A tree shrew model for steroid-associated osteonecrosis

Dear Editor,

Osteonecrosis is a common human disease in orthopedics. It is difficult to treat, and half of patients may need artificial joint replacement, resulting in a considerable economic burden and a reduction in quality of life. Hormones are one of the major causes of osteonecrosis and high doses of corticosteroids are considered the most dangerous factor. Because of the complexity of treatment, we still need a better animal model that can be widely used in drug development and testing. Tree shrews are more closely related to primates than rodents. As such, we constructed a successful tree shrew model to establish and evaluate steroid-associated osteonecrosis (SAON). We found that low-dose lipopolysaccharide (LPS) combined with high-dose methylprednisolone (MPS) over 12 weeks could be used to establish a tree shrew model with femoral head necrosis. Serum biochemical and histological analyses showed that an ideal model was obtained. Thus, this work provides a useful animal model for the study of SAON and for the optimization of treatment methods.

SAON is a multifactorial disease, with high-dose corticosteroid administration thought to be the greatest risk factor (Amanatullah et al., 2011; Mont et al., 2006). SAON is a common refractory disease in the field of orthopedics, and its etiologies and non-operative and operative methods of treatment are complex (Wang et al., 2018). To improve preclinical research on SAON, an ideal disease animal model is urgently needed.

Animal models play key roles in identifying treatments for various types of disease, including SAON (Xu et al., 2018). In the present study, we developed a new animal model of steroid-induced femoral head necrosis using tree shrews (Tupaia belangeri, Mammalia, Scandentia, and Tupaiidae) found in the Yunnan region of China (Wang, 1987). Although rats, mice, rabbits, chickens, pigs, and emus have been used to establish various models of SAON (Beckmann et al., 2014; Bekler et al., 2007; Cui et al., 1997; Ryoo et al., 2014; Sun et al., 2011; Xi et al., 2017; Zheng et al., 2013), tree shrews are much more closely associated with primates in comparison at the behavioral, anatomical, genomic, and evolutionary levels (Bekler et al., 2007; Petruzziello et al., 2012; Xu et al., 2018; Zhang et al., 2013). Tree shrews have short reproductive and life cycles, high reproductivity, moderate size, and are easy to feed. They possess many features similar to those of humans and are frequently used as an experimental model in biomedical research (Xing et al., 2015; Yao, 2017; Ye et al., 2016). For example, tree shrews have been used in models of hepatitis virus infection, myopia, social stress, depression, metabolic diseases, and osteoporosis, and have shown many unique advantages (Wang et al., 2019; Xiao et al., 2017). With the development of genetic technology, the species will be increasingly used (Li et al., 2017; Yao, 2017). Whole-genome sequencing has revealed that tree shrews have a higher homology with humans than mice, rats, and dogs (Fan et al., 2013, 2019).

In this study, we used a low dose of LPS combined with a high dose of MPS to induce a model of femoral head necrosis in tree shrews. The model was established in 12 weeks as determined by biochemical analysis, micro-CT examinations, histological analyses, and scanning electron microscopy (SEM) (Supplementary Methods). A total of 12 healthy male tree shrews (six months old) were used in this study. All animal experiments were conducted in accordance with the guidelines created by the Kunming University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Kunming University Laboratory Animal Management Ethics Committee. Both the Guide for the Care and Use of Laboratory Animal (2011) (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2012) were followed.

Received: 24 April 2020; Accepted: 22 July 2020; Online: 29 July 2020

Open Access

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright ©2020 Editorial Office of Zoological Research, Kunming Institute of Zoology, Chinese Academy of Sciences

DOI: 10.24272/j.issn.2095-8137.2020.061
To detect changes in blood biochemical indicators, the levels of bone alkaline phosphatase (BALP), bone GLA protein (BGP), N-terminal propeptide of type I collagen (P1NP), and C-terminal propeptide of type I collagen (P1CP) in serum samples from two tree shrew groups were determined using appropriate assay kits (Supplementary Figure S1). Results showed that the levels of BALP in the SAON group were significantly higher than those in the control group ($P<0.001$; Supplementary Figure S1A). Similarly, the levels of BGP, P1NP, and P1CP in the SAON group were significantly increased compared to those in the control group ($P<0.05$; Supplementary Figure S1B–D). These results indicated that there was a significant difference between the SAON and control groups in certain aspects of blood biochemical indicators, and the increases in bone metabolism markers reflected a high bone turnover rate in the SAON group.

Micro-CT examination showed that the shape of the femoral head had changed in the SAON group, with evidence of subchondral trabecular bone deterioration (Figure 1A). Bone mineral density (BMD), bone tissue volume fraction (BV/TV), and trabecular number (Tb. N) in the SAON group were all significantly lower than those in the control group (all $P<0.05$) (Table 1; Figure 1B), whereas trabecular separation (Tb. Sp) in the SAON group was significantly higher than that in the control group ($P<0.05$) (Table 1; Figure 1B). Mean trabecular thickness (Tb. Th) in the SAON group was lower than that in the control group, but the difference was not significant ($P>0.05$; Table 1; Figure 1B). In addition, the mean bone surface/volume ratio (BS/BV) in the SAON group was higher than that in the control group, but the difference was not significant ($P>0.05$; Table 1; Figure 1B).
Table 1 Micro-CT evaluation of control and SAON groups (n=6, ±S)

<table>
<thead>
<tr>
<th>Group</th>
<th>BMD value</th>
<th>BV/TV (%)</th>
<th>BS/BV (mm)</th>
<th>Tb. Th (mm)</th>
<th>Tb. Sp (mm)</th>
<th>Tb. N (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.541±0.0.033</td>
<td>45.43±3.87</td>
<td>28.72±4.69</td>
<td>0.11±0.01</td>
<td>0.21±0.01</td>
<td>4.28±0.22</td>
</tr>
<tr>
<td>SAON</td>
<td>0.470±0.049</td>
<td>38.03±5.05</td>
<td>32.11±5.53</td>
<td>0.10±0.02</td>
<td>0.25±0.02</td>
<td>3.77±0.27</td>
</tr>
</tbody>
</table>

BMD: Bone mineral density; BV/TV: Bone tissue volume fraction; BS/BV: Bone surface/volume ratio; Tb. Th: Trabecular thickness; Tb. Sp: Trabecular separation; Tb. N: Trabecular number. Compared with control group, *: P<0.05; **: P<0.01; ***: P<0.001.

Examination of tissue sections showed that in the control group, the bone trabeculae were dense, intact, rich in bone marrow cells, and contained only a few adipose cells. In contrast, the trabecular bone in the SAON group appeared to be thinner and sparser, somewhat fractured, and displayed a disordered cellular structure; furthermore, the adipose cells were fused into vacuoles (Figure 1C). In addition, there were more empty lacunae in the SAON group than in the control group (Figure 1D). SEM also showed that in the control group, the bone trabeculae were dense, trabecular spacing was small (at low magnification, Figure 1E), trabecular surface was smooth, and bone fibers showed a dense appearance (at high magnification, Figure 1F). In the SAON group, the trabeculae were much sparser, trabecular spacing was increased (at low magnification, Figure 1G, I), and the trabecular bone surface was disorder (at high magnification, Figure 1H, J).

TUNEL staining results showed that in the control group, there were more cells in each tissue section, but fewer cells were stained green (Supplementary Figure S2A). In the SAON group, there were fewer cells in each section, but more cells were stained green (Supplementary Figure S2B). These findings suggest that more cells in the SAON group were undergoing apoptosis.

Our study employed a combined pulsed LPS and MPS induction protocol previously used for measuring steroid-induced femoral head necrosis in numerous animal models (Qin et al., 2006; Zheng et al., 2013, 2018). This induction protocol has been successfully applied to establish a rabbit model of SAON and involved a single injection of low-dose (10 μg/kg) LPS, followed by three injections of high-dose (20 mg/kg) MPS, resulting in a high incidence SAON but low rate of mortality in the treated rabbits (Qin et al., 2006). SAON has also been successfully induced in Sprague-Dawley rats by an intravenous injection of LPS (0.2 mg/kg), followed by three intraperitoneal injections of MPS (100 mg/kg) over a 24 h interval, and further intraperitoneal injections of MPS (40 mg/kg) three times per week for six weeks (Zheng et al., 2018). An emu SAON model was successfully established after 24 weeks using two intravenous injections of LPS (8 mg/kg body weight) via the jugular vein, followed by three intramuscular injections (10 mg/kg body weight into the gluteus muscle) at an interval of two days (Zheng et al., 2013). In this study, we induced SAON in tree shrews by giving one intravenous injection of LPS (300 μg/kg), followed by three intraperitoneal injections of MPS (130 mg/kg) over a 24 h interval; after which, MPS (130 mg/kg) was intraperitoneally injected two times per week for 12 weeks. The dosages and induction times of LPS and MPS are different from those used for other animals.

As biochemical markers of bone formation, BALP, BGP, P1NP, and P1CP can be used to detect osteoporosis and femoral head necrosis (Mohamed et al., 2014). BGP is secreted by bone cells (Cantatore et al., 1991), osteoblasts, and osteoclasts (Quan et al., 2012), BALP is a specific and sensitive biochemical indicator of bone metabolism, whereas P1NP and P1CP reflect osteoblast activity and bone formation. While these indicators all exist in normal blood, their levels are elevated in osteoporotic and osteoarthritic patients (Garnero et al., 1996; Szulc & Delmas, 2008). The elevated levels of these bone formation markers in our study may be due to viable osteoblasts attempting to replenish lost bone tissue (Chan et al., 2006).

As described previously, femoral head necrosis is a type of systemic skeletal disease characterized by bone loss and degradation of bone tissue microstructure, accompanied by increased bone fragility and fracture susceptibility (De Ruiter et al., 2013). It is widely accepted that BMD in the proximal femur decreases after osteonecrosis occurs (Fazzalari et al., 2002). The bone loss that occurs in the proximal femur after osteonecrosis in a remodeled femoral head results from stress shielding due to stiffness of the implant caused by bone during growth (Calder et al., 2001). Our results showed that BMD of the right femoral heads in the SAON group decreased significantly compared with that in the control group. Furthermore, examination of bone microarchitecture in the SAON group revealed rarefaction and fracture of trabecular bone due to decreased cortical bone thickness and trabecular bone area. These results suggest that bone quality was significantly lower in the SAON group.

Histological and micro-electron microscopy analyses of the femoral heads were performed, especially in the central portions of the femoral heads (Kim & Kim, 2004). Micro-CT evaluations of the femoral heads, where bone structure collapse was demonstrated, indicated that the pulsed LPS-MPS induction protocol produced femoral head necrosis, as differences in bone structure and BMD were found between the SAON and control groups in regions that were distant from the subchondral bone. This finding is similar to that reported in another bone densitometry study conducted in a steroid-induced osteonecrosis model (Janke et al., 2013).

In short, we successfully replicated a tree shrew model of human hormone-induced osteonecrosis. Thus, this work provides a useful animal model for the study of SAON and optimization of treatment methods.

**SUPPLEMENTARY DATA**

Supplementary data to this article can be found online.
COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

Z.X.M., M.H., and Y.J.L. conceived and designed the study. B.L.L., T.K.M., P.F.B., X.F.W., C.T.Y., and D.P.M. performed the experiments. Q.C., Z.X.M., H.S., and W.L.W. wrote the initial manuscript. Z.N.Y., L.B.X., Y.Y.H., and W.G.W. were responsible for data and statistical analyses. D.X.K. and J.J.D. reviewed the findings and R.P.Z. checked the acquired data, helped to analyze and interpret the data, and edited the manuscript. All authors read and approved the final version of the manuscript.

Qi Chen1,†, Zhao-Xia Ma2,†, Li-Bin Xia3,†, Zhen-Ni Ye3, Bao-Ling Liu4, Tie-Kun Ma5, Peng-Fei Bao2, Xing-Fei Wu2, Cong-Tao Yu4, Dai-Ping Ma3, Yuan-Yuan Han6, Wen-Guang Wang5, De-Xuan Kuang5, Jie-Jie Dai5, Rong-Ping Zhang6, Min Hu2, Hong Shi7,†, Wen-Lin Wang7,†, Yan-Jiao Li2,†.

1 Yunnan Key Laboratory of Primate Biomedical Research, Institute of Primate Translational Medicine, Kunming University of Science and Technology, Kunming, Yunnan 650500, China
2 Yunnan Key Laboratory for Basic Research on Bone and Joint Diseases & Yunnan Stem Cell Translational Research Center, Kunming University, Kunming, Yunnan 650214, China
3 Department Obstetrics, Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650101, China
4 Department of Nuclear Medicine, First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650031, China
5 Center of Tree Shrew Germplasm Resources, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming, Yunnan 650118, China
6 School of Chinese Materia Medica, Kunming University of Chinese Medicine, Kunming, Yunnan 650500, China
7 Kunming Medical University, Kunming, Yunnan 650500, China

*Authors contributed equally to this work.
†Corresponding authors, E-mail: shih@kust.edu.cn; wenlinwang331@163.com; 391910123@qq.com

REFERENCES

Zoological Research 41(5): 564–568, 2020 567


Supplementary Materials

Supplementary Materials and Methods

Animals, groupings, and treatment
A total of 12 healthy male tree shrews (six months old) were used in this study. The animals were purchased from the Institute of Medical Biology, Chinese Academy of Medical Sciences, Kunming, China. The tree shrews were provided with special feed and apples, with adaptive feeding for two weeks in a clean environment (temperature: 23–25 °C, relative humidity: 40%–70%, illumination time: 12 h, light intensity: ≤900 Lx, noise: ≤60 dB) (Ma et al., 2015). The animals were then randomly assigned to the experimental SAON group (n=6) or control group (n=6). Animals in the SAON group received one intravenous injection of LPS (300 μg/kg), followed by three intraperitoneal injections of MPS (130 mg/kg) over a 24 h interval, with subsequent intraperitoneal injections of MPS (130 mg/kg) two times per week until sacrifice. Animals in the control group were injected with the same volume of saline at the same time points. The tree shrews were sacrificed at 12 weeks post-induction via an intraperitoneal injection of pentobarbital sodium (100 mg/kg).

Biochemical analysis of blood samples
After the tree shrews were sacrificed, samples of blood were taken and incubated at room temperature for 2 h; after which, they were centrifuged at 3000 r/min for 10 min at 4 °C. The serum fractions were collected for biochemical analysis. Enzyme linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-Linked, China) were used to measure the levels of bone alkaline phosphatase (BALP; Cat. No. ml 627904), bone GLA protein (BGP; Cat. No. ml 625695), N-terminal propeptide of type I collagen (P1NP; Cat. No. ml 6038002), and C-terminal propeptide of type I collagen (P1CP; Cat. No. ml 6036832) in tree shrew serum.

Micro-CT scanning and analysis
After the tree shrews were sacrificed, the right femoral heads and femoral necks were removed as regions of interest (ROI), with micro-CT testing (Skyscan 1272, Belgium) then performed at the National & Regional Engineering Laboratory of Tissue Engineering, Third Military Medical University (Chongqing, China).

Bone mineral density (BMD), bone tissue volume fraction (BV/TV), bone surface/volume ratio (BS/BV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp) of the femoral heads and femoral necks were determined separately with a CT analyzer.

Histological observations
After micro-CT scanning, the right femoral heads were fixed in 4% paraformaldehyde for 72 h, and then decalcified by soaking in 25% formic acid for 3 d. Cross-sections of paraffin-embedded tissue samples were cut for hematoxylin-eosin (H&E) and TUNEL
staining. An H&E staining kit was purchased from Beijing Solarbio Sciences & Technology Co., Ltd. (China; Cat. No. G 1120). In brief, the sections were de-paraffinized, washed for 2 min, and then stained with hematoxylin for 1 min. Subsequently, the samples were washed with purified water and differentiation solution for 6 s at room temperature, then counterstained with eosin for 1 min, washed with absolute ethanol, sealed with neutral gum, and finally examined by microscopy. An in-situ cell death detection kit (Beyotime, Shanghai, China, Cat. No. C1086) was used to perform TUNEL assay according to the manufacturer’s instructions. Green fluorescent apoptotic cells were viewed under a fluorescence microscope (Nikon Ci, Japan).

Scanning electron microscopy observations
The left femoral heads were removed from both groups and bone tissues were fixed in 2.5% glutaraldehyde for 48 h. The tissues were then dehydrated with a gradient alcohol series, dried with tert-butanol, vacuum-plated, and examined by scanning electron microscopy (S-3400N, Hitachi, Tokyo, Japan).

Statistical analysis
All data were analyzed using SPSS Statistics for Windows v17.0 (SPSS, Inc., Chicago, IL, USA), and results are expressed as mean±standard deviation (SD). Student’s t-test was used to analyze statistically significant differences between groups. P-values of <0.05 were considered statistically significant.

REFERENCES

Supplementary Table S1 Micro-CT evaluation of control and SAON groups (n=6, X±S)

<table>
<thead>
<tr>
<th>Group</th>
<th>BMD value</th>
<th>BV/TV (%)</th>
<th>BS/BV (/mm)</th>
<th>Tb. Th (mm)</th>
<th>Tb. Sp (mm)</th>
<th>Tb. N (/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5410±0.0333</td>
<td>45.43±3.87</td>
<td>28.72±4.69</td>
<td>0.11±0.01</td>
<td>0.21±0.01</td>
<td>4.28±0.22</td>
</tr>
<tr>
<td>SAON</td>
<td>0.4709±0.0492*</td>
<td>38.03±5.05*</td>
<td>32.11±5.53Δ</td>
<td>0.10±0.02Δ</td>
<td>0.25±0.02**</td>
<td>3.77±0.27**</td>
</tr>
</tbody>
</table>

BMD: Bone mineral density; BV/TV: Bone tissue volume fraction; BS/BV: Bone surface/volume ratio; Tb. Th: Trabecular thickness; Tb. Sp: Trabecular separation; Tb. N: Trabecular number. Compared with control group, Δ: P>0.05, *: P<0.05, **: P<0.01.
Supplementary Figure S1 Blood biochemical indicators in tree shrews from SAON and control groups
BALP (A); BGP (B); P1NP (C); P1CP (D) were detected. All data are presented as mean±SD (n=6). *P<0.05 and ***P<0.001 vs. control group. BALP, bone alkaline phosphatase; BGP, osteocalcin; P1CP, procollagen type I C terminal propeptide; P1NP, procollagen type I N terminal propeptide.

Supplementary Figure S2 Photomicrographs of TUNEL reactions indicating presence of apoptotic cells in necrotic zones
A: There were more total cells in each tissue section, but fewer apoptotic cells of bone marrow and bone trabecula in control group.
B: There were fewer total cells in each tissue section, but more apoptotic cells of bone marrow and bone trabecula in SAON group.