using micro-computed tomography (micro-CT). Choose an oval area 4.8 mm diameter and 9.6 mm long as the region of interest (ROI). Reconstruct 3D model images using bitmap data.

7.4. Choose scores of the ratio of bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecula number (Tb.N) and trabecular separation (Tb.Sp)from the3D models to assess bone regeneration.

REPRESENTATIVE RESULTS:

Evaluation of Bone Infection Model

After infection with *S. aureus*, the pathological manifestations of rabbits were similar to the representative symptom of chronic osteomyelitis in the clinic. In our study, 30 rabbits were infected, and subjected as a model group, and 10 rabbits were subjected as control animals. All the model rabbits have infected sinuses of the tibia local site, with white and yellow pus over flow from the sinuses (**Figure 3A**). The results of H&E staining indicate that bacterial aggregates are located around dead bone in the model group, and normal osteocytes cannot be identified. The levels of CRP and WBC are higher in the model group than the control group (**Figure 3B**). The necrotic bone marrow lysates are streaked on agar plates, which result in an increased number of colonies for the model group after infection (**Figure 3C**). At the end of modelling, there were 3 rabbits dead because of serious infection. The remaining infected rabbits were identified as the bone infection model and were divided into 3 groups: model group, VCS group, and VCS-AB group.

Assessments of Antibiotic Activity and Bone Regeneration

At 2, 4, 6, and 8 weeks after treatment with VCS or VCS-AB, the levels of CRP and WBC are reduced significantly (**Figure 4A**). After 12 weeks of implantation of VCS and bone allograft, tibial defects of the VCS-AB group seemed fully coalescent. Tibial plateau surfaces of the VCS-AB group are flatter than that of the VCS group (**Figure 4B**). 2D reconstruction images indicate a progressive increase in bone volume during the 12 week period after treatment with VCS-AB and VCS, while bone loss is significant in the model group. To analyze the bone regeneration quantitative indexes, an oval area 4.8 mm diameter and 9.6 mm long was chosen as the region of interest (ROI) **(Figure 4C**), and 3D model images were reconstructed using bitmap data. The micro-CT indexes BV/TV in the model group were significantly lower than that in the VCS and VCS-AB groups. The Tb.N and Tb.Th scores in the VCS-AB group were significantly higher than those in the model and VCS group. Moreover, the Tb.Sp scores in the VCS-AB group are markedly lower than that in the model group and VCS group (**Figure 4D**).

These results suggest that infection with *S. aureus* causes increasing WBC and CRP in the model group, which can be decreased using the VCS. The implantation of VCS is regarded as the optimal antibiotic treatment. However, the bone defect is observable in the VCS group. The VCS and autogenous bone treatment increase the trabeculae thickness and trabeculae number and decrease the trabecular separation. The VCS-AB treatment showed capability of promoting bone healing.

FIGURE AND TABLE LEGENDS:

Figure 1. Surgical preparation of the bone infection model. (**A**) Shows the punching position on the tibia. The distance between the drilling hole position for injection with *S. aureus* to the upper end of tibia is 1.5 cm. (**B**) Incision made in the skin to expose the periosteum. (**C**) Shows incision made through the periosteum to expose the tibia. (**D**) Punch a 2-mm diameter hole in the tibia. (**E**) Fill the 2 mm diameter hole full of bone wax. (**F**) Cut off spare bone wax. (**G**) Show the bone wax filling the bone defect. (**H**) Sew up the periosteum and skin. (**I**) Inject with *S. aureus* solution.

Figure 2. Preparation of bone allograft and antibiotic treatment. (**A**) Incision made in the skin to expose periosteum. (**B**) Punch two adjacent 4 mm diameter holes. (**C**) Debride necrotic bone and inflammatory bone marrow. (**D**) Scrape and clean the bone tissue between the two holes to make a long circle of 4 mm diameter and 8 mm long. (**E**) Fill the hole with VCS and bone allograft. (**F**) Sew up the periosteum and skin.

Figure 3. Evaluation of bone infection model. (**A**) Appearance features of the rabbit legs infected with *S. aureus*, and typical histopathology images of the rabbit tibia in the model and control groups. Blue arrow: osteocyte; pink arrow: bone trabeculae; yellow arrow: bacterial aggregates; green arrow: dead bone. (**B**) The WBC and CRP results in the rabbit serum at the time points of before modelling, 7, 14, 21 and 28 days after infection. The columns represent the mean ±SE, $*p < 0.05$ *vs.* the control group. (**C**) The number of bacterial colonies in the tibia marrow counted following overnight incubation. The columns represent the mean ±SE, $*p < 0.05$ *vs.* the colony count at day 0.

Figure 4. Assessments of antibiotic activity and bone regeneration. (**A**) The results of WBC and CRP in rabbit serum at the time points of 2, 4, 6 and 8 weeks after implantation of VCS and VCS-AB, the points represent the mean ±SE, $^{\#}p < 0.05$ and $^{*}p < 0.05$ compared to the model group. (**B**) The coronal section images of tibia analyzed by micro-CT. (**C**) The location of ROI. (**D**) The histograms show the bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecula number (Tb.N), and trabecular separation (Tb.Sp) scores of ROI from five rabbits per group. The columns represent the mean ±SE, $^{*}p < 0.05$ *vs.* the control group or the model group.

Figure 5. The timeline of all the procedures.

DISCUSSION:

In the previous studies, various kinds of animal models were constructed to study both acute and chronic bone infection; however, the search for the ideal model still persists[17,18]. In addition, the ideal bone infection model is expected to simulate the pathological characteristics of bone infection in clinical setting, while the modelling periods, remain low cost and easy to carry out. So far, the rabbit bone infection model is the most common model in inflammatory bone disease research, as the rabbits are available, feasible and inexpensive. In our previous study, we compared the death rate and infection rate of rabbits with varying body weights. The results showed that the body weight should be more than 3 kg; otherwise, there would be a high death rate or a high incidence of haematosepsis and a higher mortality rate after surgery.

Unlike earlier studies, the rabbit bone infection models and antibiotic treatment in our study are consistent with the pathological characteristics of human disease and the surgical therapy. In the previous study, animals injected with sodium morrhuate and *S. aureus* did not have pathologic status more than 60 days. Furthermore, the death rate was more than 20%[12,19]. The overflow *S. aureus* solution from bone defects had been proven to induce a low infection rate. We used bone wax to fill the 2 mm hole on the tibia, in order to block the *S. aureus* solution in the bone marrow and ensured that the holes were full of bone wax by checking the hole with or without blood overflow. As the thickness of a rabbit tibia was 2 mm, we pressed a cylinder of 2 mm diameter and 2 mm height bone wax into the 2 mm diameter holes, which ensured the bone wax filled the hole and could not infiltrate into the bone marrow. Moreover, as the bone wax was flexible and stable, it filled the holes and could not melt or react with bone marrow. In our study, at the 28th day after infection, the bone wax was still complete and filled the holes fully. As the weights of rabbits were more than 3 kg and less than 3.2 kg, the volume of bacteria suspension was 900 µL to 960 µL. Because of the slow speed of injection and blocking function of bone wax, this volume of bacteria suspension could be injected into bone marrow without high pressure. The results showed that this protocol ensures the quantity of *S. aureus* infected in bone marrow. A 2 mm diameter hole was punched in the tibia to ensure that the distance to the upper end of the tibia is 1.5 cm, which locates the hole at the tibial plateau, ensuring sufficient space to debride and implant VCS beads and autogenous bone in the following treatment. During the modelling process, 3 rabbits died because of serious infection. The remained rabbits identified as bone infection rabbits, and the infection rate in the remaining

rabbits was 100%. Compared with other bone infection protocols, such as the implantation of sponges soaked with *S. aureus* or the implantation of foreign matter, our protocols closely simulate bone infection in clinical setting, and have little effect on procedures, such as debriding necrotic bone and antibiotic treatment.

Diagnosing bone infection is a challenge to surgeons. The laboratory test results, including serum inflammation marker detection, microbiology analysis and histopathology analysis were used to evaluate bone infection in clinical settings[20]. Also, diagnostic imaging, such as ultrasound, radiology, computed tomography, magnetic resonance imaging or Raman spectroscopy were applied to detect bone infection[21]. Unfortunately, diagnosing osteomyelitis through imaging methods is often delayed because bone necrosis is difficult to detect by plain radiography until week 3 of infection. In our study, we used serum inflammation marker detection and histopathology analysis to evaluate bone infection models, as these methods were effective, operable and the indexes were sensitive. The following were the most important steps of the surgical procedure to create a bone infection model in our study. Choose the rabbits with appropriate body weight to perform the surgery and treatment. Maintain a sterile environment during the raising and surgical procedure, and ensure warm conditions following the surgical procedure protocol. Punch a 2 mm diameter hole in the tibia, and ensure the distance to the upper end of the tibia is 1.5 cm. Fill the hole with bone wax, and sew up the periosteum and skin in order to block the bacteria solution. The most important steps of antibiotic treatment are the following. Ensure the pathological bone infection of the rabbits by detecting WBC in whole blood and CRP in the serum. Debride necrotic bone completely, punch two adjacent 4 mm diameter holes and scrape and clean the bone tissue between the 2 holes. Implant 4 pieces of VCS beads and 8 pieces of autogenous bone into bone marrow and bone defect.

After antibiotic treatment, we observed that the rabbits in the VCS-AB group had a higher osteogenic potential than that in the VCS group. This may be because the autogenous bone contains activated osteocytes and bone formation growth factors, which produce bone matrix throughout the graft surface, whereas degradation of VCS induces scarce bone matrix at the defect region. Our results indicate that the autogenous bone has the superior osteogenic capability. Although autogenous bone harvesting is limited in volume and entails morbidity to the donor site, the importance of autogenous bone is not negligible[22,23,24]. In our study, the autogenous bone was acquired from the tail bone, which avoids systemic injury, and decreases death rate in comparison to gaining autogenous bone from iliac bone. Autograft bone from the tail bone is might be the preferred grafting material in the animal study of bone infection.

In conclusion, an improved rabbit model of bone infection was established in this study. Inflammation indexes and blood biochemical indexes were used to estimate the bone infection model. Also, after antibiotic treatment, multi-level analyses were carried out to detect antibiotic activity and bone regeneration capability. The modeling, treatment protocols and evaluation methods are feasible and reliable. Further studies will be focused on taking advantage of multi-modality visual devices to monitor the pathologic process of bone infection and the bone repair process.

ACKNOWLEDGEMENTS:

This work was supported by the National Natural Science Foundation of China (81803808, 81873062), Zhejiang Provincial Medical and Health Science and Technology Fund (2017KY271) and the Science and Technology Project of Zhejiang Province (2017C37181).

DISCLOSURES:

The authors report no conflicts of interest in this work.

REFERENCES:

1. Malizos, K. N. Global Forum: The Burden of Bone and Joint Infection: A Growing Demand for More Resources. *Journal of Bone and Joint Surgery-American Volume*. **99**, e20 (2017).
2. Peeters, O. Teicoplanin - based antimicrobial therapy in Staphylococcus aureus bone and joint infection: tolerance, efficacy and experience with subcutaneous administration. *BMC Infectious Diseases*. **16**, 622 (2016).
3. Sugaya, H. et al. Percutaneous autologous concentrated bone marrow grafting in the treatment for nonunion. *European Journal of Orthopeadic Surgery and Traumatology.* **24**, 671-678 (2014).
4. Birt, M.C., Anderson, D.W., Bruce, T.E., Wang, J. Osteomyelitis: Recent advances in pathophysiology and therapeutic strategies. *Journal of Orthopeadics*. **14**, 45-52 (2017).
5. Walter, G., Kemmerer, M., Kappler, C., Hoffmann, R. Treatment algorithms for chronic osteomyelitis. *Deutsches Arzteblatt International.* **109**, 257-264 (2012).
6. Henriksen, K., Neutzsky-Wulff, A.V., Bonewald, L.F., Karsdal, M.A. Local communication on and within bone controls bone remodeling. *Bone*. **44**, 1026-1033 (2009).
7. Mendoza, M.C. et al. The effect of vancomycin powder on bone healing in a rat spinal rhBMP-2 model. *Journal of Neurosurgery Spine.* **25**, 147-153 (2016).
8. Cohn Yakubovich, D. et al. Computed Tomography and Optical Imaging of Osteogenesis-angiogenesis Coupling to Assess Integration of Cranial Bone Autografts and Allografts. *Journal of Visualized Experiments.* **106**, e53459 (2015).
9. Brecevich, A.T. et al. Efficacy Comparison of Accell Evo3 and Grafton Demineralized Bone Matrix Putties against Autologous Bone in a Rat Posterolateral Spine Fusion Model. *Spine Journal*. **17**, 855-862 (2017).
10. Jensen, L.K. et al. Novel porcine model of implant-associated osteomyelitis: A comprehensive analysis of local, regional, and systemic response. *Journal of Orthopeadic Research*. **35**, 2211-2221 (2016).
11. de Mesy Bentley, K.L. et al. Evidence of Staphylococcus Aureus Deformation, Proliferation, and Migration in Canaliculi of Live Cortical Bone in Murine Models of Osteomyelitis. *Journal of Bone and Mineral Research*. **32**, 985-990 (2017).
12. Norden C.W., Kennedy E. Experimental osteomyelitis. I: A description of the model. *Journal of Infectious Diseases*. **122**, 410-418 (1970).
13. Mistry, S. et al. A novel, multi-barrier, drug eluting calcium sulfate/biphasic calcium phosphate biodegradable composite bone cement for treatment of experimental MRSA osteomyelitis in rabbit model. *Journal of Controlled Release*. **239**, 169-81 (2016).

14. Nicholas M. Bernthal. et al. Combined In vivo Optical and μCT Imaging to Monitor Infection, Inflammation, and Bone Anatomy in an Orthopaedic Implant Infection in Mice. *Journal of Visualized Experiments.* **92**, e51612 (2014).
15. Laura M. Koeth, Jeanna M. DiFranco-Fisher, Sandra McCurdy. A Reference Broth Microdilution Method for Dalbavancin In Vitro Susceptibility Testing of Bacteria that Grow Aerobically. *Journal of Visualized Experiments.* **103**, e53028 (2015)
16. Uttra AM. et al. Ephedra gerardiana aqueous ethanolic extract and fractions attenuate Freund Complete Adjuvant induced arthritis in Sprague Dawley rats by downregulating PGE2, COX2, IL-1β, IL-6, TNF-α, NF-kB and upregulating IL-4 and IL-10. *Journal of Ethnopharmacology.* **224**, 482-496 (2018).
17. Harrasser, N. et al. A new model of implant-related osteomyelitis in the metaphysis of rat tibiae. *BMC Musculoskeletal Disorders*. **17**, 152 (2016).
18. Abedon, S.T. Commentary: Phage Therapy of Staphylococcal Chronic Osteomyelitis in Experimental Animal Model. *Frontiers in Microbiology*. **7**, 1251 (2016).
19. Tan, H.L., Ao, H.Y., Ma, R., Lin, W.T., Tang, T.T. In vivo effect of quaternized chitosan-loaded polymethylmethacrylate bone cement on methicillin-resistant Staphylococcus epidermidis infection of the tibial metaphysis in a rabbit model. *Antimicrobial Agents and Chemotherapy.* **58**, 6016-23 (2014).
20. Chiara Lauri. et al. Detection of Osteomyelitis in the Diabetic Foot by Imaging Techniques: A Systematic Review and Meta-analysis Comparing MRI, White Blood Cell Scintigraphy, and FDG-PET. *Diabetes Care*. **40**, 1111-1120 (2017).
21. Khalid M. et al. Raman Spectroscopy detects changes in Bone Mineral Quality and Collagen Cross-linkage in Staphylococcus Infected Human Bone. *Scientific Reports.* **8**, 9417 (2018).
22. Putters, T. F., Schortinghuis, J., Vissink, A., Raghoebar, G. M. A prospective study on the morbidity resulting from calvarial bone harvesting for intraoral reconstruction. *International Journal of Oral and Maxillofacial Surgery*. **44**, 513-7 (2015).
23. Yin, J., Jiang, Y. Completely resorption of autologous skull flap after orthotopic transplantation: a case report. *International Journal of Clinical and Experimental Medicine*. **7**, 1169-1171 (2014).
24. Takehiko, S. et al. Preliminary results of managing large medial tibial defects in primary total arthroplasty: autogenous morcellised bone graft. *International Orthopaedics*. **41**, 931-937 (2017).

Figure 1

Click here to access/download;Figure;Fig 1.tif

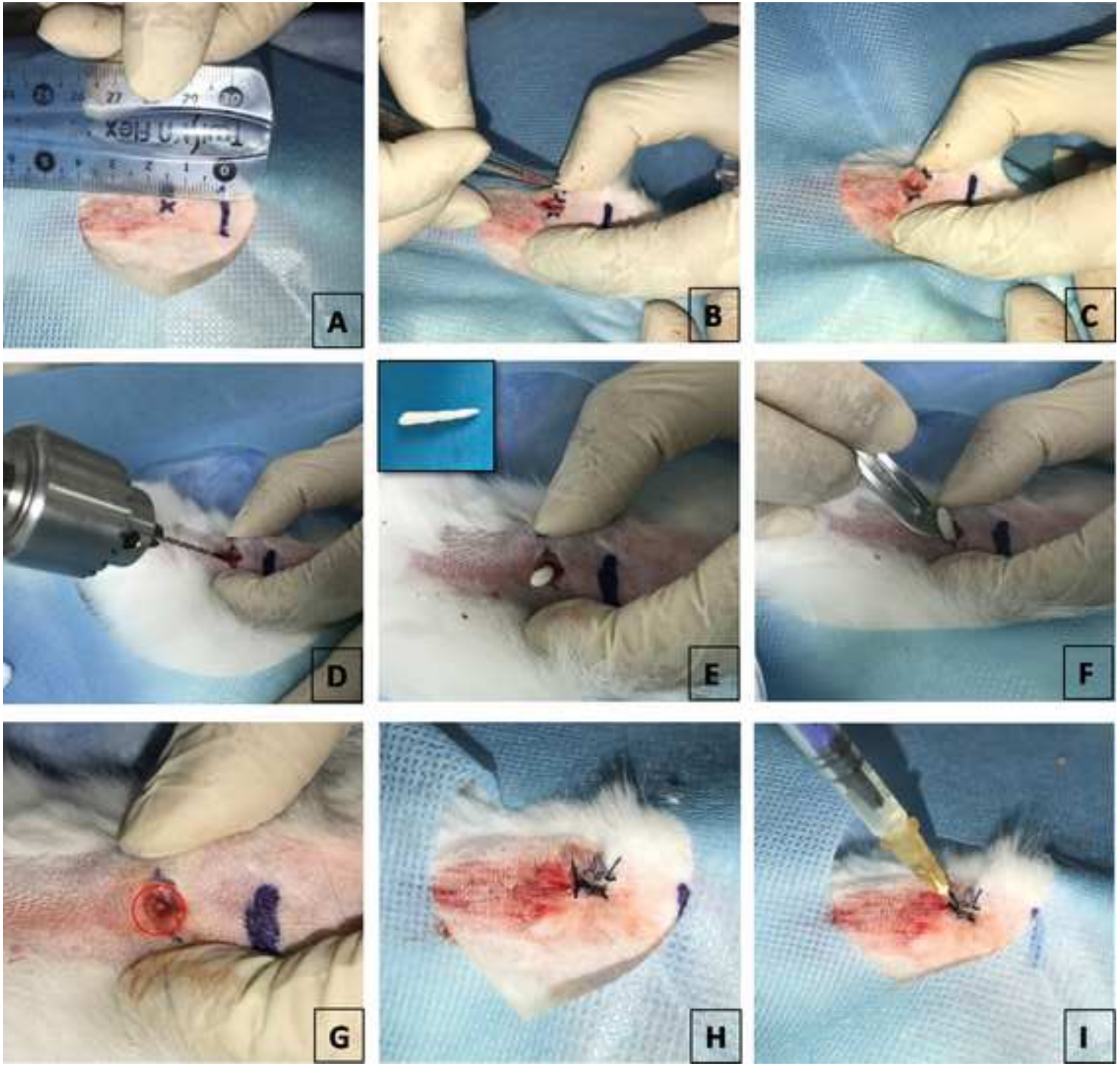

Figure 2

Click here to access/download;Figure;Fig 2.tif

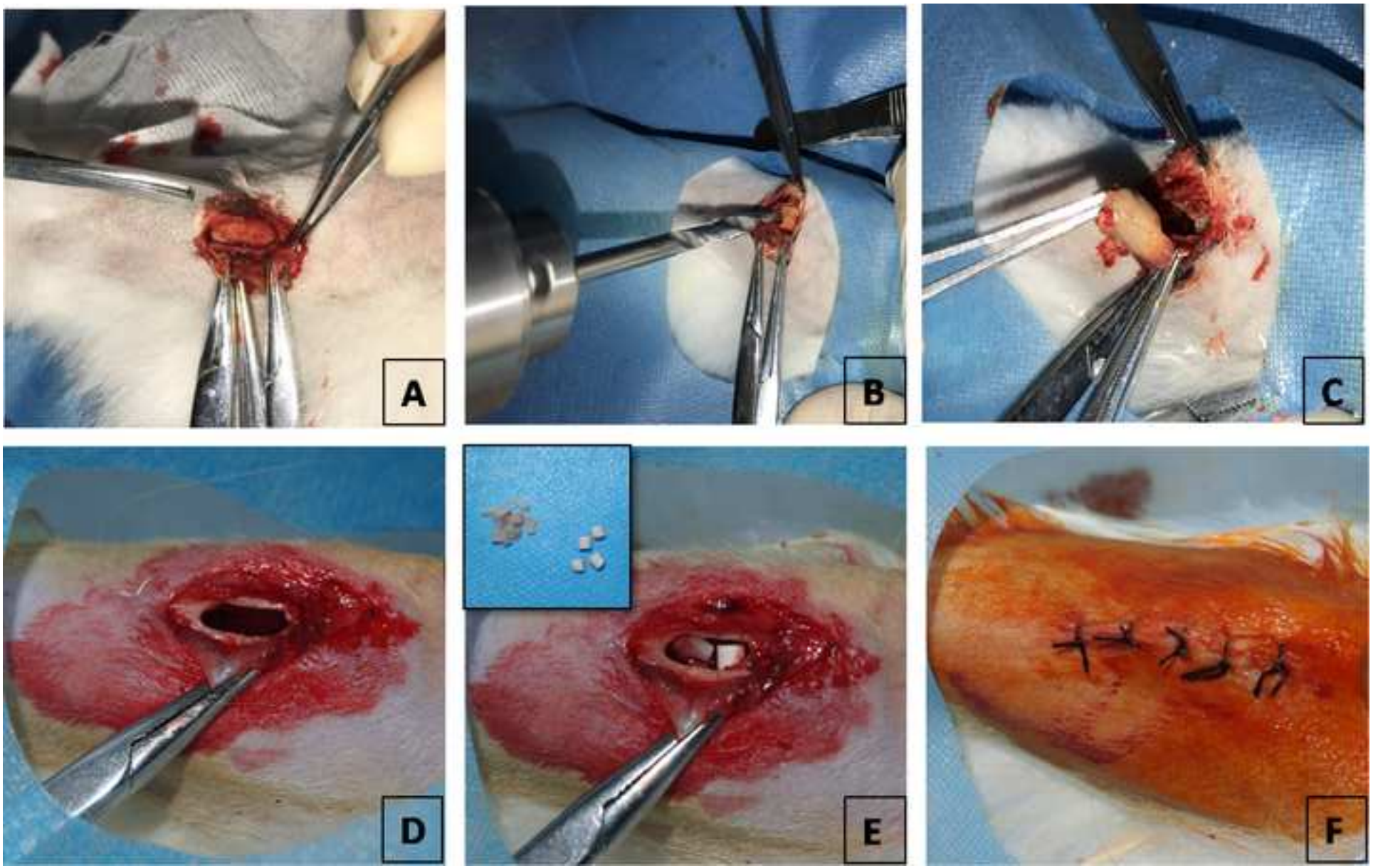

Figure 3

Click here to access/download;Figure;Fig 3.tif

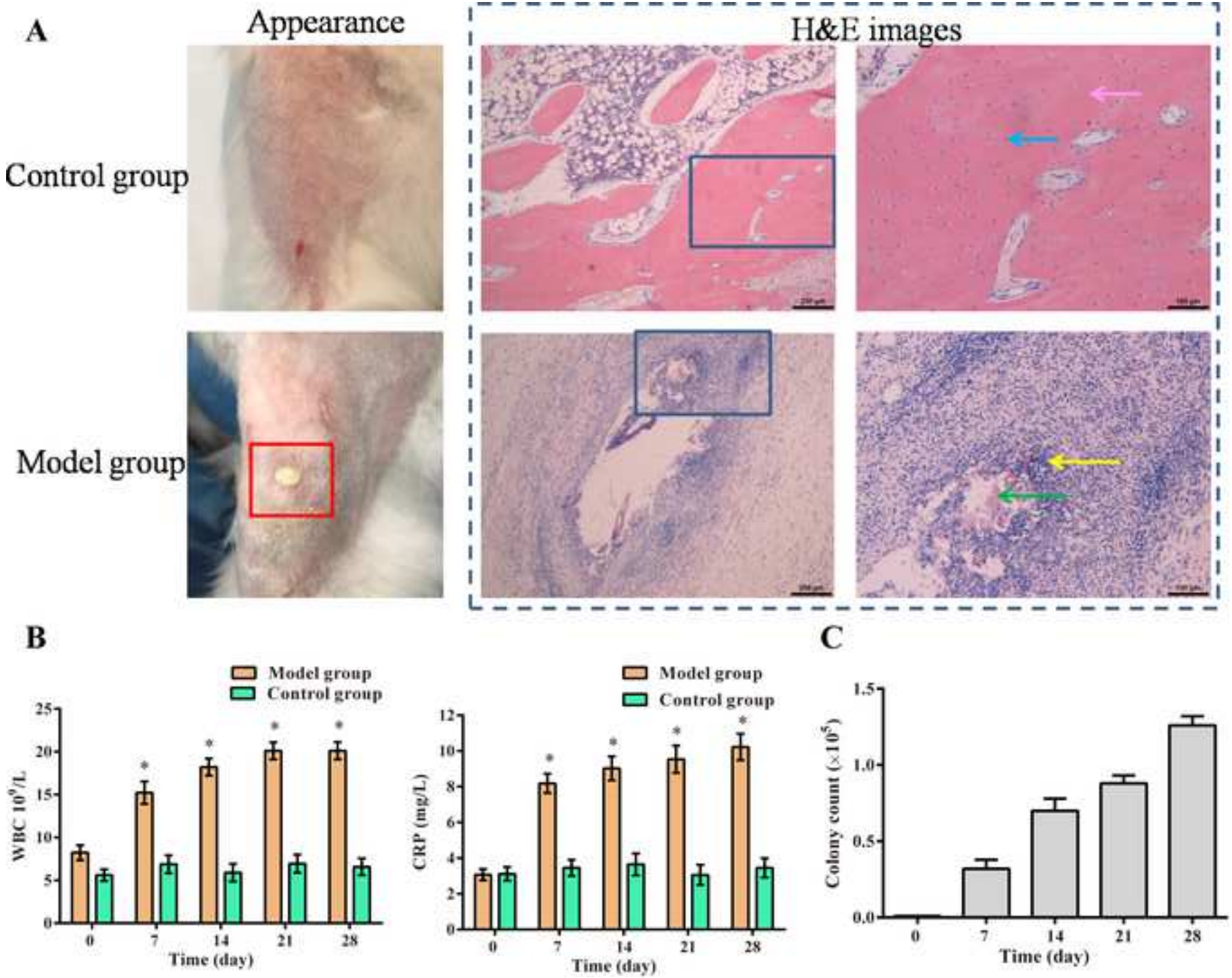

Figure 4

Click here to access/download;Figure;Fig 4.tif

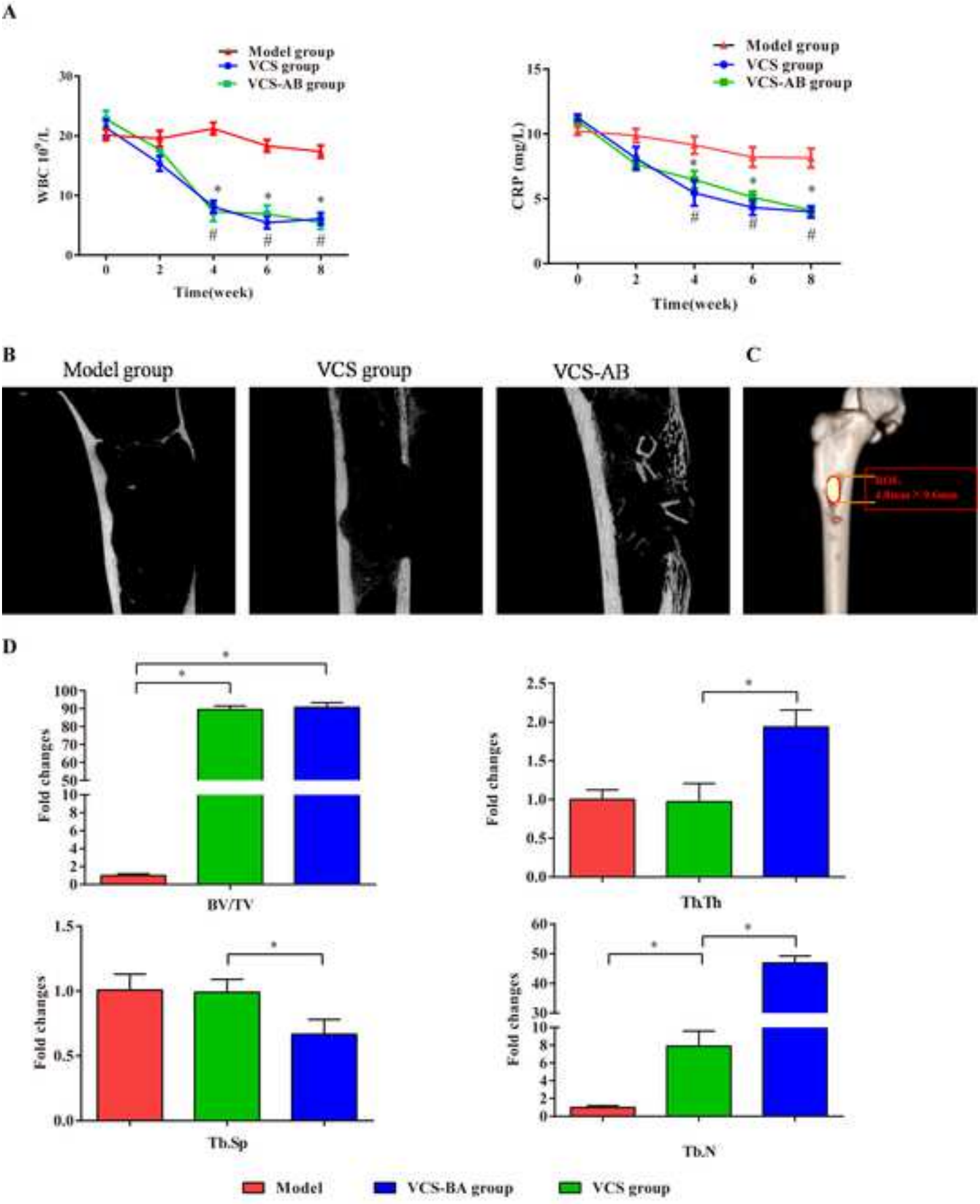

Figure 5

Click here to access/download;Figure;Fig 5.tif

Click here to access/download;Table of Materials;JoVE_Materials revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
absorbable surgical suture	Jinghuan	18S0604A	
asepsis injector	Jinglong	20170501	
bone wax	ETHICON	JH5CQLM	
CCD camera	Olympus	DP72	
EDTA-K2 anticoagulant blood vessel	XINGE	20170802	
Electric bone drill unit	Bao Kang	BKZ-1	
Electric shaver	Codos	3800	
flexible silica gel mold	WRIGHT	1527745	
Hematoxylin and Eosin Staining Kit	Beyotime	20170523	
Luria-Bertani culture medium	Baisi Biothchnology	20170306	
Medical-grade calcium sulphate	WRIGHT	1527745	
microcomputed tomography (micro-CT)	Bruker	SkyScan 1172	
Microscope	Olympus	CX41	
New Zealand white rabbits	Zhejiang Experimental Animal Center	SCXK 2014-0047	
No. 11 scalpel	Yuanlikang	20170604	
normal saline	Mingsheng	20170903	
PBS	TBD(Jingyi)	20170703-0592	
pentobarbital sodium	Merk	2070124	
povidone-iodinesolution	Lierkang	20170114	
S. aureus freeze drying powder	China General Microbiological Culture Collection Center	ATCC 6538	
sheep blood agar	HuanKai Microbial	3103210	
tryptic soy agar plates	HuanKai Microbial	3105697	
tryptic soy broth tubes	HuanKai Microbial	3104260	
Vancomycin	Lilly	C599180	

Author License Agreement

Click here to access/download;Author License Agreement (ALA);Author_License_Agreement.pdf

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Establishment of an improved rabbit model of bone infection with Staphylococcus aureus at tibia site and evaluation of combined treatment with vancomycin loaded calcium sulfate and autogenous bone

Author(s): Yang Zhang[1*], Lifeng Shen[2*], Ping'er Wang[3], Weixing Xi[4], ZhongmingYu[1], Xiaowen Huang[1], Ying Hu[1], Dan Shou[1]

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☐ Standard Access ☐ Open Access

Item 2 (check one box):

☐ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Dan Shou

Department: Department of Medicine

Institution: Zhejiang Academy of traditional Chinese Medicine

Article Title: Establishment of an improved rabbit model of bone infection with Staphylococcus aureus at tibia site and evaluation of combined treatment with vancomycin loaded calcium sulfate and autogenous bone

Signature: Dan Shou Date: 9.11.2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Click here to access/download;Rebuttal Letter;JOVE Respond.docx

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have checked the manuscript in this revised manuscript.

2. Keywords: Please provide at least 6 keywords or phrases.

Six keywords, "Bone infection, rabbit model, Staphylococcus aureus, tibia, vancomycin loaded calcium sulfate, autogenous bone" were provided in "line 34".

3. Please shorten the Short Abstract to no more than 50 words.

The short abstract has been reduced to 45 words, "line38-42" .

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

The numbering of the "Protocol" has been adjusted follow the "JoVE Instructions for Authors".

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

The "Protocol" has been revised in the imperative tense, and discussion about the protocol has been added to the "Discussion" section.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have revised the details of the protocols.

7. Lines 102-103: Please specify the mass of s. aureus freeze drying powder and volume of LB medium used.

The mass of s. aureus freeze drying powder and volume of LB medium have been added in " lines 102-103 " .

8. Lines 106-107: How to confirm that the culture has reached the mid-logarithmic growth phase.

We confirmed the mid-logarithmic growth phase by detecting the OD value of bacteria suspension, and drawing growth curve. In mid-logarithmic growth phase, the quantities of bacteria increase in logarithmic growth manner. We performed the protocol following the reference 14.

9. Line 108: Are the bacteria transferred from the agar plates to a centrifugation tube? Or the plates are directly centrifuged? Please specify.

The bacteria transferred into centrifugation tube. This step has been specified in "line

110".

10. Line 109: What volume of PBS is used?

The volume of PBS has been added in "line 112".

11. Line 110: Please describe how to estimate the bacteria concentration using McFarland's turbidimetry.

We used McFarland's turbidimetry to estimate the bacteria concentration, following the reference 15. The protocols have been added in "lines 113-116".

12. Line 113: How to verify the concentration of bacteria suspension? Culturing the bacteria on what plates and at what conditions? Please specify.

We transferred bacteria suspension into agar plate, incubated the plate at 37 ℃, and counted the bacteria colonies to verify the concentration of bacteria suspension. The specific protocols have been revised in the "lines 121-123 ".

13. Line 127: What is used to mark different positions?

The " ..., with marker pen and ruler " has been added in "line 138".

14. Line 147: What volume of blood is drawn?

The " ..., 2 mL ...., and 1mL... " has been added in "line 162".

15. Lines 108, 148, etc.: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speeds have been revised in "line 111 and line 163"

16. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

The protocol steps have been revised.

17. Please include single-line spaces between all paragraphs, headings, steps, etc.

All the paragraphs has been adjusted to single-line spaces.

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The protocol that identifies the essential steps, and protocol for the video have been highlighted with yellow background.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

The highlighted part has been checked.

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The highlighted protocols and sub-steps have been marked.

21. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently Figure 3 (line 175) is introduced before Figure 2 (line 210).

The figures have been renumbered in the sequence in which refer to them in the manuscript text. Specifically, figure 2 and figure 3 has been exchanged order, also figure captions have been revised.

22. Figures 2 and 4: Please define the scales bars and error bars in the figure legend.

The error bars have been added in the figure 3 (Figure 2 and figure 3 has been exchanged order) and figure 4 legends, "line 314 and 316", and "line 321 and 325".

23. References: Please do not abbreviate journal titles.

The full names of journal titles have been added in "References" section.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This study presented an improved rabbit model infected with Staphylococcus aureus, by blocking the same amount bacteria in bone marrow and then treated using vancomycin loaded calcium sulfate and autogenous bone.

Minor Concerns:

This manuscript is suitable for publication in JOVE. However, the english language is poor and should be enhance. Moreover, the authors have to compare their procedure with other similar methods, clearly. Why this method is more suitable than other one?

Thank you for your comment. In the previous studies, the rabbits were punched a hole and injected *s. aureus* solution. We prepared bone infection models followed the previous references, however, we found the *s. aureus* solution overflowed from the holes, which induced inaccurate dosages of *s. aureus.* In our study, a bone wax was used to fill the 2-mm hole on the tibia before injecting *s. aureus,* in order to block the s. aureus solution in the bone marrow. This protocol ensured accurate dosages of *s. aureus* injected into bone marrow. The specific description has been provided in the first two paragraphs in the "Discussion" section.

Reviewer #2:

Manuscript Summary:

In this manuscript the authors endeavor to describe a new rabbit model for bone infection, which uses successive survival surgeries to develop and then treat a S.aureus infection using vancomycin loaded calcium sulfate beads vs autogenous bone harvested from the tail of the rabbit. Although this is a very important topic, the method described lacked many details necessary to allow not only a scientific assessment of the representative results shown, but more importantly, allow it to be reproduced. Additionally, the manuscript requires extensive copy-editing, which made several areas unclear. Several points are outlined below:

Major Concerns:

1. Preparing the bacterial suspension:

a. How much LB broth are you using to dissolve the freeze dried bacteria?

0.3 mL Luria-Bertani culture medium was used to dissolve the freeze dried bacteria.

The volume has been add in "line 103".

b. What OD600 was defined as mid-log phase?

We confirmed the mid-logarithmic growth phase by detecting the OD value of bacteria suspension, and drawing growth curve. In mid-logarithmic growth phase, the quantities of bacteria increased in logarithmic growth manner. We performed the protocol following the reference 14. In the step 1.5, we used McFarland's turbidimetry to estimate the bacteria concentration, following the reference 15.

c. Where the bacteria centrifuged at room temperature or 4 C?

The bacteria centrifuged at 4℃. The specific description has been added in "line 111".

2. It is unclear if 2 or three surgeries are being performed. Please clarify.

In our study, we prepared moderate and enough bacteria suspension for the following experiments. For every rabbit, the dosage of bacteria suspension was 30μL/100 g of body weight, the volume of bacteria suspension injected into bone marrow was less than 1mL. The emphasis of enough volume of bacteria suspension for more than one rabbits surgery has been added in "line 119".

3. Preparation of bone infection models:

a. How long does the anesthesia last?

The rabbits were anaesthetized by intraperitoneal injecting with pentobarbital sodium (3mg/100g of body weight). Under this dosage, the anesthesia was lasting 1 hour. The duration of molding has been added in "line 134".

b. Why should a distance of 1.5 cm be used? How large was the incision?

In our experience, the distance of 1.5 cm from the upper end of the tibia to the hole " makes the hole locate at the tibial plateau, which ensures enough space to debride and implant VCS beads and autogenous bone in the following treatment. The specific description of the reason has been added in "lines 357-360". The "1 cm incision" has been added in "line 140".

c. In step 6, are more than 1* 2-mm hole being drilled? How much bone wax was used? What was the temperature of the bone wax? How do you check if the 2-mm hole is full of bone wax and that it has not infiltrated into the marrow space? What happens if it infiltrates into the marrow space?

In step 2.6, "line 140", we punched a 2-mm diameter hole in the tibia by using electric bone drill unit. In step 2.7, "line 143", the 2-mm diameter and 2-mm height bone wax was used to fill the hole. All of the operations were under room temperature (25 ℃). We ensured the holes were full of bone wax by checking the hole with or without blood overflow. As the thickness of rabbits tibia was 2 mm, we pressed a cylinder of 2-mm diameter and 2-mm height bone wax into the 2-mm diameter holes, which ensured the bone wax filled the hole and could not infiltrate into the bone marrow. Moreover, as the bone wax was flexible and stable, it filled the holes fully and could not melt or react with bone marrow. In our study, at the 28^{th} days after infection, the bone wax was still complete and filled the holes fully. The specific discussion were added in "line 347-352".

d. What suture pattern was used? In our experience, a mattress suture is needed to prevent the animal from chewing the sutures.

The suture pattern has been added in "line 149 and 206".

e. 30 microliters/ 100 grams of weight for the suspension seems like a very large amount? What is maximum volume that can be injected? Does it put additional pressure on the marrow space?

The dosage of bacteria suspension was 30μL/100 g of body weight ("line 150"). As the weights of rabbits were more than 3000g and less than 3200g, the volume of bacteria suspension was 900μL to 960μL. Because of the slow speed of injection and blocking function of bone wax, this volume of bacteria suspension could be injected into bone marrow without high pressure. The description was added in "line 352-356".

4. Evaluation of the Bone Infection Model:

a. Step 3: How much bone marrow was plated? What happens to the bone wax? Can it hinder bone regrowth? What is the debridement procedure?

In our study, We cleaned bone wax before debridement necrotic bone. The bone wax was used to avoid bacteria suspension overflow the 2-mm holes in the molding process. Actually, in the modeling process, bone regrowth was slight that can be ignored. 1mL bone marrow was spread on top of sheep blood agar plates. The debridement procedure has been revised in "line 166-175 ".

5. Preparation of VCS Beads and Autogenous Bone

a. Was this procedure done at 28 days after the evaluation or was the evaluation done and sewn up prior to bone retrival?

VCS beads preparation was carried out before antibiotic treatment and implantation of autogenous bone. The description of autogenous bone preparation has been moved to steps 5.2- 5.5.

b. Was there any sterilization of the tail bone fragments? Was this done as a sterile procedure?

The process of molding, preparation of autogenous bone and implantaion of VCS beads and autogenous bone were under sterile environment.

c. What kind of sutures were used for the tail region to avoid chewing?

The suture was added in "line 206".

6. Antibiotic Treatment and Implantation of Autogenous Bone

a. What size incision was made in the periosteum?

A 2-cm incision in the periosteum was made, "step 3.3 , line 166" (the procudure of debride necrotic bone were added in the step 3.3).

b. Step 3: Using 2 adjacent 4-mm diameter holes seems like it would mechanically destabilize the tibia. What was the incidence of spontaneous fracture?

As 2 adjacent 4.8-mm diameter holes were punched on the tibial plateau, and the width of tibial plateau was more than 10 mm. The rabbits were housed in individual cage, which avoided collision damage on tibia. As a result the incidence of spontaneous fracture was low that can be ignored. In fact, in our study, spontaneous fracture did not happen in the infected rabbits.

c. Step 4: What is the size and shape of the implant site after debridement?

The VCS bead was cylinder of 4.8-mm diameters and 4.8-mm heights. The tail bone was detached at each joint, each autogenous bone implanted into bone defect was cylinder of 2-mm diameters and 4-mm heights. The specific sizes and shapes

were added in "lines 190, 2156 and 219".

d. Step 5: How much autogenous bone was used to fill the defect?

The bone defect was filled with 8 pieces of autogenous bones. The description was added in "line 219".

e. Step 7: Was there temperature control during the surgery? How long was the surgery?

The temperature was 25 ℃,during the surgery, see "line 223". The surgery was lasting less than 30min. After surgery, the rabbits were housed in warm cages to avoid heat loss.

7. Assessments of Antibiotic Activity:

a. Step 3: What is necrotic bone marrow? Why was there still necrosis after treatment? How much bone marrow is spread on the agar plate? What happened to the VCS beads?

In our study, the WBC and CRP indexes were used to assess antibiotic activity. As the bacteria colonies count should be carried out after euthanasia, it was not feasible to assess bacteria colonies. The "step 3" has been deleted.

i. No data or procedure found for colony counts.

The protocol of bacteria colonies counting has been deleted.

8. Assessments of Bone Regeneration:

a. How were tibia specimens extracted?

The specific protocol of tibia specimens extraction has been revised in "line 243".

b. After so much debriding wasn't bone formation compromised?

In our study, debriding necrotic bone marrow was essential for clear infection lesions. And also, 2 adjacent 4.8-mm diameter holes were punched for debriding dead bone and make the bone defects consistent. As the results showed that these holes did not affect bone formation at the end of 12 weeks after treatment. However，different sizes of bone defects will be performed in bone infection models in the further studies.

c. It is difficult to understand the timeline of all the procedures done. An overall timeline would be helpful.

The timeline of all the procedures has been added in figure 5.

d. No description of histology methods, but results shown.

The introduction of micro-CT analyze has been added in "lines 245-250".

e. Was there any determination of residual bacteria?

As the indexes of WBC and CRP were reduced significantly, at the 8 weeks after treatment, the determination of residual bacteria did not perform in our study. However, in the further study, the residual bacteria detection will be added.

9. Results:

a. There was no description of the number of rabbits in each group or the groups used. Was a power analysis done? There is no method described for statistical analysis.

In our study, 30 rabbits were infected, and subjected as model group, 10 rabbits subjected as control animals. At the end of molding, there were 3 rabbits dead because of serious infection. The remained infected rabbits were divided into three

groups, model group, VCS group, and VCS-AB group. The number of rabbits in each group, and group information have been added in "lines 255 and 262-265".

b. There was no mention of the CRP and WBC done at the 56th day in the methods, but it was shown in the results.

The CRP and WBC indexes were detected at the 2 , 4, 6, 8 weeks after treatment. The step 6.1 has been revised in "line 231".

c. Bacterial Counts weren't mentioned in the results.

The bacterial counts in the model group has been described in line "261".

d. Is VCS-AB the same as VCS-BA (line 223)?

The "VCS-BA" has been revised as "VCS-AB" in "line 273".

e. Although at least 9E7 CFU of bacteria were inoculated after 7 days, less than 5E3 CFU was found. Did the rest leak out even though bone wax was used? Were the bacteria killed by the vancomycin?

In the "preparing of bone infection models", less than 1×10^8 cfu/mL *s. aureus* solutions were injected into bone marrow, and less than 0.5×10^5 colony count could be detected in the bone marrow at the end of 7^{th} day after infection. The different amounts of bacteria might because the invasion of *s. aureus* inducing activation of autoimmune response, which reduced the amounts of active bacteria. However, at the end of 28^{th} day after infection, the colony count was more than 1.0×10^5, which illustrated the bacteria were proliferated in the closed bone marrow cavity.

f. How much bone was used to get the colony count?

1mL bone marrow was spread on top of sheep blood agar plates to calculate the bacteria colony, as concerned in "line 174".

10. Figure 4: The legend only mentions 2 data points, but more data points are shown.

The legend has been revised in "line 320".

11. Discussion:

a. What age rabbits were used? This could significantly impact the rate of bone healing.

In our study, three months rabbits were used. The animal information has been added in the "line 126".

b. More explanation is needed for the assertion that the model presented is consistent with the pathological characteristics of human disease and the surgical therapy (lines 275-276 and 284). How is infection clinically diagnosed? Just through CRP and WBC (line 292)?

Actually, there were several indexes to evaluate the bone infection pathological, and pathological characteristics in clinic. However, the serum inflammation markers and histopathology results were the most important indexes. The discussion were revised in "line 366-374".

c. What happens if chunks of bone are broken off and displaced into the marrow during the surgery and/or debridement?

The step of bone tissue cleaning has been added in "lines 171-172".

d. More detail needs to be included about how many VCS-beads should be implanted (line 295).

In our study, 4 pieces of VCS beads were implanted into the marrow. The details of VCS-beads implantation has been revised in the "lines 384-385".

e. Assertion of allograft bone being the preferred grafting material (line 305-306) seems inconsistent with the study and needs better justification.

The "allograft bone" has been revised as "autograft bone", in "line 394".

f. What is the rate of infection creation for this model?

In our study, there were 3 rabbits dead because of serious infection during modeling process. The remained rabbits identified as bone infection rabbits, and the infection rate in the remained rabbits was 100%. The specific clarification has been added in "lines 360-362".

In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.