Original Article

A canine model of osteonecrosis of the femoral head induced by MRI guided argon helium cryotherapy system

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Abstract: Objective: This study is to identify the reliability of osteonecrosis of the femoral head (ONFH) modeling established by MRI guided argon helium cryotherapy system in beagles. Methods: A total of 15 beagles were used to establish the ONFH model. The left femoral heads of the beagles received two cycles of argon helium freezing-thawing under MRI guidance and were considered as experimental group while the right femoral heads received only one cycle of argon helium freezing-thawing and were considered as the control group. X-ray, MRI, general shape and histological examinations were performed so as to identify the effect of modeling. Results: At 4 week after modeling, MRI showed obvious bilateral hip joint effusion and marked femoral head bone marrow high signal. At 8 week after surgery, abnormal signal appeared in bilateral femoral heads. T1WI showed irregular patchy low signal, T2WI showed irregular mixed signals and the joint capsule effusion showed long T1 and T2 changes. Twelve weeks after operation, T1WI showed a low signal strip with clear boundary and T2WI showed intermediate signal. The changes of the left femoral heads were significant while compared with those of the right sides. The lacunae rates of femoral heads in the experimental group at 4, 8, and 12 week after surgery (40.75 ± 3.77, 57.46 ± 4.01, 50.27 ± 2.98) were higher than those in control group (30.08 ± 3.61, 49.43 ± 2.82, 40.56 ± 2.73). Conclusion: Canine model of ONFH was successfully established using an argon helium cryotherapy system.

Keywords: Osteonecrosis, femoral head, magnetic resonance imaging, cryotherapy, canine model

Introduction

At present, three methods are commonly used to establish the animal model for research on the etiology and treatment of avascular necrosis of the femoral head [1-3], namely hormone induction method, alcohol induced method [4, 5] and liquid nitrogen freezing method [3, 6]. Hormone induction method and alcohol induced method are usually used for analyzing etiologies of hormones or alcohol, respectively, while liquid nitrogen freezing method is used for studying the treatment of avascular necrosis of the femoral head. However, shortcomings including time-consuming, low success rate, severe trauma and high animal mortality are found in these model establishing methods. In previous study [7], we successfully established New Zealand rabbit model of osteonecrosis of the femoral head (ONFH) using MRI guided argon helium cryotherapy system. The purpose of this study is to further identify the reliability of ONFH modeling established by MRI guided argon helium cryotherapy system in beagles.

Materials and methods

Model preparing

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Animal Care and Use Committee of Shandong University (Jinan, China).

A total of 15 healthy adult beagles weighted 10.4 ± 2.1 kg and regardless of gender were purchased from Shenyang Kangping Experimental Animal Institute. The beagles were maintained in standard conditions with free
access to food and water for one week so as to keep normal body temperature, respiration, pulse and a good mental state. Lateral longitudinal hip incision about 2 cm was performed taking the greater trochanter of femur as the center after preoperative bilateral hip skin preparation. Each layer of related tissue was separated and the trochanter was fully exposed. Miniature probe of Ipath 200 optical tracking system (Panorama; Philips Medical Systems, Vantaa, Finland) was planted 1 cm under the trochanter and a Kirschner wire with the diameter of 2 mm was drilled in parallel to the probe to a depth of 5 mm under the cartilage of the femoral head (Figure 1). Implantation of argon helium targeted probe (diameter 1.47 mm) was performed when field echo sequence (FE) and fast spin echo sequence (FSE) MRI scanning showed that the miniature probe was located well. The cryoablation parameters used were 100% refrigeration power for 10 minutes and rewarming for a minute. The left femoral heads of each beagle were applied to 2 freeze-thaw cycles and were taken as the experimental group. Meanwhile, the right femoral heads were applied to one freeze-thaw cycle and considered as the control group. After washed thoroughly with sterile saline, the incision was sutured. At the first 3 days after modeling, intra-muscular injection of cefuroxime sodium with the dosage of 1.5 g for each beagle was performed. General conditions including hip joint change, infection occurrence, mental state, weight and diet were observed.

**X-ray inspection**

At 4 week, 8 week and 12 week after operation, 5 beagles were randomly selected each time for X-ray inspection and a series of other tests. After general anesthesia, the beagles were applied to X-ray inspection in a prone position. The midpoint of both sides of the hip joint was considered as the center and bilateral hip anteroposterior scanning was performed. Imaging changes including structure and bone density of trabecular bone, bilateral femoral head appearance, hip joint space and bone cystic degeneration were detected.

**MRI scanning**

After X-ray scanning, the beagles were applied to 1.5 T nuclear magnetic resonance detection platform in lateral position. Bilateral hip MRI scanning was then performed with the hip joint positioned as the center. The conditions including femoral head signal change, bone marrow edema signal existence, hip joint space and osteoarthritic changes were observed.

**General morphological analysis of the femoral head**

After the animals were sacrificed, the hip joint capsule was exposed and cut open, and then the round ligament of femur was exposed and cut off. After that, the muscles around the femoral head and femoral shaft were removed and the femur was taken out completely. The general shape of the femoral head and the articular cartilage stripping conditions were analyzed.

**Hematoxylin and eosin (HE) staining**

With the greater trochanter of femur as the center, the removal femoral head was cut off longitudinally and was washed with saline 3 times. Then the femoral heads were fixed with 10% formalin solution for 48 hours and treated with mixed decalcifying fluid. After dehydration with alcohol the femoral heads were embedded with paraffin, cut into 4 μm sections and stained with HE. Articular cartilage, empty lacuna cell rate and trabecular bone tissue morphology changes were detected under light microscope.

**Statistical analysis**

All the statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Unpaired t-test was performed to compare the percentage of empty lacunae in 4, 8 and 12 week after modeling. \( P < 0.05 \) was considered as statistically significant.

**Results**

**General situation of the animals**

To testify the success of the modeling, general conditions of the beagles were examined. Unilateral left hind limb dared not touch the ground appeared in 5 dogs and this situation improved 2 days to 3 days after operation. The other beagles were with normal mental state, gait, diet and were without diarrhea within 24 h after surgery. Most of the wound sutures fell off
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on their own and the wounds all were primary healing. No dislocation of the hip was found in all the beagles. In conclusion, the models were successfully established.

X-ray inspection

To test the modeling result, X-ray inspection was performed. Four weeks after modeling, no obvious changes in the shape and bone density of the femoral head were found in both groups (Figure 1A). At 8 weeks after surgery, the cortical bone was complete in both groups and no obvious stenosis was found in the joint space, however, uneven bone density appeared in both groups and it was more significant in the experimental group (Figure 1B). Twelve weeks after modeling, part of the cortical bone of femoral head collapsed and the joint space became narrowed in the experimental group (Figure 1C). In the control group, the cortical bone was complete and the joint space was clear, how-

Figure 1. X-ray imaging of the beagles at different times after modeling. A. At 4 week after surgery, bilateral femoral head shape and bone density had no significant change. B. Eight weeks after modeling, bilateral femoral bone had no obvious change in shape, however, bone density was uneven and this situation was more obvious in the left side. C. At 12 week after operation, for the experimental group, the hip joint space narrowed, the cortical bone was not complete, cystic change appeared and bone density increased, however for the control group, the femoral head joint space was clear. D. For the experiment group, the cystic change was obvious.
ever, cystic changes appeared below the articular cartilage (Figure 1D). To sum up, X-ray imaging showed that bilateral ONFH model was successfully established and femur head necrosis in experimental group was more serious than in the control group.

**MRI examination**

To further determine the modeling condition, MRI examination was performed. At 4 weeks after modeling, obvious bilateral hip joint effusion and patchy high signal in femoral head in STIR phase (B). Linear low signal on the left side of the femoral head and joint effusion in STIR phase at 8 week after operation (C). After 12 weeks, STIR showed that the left femoral cortical bone was incomplete with low signal cavity formation (D).

Figure 2. MRI scanning of the beagles at different times after modeling. Four weeks after modeling, T1W showed low signal of bilateral femoral head (A) and bilateral hip joint effusion and patchy high signal in femoral head in STIR phase (B). Linear low signal on the left side of the femoral head and joint effusion in STIR phase at 8 week after operation (C). After 12 weeks, STIR showed that the left femoral cortical bone was incomplete with low signal cavity formation (D).
X-ray results, the changes of the left femoral heads was significant while compared with those of the right sides (Figure 2D). Together, MRI examination further indicated that bilateral ONFH model was successfully established.

**General shape of the femoral head observation**

To distinguish the differences between the two groups so as to identify the modeling result, general shape of the femoral heads was observed. Four weeks after modeling, the general shape of the canine femoral heads was rather normal, round and smooth. The right articular cartilage was white, whereas the left articular cartilage became dim (Figure 3A). At 8 week after operation, the femoral heads in both groups were complete, with slight cartilage denudation, and the articular cartilage was dark in color (Figure 3B). At 12 week after modeling, articular cartilage color of the left femoral head surface was uneven and the spherical surface of femoral head disappeared. Weight-bearing area of the left femoral head collapsed and the edge of the femoral head-neck junction was rough. The same situation appeared in the right femoral head, however, the condition was not that much worse (Figure 3C). These results indicated that the model was successfully established.

**Histological analysis**

To investigate the histological changes of the femoral head in both groups, histological analysis was performed. Four weeks after modeling, femoral head trabecular bone structure of the experimental group was complete, thick and arranged in neat rows. There was some empty bone lacuna and the cartilage layer structure was integrity with cartilage cells arranged in neat rows. The trabecular bone structure of the control group was similar to that of the experimental group, whereas the number of empty bone lacuna was significantly less than the experimental group (Figure 4A and 4B).

Eight weeks after surgery, cortical bone of the experimental group was integrity with slight cartilage denudation. The cartilage thickness thinned and the cartilage cells were arranged in disorder. The trabecular bone thinned, the structure was disordered and some debris was visible. Empty bone lacuna was significantly more than 4 weeks after modeling and hematopoietic tissue in bone marrow decreased, with a small amount of fibrous tissue in the necrotic area. In the control group, cortical bone was complete, cartilage cells were arranged in disorder and the trabecular bone thinned, however, these situations were lighter than those of the experimental group. Similarly to the experimental group, the rate of empty lacuna increased significantly (Figure 4C and 4D).

At 12 week after modeling, articular surface in the experimental group was damaged, the organization structure was disordered and broken, the articular cartilage surface fell away, the cartilage cells were arranged in disorder and the cartilage tidal line disappeared. A small amount of new bone tissue was found in the necrosis area, and the trabecular bone thinned.
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Figure 4. HE staining of beagles at different times after modeling. A and B. At four week after modeling, femoral head trabecular on both sides were arranged in neat rows and there were some empty bone lacuna both sides of femoral heads. The empty bone lacuna rate on the left side was larger than that of the right side. C. Eight weeks after modeling, femoral head trabecular bone of the left side fractured and was arranged in disorder. Hematopoietic tissue in bone marrow decreased, fat cell fusion in bone marrow was found and newly bone appeared. D. The right femoral head cartilage cells were arranged well and the tide line was absent or interrupted. E. At 12 weeks after modeling, the left femoral head cartilage cells arranged disorderly, the cartilage surface was not smooth and the tide line disappeared completely. F. The right femoral head trabecular was completely disordered, the fat cells fused into a bubble and new bone creeping substitution appeared.

Table 1. Comparison of empty bone lacuna rate between two groups at the different times

<table>
<thead>
<tr>
<th>Time</th>
<th>Empty bone lacuna rate (%)</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Experimental group</td>
<td>Control group</td>
</tr>
<tr>
<td>Week 4</td>
<td>40.75 ± 3.77</td>
<td>30.08 ± 3.61</td>
</tr>
<tr>
<td>Week 8</td>
<td>57.46 ± 4.01</td>
<td>49.43 ± 2.82</td>
</tr>
<tr>
<td>Week 12</td>
<td>50.27 ± 2.98</td>
<td>40.56 ± 2.73</td>
</tr>
</tbody>
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and was even shatter and disordered (Figure 4E and 4F). In the control group, articular surface damage was lighter than the experimental group, bone formation was obvious in necrosis area and trabecular bone was complete. As shown in Table 1, empty bone lacuna rate in the experimental group was significantly higher than that of the control group.

Discussion

It is shown that liquid nitrogen and alternating hot and cold can cause bone necrosis [8-12], meanwhile, studies have shown that argon helium cryotherapy can lead to osteonecrosis [13-17]. In this study, on the basis of previous research [7], argon helium cryotherapy system was used for canine ONFH model establishment. In the process of modeling, only the greater trochanter was exposed but without opening the joint capsule. MRI was used for localizing the center of the femoral head so as to avoid the position deviation and inaccurate caused by manual operation. This system not only could accurately locate the operation area, but also could control the temperature and the freezing thawing cycle time, which brought better repeatability for the future study.

In this study, at 4, 8 and 12 week after modeling, examinations including X-ray, NMR, general shape and microstructure of canine femoral head manifestations were performed so as to elaborate the evolution of argon helium cryotherapy induced canine ONFH from multiple angles. The results showed that 4 weeks after modeling, articular cartilage of the left femoral head began to darken, whereas articular cartilage of the right was normal. X-ray showed that the femoral head had no obvious change while MRI imaging showed varying degrees of joint
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effusion and femoral bone marrow edema signal in both groups. Histopathological observation found that cartilage cells on both sides were normal without interruption or absence of the tide line, the trabecular bone was arranged in neat rows, and some empty bone lacuna were found without tidal line interruption.

At 8 week after surgery, articular cartilage on the right side of the femoral head became dark and cartilage denudation was found on both sides of the femoral heads. X-ray found that bone density of the femoral head in both groups increased and was uneven. The femoral head showed zonal or focal inhomogeneity low signal intensity in MRI imaging and the left side was obvious than the right side. Histopathology showed that the left femoral head cartilage cell arrangement became sparse and part of the tide line was absent. Trabecular bone fractured and was arranged irregularly with disordered shape. Large empty bone lacuna was found and bone marrow fat cell fused into vesicles. Bone marrow mesenchymal hematopoietic cells reduced and some new bone tissue appeared. Cartilage cells on the right side arranged normal, the tide line was absent or interrupted, and the situation of trabecular bone was similar to that of the left side.

At 12 week after modeling, the femoral head surface gradually disappeared and bilateral femoral head collapsed when observed with naked eye. The left femoral head collapsed more obviously than the right femoral head and the left femoral head-neck junction edge was rough. X-ray showed that the femoral head collapsed, the femoral head surface disappeared, and there was large cystic change in left articular cartilage. Histopathology showed that the cartilage cells of left femoral head arranged disorderly, the cartilage surface was not smooth, the tide line was absent or interrupted, and the situation of trabecular bone was completely disordered. Bone repair conditions of granulation tissue and fibrous tissue around the fractured bone fragments and newly formed bone were found in the medullary cavity. The right femoral head cartilage cell arrangement was sparse with the tide line interrupted. The trabecular bone was arranged irregularly, the fat cells fused into vesicles and tissue repair of newly bone creeping substitution appeared. Totally, bone necrosis of the left femoral heads was more serious than that on the right femoral heads.

The overall pathological changes of the femoral head necrosis induced by this method was from the beginning of bone cell and cartilage cell nuclear pyknosis lysis necrosis to fat cells necrosis within the bone marrow and then to the repair process of the appearance of granulation tissue and new bone. This process was in accordance with the situations of the pathological changes of normal human femoral head necrosis. When the bone trabecula necrosy occurred, empty bone lacuna rate was high. And the severity of bone necrosis was reflected by the ratio of empty lacuna. In this study, by comparing empty bone lacuna rate between the two groups at different times, it was found that two cycles of freeze-thaw using argon helium cryotherapy system could well establish canine ONFH model.

In conclusion, argon helium cryotherapy system was successfully in establishing ONFH animal model. It had the characteristics of small operation wound, controlled necrotic area, low mortality, high success rate, repeatability and easy for standardization, which, may be used for the preparation of ONFH animal model.

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Disclosure of conflict of interest

None.

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