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COX-2/PGE2 facilitates fracture healing by activating the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the influence of cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) on fracture healing by activating the Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS: In this study, 36 adult Sprague-Dawley (SD) rats raised in our laboratory were selected as research objects. The rats were subjected to fracture surgery on the middle part of the right femoral shaft. Subsequently, they were randomly divided into the control group and experimental groups (including experimental group A and experimental group B). Rats in experimental group A were injected with PGE 2 or COX-2 selective inhibitor NS-398, while rats in experimental group B were injected with PGE2 (5 μmol/L). Meanwhile, rats in the control group were injected with the same amount of normal saline. After that, the transcriptional levels of PEG2, COX-2, vascular endothelial growth factor (VEGF) and β-catenin in rats of the experimental group A, experimental group B and control group were detected via fluorescence quantitative Polymerase Chain Reaction (PCR) assay. Enzyme-linked immunosorbent assay (ELISA) and Western blotting were conducted to determine the changes in protein levels of PEG2, COX-2, VEGF and β-catenin in rats of the experimental group A, experimental group B and control group. The expression level of VEGF in bone tissues at fracture ends of rats in the experimental group A, experimental group B and control group was observed through the hematoxylin-eosin (HE) staining. Furthermore, micro-computed tomography (CT) was employed to evaluate callus formation.

RESULTS: The transcriptional and translational levels of COX-2, β-catenin and VEGF in rats of experimental group A treated with COX-2 inhibitors were significantly down-regulated when compared with those of the control group, showing statistically significant differences ($p<0.05$). However, the levels of these genes were markedly elevated in the experimental group B treated with PGE2 in comparison with those in the control group, and the differences were statistically significant ($p<0.05$). After 6 weeks, HE staining showed that the expression level of VEGF in rats of the experimental group B was remarkably higher than that of the experimental group A ($p<0.05$). Micro-CT results revealed that the mean trabecular plate density (MTPD) of rats in the experimental group B (73.29±5.4) was markedly higher than the number of osteoblasts (49.6±3.9) in the experimental group A, showing a statistically significant difference ($p<0.05$).

CONCLUSIONS: COX-2/PGE2 facilitates fracture healing by activating the Wnt/β-catenin signaling pathway.

Key Words:
COX-2, PGE2, Wnt/β-catenin, Signaling pathway, Fracture healing.

Introduction

Fracture, a severe injury caused by external factors, is common in daily lives. It may exert many adverse effects on patients in life[1]. Due to the lack of nutrients in bone tissue and self-repair mechanism with advanced age, the proportion of fractures in the middle-aged and elderly population has significantly increased[2]. Besides, the hard healing after fracture gives adverse impacts on families and individuals. Fracture healing is a comprehensive result of bone tissue cytology, morphology, and a series of complex processes including *in vivo* immune regulation[3-5]. Therefore, the mechanism of fracture remains uncertain at present.

Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid (AA) to prostaglandins. Recent studies have discovered

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that COX can maintain and regulate physiological functions of blood vessels, kidneys and other organs by promoting the production of substances, such as prostaglandin E2 (PGE2). Meanwhile, another study has found that COX-2 is significantly inhibited in various malignant tumors and cancer cells *in vivo*. These results suggest that COX-2 may be associated with the above diseases[6]. In recent years, studies have discovered that COX-2 is closely correlated with the Wnt/β-catenin signaling pathway. For instance, in tumors of the intestine, both COX-2 and the Wnt/β-catenin signaling pathway are blocked. Meanwhile, vascular endothelial growth factor (VEGF) is markedly down-regulated in diseased tissues[7].

In this work, we first investigated whether COX-2/PGE2 was involved in fracture healing through the Wnt/β-catenin signaling pathway. Our findings might provide important theoretical and experimental bases for subsequent interpretation of the mechanism of fracture healing.

Materials and Methods

General Data

In this study, 36 adult Sprague-Dawley (SD) rats (male) raised in our laboratory were selected as research objects. All rats were subjected to fracture surgery on the middle part of the right femoral shaft. Subsequently, they were randomly divided into the control group and experimental groups (including experimental group A and experiment group B). Rats in the experimental group A were injected with PGE2 or COX-2 selective inhibitor NS-398, while those in the experimental group B were injected with PGE2 (5 μmol/L). Meanwhile, rats in the control group were injected with the same amount of normal saline. This study was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine Animal Center.

Main reagents in this experiment: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Roche (Basel, Switzerland); 0.25% trypsin and EDTA (ethylenediaminetetraacetic acid) reagent from Invitrogen (Carlsbad, CA, USA); PEG2, COX-2, VEGF, β-catenin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies and MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay kits from Roche (Basel, Switzerland); animal cell intracellular total protein extraction kits and hematoxylin-eosin (HE) staining kits from Thermo Fisher Scientific (Waltham, MA, USA); fluorescent quantitative Polymerase Chain Reaction (PCR) kits and intracellular ribonucleic acid (RNA) extraction kits from Axygen (Beijing, China).

RNA Extraction

RNA extraction was carried out in accordance with the instructions of Axygen kits (Beijing, China), shown as follows:

(1) Cryopreserved tissue samples (about 0.1 g) were taken out from liquid nitrogen, dissolved on ice and added with 0.45 mL of RNA Plus. After being ground in a pre-cooled mortar, the tissues were transferred to a 1.5 mL Eppendorf (EP; Hamburg, Germany) tube. Thereafter, additional 0.45 mL of RNA Plus was added to the mortar, washed and transferred to a new tube. (2) 200 μL of chloroform was added to the tube, followed by vigorous shaking for 15 s and incubation on ice for 15 min. (3) Then, samples were centrifuged at 12000 rpm and 4°C for 15 min. (4) The supernatant was transferred to a RNase-free EP tube and added with the same volume of isopropanol. After mixing by overturning, the mixture was incubated on ice for 10 min. (5) Subsequently, the mixture was centrifuged at 12000 rpm and 4°C for 10 min. (6) After discarding the supernatant, the mixture was added with 750 μL 75% ethanol, gently mixed and centrifuged at 12000 rpm and 4°C for 10 min. (7) The supernatant was discarded and the residual ethanol was removed to the greatest extent. (8) An appropriate amount of RNase-free water was added, and the quality of extracted RNA was determined. The remaining was used for reverse transcription[8].

Fluorescence Quantitative Polymerase Chain Reaction

Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). Quantitative Polymerase Chain Reaction (qRT-PCR) conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of target genes was expressed by the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference for quantitative analysis of COX-2, PGE2, β-catenin and VEGF expression. The experiment was repeated 3

times. Primers used in this study were shown in Table I.

Detection of Protein Expression Levels Through Western Blotting

In this study, total protein was extracted from samples according to the instructions of animal cell protein extraction kit (Roche, Basel, Switzerland), with some improvements[8]. The antibodies were diluted to the final ratio of 1:5000. Dilution and relevant operations were carried out in accordance with Molecular Cloning Manual[9].

Determination of Protein Expression Levels via Enzyme-Linked Immunosorbent Assay (ELISA)

This assay was carried out in accordance with the instructions of ELISA kits (TaKaRa, Otsu, Shiga, Japan), with some improvements[10]. ELISA standard protein samples were diluted with Assay Buffer at a ratio of 1:50. A standard curve was then plotted according to the instructions. Samples to be tested were diluted with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA; pH 7.2) at a ratio of 1:100, and then dispensed (100 μL/each well). Thereafter, 50 μL of test solution was added to each well, followed by incubation at room temperature for 2 h. Subsequently, tetramethylbenzidine (TMB) chromogenic substrate was added, and the absorbance at 495 nm was measured. Finally, the content and concentration of PEG2, COX-2, VEGF, β-catenin and GAPDH antibodies in each sample were calculated according to the standard curve[11].

Observation of Callus by Micro-Computed Tomography (CT)

During the experiment, SD rats were killed with anhydrous ether. Soft tissues around the femur were removed, and calluses were kept. Subsequently, the Kirschner wire was removed, and fracture specimens were collected. The specimens were immersed in 10% neutral formaldehyde for 24-48 h of fixation. They were placed in a Micro-CT machine for scanning along the long axis of the femur specimen (current: 145 lI A, energy: 55 KVP, time interval: 200 ms). The 10r site of the fracture was selected as the region of interest for analysis. Finally, parameters, including trabecular bone area (BA), sample area (SA) and mean trabecular plate density (MTPD) per unit area in each cross-sectional view were obtained. Three-dimensional reconstruction of fracture specimens in the region of interest was performed as well[11].

Table I. Primers for fluorescence quantitative PCR.

Gene	Primer sequence
COX-2	F:5'-CGCGCTAGCATCGATCAGCTAGC-3'
	R:5'-CGGGCTAGCTACGATCGCTACG-3'
PGE2	F:5'-CGGGCATCGATCGATAAGCTAC-3'
	R:5'-CGGCGCATGCTACGATCGACTCG-3'
β-catenin	F:5'-GGCGCTAGCGATCGATCGATCG-3'
	R:5'-CGGCGCTAGCTACGATCGATCG-3'
VEGF	F:5'-GGCGCTAGCGATCGATCGATCG-3'
	R:5 '-CGGCGCTAGCTACGATCGATCG-3'
GAPDH	F:5'-TCATGGGTGTGAACCATGAGAA-3'
	R:5'-GGCAGGACTGTGGTCATGAG-3'

HE Staining

Positive results of immunohistochemical staining referred to that yellow particles were observed in the cell membrane or cytoplasm. Specific evaluation criteria for immunohistochemistry was as follows[12]: negative for stained membrane <10% or no staining, and positive for stained cell membrane only or >10% stained tumor cell membrane. The results were quantitatively determined according to LI index. KI index referred to the number of positive cells in each field of view.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as ($\chi \pm s$). One-way analysis of variance (ANOVA) test was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). *t*-test was used to compare the difference between the two groups, and *q* test was employed for pairwise comparison among groups. $p<0.05$ was considered statistically significant.

Results

Transcriptional Levels of COX-2, β-Catenin and VEGF in Experimental and Control Groups Determined via Fluorescence Quantitative Polymerase Chain Reaction

To investigate the relationship between COX-2 and fracture healing, the transcriptional levels of COX-2, β-catenin and VEGF in different

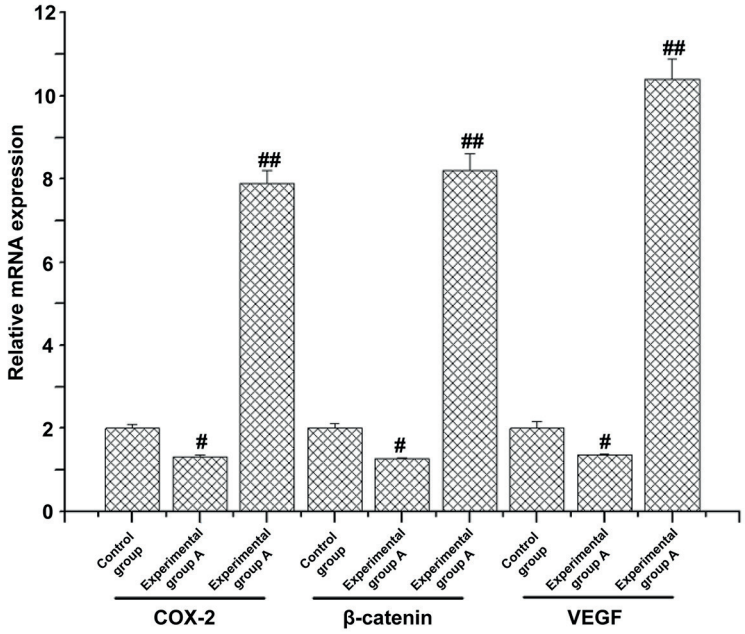

Figure 1. Transcriptional levels of COX-2, β-catenin and VEGF in experimental and control groups determined via fluorescent quantitative PCR. The results indicated that compared with the control group, experimental group A showed markedly declined transcriptional levels of COX-2, β-catenin and VEGF, with statistically significant differences (p<0.05). However, experimental group B exhibited remarkably increased transcriptional levels of COX-2, β-catenin and VEGF, showing statistically significant differences (p<0.05). The results implied that decreased COX-2 could evidently repress the expressions of β-catenin and VEGF. # represented that the difference was significant (p<0.05).

samples were firstly measured in this study. The results (Figure 1) showed that the transcriptional levels of COX-2, β-catenin and VEGF in rats of the experimental group A were evidently lower than those of the control group, displaying statistically significant differences (p<0.05). However, the transcriptional levels of COX-2, β-catenin and VEGF in rats of the experimental group B were markedly higher than those of the control group, showing statistically significant differences (p<0.05). These results suggested that inhibition of COX-2 was able to decrease the transcriptional levels of β-catenin and VEGF.

Translational Levels of COX-2, β-Catenin and VEGF in Experimental and Control Groups Detected Through ELISA

Fluorescence quantitative PCR results revealed that COX-2 inhibition resulted in significantly down-regulated expression levels of β-catenin and VEGF. To verify this finding at the protein level, the translational levels of COX-2, β-catenin and VEGF in rats of different groups were detected *via* ELISA. The results suggested that the translational levels of COX-2, β-catenin and VEGF in rats of the experimental group A were markedly lower those of the control group, showing significant differences (p<0.05). However, they were remarkably higher in the experimental group B than the control group, displaying significant differences (p<0.05). The above results indicated that the inhibition of COX-2 could decrease the transcriptional levels of β-catenin and VEGF (Figure 2).

Protein Levels of COX-2, β-Catenin and VEGF in Experimental and Control Groups Measured via Western Blotting

Western blotting results showed that compared with the control group, the protein levels of COX-2, β-catenin and VEGF decreased significantly in the experimental group A and there were markedly significant differences (p<0.05). However, the protein levels of COX-2, β-catenin and VEGF were remarkably up-regulated in the experimental group B when compared with the control group (p<0.01). These results implied that the down-regulation of COX-2 gene was capable of inhibiting the protein expressions of β-catenin and VEGF (Figure 3).

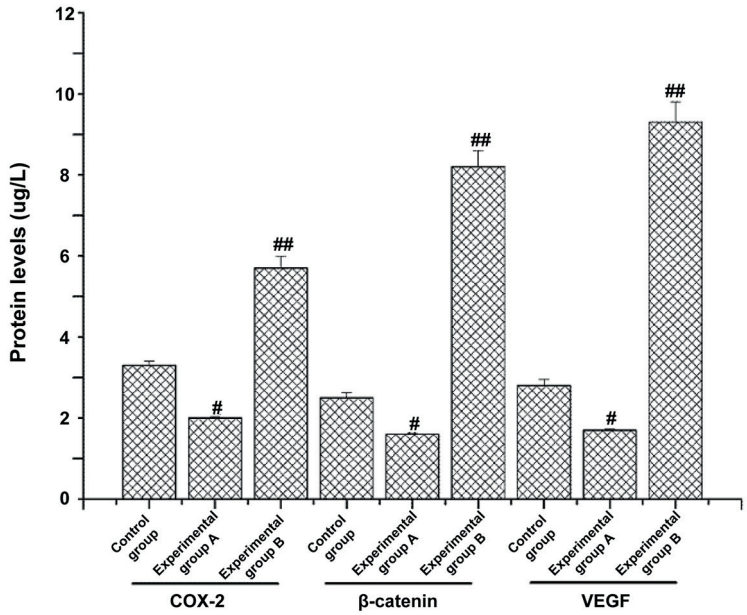

Figure 2. Translational levels of COX-2, β-catenin and VEGF in experimental and control groups detected through ELISA. The results suggested that compared with the control group, the translational levels of COX-2, β-catenin and VEGF in rats of experimental group A were significantly lowered ($p<0.05$). However, the translational levels of COX-2, β-catenin and VEGF were remarkably elevated in experimental group B when compared with the control group, displaying statistically significant differences ($p<0.05$). These results indicated that down-regulation of COX-2 remarkably suppressed the expressions of β-catenin and VEGF.

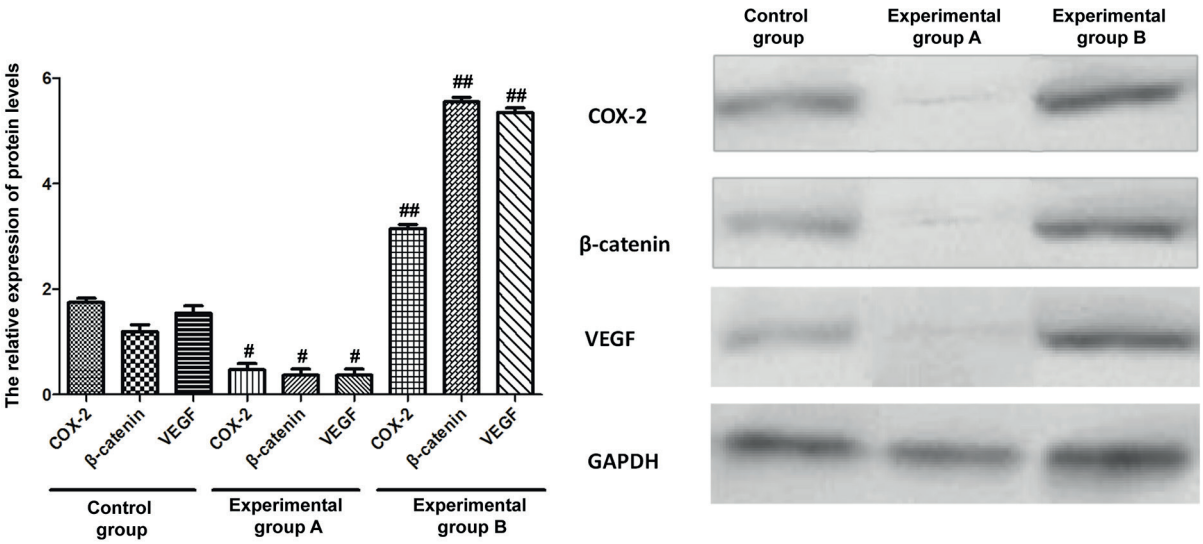

Figure 3. Protein levels of COX-2, β-catenin and VEGF in experimental and control groups measured via Western blotting. #: $p<0.05$ *vs.* control group, ##: $p<0.01$ *vs.* control group.

VEGF Expression Level in Experimental and Control Groups Observed Through HE Staining

Recent studies[13] have manifested that VEGF not only facilitates the repair of damaged blood vessels *in vivo*, but also participates in the formation of osteogenic factors. To investigate the correlation between PGE2/COX-2 and bone repair, HE staining was applied to observe the protein expression level of VEGF in different groups.

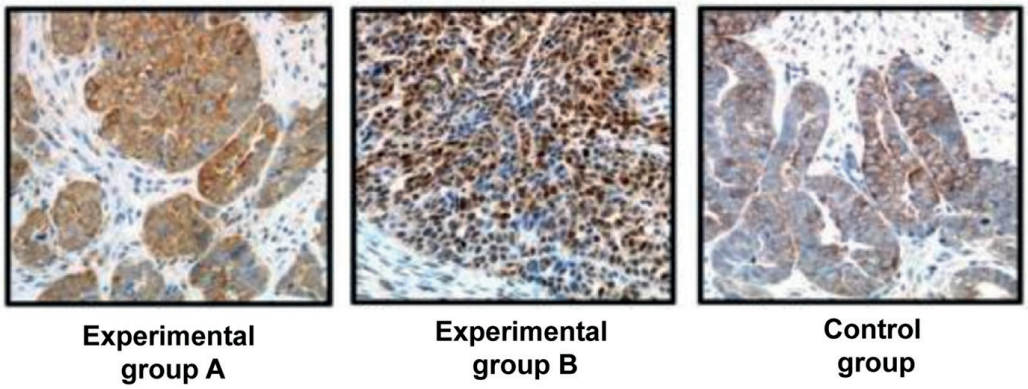

Figure 4. VEGF expression level in different samples observed through HE staining (Magnification × 40). HE staining results suggested that the expression level of VEGF in the experimental group A was markedly lower than that of the experimental group B, showing a statistically significant difference ($p<0.05$).

VEGF expression represented the degree of fracture healing. The results shown in Figure 4 suggested that compared with the control group, the experimental group A showed evidently lowered VEGF expression level, and the difference was statistically significant ($p<0.05$). However, the experimental group B exhibited markedly increased VEGF expression level when compared with the control group ($p<0.05$). The above results suggested that overexpression of COX-2 promoted the protein expression of VEGF (which was consistent with the findings of fluorescence quantitative PCR), thus facilitating the production of osteoblasts and promoting fracture healing.

The Formation of Calluses in Experimental and Control Groups Evaluated via Micro-CT

The above fluorescence quantitative PCR, ELISA, Western blotting and HE staining indicated that COX-2 could affect fracture healing by regulating the Wnt/β-catenin signaling pathway at the molecular level. For further investigation, fracture healing of experimental rats in different treatment groups was observed *via* Micro-CT. The results (Figure 5) showed that new bone tissues were observed at the fracture site of rats in the control group, experimental group A and experimental group B. However, in comparison with the control group, experimental group A showed significantly less new bones and thinner calluses at the fracture end of rats ($p<0.05$). However, experimental group B exhibited clearly more new bones and thicker calluses at the fracture end of rats, showing statistically significant differences ($p<0.05$). The above results suggested that overexpression of COX-2 promoted fracture healing by facilitating the formation of calluses by up-regulating the Wnt/β-catenin signaling pathway.

Discussion

Fracture healing is the result of a series of complicated biochemical reactions, which is often associated with age, gender and health status. At the cellular level[14], fracture healing refers to the regeneration of bone cells, which is promoted by related cytokines and nutrients secreted by tissues and cells surrounding the fracture site. In recent years, certain advances have been made in exploring the molecular mechanism of fracture healing. However, its specific signaling pathways and related gene functions during signal transduction remain unclear[15-17]. A study has found that COX-2 can catalyze and produce AA in the body. In case of external stimuli, cells will secrete growth factors, cytokines and other inflammatory factors, including PGE2. PGE2 is known as the main product of COX-2-catalyzed AA, which can participate in an inflammatory response in the body[18]. Recent follow-up treatment of fracture healing has suggested that fracture healing can be divided into four main stages, namely, inflammatory response (during which massive inflammatory factors are produced), soft callus formation (during which massive fibroblasts in granulation tissues are proliferated), callus formation and callus remodeling. Among the 4 stages, the first and second stages are critical for the formation of calluses 2[19,20].

In this study, we first demonstrated that COX-2 was involved in fracture healing by regulating the Wnt/β-catenin signaling pathway. The results showed that compared with the control group, inhibition of COX-2 (i.e., experimental group A) markedly decreased the transcriptional and translational levels of key gene β-catenin in the Wnt/β-catenin signaling pathway. However, activation of COX-2 (i.e., experimental group B) resulted in significantly elevated transcriptional and translational levels of key gene β-catenin in the Wnt/β-catenin signaling pathway. This indicated that the Wnt/β-catenin signaling pathway was regulated by COX-2 during fracture healing. Furthermore, ELISA and Western blotting revealed that the activation of COX-2 promoted the production of VEGF in cells. As a cytokine, VEGF plays an important regulatory role in the first stage of fracture healing. Studies have manifested that in the case of brain injury, the brain produces a series of cells and related molecules, including cytokines. Meanwhile, these related molecules released from the central nervous system are able to accelerate fracture healing by promoting the production of osteogenic factors. In the present study, HE staining assay showed that overexpression of COX-2 in experimental group B significantly increased the expression level of VEGF at the fracture site. Micro-CT results also suggested that experimental group B with COX-2 overexpression showed more rapid formation of calluses at fracture end. All of the above results implied that PGE2/COX-2 could accelerate the production of substances (including cytokines and osteogenic factors) in the body by promoting the Wnt/β-catenin signaling pathway during fracture healing. Ultimately, this might facilitate the formation of calluses at the fracture site and promote fracture healing. In this study, the results found that PGE2/COX-2 promoted the transcription and translation of key gene β-catenin in the Wnt/β-catenin signaling pathway. However, there was no in-depth study on the mechanism by which the Wnt/β-catenin signaling pathway promoted the production of cytokines, such as VEGF. Meanwhile, our findings indicated that PGE2/COX-2 accelerated fracture healing by promoting the proliferation of osteoblasts, which was in accordance with the Nagano et al study[21]. However, samples with COX-2 overexpression showed markedly severer inflammatory responses and autophagy at the injury site in comparison with the control group during fracture healing, especially in the early stage of fracture healing. The mecha-

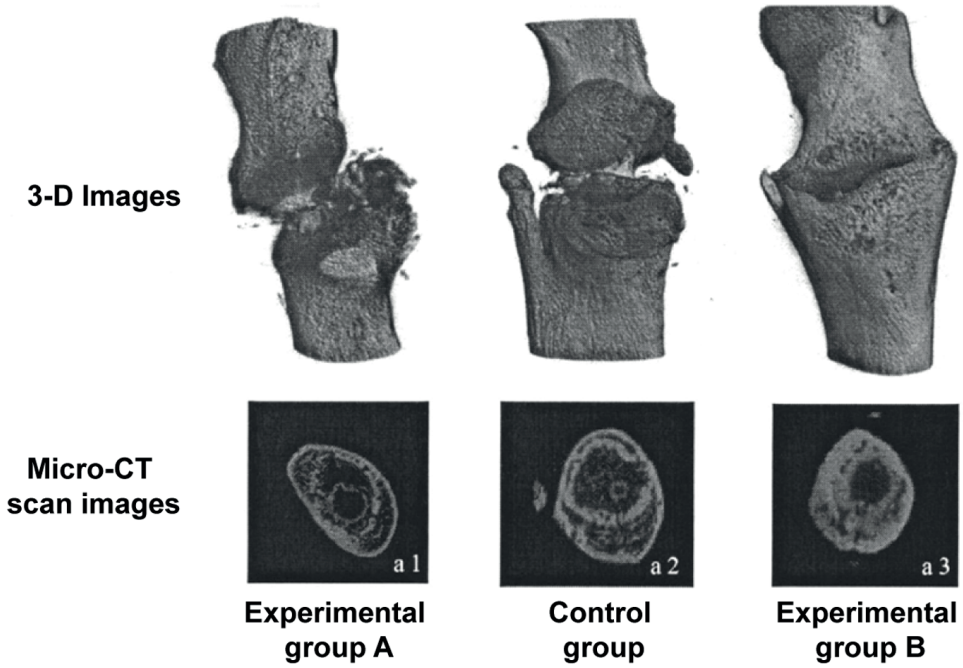

Figure 5. The formation of calluses in experimental and control groups evaluated via Micro-CT. The callus at the fracture end in the experimental group A was thinner than that of the experimental group B, and there was a statistically significant difference ($p<0.05$).

nism of this finding was unclear, which would be the focus in future researches.

Conclusions

This study shows that COX-2/PGE2 facilitates fracture healing by activating the Wnt/β-catenin signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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