Celecoxib alleviates traumatic myositis ossificans by inhibiting bone morphogenetic protein-4 in rats

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Abstract

Background: Bone morphogenetic protein-4 (BMP-4) is one of the important molecules regulating the formation and differentiation of bone tissue. It is the main active medium to induce ectopic ossification, and is mainly involved in bone growth and repair of wounds.

Methods: To investigate the expression and significance of BMP-4 in rat model of traumatic myositis ossificans (TMO), 60 SD rats aged 4-6 weeks were randomly divided into control group, model group and celecoxib group, and TMO model was established at the right Achilles tendon. Each rat in the celecoxib group was intragastrically administered with 10 mg of celecoxib per kilogram per day. Each rat in the other group was intragastrically administered with 2 mL of physiological saline per day. At the 5th week and the 10th week after operation, 30 rats were killed. X-ray film was used to examine the formation of new bone, and skin temperature of the affected side was measured before execution. BMP-4 protein and mRNA level, and IL-2 mRNA level in the Achilles tendon tissues were detected by western blotting and RT-qPCR. Before execution, 2 mL of venous blood was taken from rats, and the level of IL-2 in serum was detected by ELISA.

Results: The results showed that compared with the model group, in the celecoxib group, the swelling of the affected side was significantly alleviated, the overall formation rate was slower, the osteogenesis time was later, the ossification degree was lighter, the BMP-4 mRNA and protein levels in the Achilles tendon tissues of the affected side were significantly lower, and the IL-2 content in the blood and the mRNA of IL-2 in the tissues were significantly lower.

Conclusions: In conclusion, celecoxib can effectively alleviate the occurrence of TMO after Achilles tendon surgery in rats by inhibiting BMP-4 and IL-2 expression.

Background

Traumatic myositis ossificans (TMO) is a kind of heterotopic ossification secondary to trauma or surgery, and its pathological features are local fibrous hyperplasia, calcification and metaplasia, accompanied by heterotopic new bone formation[1]. The current studies suggest the occurrence mechanism of TMO is that after trauma and surgical stimulation, local bleeding, oozy and inflammatory cell infiltration, all kinds of active cells, and bone growth factor are involved in hematomas gradually turning into bone tissue by the induction of the internalization of bone or cartilage membrane internalization of bone, which affects the function of muscle contraction and leads to anchylosis[2].

Bone morphogenetic protein (BMP) whose target cells are undifferentiated active mesenchymal cells, is the main active medium inducing heterotopic ossification, and is mainly involved in bone growth, development and wound repair through the induction of mesenchymal cells around the muscles and blood vessels, and the irreversible differentiation of chondrocyte and osteocyte[3]. Bone morphogenetic protein-4 (BMP-4) is one of the important molecules to regulate the formation and differentiation of bone

tissue[4]. In addition, our previous studies demonstrated the efficacy of celecoxib in the prevention and treatment of TMO[5].

Based on the anti-inflammatory effect of celecoxib and osteogenesis effect of BMP-4, TMO rats model was constructed in the study to observe the inflammatory reaction, determinate the expression of BMP of Achilles tendon tissue in rat models, and analyze the expression and the significance of BMP from the level of gene, so as to provide experimental basis for the prevention and treatment of TMO.

Methods

Animal experiment

All animal experiments were performed by senior attending physicians, were approved by the Institutional Ethics Committee of Quanzhou First Hospital Affiliated to Fujian Medical University and were performed in accordance with a protocol approved by the Animal Care and Use Committee of Fujian Medical University. 60 male SD rats purchased from Animal Experiment Center of Shandong University were housed individually with free access to water and chow with constant 12-hour light-dark cycle in transparent plastic cages that were free of specific pathogens. They were all acclimatized for 1 week before the experiment began. At the end of the study, all SD rats used in this study were placed in the euthanasia chamber and then euthanized by introducing 100% CO_2 gas at a flow rate of 20% to 30% of the chamber volume per minute.

They were randomly divided into two batches, 5th-week batch and 10th-week batch, which was divided into the control group, the model group and the celecoxib group in each batch. There were 10 SD rats in each group. After anesthetized with isoflurane (Attane vet, 1000 mg/g, Oiramal Healthcare, UK), the rats were fixed on the operating table. After hair removal on the surgery area and disinfection with iodine, the skin of the rat right Achilles tendon was cut lengthwise through a 1 cm incision by a sterile surgical blade, revealing the end of the Achilles tendon. The Achilles tendon nearly the end was cut laterally. The whole incision was sutured postoperatively. Starting from the first day after the operation, the rats in the celecoxib group were given celecoxib (Gibco, USA) 10 mg/ (kg/d) by gavage. The first batch was given gavage for 5 weeks, while the second batch was given gavage for 10 weeks. The model group and the control group were given 2 mL normal saline by gavage. The groups were given a gavage once a day. Observation was made once every other day after the operation, and observation was made once a week after the first week.

Observation and X-ray results of TMO model in rats

Skin temperature and swelling of limbs of rats in each group were observed at 5th or 10th postoperative week, and the formation of heterotopic ossification was observed by taking films under anesthesia. Swelling at the injury point was counted as 1 point, at knee joint was counted as 2 points, above knee joint was counted as 3 points, and no swelling was counted as 0 point. The ossification (or calcification) of rat Achilles tendon was evaluated by referring to the classification method of ossification degree by

Weiliang Chen et al[6]. After the completion of the film, bilateral Achilles tendon tissues were revealed again, and the color, thickness, elasticity, number and location of ectopic bone of the Achilles tendon of the rats were compared and observed.

Real-time quantitative PCR(RT-qPCR)

The Achilles tendon was cut off at the junction between the tendon and gastrocnemius muscle and the junction with the calcaneus bone. The Achilles tendon specimens were removed completely and other tissues were removed. After washing with normal saline, the rat Achilles tendon was divided into three parts along the sagittal axis of the Achilles tendon. The ossification site tissues were milled with liquid nitrogen. RNA was extracted with Trizol (Invitrogen, USA), complementary cDNA was synthesized by Super Script III reverse transcriptase (Life Technologies, USA), and BMP-4 gene and IL-2 gene expression was quantitatively analyzed using Power SYBR Green PCR Master Mix and StepOnePlus Real Time PCR System (Thermo Fisher Scientific, USA). The primer sequences used are shown in table 1.

Western blot analysis

After surgery 5th or 10th week, some Achilles tendon tissues were cut in RPA lysate at low temperature, incubated on ice for 30 min, centrifuged at 12000 r/min at 4℃ for 30 min, and then the supernatant was obtained. BCA kit (Beyotime, China) was used for protein quantification. The protein samples were added appropriate volume of 5×SDS sample buffer (including β-mercaptoethanol), mixt well, boiling water bath for 10 min, and made fully denatured. The expression of BMP-4 protein was detected by Western blotting. The antibody anti-BMP-4（1:500, #ab39937, abcam, USA）and anti-β-actin (1:1000, #AA128, Beyotime, China) were as primary antibody. Anti-rabbit IgG HRP-linked antibody (1:2000, #7074, CST, USA) and anti-mouse IgG HRP-linked antibody (1:2000, #7076, CST, USA) were as secondary antibody.

Immunohistochemical examination

After fixed with 4% paraformaldehyde fixative at room temperature and embedded in paraffin, the part of the tissue was sliced. After dewaxed for 5 min at 60℃ in a baking machine, the slices were washed with PBS twice. Subsequently, the slices were soaked in a dye box filled with citrate buffer, heated in the microwave for 30 minutes, cooled to room temperature, and washed with PBS 3 times. After sealed with 5% donkey serum, the slices were incubated with primary antibody overnight at 4℃. After incubated the secondary antibody, the slices were observed under microscope.

Determination of IL-2 in serum

2 ml of venous blood was extracted from rats at 5th and 10th week after the operation, and the serum was separated by centrifugation. The determination of IL-2 in serum was performed in accordance with the kit instructions (EK0399, BOSTER, China).

Statistical analysis

SPSS13.0 statistical software package was used for analysis. The measurement data were expressed as mean±standard deviation, and one-way ANOVA was used for comparison between groups. The statistical differences were detected among control group, model group and celecoxib group. Statistical significance was accepted at $P < 0.05$.

Results

Statistical results of body temperature and swelling as well as image results

In the control group, there was no significant change in the whole process. At the 5th week after surgery, the skin temperature of the rat in the celecoxib group was higher than that in the model group (Table 2). Compared with the model group, the affected side of the rat in the celecoxib group swelled obviously, and the difference was significant ($P<0.05$) (Table 3 and Figure 1). At the 10th week after the operation, compared with the model group, in the celecoxib group, the skin temperature and the swelling was significantly alleviated (Table 2 and Figure 1).

At the 5th week after surgery, in the model group, the Achilles tendon repaired itself and there was adhesion, congestion and edema at the affected side of Achilles tendon. Compared with the uninjured side, the diameter of the affected side thickened, and elasticity decreased and hardened. A transparent fusiform expansion can be seen at the anastomosis, the activity is poor, and the X-ray suggests heterotopic ossification (Level II). Compared with the model group, the celecoxib group had less congestion and edema, and the anastomosis is connected by tendon tissue. The sliding ability of the Achilles tendon in celecoxib group was better than that of the model group, and the X-ray showed heterotopic ossification (Level I). At the 10th week after surgery, in the model group, hard bone-like nodules were reached at the Achilles tendon, the activity was poor, and the swelling was obvious compared with the contralateral side. At the distal end of the Achilles tendon, the X-rays all indicated heterotopic ossification (Level III). In the celecoxib group, the degree of hemorrhage and edema was significantly reduced, and the adhesion of the sputum was obvious. The sliding ability of the Achilles tendon was better than that of the control group. In the celecoxib group, 3 rats were heterotopic ossified grade III, and the remaining heterotopic ossification was grade II. With the increase of the experimental period, the Achilles tendon of rats in the model group and the celecoxib group gradually thickened, and there was ectopic bone formation. However, the overall formation rate of the osteogenesis in the model group where the osteogenesis time was earlier was faster than that in the celecoxib group, and the difference was significant ($p<0.05$). The general observation is shown in Figure 1. The X-ray imaging observation is shown in Figure 2. The statistical results are shown in Table 4.

RT-qPCR detection of BMP-4 gene expression in rat Achilles tendon tissue and protein western blot results

At the 5th week after operation, in the model group, the expression of BMP-4 gene in the right Achilles tendon of the rats was significantly higher than that in the left Achilles tendon, and compared with the model group, the BMP-4 gene expression in the right axillary tissue of the rats is significantly reduced in the celecoxib group (Figure 3A). With the extension of the administration time, the expression of BMP-4

gene in the Achilles tendon tissue of the rats in the celecoxib group was significantly different from that in the model group (Figure 3B). This indicated that celecoxib can significantly inhibit the expression of BMP-4 gene in a heterotopic ossification rat model. Next, we used western blotting to examine the expression of BMP-4 protein in tissues. It was also found that the BMP-4 protein in the ossified tissue of rats fed with celecoxib was significantly lower than that of the model group (Figure 3C-3D).

Immunohistochemical analysis of BMP-4 expression in Achilles tendon tissue

At the 5th week after operation, the positive rate of BMP-4 protein expression in the surgical site was significantly higher than that of the left leg at the same rat in the model group. Compared with the model group, the positive rate of the BMP-4 expression in the Achilles tendon tissue of the rats was significantly decreased in the celecoxib group (**$p<0.01$) (Figure 4A). What's more, the positive rate of BMP-4 expression in the Achilles tendon tissue of the celecoxib group was significantly different from that of the model group as the administration time prolonged (***$p<0.001$) (Figure 4B). These data further demonstrated that celecoxib may inhibit ossification by reducing the expression of BMP-4 protein in in rat Achilles tendon tissue.

Changes and comparison of serum inflammatory factors between model group and celecoxib group

In order to detect the changes of inflammatory factors in blood and tissues of rats, we used ELISA to detect the content of IL-2 in the blood of rats. It was found that the levels of IL-2 in blood were obvious increased at 5th week after operation in the model group. In addition, the IL-2 content in the blood of the rats in the celecoxib group was significantly decreased, and there was a significant difference ($p<0.01$). The result is shown in Figure 5A-5B. Next, we analyzed the expression of IL-2 gene in Achilles tendon tissue by RT-qPCR. The results are shown in the Figure 5C and 5D. Compared with the control group, IL-2 gene expression was significantly increased in the model group where IL-2 expression was significantly higher in the Achilles tendon tissue at the celecoxib group ($p<0.01$), and this difference was more pronounced with the prolonged administration time. The above results indicated that celecoxib may affect ossification by inhibiting the expression of inflammatory factor IL-2 in rats.

Discussion

BMP is a low molecular weight hydrophobic glycoprotein that induces mesenchymal stem cells to differentiate into osteoblasts, is transformed into osteoblasts by transforming mesenchymal tissue, induces heterotopic ossification, and promotes the deposition of hydroxyphosphate and calcium[7]. It is a cytokine that induces bone formation and has an important effect on bone growth and remodeling. Therefore, when exploring the mechanisms involved in bone growth and remodeling, it is necessary to detect the BMP levels in different periods of bone growth and remodeling to understand the expression and distribution of BMP[8]. BMP-4 was purified and cloned by Wozney et al in 1988. Since its DNA sequence is similar to that of BMP-2, it was originally called BMP-2B[9]. Kuroda's studies showed that mouse-derived stem cells expressing BMP-4 produce hyaline cartilage in articular cartilage defects of rodent and do not degrade or ossify even after 6 months[10]. Experimental studies by domestic scholars

have shown that BMP-4 recombinant adenovirus can successfully induce heterotopic ossification in the muscle of nude mice, further verifying the osteogenic effect of BMP-4[11, 12]. It has been reported that by inhibiting the synthesis of BMP-4, the formation of traumatic ectopic ossification could be inhibited, which confirmed the role of BMP-4 in ectopic ossification from the reverse side[13].

Studies have shown that Achilles tendon severance, subsequent tissue necrosis and local hypoxic environment lead to macrophage aggregation[14], while reducing the differentiation and proliferation of mesenchymal stem cells[15]. Macrophages reactively release some inflammatory mediators such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), which can up-regulate the expression of cyclooxygenase 2 (COX-2) mRNA in monocytes, vascular endothelial cells, chondrocytes, and osteoblasts, leading to the release of prostaglandins[16]. Inflammatory mediators can also lead to increased release of BMPs, prolactin, bFGF, etc[17]. Prostaglandins, BMPs, prolactin and bFGF play an important role in formation, repair and reconstruction of bone, and even in heterotopic ossification, which promotes bone formation by stimulating the proliferation and differentiation of mesenchymal stem cells and osteoblasts, stimulating the synthesis and mineralization of extracellular matrices, and stimulating angiogenesis[18]. Ossification is caused by collagen metaplasia of connective tissue, which is thought to originate from pluripotent stem cells in soft tissues, mainly mesenchymal stem cells[19, 20]. Under the action of stimulating factors, local osteogenic factors are secreted, and in a suitable osteogenesis environment, mesenchymal stem cells gradually differentiate into osteoblasts, eventually forming new bone in the tissue[21]. However, studies have directly demonstrated that BMP-4 promotes the expression of germ cell-specific genes in bone marrow mesenchymal stem cells[22]. In summary, inflammation is the starting point of heterotopic ossification. Inflammatory factors in macrophages can promote the release of active TGFβ. BMPs are an important component of the TGFβ family. TGFβ can drive ectopic ossification and inhibition of TGFβ can inhibit the progression of heterotopic ossification. Therefore, inhibition of inflammatory response is an important measure to inhibit heterotopic ossification. Currently, non-selective inhibitors (indomethacin) and selective inhibitors (celecoxib) are commonly used for clinical prevention[23]. Non-selective inhibitors are controversial in terms of application effect, and the effect is not clear[24]. The selective inhibitors represented by celecoxib have clear effects in clinical application and scientific research, but the mechanism is not completely clear[25–28].

The method of Achilles tendon severing successfully induces TMO in a short period of time. This method is simple, effective and easy, and the results are stable and reproducible. The animal model is similar in morphology and radiology to the pathological state of TMO caused by clinical trauma or surgery, which is helpful for studying the pathology and pathogenesis of TMO. In particular, it lays the foundation for judging the degree of ossification activity for early diagnosis, which is consistent with the results of relevant foreign studies[29]. In this experiment, the TMO animal model was established by cutting off the Achilles tendon, and the selective inhibitor (celecoxib) was used to inhibit the local inflammatory reaction. The clinical symptoms were observed by gross and imaging. The BMP-4 content of the sample was detected by western blot and RT-qPCR. The process of inhibition of ossification by celecoxib was observed by immunohistochemistry and serum IL-2 levels. According to the experimental results, the expression of serum IL-2 was inhibited, the local inflammatory reaction was reduced and ossification was

inhibited by celecoxib in the celecoxib group. The expression of BMP-4 gene in the celecoxib group was significantly lower than that in the model group, indicating that celecoxib can effectively inhibit the expression of BMP-4 gene, thereby inhibiting the formation of heterotopic ossification in rats.

Conclusions

Based on the above research and discussion, our study found that the expression of BMP-4 in TMO rat model is positively correlated with the degree of ossification, and celecoxib can effectively alleviate the postoperative inflammatory response and inhibit the expression of BMP-4 in rats, thereby slowing the occurrence of TMO, which provided an experimental basis for the prevention and treatment of TMO.

Abbreviations

BMP: bone morphogenetic protein; TMO: traumatic myositis ossificans; COX-2: cyclooxygenase 2

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of Quanzhou First Hospital Affiliated to Fujian Medical University. Each participant signed an informed consent form. All experiments using animals were performed in accordance with a protocol approved by the Animal Care and Use Committee of Fujian Medical University.

Consent to publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ML and RZ designed the study, raised experimental animals, performed the animal experiments, analyzed the data, and drafted the manuscript. JS was responsible for RT-qPCR and Western blot. JX was responsible for immunohistochemical examination and ELISA. It should be noted that ML and RZ contributed equally to this work. All authors discussed the results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript for publication.

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Not applicable

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Tables

Table 1. The primer sequences

Gene name	Primer	Sequences
β-actin	forward	5′-GATGGTGGGTATGGGTCAGAAGGA-3′
	reverse	5′-AGTTGGTGACAATGCCGTGTTCAA-3′
BMP-4	forward	5′-GCCAAGCGTAGTCCCAAGCATC-3′
	reverse	5′-TTCCAGCCCACGTCACTGAAGT-3′
IL-2	forward	5′-GCTTTCACTTGGAAGACGCTGGA-3′
	reverse	5′-GGCTCATCATCGAATTGGCACTCA-3′

Table 2. The skin temperature after operation (℃) (n=10)

Time	Celecoxib group	Model group	T value	P value
5th week	37.05±0.51℃	37.61±0.32℃	2.941	0.009
10th week	36.3±0.41℃	37.12±0.65℃	3.333	0.004

Table 3. The degree of swelling after operation(n=10)

Time	Celecoxib group	Model group	T value	P value
5th week	0.54±0.13	1.26±0.31	3.557	0.002
10th week	0.85±0.21	1.98±0.56	3.969	0.001

Table 4. Statistical results of ossification degree

Time	5th week				10th week			
Level	0	□	□	□	0	□	□	□
Celecoxib group	3	6	1	0	0	1	8	1
Model group	1	1	8	0	0	1	2	7
X value	X=17.629				X=17.880			
P value	<0.001				<0.001			

Figures

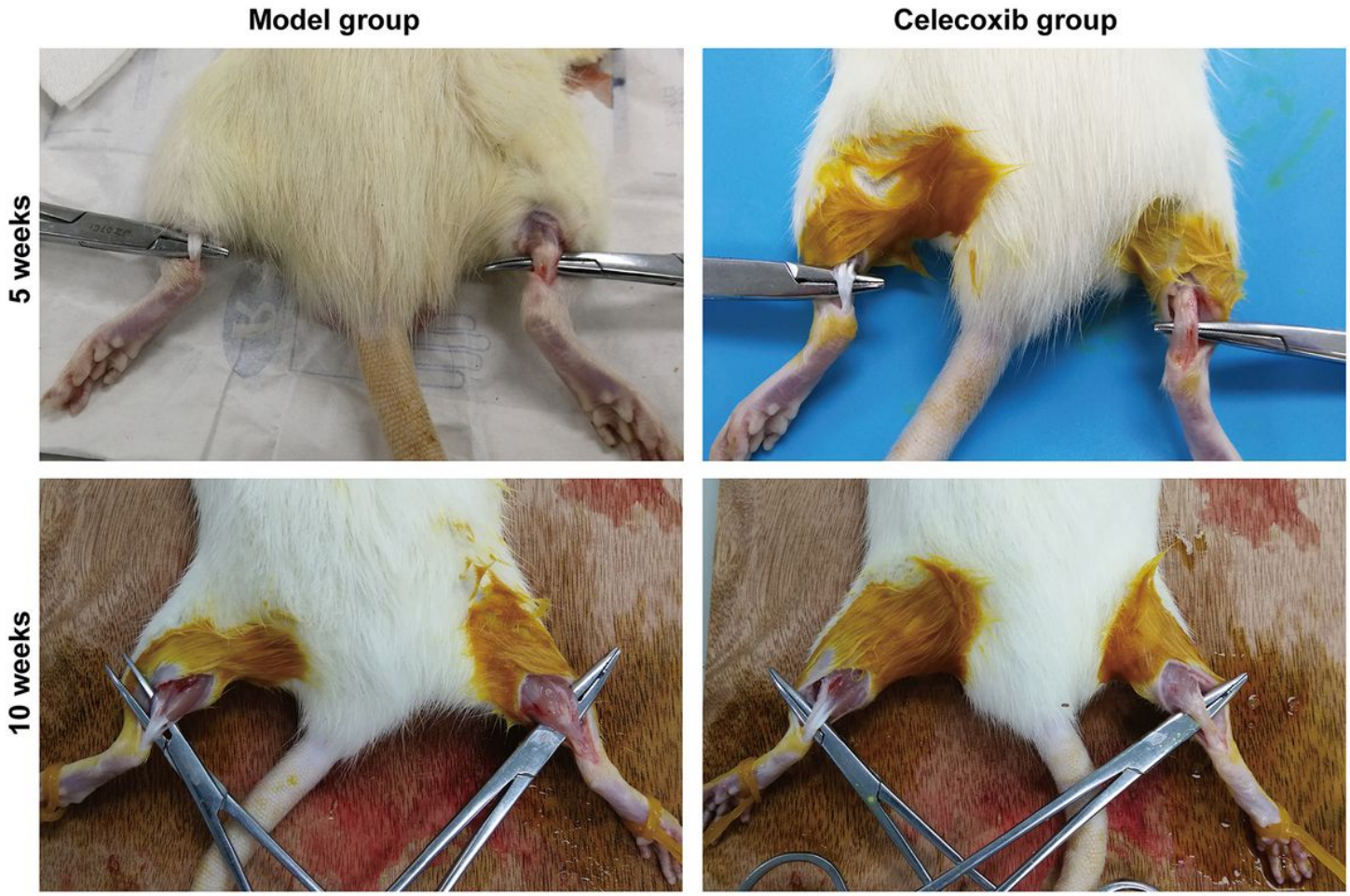

Figure 1

The swelling of Achilles tendon at the 5th and 10th week after operation(n=10). At the 5th and 10th week after operation, general contrast between the left and the right Achilles tendons in the model group and the celecoxib group. At the 10th week after operation, the right Achilles tendons of rats in the model group showed obvious heterotopic ossification.

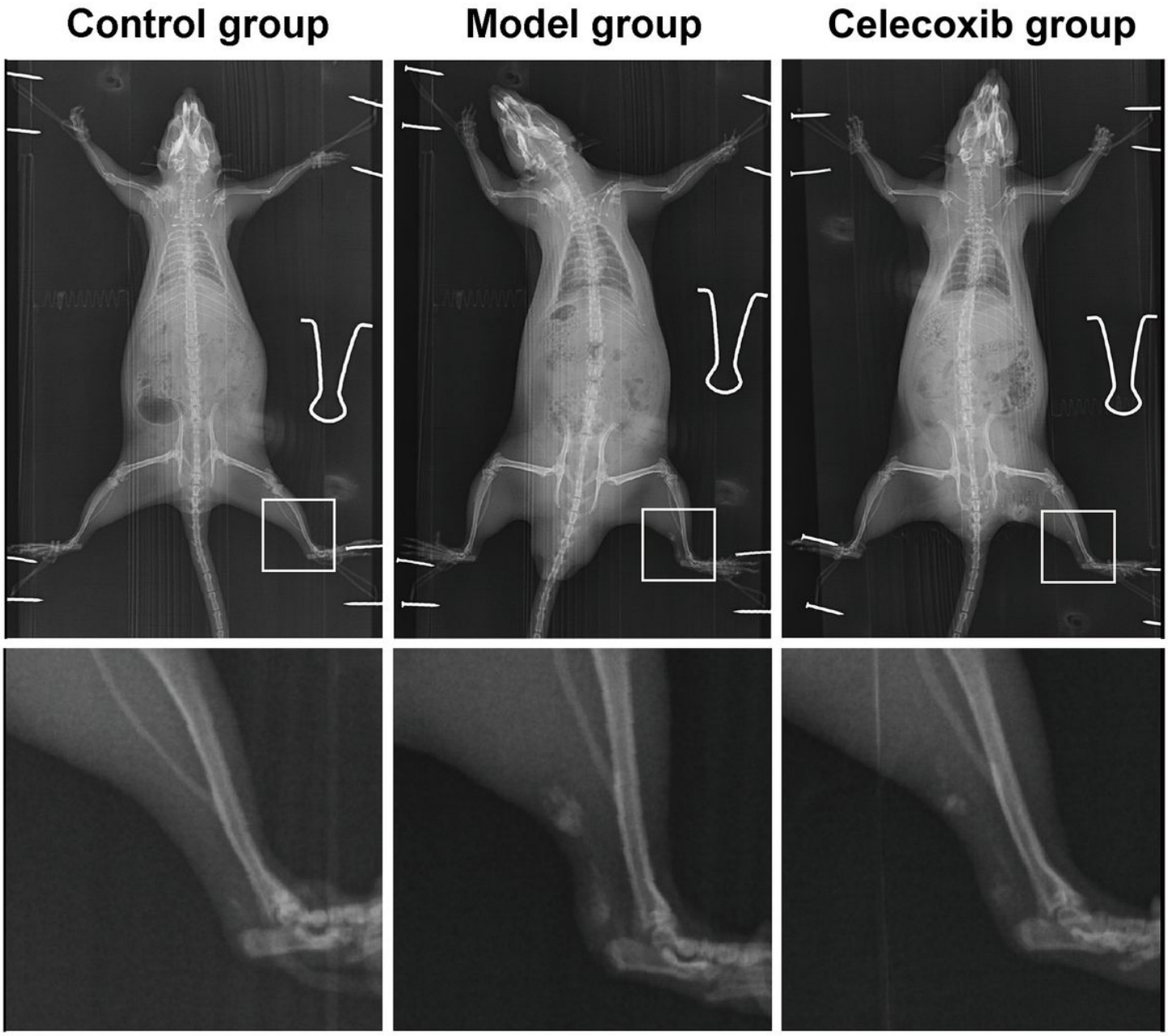

Figure 2

The formation of ectopic ossification at the 10th week after operation(n=10). Left: no ossification occurred in the control group; middle: ossification formation in the model group; right: ossification formation in the celecoxib group.

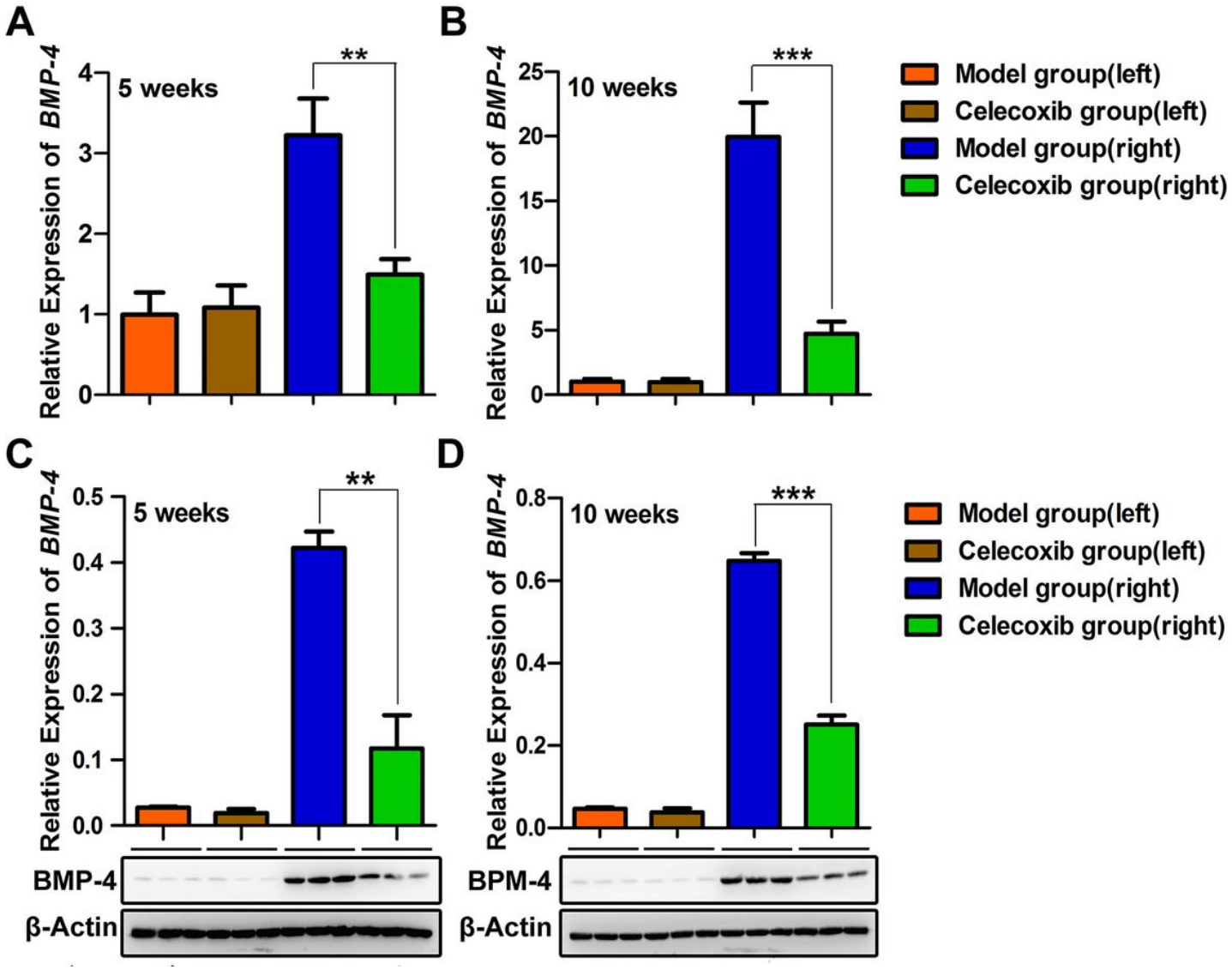

Figure 3

Celecoxib inhibits the expression of BMP-4 gene and protein(n=10). A: At 5th week postoperatively, the effect of celecoxib on the mRNA of BMP-4 in heterotopic ossification model of rats was detected by RT-qPCR. B: At 10th week postoperatively, the effect of celecoxib on the expression of BMP-4 gene in heterotopic ossification model of rats was detected by RT-qPCR. C and D: At the 5th week and the 10th week after operation, the expression of BMP-4 protein in the Achilles tendon tissues was detected by western blotting. The gray scale of the bands was analyzed by Image J. All data represent the mean ± standard deviation; **P<0.005, ***p<0.001 by one-way ANOVA.

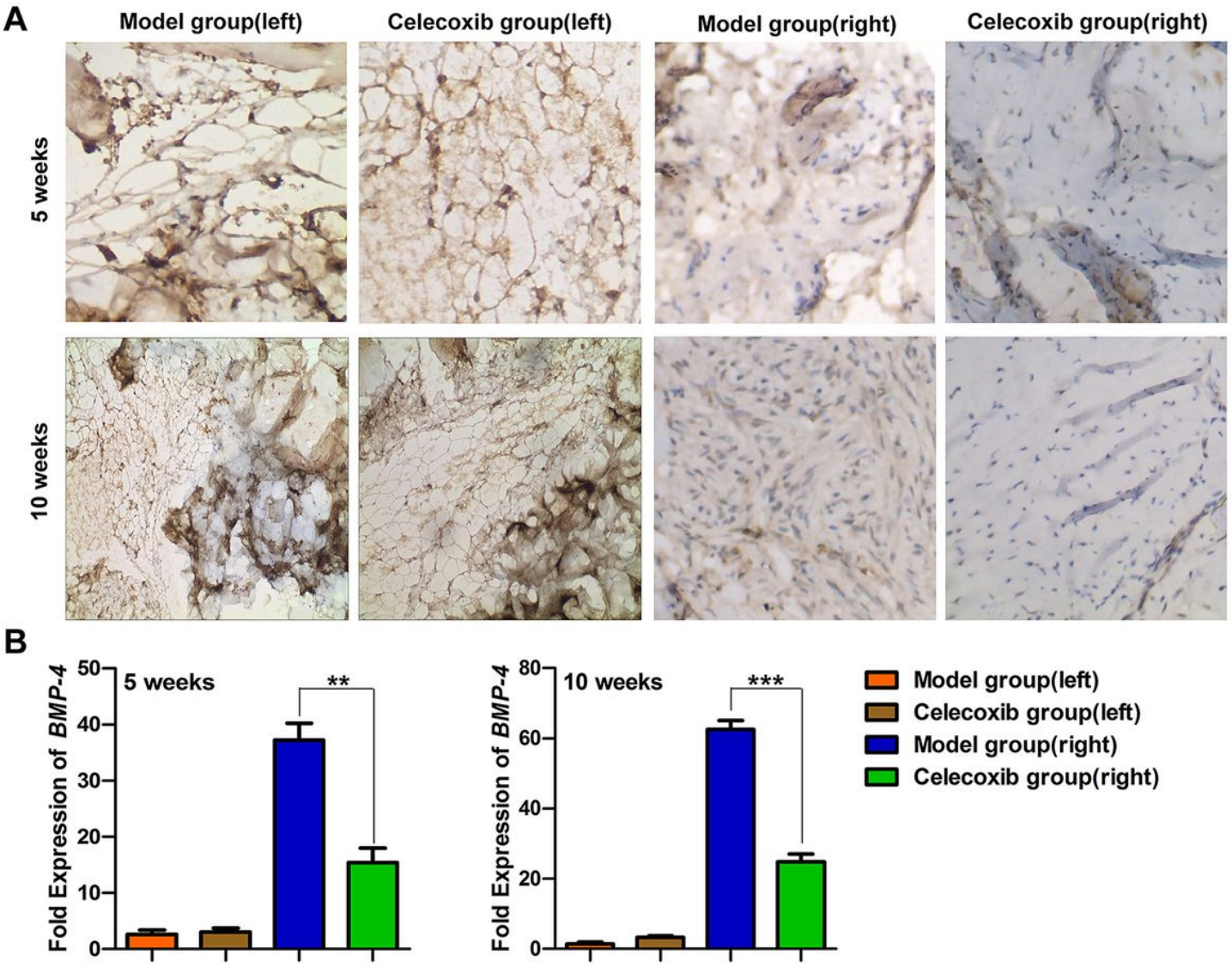

Figure 4

Immunohistochemical analysis of BMP-4 expression in Achilles tendon tissue(n=10). A: At 5th week or 10th week after operation, the expression of BMP-4 protein in rat Achilles tendon tissues was detected by immunohistochemistry (200×); B: The positive rate of BMP-4 protein was analyzed by Image J. All data represent the mean ± standard deviation; **P<0.005, ***p<0.001 by one-way ANOVA.

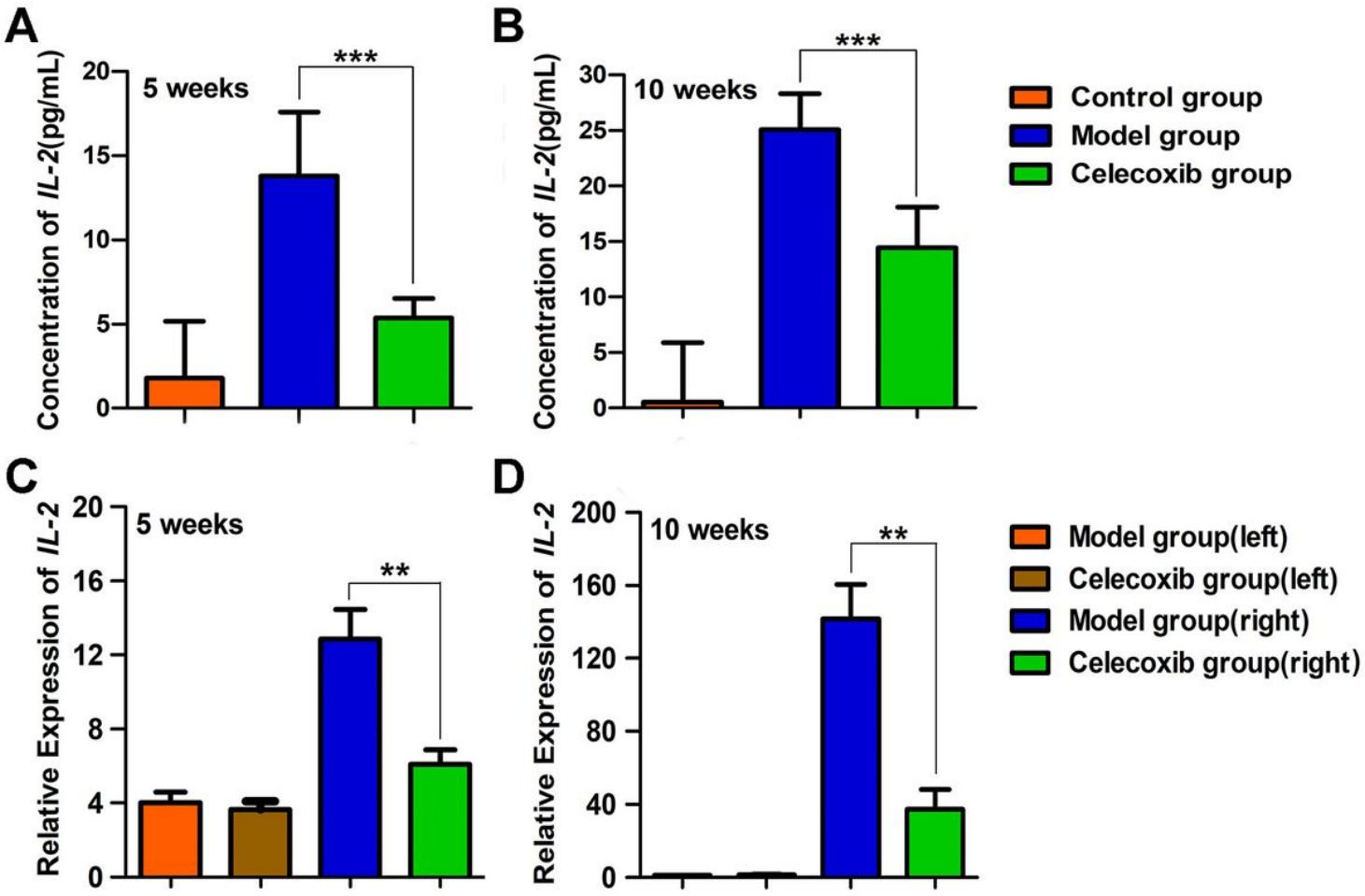

Figure 5

Effect of celecoxib on IL-2 expression in a heterotopic ossified rat model(n=10). A: At the 5th week after surgery, the level of IL-2 in the blood of rats was detected by ELISA; B: At the 10th week of surgery, the level of IL-2 in the blood of rats was detected by ELISA; C and D: At the 5th week(C) and 10th week(D) after surgery, RT-qPCR was used to detect IL-2 gene expression in the Achilles tendon tissue of rats in heterotopic ossification model. All data represent the mean ± standard deviation; **P<0.005, ***p<0.001 by one-way ANOVA.

Supplementary Files

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