**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 28th 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 1×108 cfu/mL *s. aureus* solutions were injected into bone marrow, and less than 0.5×105 colony count could be detected in the bone marrow at the end of 7th 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 28th day after infection, the colony count was more than 1.0×105, 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".  
  
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