Video Article

Construction and Evaluation of a Murine Calvarial Osteolysis Model by Exposure to CoCrMo Particles in Aseptic Loosening

Hui Jiang^{*1}, Yicun Wang^{*1}, Zhantao Deng^{*1,2,3}, Jiewen Jin^{2,3}, Jia Meng¹, Shuo Chen¹, Jun Wang¹, Yang Qiu¹, Ting Guo¹, Jianning Zhao¹

¹Department of Orthopedics, Jinling Hospital, School of Medicine, Nanjing University

²Center for Translational Medicine, Nanjing University Medical School

³Jiangsu Key Laboratory for Molecular Medicine, Nanjing University Medical School

*These authors contributed equally

Correspondence to: Zhantao Deng at dengzhantao_med@163.com

URL: <https://www.jove.com/video/56276>

DOI: [doi:10.3791/56276](https://doi.org/10.3791/56276)

Keywords: Medicine, Issue 132, osteolysis, aseptic loosening, murine model, CoCrMo particle, total hip arthroplasty, total knee arthroplasty

Date Published: 2/17/2018

Citation: Jiang, H., Wang, Y., Deng, Z., Jin, J., Meng, J., Chen, S., Wang, J., Qiu, Y., Guo, T., Zhao, J. Construction and Evaluation of a Murine Calvarial Osteolysis Model by Exposure to CoCrMo Particles in Aseptic Loosening. *J. Vis. Exp.* (132), e56276, doi:10.3791/56276 (2018).

Abstract

Wear particle-induced osteolysis is a major cause of aseptic loosening in arthroplasty failure, but the underlying mechanism remains unclear. Due to long follow-ups necessary for detection and sporadic occurrence, it is challenging to assess the pathogenesis of particle-induced osteolysis in clinical cases. Hence, optimal animal models are required for further studies. The murine model of calvarial osteolysis established by exposure to CoCrMo particles is an effective and valid tool for assessing the interactions between particles and various cells in aseptic loosening. In this model, CoCrMo particles were first obtained by high-vacuum three-electrode direct current and resuspended in phosphate-buffered saline at a concentration of 50 mg/mL. Then, 50 μ L of the resulting suspension was applied to the middle of the murine calvaria after separation of the cranial periosteum by sharp dissection. After two weeks, the mice were sacrificed, and calvaria specimens were harvested; qualitative and quantitative evaluations were performed by hematoxylin and eosin staining and micro computed tomography. The strengths of this model include procedure simplicity, quantitative evaluation of bone loss, rapidity of osteolysis development, potential use transgenic or knockout models, and a relatively low cost. However, this model cannot be used to assess the mechanical force and chronic effects of particles in aseptic loosening. Murine calvarial osteolysis model generated by exposure to CoCrMo particles is an ideal tool for assessing the interactions between wear particles and various cells, e.g., macrophages, fibroblasts, osteoblasts and osteoclasts, in aseptic loosening.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56276/>

Introduction

Aseptic loosening is the most common cause of total hip arthroplasty (THA) and total knee arthroplasty (TKA) failure, which requires revision surgery¹. However, the underlying mechanism remains unclear². A long follow-up is required to detect particle-induced osteolysis, whose occurrence is rare; therefore, it is challenging to explore its pathogenesis in clinical cases. Hence, further studies focusing on complex cellular and tissue mechanisms require both *in vivo* experiments in wear particle-induced osteolysis models and *in vitro* assays in cells related to bone homeostasis³. A valid animal model is important in revealing the effects of wear particles on bone loss, providing evidence for further cellular assays.

A murine calvarial osteolysis model constructed by exposure to CoCrMo particles is an effective and valid method for assessing the interactions between particles and various cells in aseptic loosening. In this model, CoCrMo particles cause calvarial osteolysis by inducing inflammatory cytokines in macrophages, activating osteoclasts, inhibiting osteoblast proliferation, and promoting osteoblast apoptosis.

It only takes two weeks to establish this model. Osteolysis can be visualized and quantified by hematoxylin and eosin (H&E) staining and micro computed tomography (micro-CT)². In addition, this model has a relatively low cost, and transgenic and knockout mouse models can be used to screen a large number of compounds at various doses³.

The procedure to establish and evaluate this model is simple. First, CoCrMo particles were obtained by high-vacuum three-electrode direct current and resuspended in phosphate-buffered saline (PBS) at a concentration of 50 mg/mL. Then, 50 μ L of the resulting suspension was applied to the middle of the murine calvaria after separation of the cranial periosteum by sharp dissection. The mice were sacrificed after two weeks, and calvaria samples were harvested; qualitative and quantitative analyses were performed by H&E staining and micro-CT.

A murine calvarial osteolysis model constructed by exposure to CoCrMo particles is an ideal tool for assessing the interactions between CoCrMo particles and various cells, such as macrophages, fibroblasts, osteoblasts, and osteoclasts, in aseptic loosening.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University.

1. CoCrMo Particle Preparation

1. Obtain CoCrMo particles by using a fabricated high-vacuum three-electrode direct current⁴. Place CoCrMo alloy in the instrument under 10^{-3} Pa vacuum, 0.04 MPa argon and hydrogen 3:2 (v/v), and 650 A cathode current.
2. Measure the diameters of CoCrMo particles.
 1. Add 1 mg of CoCrMo particles into 1.5 mL of anhydrous ethanol.
 2. Resuspend CoCrMo particles in anhydrous ethanol by ultrasonic shaking at 28 kHz and 600 W for 5 min.
 3. Apply one drop (about 20 μ L) of the resulting suspension on the objective table of a transmission electron microscope (TEM). Capture series of TEM photos at 200 kV acceleration voltage and 0.24 nm resolution.
 4. Use the provided software to calculate the mean diameter and particle size distribution in TEM micrographs.
3. Decontaminate endotoxins
 1. Autoclave 50 g of particles for 15 min at 121 °C and 15 psi.
 2. Detect endotoxins by a quantitative Limulus Amebocyte Lysate (LAL) Assay (<0.25% EU/mL was considered to indicate endotoxin absence)⁵.
4. Resuspend the particles in phosphate-buffered saline (PBS) at a concentration of 50 mg/mL as stock solution⁶.

2. Construction of the Calvarial Osteolysis Model

1. Anesthetize 6 week old C57BL/J6 mice (six mice per group) with pentobarbital (50 mg/kg). Use the pinch test to assess the level of anesthesia. Prevent drying of eyes with normal saline.
2. Place mice in the prone position. Remove the fur on the cranium with a shaver and disinfect the skin using medical cotton balls containing 75% ethanol.
3. For point localization, identify two points, including the midpoints between the two eyes and ears, respectively. Then, determine the line between the two above points, and incise the skin along the above line with scissors (**Figure 1A**).
4. Remove the cranial periosteum from the calvaria with a scalpel (**Figure 1B**)⁶.
5. Suture skin at both ends with simple interrupted suture.
6. Make a suture line through the middle of the incision without knotting. Hold the two ends of the suture line.
7. Embed 50 μ L of CoCrMo particle suspension (50 mg/mL, in PBS) in the middle of the calvarias (**Figure 1C**)².
8. Knot the last stitch within simple interrupted suture (**Figure 1D**).
9. Maintain mice for another 2 weeks.

3. Evaluation of Calvarial Osteolysis Model by Micro-CT Scanning

1. Sacrifice the mice with carbon dioxide. Decapitate the mice in the horizontal plane. Remove the brain tissue inside, and the skin and fur outside. Harvest the calvarias for further experiments.
2. Gently clear up all the soft tissue on the calvaria with tweezers. Fix the cleared calvarias in 4% paraformaldehyde at 4 °C for 24 h. Immerse the calvarias in PBS 24 h before micro-CT scanning.
3. Analyze the mice calvarias by high-resolution micro-CT at an isometric resolution of 18 μ m and X-ray energy settings of 45 kV and 550 mA.
4. Conduct three-dimensional reconstruction of micro-CT data with the software.
5. Qualitative and quantitative analysis.
 1. First, select the square region around the midline suture as the region of interest.
 2. Secondly, measure bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation/spacing (Tb.Sp), and percentage of total porosity with the provided software for micro-CT.
 3. Thirdly, compare the three groups for various measurements by one-way ANOVA. For post hoc analysis of variance, apply the Bonferroni method².

4. Evaluation of Calvarial Osteolysis Model by H&E Staining

1. Decalcify calvaria samples in 15% ethylene diamine tetraacetic acid (EDTA)-PBS at 4 °C. Change the decalcification solution every day for 3 weeks.
2. Embed the decalcified samples in paraffin for a 2 cm x 1 cm x 1 cm cube, and slice them into 2 μ m sections in the area of particle deposition.
3. Stain the sections with hematoxylin and eosin as previously described⁷.
4. Capture micrographs of the overall pathomorphism by light microscopy.

Representative Results

The in-house produced nanoscale CoCrMo particles were around 50 nm (standard error of 3.56) in diameter, as quantified by TEM (**Figure 2**). After exposure of mouse calvarias to CoCrMo particles, the animals ($n=6$ per group) were maintained for another two weeks. Within the two weeks, the calvarial incision was completely healed, and the suture may fall. Any local infection or nonunion may affect bone loss assessment. After mouse sacrifice, calvaria samples were harvested. Then, all the soft tissue was gently cleared up, and micro-CT was used to quantify bone loss. From both three-dimensional reconstruction images and representative coronal photographs at the cross-section, significant bone loss was observed in mice treated with CoCrMo particles (**Figure 3**). Bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were significantly reduced, while total porosity and trabecular separation/spacing (Tb.Sp) were significantly increased in the CoCrMo group compared with the control and sham operation groups (**Figure 4**). Student *t* test was used to assess differences between groups, and $p<0.05$ was considered statistically significant. Furthermore, H&E staining of calvaria sections confirmed bone loss in mice treated with CoCrMo particles (**Figure 5**).

Figure 1: Schematic of the particle-induced osteolysis (PIO) mouse model. The left side shows the mouse position in modeling. (A) Point localization. Determine the midpoints between the two eyes and ears, respectively, and incise the skin along the line between them. (B) Expose and remove the cranial periosteum from the calvaria. (C) Embed CoCrMo particle suspension in the middle of the calvaria. (D) Suture skin in simple interrupted suture. [Please click here to view a larger version of this figure.](#)

Figure 2: Transmission electron microscopy scanning of CoCrMo particles. (A) Representative Transmission electron microscopy images of CoCrMo particles. (B) Particle size distribution of CoCrMo particles was quantified with the software. Each bar represents frequency normalized to the total number of particles. [Please click here to view a larger version of this figure.](#)

Figure 3: Micro-CT analysis with 3-dimensional reconstruction of samples from control mice and those treated with PBS (sham operation) and CoCrMo particles. The white horizontal line indicates the location of the cross-section image. The white arrow indicates bone loss in the CoCrMo implantation group. [Please click here to view a larger version of this figure.](#)

Figure 4: Quantitative analysis of micro-CT images after three-dimensional reconstruction. Quantification of bone mineral density (BMD) (A), bone volume/total volume (BV/TV) (B), percentage of total porosity (C), trabecular number (Tb.N) (D), trabecular thickness (Tb.Th) (E), and trabecular separation/spacing (Tb.Sp) (F), in mean±standard error. Student t test was used to assess differences between the groups, with $p < 0.05$ considered statistically significant. **, $P < 0.01$; ***, $P < 0.001$. $n = 6$ mice per group. [Please click here to view a larger version of this figure.](#)

Figure 5: Representative images of H&E staining of calvaria samples (10×) from control mice and those treated with PBS (sham operation) and CoCrMo particle. Red arrow indicates osteolysis. [Please click here to view a larger version of this figure.](#)

Discussion

There are two main methods for wear particle-induced osteolysis in mice: the air-pouch model and the calvarial osteolysis model. In the air-pouch model, a subcutaneously generated air-pouch is first established, followed by wear particle introduction and implantation into the bone tissue⁸. The pouch wall mimics the periosteum in aseptic loosening. However, bone implantation is nonvascular with no biological activity, which makes it difficult to assess direct interactions between particles and the bone tissue. The calvarial osteolysis model has several advantages over the air-pouch counterpart. First, wear particles are directly exposed to the calvaria, making it possible to assess interactions between wear particles and bone homeostasis, including bone resorption, and osteoblast, osteoclast and macrophage activities^{9,10}. Secondly, quantitative measurements of bone loss are available, allowing the assessment of various potential genetic approaches and biological agents in bone loss prevention¹¹. Thirdly, it is possible to assess the relationship between wear particles and bone loss in various genetic backgrounds, including transgenic and gene knockout mice^{12,13}. Fourthly, it can be used to screen a large number of compounds at various doses. However, the success rate of the traditional calvarial osteolysis model is relatively low, and using bone histomorphometry to measure osteolysis makes the results less objective¹.

To improve the success rate of the model and render results more objective, several modifications have been made. First, nanoscale particles were used to enhance the interactions between the calvaria and wear particles. Indeed, the interactions between nanoscale particles and the calvaria is enhanced compared with commercially alloy particles, with a mean diameter of 1.5 µm^{14,15}. Secondly, a 1.0 cm² area on the calvaria was delineated to achieve adequate exposure of the calvaria. Thirdly, micro-CT and three-dimensional reconstruction were used to quantify bone loss.

There are several limitations in the present model. First, micro-CT equipment for mice is not widely available for researchers, and technicians are required for scanning and three-dimensional reconstruction. Secondly, the CoCrMo nanoparticles used in the present model are not commercially available, and their production relies on support from materials science technicians. Thirdly, this model does not represent chronic effects of particles on bone mass, and lacks non-biological factors related to osteolysis, such as oscillatory fluid pressure or mechanical forces. The efficacy of the model could be significantly increased with the help of nanoscale particles and enough exposure of the calvaria. Bone loss could be quantified, and more objective data obtained with micro-CT.

A murine calvarial osteolysis model established by exposure to CoCrMo particles is an ideal tool to assess the interactions between CoCrMo particles and different cells such as macrophages, fibroblasts, osteoblasts and osteoclasts in aseptic loosening. In addition, a series of medications can be tested of their effects on aseptic loosening using this model.

There are many critical steps of this procedure. The first is the application of CoCrMo nanoparticles with a mean diameter of 50 nm. Secondly, adequate exposure of the calvaria was achieved. A 1.0 cm² area on the calvaria was enough in this model, and the periosteum on the calvaria should be dissected carefully and completely. A clear dissection of the periosteum intensifies the interactions between the particles and calvaria. Thirdly, a quantitative measurement of bone loss by micro-CT is possible. Quantitative measurements of bone loss from three dimensional reconstruction, such as BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp and percentage of total porosity, make it easier to distinguish bone loss differences in various treatments, and provide solid evidence of osteolysis compared with the traditional bone histomorphometry.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81572111), the Clinical Science and Technology Project Foundation of Jiangsu Province (BL2012002), the Scientific Research Project of Nanjing (201402007), the Natural Science Foundation of Jiangsu Province (BK20161385), and the Special Foundation of Chinese Medical Doctor Association (2015COS0810).

References

1. Dong, L. *et al.* Antisense oligonucleotide targeting TNF- α can suppress Co-Cr-Mo particle-induced osteolysis. *J Orthop Res.* **26** (8), 1114-1120 (2008).
2. Deng, Z. *et al.* SIRT1 protects osteoblasts against particle-induced inflammatory responses and apoptosis in aseptic prosthesis loosening. *Acta Biomater.* **49** 541-554 (2017).
3. Langlois, J., & Hamadouche, M. New animal models of wear-particle osteolysis. *Int Orthop.* **35** (2), 245-251 (2011).
4. Wang, P., Zhao, F. X., & Zhang, Z. Z. Preparation of ultrafine zinc powders by DC arc plasma evaporation method. *Chinese Journal of Nonferrous Metals.* **21** (9), 2236-2241 (2011).
5. Wang, Z. *et al.* The fibroblast expression of RANKL in CoCrMo-particle-induced osteolysis is mediated by ER stress and XBP1s. *Acta Biomater.* **24** 352-360 (2015).
6. Wang, Z. *et al.* Autophagy mediated CoCrMo particle-induced peri-implant osteolysis by promoting osteoblast apoptosis. *Autophagy.* **11** (12), 2358-2369 (2015).
7. Wang, R. *et al.* Particle-induced osteolysis mediated by endoplasmic reticulum stress in prosthesis loosening. *Biomaterials.* **34** (11), 2611-2623 (2013).
8. Yang, S. Y. *et al.* Adeno-associated virus-mediated osteoprotegerin gene transfer protects against particulate polyethylene-induced osteolysis in a murine model. *Arthritis Rheum.* **46** (9), 2514-2523 (2002).
9. Liu, N. *et al.* Autophagy mediated TiAl(6)V(4) particle-induced peri-implant osteolysis by promoting expression of TNF- α . *Biochem Biophys Res Commun.* **473** (1), 133-139 (2016).
10. Wang, Z. *et al.* ER Stress Mediates TiAl6V4 Particle-Induced Peri-Implant Osteolysis by Promoting RANKL Expression in Fibroblasts. *PLoS One.* **10** (9), e0137774 (2015).
11. Wang, Z. *et al.* TiAl6V4 particles promote osteoclast formation via autophagy-mediated downregulation of interferon-beta in osteocytes. *Acta Biomater.* **48** 489-498 (2017).
12. Chen, S. *et al.* Lycorine suppresses RANKL-induced osteoclastogenesis in vitro and prevents ovariectomy-induced osteoporosis and titanium particle-induced osteolysis in vivo. *Sci Rep.* **5** 12853 (2015).
13. Neuerburg, C. *et al.* The role of calcitonin receptor signalling in polyethylene particle-induced osteolysis. *Acta Biomater.* **14** 125-132 (2015).
14. Catelas, I., & Jacobs, J. J. Biologic activity of wear particles. *Instr Course Lect.* **59** 3-16 (2010).
15. Liu, A. *et al.* The biological response to nanometre-sized polymer particles. *Acta Biomater.* **23** 38-51 (2015).