

Original Article

Establishment of heterotopic ossification via sharp instrument injury in rats

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Abstract

Objective: To establish an animal model for heterotopic ossification (HO) induced by sharp instrument injury in Sprague-Dawley (SD) rat and to investigate its possible mechanism. **Materials and methods:** A total of 48 male SD rats were divided into 3 groups (n=16). In sham group, incision and suture were performed only in the left leg. Partial tenotomy was performed in the left Achilles tendons in the PAT group. In Achilles' tenotomy (AT) group, tenotomy was performed in the left Achilles tendons to establish animal model of EO. X-ray and histological examinations were made at 6 and 10 weeks after operation. **Results:** No HO occurred in the sham and PAT groups. In AT group, X-ray results on 4 rats showed cartilage and bone formation while the remaining 4 rats showed chondrification in histological examination at 6 weeks after operation. At 10 weeks all rats showed bone formation with trabecular bone. This kind of HO usually develops through a process of endochondral ossification. **Conclusion:** Tenotomy is a simple, effective and feasible method to induce HO.

Keywords: Heterotopic Ossification, Mechanism, Endochondral Ossification

Introduction

In recent years, with wide application of artificial joints and popularity of open reduction internal fixation of fractures, the incidence of heterotopic ossification (HO) has remarkably increased. Its cause and pathogenesis have received widespread concerns because of the unclear pathogenesis, lack of ideal prevention and treatment methods and high disability rate. The current studies on HO are mostly concentrated on clinical prevention and treatment. Animal models are the beneficial tools for studying the bone shapes, structures and growth metabolism characteristics. This research aims to establish an effective way of inducing HO animal models and having a preliminary discussion about its formation mechanism.

Materials and methods

Experimental animals

We used 120 g to 150 g (average=140 g) male SD rats (6-8 weeks old). Animals were provided by Guangzhou University of Chinese Medicine Laboratory Animal Center.

Experimental design

A total of 48 rats were divided randomly into three groups: (i) the sham group (n=16), (ii) the partial Achilles' tenotomy (PAT) group (n=16) and (iii) the Achilles' tenotomy (AT) group (n=16). Rats were anesthetized, under aseptic conditions, with an injection of 30 mg/kg pentobarbital in the abdominal cavity. We used the left crus posterolateral approach. In the sham group we cut the skin and stitched after exposing the Achilles tendon. In the PAT group, at the 1/2 cross section of Achilles tendon midpoint we stitched the skin. In AT group, at the whole cross section of the midpoint of Achilles tendon, on both sides of fractured Achilles tendon, we used vascular clamp to repeatedly clamp 5 times, in order to cause trauma, and left Achilles unstitched, and then stitched the skin incision. Rats were fed normally and were observed after operation.

The authors have no conflict of interest.

The five authors contributed equally to this article.

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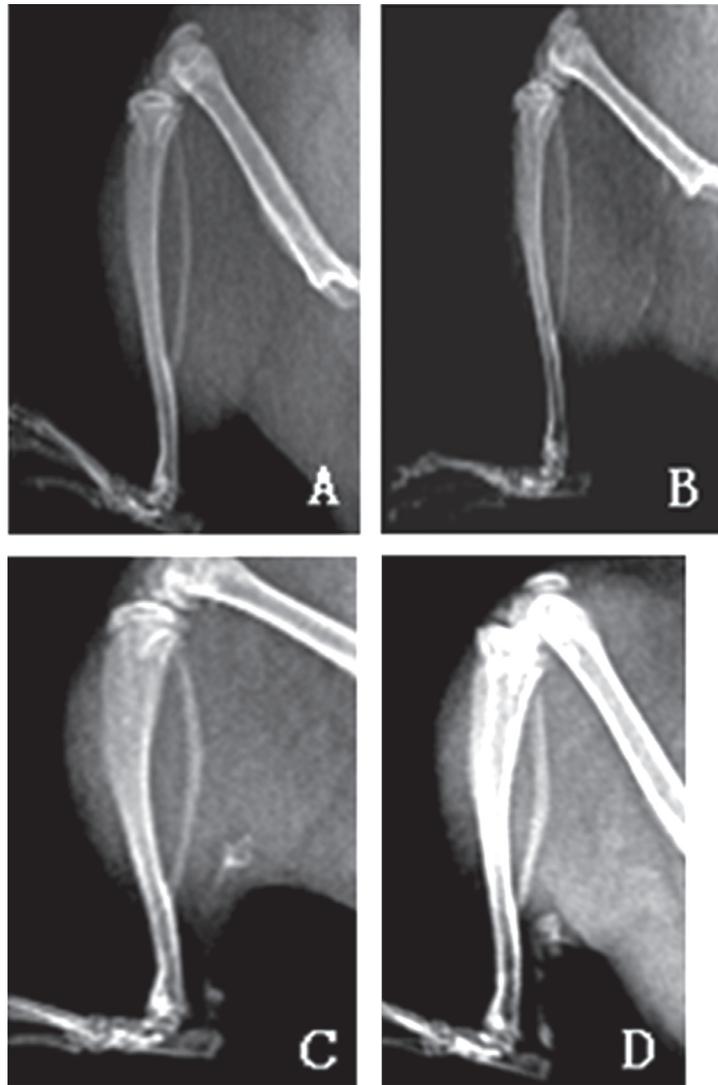


Figure 1. X-ray examination results: (A) X-ray from an animal in the sham group at the 10th week after operation; (B) X-ray from an animal in the PAT group at the 10th week after operation; (C) X-ray from an animal in the AT group at the 6th week after operation, Two high density shadow appeared at Achilles tendon section; (D) X-ray from an animal in the AT group at the 10th week after operation, high density linear shadow appeared.

Observation index

X-ray check

In order to verify whether there was any heterotopic bone formation, after the rats were anesthetized, Achilles tendon in the operated sections were examined with X-ray at 6th and the 10th postoperative week.

Naked-eye observation and histological examination of Achilles tendon

After completing X-ray examinations, the rats were sacrificed using cervical dislocation. Next we extracted materials from their Achilles tendons and examined the color, thickness,

elasticity, firmness and heterotopic bone location in rats' Achilles tendon. 10% neutral formaldehyde was used in order to fix the samples for 24 hours. Samples were then decalcified for one week in 15% EDTA. Subsequently, samples were dehydrated using gradient ethanol and embedded in transparent paraffin. We then prepared 5 μ m slices, dyed them with HE and Masson and observed samples with light microscope.

Statistical analysis

All quantitative data were expressed as mean \pm standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Sig-

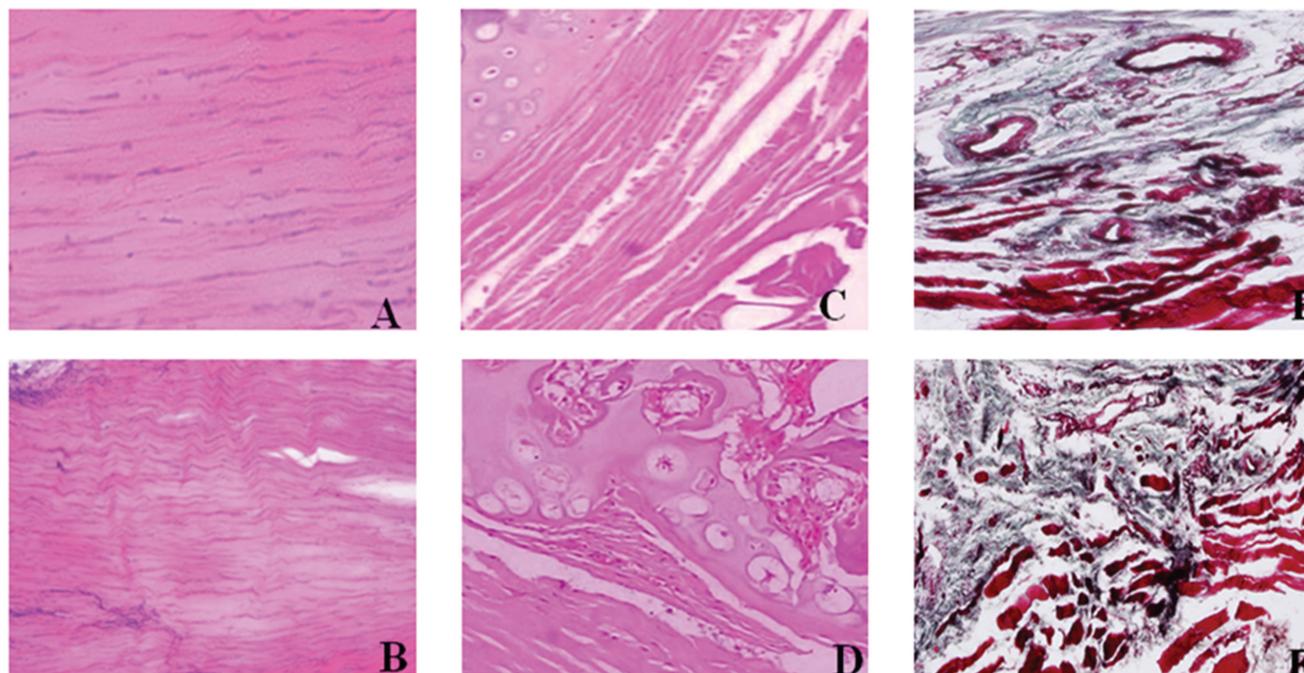


Figure 2. Histological examination results: (A) Sham group at the 10th week after operation, normal Achilles tendon tissues (HE, 400X); (B) PAT group at the 10th week after operation, degenerated Achilles tendon tissues (HE, 400X); (C) AT group at the 6th week after operation, cartilage cells seen inside degenerated Achilles tendon (HE, 400X); (D) AT group at the 10th week after operation, mature bone tissues, trabecular structure and marrow cavity could be seen (HE, 400X); (E) AT group at the 6th week after operation, bone lacuna and trabecular bone structure could be seen (Masson, 400X); (F) AT group at the 10th week after operation, mature lamellar bone structure could be seen (Masson, 400X).

nificant Difference). P values < 0.05 were considered statistically significant.

Results

The general situations of rats

On the first postoperative day, two rats in the AT group and one in PAT group died, which were replaced. The remaining animals were in good conditions and no incision infection was observed.

Result of Achilles tendon naked-eye examination

In the sham group, at the 6th and the 10th postoperative week Achilles tendon appeared white, approximately 2 to 4 mm wide and 1 mm thick. In the PAT group, at the 6th postoperative week, the color of the fracture area of Achilles tendon appeared darker, slightly swelled and coarse. At the 10th week, linked by hyperplastic fibrous connective tissues, it was hardened. In AT group, at the 6th postoperative week, the color of Achilles tendon became darker, appeared beige, the fracture edges showed signs of swelling and coarsening, it was hardened, hyperplastic tissues linked the fracture edges and the softness and elasticity reduced. At the 10th week af-

ter operation, heterotopic ossification occurred at the fracture area, extended gradually towards the two edges, adhering with the surrounding tissues.

The result of X-ray examination

At the 6th postoperative week, HO was not observed in the sham or the PAT group (Figure 1-A, B); HO was observed in 4 rats in the AT group with 2 circular high density shadows at Achilles tendon, at the middle of which there was a separating gap (Figure 1-C). At the 10th postoperative week, HO was not observed in the sham and the PAT group. In the AT group, HO occurred in all rats with linear high density shadow observed at Achilles tendon section (Figure 1-D).

Histological examination results

At the 6th and the 10th postoperative week, histological examination on the sham and PAT groups samples, demonstrated no cartilage or bone formation (Figure 2-A, B). In the AT group at 6th postoperative week, cartilage cells with bone lacuna and trabecular bone structure were observed (Figure 2-C, E); at the 10th week after operation, mature bone tissues, trabecular structure, marrow cavity and lamellar bone structure could be seen inside the slices (Figure 2-D, F).

Discussion

HO refers to the bone formation in soft tissues where bone normally does not exist. Based on the etiology, HO can be classified into 3 types: traumatic, neurogenic and genotype¹ from which the traumatic is the most common type. Since the exact mechanism of HO formation remains unclear; animal models are useful tools in any research on the HO formation mechanism. Hence, many scholars have probed into various types of modeling methods. Walton² used the repeated blunt injuring sheep thigh muscles method to induce heterotopic ossification. Histological examination in that study suggested that the heterotopic bone is formed through intramembranous ossification. Feng³ used violent massage and straight fixing rabbit knee joints to induce heterotopic ossification inside quadriceps, and histological examination proved that this kind of heterotopic bone is formed through the perichondral ossification. Xianfeng⁴ firstly induced rabbit spinal cord injury, and then adopted the method of combining long-term fixation of knee joints and intermittent negative activity to induce HO. Lin⁵ and Kan⁶ used transgenesis techniques to successfully create animal models of HO. As for the above mentioned models, most of the scholars hold positive opinion towards them while some others are still skeptical.

In our previous study, we used this method to establish an animal model of HO and we achieved good results in our subsequent experiments⁷. In the present study, we introduced this method of HO model establishment in detail. We applied Achilles tendon cutting method to induce heterotopic ossification successfully in a relatively short period of time. The method we used was very convenient, effective and doable with steady results and good repeatability. This type of animal model in respect to shape and radiology is relatively similar to the HO pathological state caused by clinical injury or surgery. Therefore, this animal model is efficient for studying the pathology and mechanisms of HO pathogenesis, which lays the foundation for early diagnosis, especially for the specification of the degree of ossification activity which can also be applied for treatment studies.

There was no difference between HO and bone callus forming in histology. Three conditions are required for HO: (i) precursor cells (target cells); (ii) osteogenesis inducing factor; (iii) suitable environment⁸. Undifferentiated mesenchymal stem cells with osteogenesis potential in soft tissues play an important role in the development process of HO, but it cannot independently transform into HO in the absence of osteogenesis inducing factors^{9,10}. According to the histology results obtained in this study, cutting the Achilles tendon in rats led to the formation of cartilage and bone during the repair process. Ossification first appeared as proliferation and swelling of cartilage cells, followed by matrix calcification and blood vessel formation. Subsequently, we observed cartilage bone transformation, reconstruction of osteoclasts and osteoblasts. We assume that the injury caused by Achilles tendon rupture created tissue necrosis and local hypoxia envi-

ronment which led to macrophage aggregation and response which were followed by the release of tumor necrosis factor (TNF), interleukin-1 (IL-1) and other inflammatory mediators. These inflammatory mediators stimulated the formation of mononuclear cells, endothelial cells, cartilage cells, osteoblasts inner oxidase 2 (COX-2) mRNA and caused the release of prostaglandin. Inflammatory mediators also induced the release of *in vivo* BMPs¹¹, prolactin and bFGF. Previous studies proved that humoral factors, such as prostaglandin¹²⁻¹⁴, BMPs, prolactin and bFGF, played a significant part in the process of bone formation, repair and reconstruction as well as in heterotopic ossification^{7,10,11,16,17}. These mediators promote the bone formation by stimulating the proliferation and differentiation of mesenchymal stem cells and osteoblasts. In some cases, inflammatory mediators also stimulate the synthesis and mineralization of extracellular matrix and stimulating the blood vessel formation¹⁸.

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