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Research article

Effect of Huoxue Jiegu compound capsule on osteoblast differentiation and fracture healing by regulating the PI3K/Akt/mTOR signaling pathway in rabbits

Yingjie Wu [1], Mingxiang Fan, Shixiang Tan, Qiucheng Guo, Hegui Xu [*]

Spine surgery, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou, 550000, China

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ABSTRACT

Objective: To examine and talk about the mechanism of the Huoxue Jiegu compound capsule's effects on osteoblasts and the PI3K/Akt/mTOR signal pathway in rabbits suffering from tibial fractures.

Method: In vitro, CCK8 was used to assess the survival rates. Alizarinred staining was used to evaluate mineralized nodules. ALP staining was used to observe the osteoblasts. qRT-PCR was used to determine the mRNA expression of the bone formation-related factors BMP-2, bFGF, and TGF-β. In vivo, three groups of nine male rabbits each were randomly assigned to three groups: the Model group, the Huoxue Jiegu compound capsule group (HXJGC group), and the inhibitor group (HXJGC+3-MA), four weeks following the intervention. HE staining was employed to examine the rabbits' bone histology. immunohistochemistry was employed to examine the relative expression of the proteins VEGF and LC3-II. Western Blot was utilized to examine the relative expression of the proteins Beclin-1, LC3-II/I, p62, p-PI3K, p-AKT, and p-mTOR.

Results: Compared to the control group, the medium- and high-dose groups exhibited considerably higher survival rates ($P < 0.05$), as well as enhanced cell proliferation and differentiation ($P < 0.05$) and more pronounced mineralized nodules. ($P < 0.05$), but the low-dose groups showed no appreciable variation. In the low, medium, and high-dose groups, there was a substantial reduction in the expression of bFGF mRNA, whereas the levels of BMP-2 and TGF-β mRNA were considerably higher than in the control group ($P < 0.05$). In vivo, after four weeks of treatment, the model control group and inhibito group had a large amount of fibrous hyperplasia accompanied by bleeding and a small amount of inflammatory cell infiltration. But in the HXJGC group, new cartilage appeared, and the surface of the cartilage was smooth and flat. Beclin-1 and LC3-II/I expression in the HXJGC+3 MA group was significantly lower than in the HXJGC and Model groups ($P < 0.05$). The HXJGC group showed lower p62 expression than the HXJGC+3 MA and model groups ($P < 0.05$). The HXJGC group exhibited significantly reduced levels of p-PI3K, p-AKT, and p-mTOR expression in comparison to HXJGC+3 MA groups ($P < 0.05$).

Conclusion: Rabbits with tibial fractures can be treated with HXJGC, which can control the expression of the PI3K/Akt/mTOR signal pathway. It can promote the differentiation and

* Corresponding author. The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, No. 71 Baoshan North Road, Yunyan Distric, Guiyang, Guizhou, 550000, China.
E-mail address: qq1721449895@163.com (H. Xu).
[1] First Author: Doc. Yingjie Wu, Spine surgery, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, No. 71 Baoshan North Road, Yunyan Distric, Guiyang, Guizhou, 550000, China. Email: qq1737579051@163.com

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maturation of osteoblasts at the fracture end of rabbits, accelerate the recovery of fractures, and achieve the purpose of treating the disease.

Abbreviations

PI3K	phosphoinositide 3-kinase
Akt	protein kinase B
mTOR	mammalian target of rapamycin
CCK8	cell counting kit-8
ALP	alkaline phosphatase
qRT-PCR	quantitative real-time polymerase chain reaction
mRNA	messenger ribonucleic acid
BMP-2	Bone Morphogenetic Protein2
bFGF	basic fibroblast growth factor
TGF-β	Transforming growth factor-β
LC3-II	light chain 3-II
RIPA	radio immunoprecipitation assay lysis buffer
BCA	bicinchoninic acid
Tris-HCL	trimethylol aminomethane hydrochloride
actin	actin(internal reference)
ECL	chemiluminescence substrate
SD	standard deviation
PVDF	polyvinylidene fluoride
HRP	horse radish peroxidase
LC3-I/II	light chain 3-II
VEGF	vascular endothelial growth factor
Hi-Fi cDNA Synthesis Kit	Universal reverse transcription kit
cDNA	complementary deoxyribonucleic acid
SYBR Green	A dye with a green excitation wavelength
ddH2O	double-distilled water
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPDH F	GAPDH forward primer
GAPDH R	GAPDH reverse primer
HE	hematoxylin-eosin
EDTA	ethylenediamine tetraacetic Acid
PBS	phosphate buffered solution
DAB	diaminobenzidine
BCA	bicinchoninic acid
ULK1	unc-51-like kinase 1

1. Introduction

Fracture healing is a complex and orderly regulatory process caused by a series of tissue and biochemical changes [1]. Osteocytes, inflammatory cells, blood supply, and cytokines around the fracture site play an important role in the whole process of fracture healing [2]. 5 %–10 % of patients still have problems with the healing of fractures, despite the fact that bone tissue has a great capacity for self-healing. This causes patients and their families considerable stress [3,4]. Consequently, there exists a strong correlation between the state of fracture healing and the patients' quality of life.

Autophagy is an important mechanism to maintain the balance between cell death and survival [5]. Autophagy is involved in the pathogenesis of many diseases, such as cancer, cardiovascular disease, neurodegenerative disease, metabolic disease, eye disease, and infection [6]. Recent research has revealed that [7,8] cell autophagy plays a significant role in bone metabolism. By controlling cell autophagy, this process protects osteoblasts and enhances rat bone metabolism. An essential intracellular signal transduction system involved in every facet of cell activity is the PI3K/Akt/mTOR signal pathway. Certain plant extracts have been shown in recent years to be able to control the PI3K/Akt/mTOR signal pathway, which in turn activates autophagy and stimulates the differentiation of bone marrow mesenchymal stem cells into osteoblasts [9–11], Simultaneously, after obstructing conduction, the PI3K/Akt/mTOR signal pathway inhibitor can considerably lower osteoclast development and decrease osteolysis. Based on the HXJGC, an empirical prescription for treating fractures in the Department of Orthopaedics and Traumatology, an affiliated hospital of Guizhou University of Traditional Chinese Medicine, the HXJGC is formed into granules. Prior research [12,13] has demonstrated that HXJGC can enhance the expression of bFGF, TGF-β, and bone morphogenetic protein-2 (BMP-2) in the bone tissue of rabbits after fracture, so as to promote fracture healing in rabbits.

This study tried to investigate the connection between HXJGC and osteoblast and bone metabolism based on the PI3K/Akt/mTOR signaling pathway, and it put forth the following theories:In rabbits with tibial fractures, HXJGC can stimulate osteoblast differentiation and maturation, hence enhancing fracture healing, by regulating the PI3K/Akt/mTOR signaling pathway. The experiment in this study was planned and executed with the purpose of validating this hypothesis.

2. Materials and methods

2.1. Experimental rabbits

Twenty-seven ordinary-grade adult male New Zealand white rabbits (weight 2.6 ± 0.23 kg), feeding environment: temperature 20–26 °C; humidity 40 %–70 %, provided by Ganzhou Institute of Animal Husbandry and Fisheries. Laboratory Animal Production License No.: SCXK (Gan) 2018-0009. Animal handling shall comply with the ethical requirements of the Experimental Animal Ethics Committee of Guizhou University of Chinese Medicine (approval number: 20230148).

2.2. Reagents and instruments

HXJGC is made of Rhizoma drynariae 12g, Dipsacus 12g, Natural copper 9g, Trionyx sinensis 9g, Salvia miltiorrhiza 12g, Speranskia tuberculata 15g, Caesalpinia sappan 9g, Panax notoginseng 12g, Borneolum syntheticum 5g, Corydalis yanhusuo 9g (The First Affiliated Hospital of Guizhou University of Traditional). Inhibitor 3-MA (M9281,sigma). RIPA cell lysate (C1053, Beijing Puli Gene Technology Co., Ltd.), BCA protein quantitative kit (BCA Protein Assay Kit), 30 % acrylamide (PAGE Pre-Solution), 1m Tris-HCL buffer (PH = 6.8), 1.5m Tris-HCL buffer (PH = 8.8), sodium dodecyl sulfate (SDS). Xilong Science Co., Ltd.), ammonium persulfate (Sodium persulfate) (Tianjin Zhiyuan Chemical Reagent Co., Ltd.), Glycine (Glycine) (G8200 less Solarbio), Sodium Chloride (Nacl) (7647-14-5, Tianjin Damao Chemical Reagent Factory), Potassium Chloride (Kcl) (Tianjin Zhiyuan Chemical Reagent Co., Ltd.), Tetramethylethylenediamine (TEMED) (T105496, Aladdin), Marker (26617, Thermo), PVDF membrane (IPVH00010, Millipore). Sealing special skim milk powder (P1622, Beijing Puli Gene Technology Co., Ltd.), Bovine Serum Albumin (BSA) (A8020, Solarbio), hypersensitive photoluminescence solution (RJ239676, Saimofi), internal reference first antibody: Mouse Anti-β-Actin (HC201, TransGen Biotech, 1/2000), second antibody: HRP-conjugated Goat Anti-Mouse IgG (Helil) (GB23301, Servicebio, 1/2000), objective first antibody: Rabbit Anti-LC3-I/II (12741T). Rabbit Anti P-PI3K (AF3241,Affinity,1/500), Rabbit Anti P-AKT (Ab38449, Abcam, 1/1000), Rabbit Anti Beclin-1 (AF5128, Affinity, 1/500), Rabbit Anti VEGF (Ab214424, Abcam, 1/1000), the second antibody: HRP conjugated Goat Anti-Rabbit IgG (HRP conjugated Goat Anti-Rabbit IgG) (GB23303, Servicebio, 1/2000). The vertical electrophoresis apparatus (DYY-6C) was from LIUYI, Beijing; the hemiDocTM XRS + System was from Bio-Rad Laboratories; the fully automated vibrating blade microtome (2235) was from Leica; and a light microscope (BX43) was from Olympus.

2.3. Rabbit medicated serum preparation

Six rabbits were administered intragastric medication twice a day (three received intragastric saline and three received intragastric HXJGC). Each group's rabbits were euthanised with a combination of 4 % isoflurane and air after seven days in a row. Blood was drawn from the abdominal aorta, centrifuged for 30 min at 3000 revolutions per minute, the supernatant (serum) was absorbed, the serum from the same group was mixed, and the complement was inactivated for 30 min at 56 °C in a water bath. Following the 0.22 μm microporous filter membrane filtering of the bacteria, the sterile test tubes were packed, labeled with the category and date, and refrigerated at −20 °C for later use.

2.4. CCK8 experiment

The drug-containing serum was administered for 48 h after the cells were adhered to the wall, and then the 96-well plate cells that were to be assessed were changed with the same medium, 100 μl per well. This is in accordance with the method mentioned above. To each well, add 10 μl of CCK8 reagent, and then incubate for 2 h. The 450 nm wavelength of the enzyme labeler allowed for the detection of each hole's light absorption value.

2.5. Alkaline phosphatase staining

First, reagent A was applied to the cell dish, which was then incubated at 37 °C for 30 min, washed in distilled water for 2 min, and then reagent B was added. After diluting reagent C by 50 times with distilled water, it was incubated for 2 min at 37 °C as an ALP vulcanization working liquid. It was then washed with distilled water for 10 min, and the glycerin gelatin was sealed and examined under a microscope.

2.6. Alizarin red staining

The samples in the petridish were covered with alizarin red dye and incubated at room temperature without light for 30 min. After that, the samples were slowly rinsed with double-steaming water for 3 min, and the glycerin gelatin was sealed and examined under a microscope.

2.7. Quantitative real-time polymerase chain reaction

The Hi-Fi cDNA Synthesis Kit was used to reverse-transcribe the total RNA in the cells to cDNA after the TRIzol reagent was used to separate the RNA. To determine relative amounts of target genes, a 20 μL qRT-PCR system was built with 10 μL of 2 × SYBR Green PCR

Master Mix, 1 μL of cDNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 8.2 μL of RNase-free ddH2O. Thermal reactions were performed at 95 °C for 10 min predenaturation, followed by 40 cycles at 95 °C for 10 s denaturation, 58 °C for 30 s annealing, and 72 °C for 30 s extension. GAPDH was used as the internal reference (the qRT-PCR primer sequence is shown in Table 1).

2.8. Establishment of a fracture model

Establishment of fracture model refer to Alhasson FA [14]: experimental rabbits were modeled by intravenous injection of anesthetic (ethyl carbamate, 20 % gravity, 4 ml/kg). After successful anesthesia, the right lower extremities of rabbits were depilated and prepared with a pet electronic shaver, and after wiping with alcohol gauze, the rabbits were placed on the animal fixation table to fix their limbs. Iodine tincture was disinfected from the right knee joint to the right ankle joint of rabbits, disinfected twice, and deiodized with alcohol in the same way, disinfected 3 times. Use disposable aseptic towels to cover towels, only expose the surgical site, and fix towel forceps. Take the 1.5 cm under the tibial plateau, cut a vertical incision of about 1.5 cm along the anterior edge of the tibia, cut the rabbit skin, continue to cut the 1 cm vertically along the anterior edge of the tibia, cut the superficial fascia and deep fascia, shield the tibial anterior muscle, and expose the tibia. A bone defect model about 5 mm `2 mm was made by using special animal rongeur and biting bone. After washing with saline and diluted iodophor, the cut tissue was sutured in layers, the skin was sutured, subcutaneous alcohol was disinfected, and the wound was bandaged with gauze. 160000 units of penicillin were injected intramuscularly, and the same unit of penicillin was injected continuously 3 days after the operation.

2.9. Drug intervention

Drug intervention methods: 27 rabbits were randomly divided into three groups: the Model group, the HXJGC group, and the HXJGC + 3-MA group. All rabbits were randomly divided into 3 groups: Model group (n = 9), HXJGC group (n = 9), and HXJGC + 3-MA group (n = 9). HXJGC group: according to the body weight of 300 mg/kg of rabbits, Once a day, the model control group was given the same amount of normal saline, Given to the autophagy inhibitor group with the 1.9 ml/kg inhibitor intervention guidelines. The drug was given continuously for 4 weeks.

2.10. HE staining

The femurs were decalcified using EDTA, sliced into 4-μm-thick sections, and stained with hematoxylin and eosin (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. Histological images were observed under a microscope (XSP-C204, China Investment Corporation).

2.11. Immunohistochemistry

Tissue paraffin sections were dewaxed to water by routine. 3 % H202 inactivates endogenous peroxidase at room temperature 5 to 10 wash again. Heat at 95 °C for 10 min to repair antigen activity; $5g{\cdot}L^{-1}$ sheep serum was closed at room temperature for 30 min; drop diluted primary antibody (1:50) at 4 °C overnight. Rinse with PBS 3 min × 3 times. Add secondary antibody (1:100) and incubate at room temperature for 10–15 min. PBS rinse, 3 min × 2 times, DAB color development. Redye, dehydrate, be transparent, and seal. Under the microscope, the cells were brown-yellow after immunohistochemical staining, and the staining was negative. Immunohistochemical staining results determined that: under the 400-fold field of view, 4 to 5 fields were randomly selected from the non-infarct area of each section for imaging.

2.12. Western bloting

The left middle femur callus tissue was isolated (bone tissue in the same position was taken from the normal control group), homogenized, and added to the RIPA protein cracking solution to extract protein. After protein quantification by BCA method, sample buffer was prepared for electrophoresis, membrane transfer, sealed with 4 % skim milk for 1h, and rabbit source Beclin-1, LC3-II, LC3-I, P-PI3K, P-AKT, P-mTOR, p62, ACTIN (internal reference) (1:400) was incubated overnight, and the secondary antibody of sheep and rabbit was added after cleaning (1: 2000), incubated at room temperature for 1 h, washed, and added ECL reagent for color

Table 1
qRT-PCR primer sequence.

Primer name	Primer sequence (5′–3′)	Product length (bp)	Annealing temperature (°C)
GAPDH F	CCACTTTGTGAAGCTCATTTCCT	140	58
GAPDH R	TCGTCCTCCTCTGGTGCTCT		
BMP-2 F	CGTGAGGATTAGCAGGTCTTTG	127	58.2
BMP-2 R	TTTCGCTTGACGCTTTTCTC		
TGF-β F	TGTCACTGGAGTTGTGAGGC	284	59.9
TGF-β R	AGCAGTTCTTCTCTGTGGAGC		
bFGF F	AAGGAAGATGGAAGACTGCTGG	134	60.0
bFGF R	GTTCGTTTCAGTGCCACATACC		

development. A gel imaging system was used to analyze the gray values of bands and calculate the relative expression levels of Beclin-1, LC3-II, LC3-I, P62, p-PI3K, P-AKT, P-mTOR, bFGF, BMP-2, and TGF-β proteins.

2.13. Statistical analysis

All data were presented as the mean ± Standard Deviation. Statistical analyses were calculated using GraphPad Prism (version 8.0) software. The Student's t-test was adopted for two-sample comparisons. A one-way ANOVA followed by Tukey's post hoc analysis was adopted for multiple comparisons. All data were determined by at least three independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. HXJGC improved the survival osteoblasts

Primary osteoblast of rabbits were shown in(Fig. 1A). The Survival rates of primary osteoblasts were detected by CCK8 after cultured cells with different concentrations of drug-containing serum for 24 h. Compared with the control group and the low-dose group, the survival rates of the medium-dose group and the high-dose group were significantly increased. ($P < 0.05$).(Fig. 1B). These results suggest that HXJGC can promote thesurvival rates of osteoblasts.

3.2. HXJGC promotes the proliferation and differentiation in osteoblasts

ALP staining was used to observe the effect of HXJGC on osteoblas. Different concentrations of HXJGC medicated serum preparation were used in co-culture with osteoblasts, and showed obvious osteoblastic morphology. Increased cell density, cell filling, and deeper nuclear lysis occurred. HXJGC can promote the differentiation of osteoblasts and the secretion of alkaline phosphatase. The cell proliferation and differentiation in the medium-dose(Fig. 2C) and high-dose(Fig. 2D) groups were significantly higher than those in the control(Fig. 2A) and low-dose groups (Fig. 2B). Therefore, HXJGC can promote the proliferation and differentiation of osteoblast.

3.3. HXJGC promotes the formation of mineralized nodules in osteoblasts

Alizarin red staining was used to look at mineralized nodules. Compared with the control(Fig. 3A) group and the low-dose(Fig. 3B) group, the middle-dose(Fig. 3C) and high-dose(Fig. 3D) groups mineralized nodules more obviously, with a significant difference ($P < 0.05$). Therefore, HXJGC can promote the proliferation and differentiation of osteoblasts.

3.4. Effects of HXJGC on mRNA expression levels of BMP-2, TGF-β and bFGF in osteoblasts

The mRNA expression levels of BMP-2(Fig. 4A) and TGF-β(Fig. 4B) in the low, middle, and high-dose groups were considerably higher ($P < 0.05$) than in the control group. There was a substantial reduction in bFGF(Fig. 4C). mRNA expression ($P < 0.05$)

3.5. HXJGC improves fracture healing in rabbits with pathological injuries

The bone histopathology of rabbits in each group was observed by HE staining. Defects of the cartilage layer in the model control

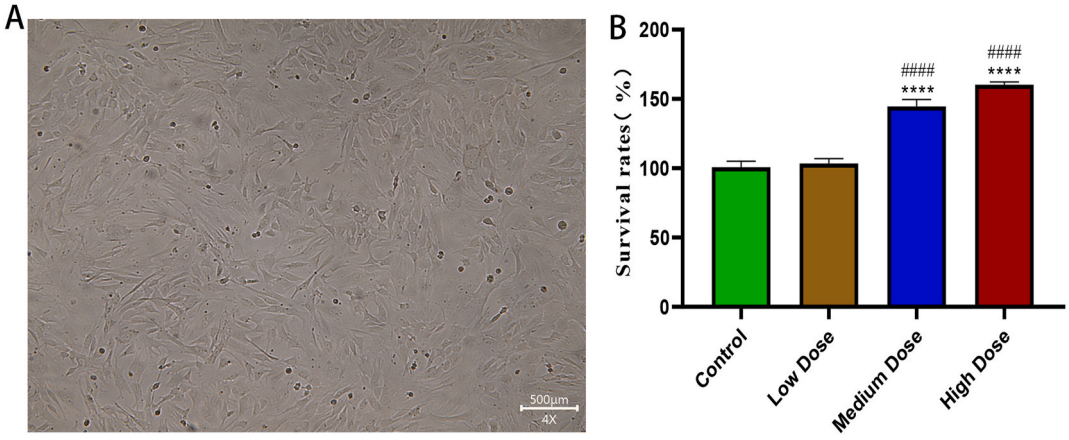

Fig. 1. A Primary.osteoblast of rabbit (× 4). B Survival rates of osteoblast with different dose drug-containing serum. *$P < 0.05$ vs. Control group, #$P < 0.05$ vs. Cell + low dose.

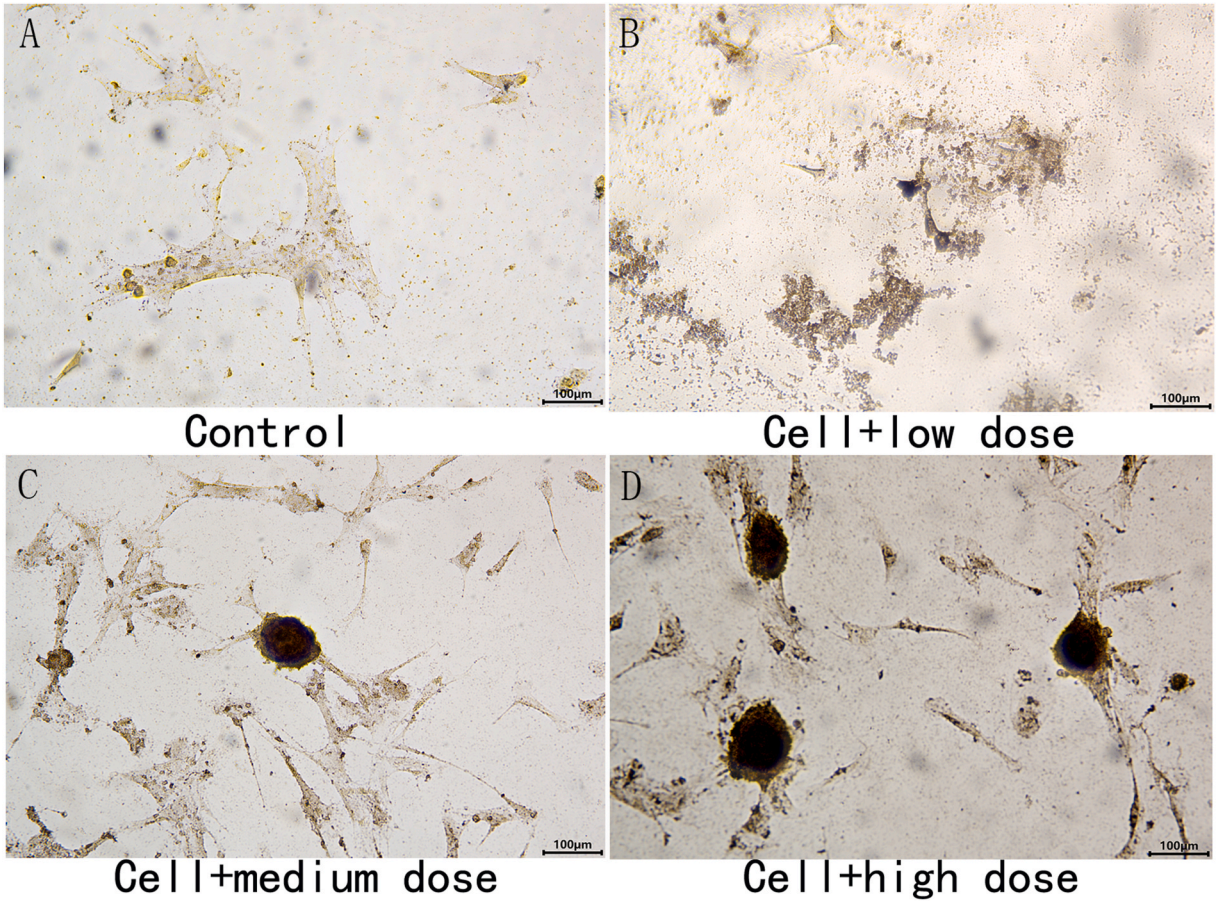

Fig. 2. Alkaline phosphatase staining of osteoblast after 7 days of HXJGC. (× 200). A Control group. B Cell + low dose group. C Cell + medium dose group. D Cell + high dose group.

group were observed under an optical microscope, with a large amount of fibrous hyperplasia accompanied by bleeding and a small amount of inflammatory cell infiltration(Fig. 5A). In the HXJGC group, new cartilage appeared, and the surface of the cartilage was smooth and flat(Fig. 5B). However, after the addition of the autophagy inhibitor 3-MA, the surface of the cartilage was not smooth, and the cartilage cells were disordered(Fig. 5C).

3.6. HXJGC impact LC3II/I and VEGF in the bone tissues of rabbits with fracture model

LC3II/I and VEGF protein expressions in rabbit bone tissues of each group were detected by immunohistochemistry. LC3II/I and VEGF protein expression increased significantly after HXJGC(Fig. 6B), (Fig. 6E) intervention compared with the model (Fig. 6A), (Fig. 6D)group ($P < 0.05$). Compared with the HXJGC group, LC3II/I and VEGF protein expression in the HXJGC+3-MA(Fig. 6C), (Fig. 6F) group were significantly decreased ($P < 0.05$). The model group compared with the HXJGC + 3-MA group, the expression levels of LC3II/I and VEGF in the HXJGC + 3-MA group with the addition of cellular autophagy inhibitors are lower than those in the model group ($P < 0.05$).

3.7. HXJGC activate autophagy to promote fracture healing in rabbits

In order to explore whether HXJGC promotes fracture healing in rabbits by activating autophagy, the expressions of autophagy related proteins Beclin-1, LC3II/I and, p62 were detected by WB(Fig. 7A). Compared with the HXJGC+3-MA group, Beclin-1(Fig. 7B) and LC3II/I(Fig. 7C) protein expressions in HXJGC group were significantly increased, while p62(Fig. 7D) protein expression was significantly decreased ($p < 0.05$). Compared with the HXJGC group, the expression of Beclin-1 and LC3II/I protein in the Model group was decreased, while the expression of p62 protein was increased($p < 0.05$), These results suggest that the effect of HXJGC on promoting fracture healing may be related to the activation of autophagy.

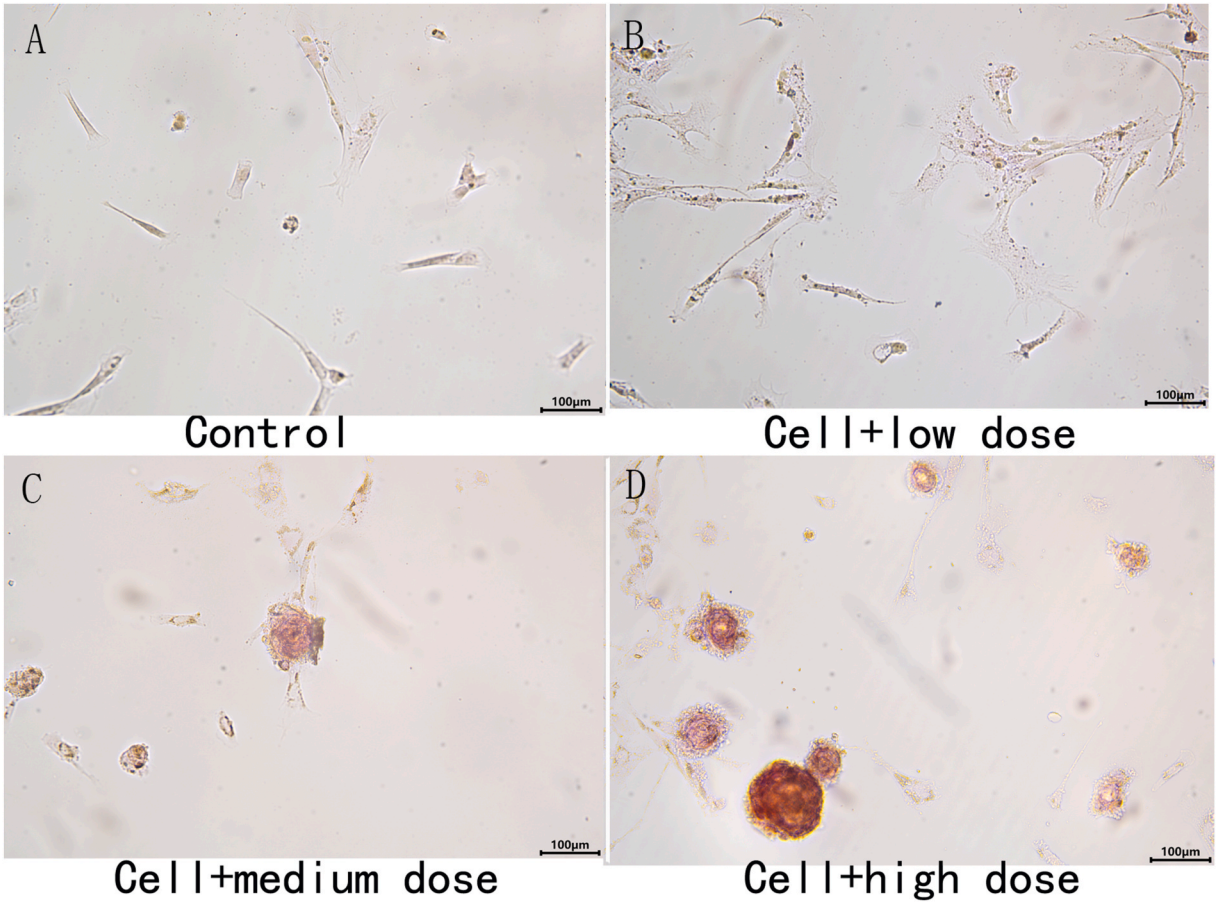

Fig. 3. Alizarin red staining of osteoblast after 21 days of mineralization induction. (× 100). A Control group. B Cell + low dose group. C Cell + medium dose group. DCell + high dose group.

3.8. HXJGC may inhibit PI3K/AKT/mTOR signaling pathway to promote fracture healing in rabbits

In order to explore the role of PI3K/AKT/mTOR pathway in rabbit fracture healing, the phosphorylation level of PI3K/AKT/mTOR pathway protein was detected by WB(Fig. 8A). Compared with HXJGC+3-MA group, the phosphorylation level of PI3K/AKT/mTOR (Fig. 8B), (Fig. 8C), (Fig. 8D) protein in HXJGC group was significantly decreased($p < 0.05$). Compared with HXJGC group, there was no significant difference in the phosphorylation level of PI3K/AKT/mTOR protein in Model group. These results suggest that the effect of HXJGC on rabbit fracture healing may be related to the inhibition of PI3K/AKT/mTOR pathway.the decrease in phosphorylation level represents the inactivation of the pathway, which leads to cellular autophagy.

4. Discussion

Fracture healing is a complex tissue engineering process involving different levels of signaling, cells, matrix, etc. Traditionally, it is divided into three stages: early inflammation and cell aggregation (hematoma mechanization stage); metaphase cell differentiation and new bone formation (callus formation stage); and late bone remodeling and cortical bone formation (heavy callus remodeling stage). Most fractures heal in time with adequate blood supply, a sterile environment, mechanical stability, and proper soft tissue management. However, when the fracture is not fully reduced or opened, the mechanical stability of intramembranous disorder and intrachondral ossification after callus formation is incomplete, which may lead to indirect or secondary healing. In the first few days after injury, intramembranous ossification produces bone directly under the periosteal, resulting in intrachondral ossification, which is prone to bone nonunion [15]. Once nonunion occurs, a second surgery is often required, but even then, nonunion may occur after a second surgery. At present, BMP-2 is the main biological agent approved for clinical use to promote fracture healing [16]. Studies have shown that ectopic ossification is more likely to occur after the use of BMP-2, and some patients may even be induced by the use of BMP-2 and have life-threatening complications [17,18].

The Huoxue Jiegu compound capsule is made of Rhizoma drynariae, Dipsacus, Natural copper, Trionyx sinensis, Salvia miltiorrhiza, Speranskia tuberculata, Caesalpinia sappan, Panax notoginseng, Borneolum syntheticum, Corydalis yanhusuo. The

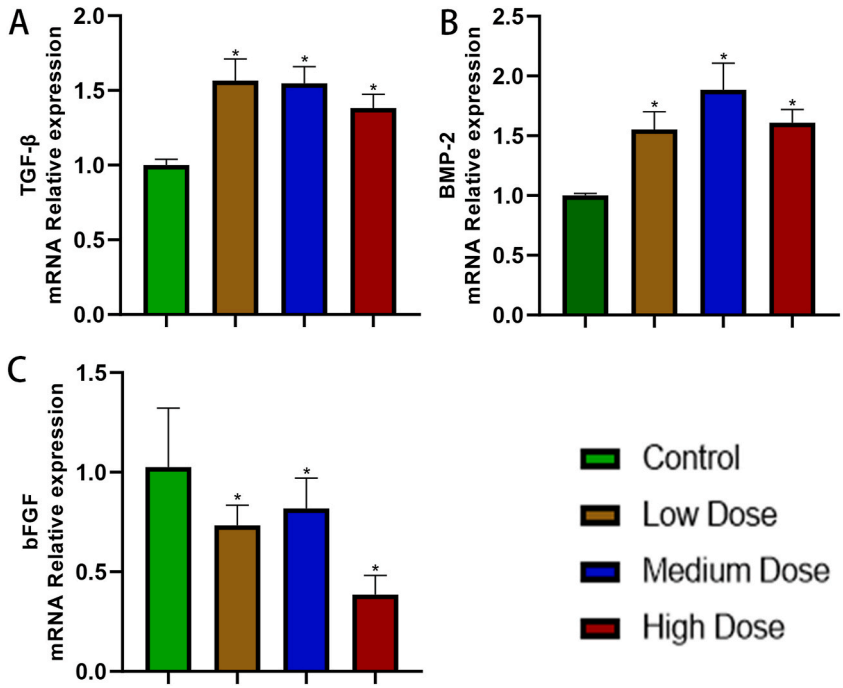

Fig. 4. A Effects of HXJGC on mRNA expression of TGF-β in osteoblast. B Effects of HXJGC on mRNA expression of BMP-2 in osteoblast. C Effects of HXJGC on mRNA expression of bFGF in osteoblast. $^{*}P < 0.05$ vs. Control group, $^{\#}P < 0.05$ vs. Cell + low dose.

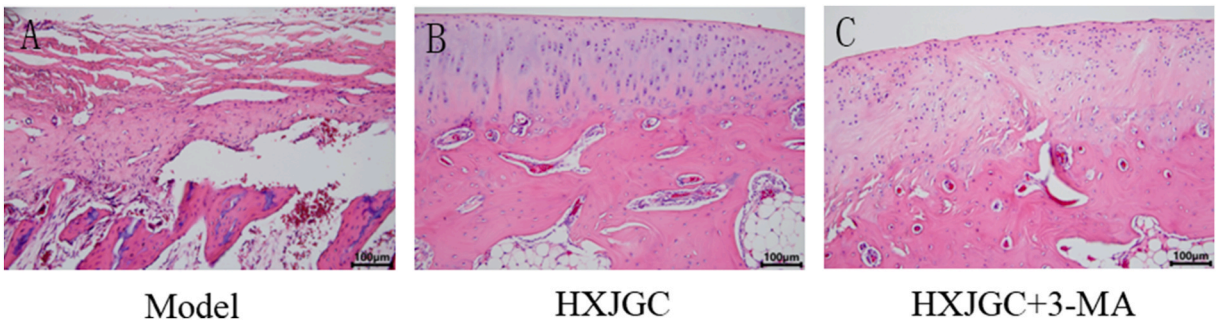

Fig. 5. HE staining results of rabbit bone tissue. (× 200). A Model group. B HXJGC group. C HXJGC+3-MA group.

characteristics and effects of each ingredient in Huoxuejigu capsules on bones. Rhizoma Drynariae: activating blood and continuing injury, tonifying kidney and strengthening bone. Dipsacus: bruises, broken bones. Natural Copper: dispersing stasis and relieving pain, reinforcing tendon and bone. Trionyx sinensis: break the blood and remove the stasis, and renew the tendons and bones. Salvia miltiorrhiza: activating blood to regulate menstruation, removing blood stasis to relieve pain, cooling blood to eliminate carbuncles, eliminating irritability and calming. Speranskia tuberculata: dispelling wind and dampness, relaxing muscles and promoting blood circulation, dispersing stasis and reducing swelling, detoxifying and relieving pain. Caesalpinia sappan: bruises, broken bones, bruises and pain. Panax notoginseng: removing blood stasis and stopping bleeding, promoting blood circulation and fixing pain. Borneolum Syntheticum: To awaken the spirit, clear heat and relieve pain. Corydalis yanhusuo: activating blood, promoting qi and relieving pain (https://www.dayi.org.cn/).

The formation, survival, differentiation, and mineralization of osteoblasts in bone further affect bone formation, and the active substances secreted by osteoblasts can regulate the recruitment, attachment, proliferation, and differentiation of osteoclasts, thus affecting bone resorption [19]. In this study, it was found that under the ALP staining smear, the bone cells were more dense and differentiated than those in the control group and the inhibitor group. These results indicated that after taking HXJGC for 4W, osteoblasts were catalyzed to mature and promote bone formation in rabbits with tibial fracture.

Autophagy is a stress defense mechanism of cells that maintains a stable state by degrading damaged cells and organelles to form autophagosomes [20]. At the same time, autophagy can also promote osteoblast formation and late osteogenesis [21]. One study found that the use of the glucocorticoid dexamethasone inhibited the expression of the PI3K/Akt signaling pathway, thereby inhibiting

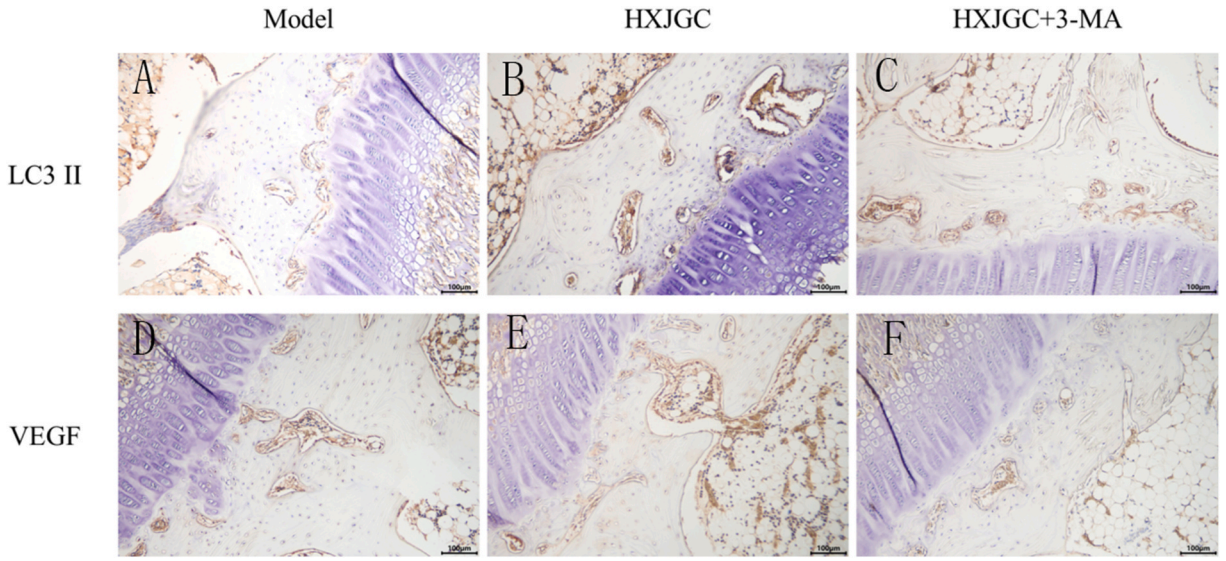

Fig. 6. A LC3II protein expression of Model group. B LC3II protein expression of HXJGC group. C LC3II protein expression of HXJGC+3-MA group. D VEGF protein expression of Model group. E VEGF protein expression of HXJGC group. F VEGF protein expression of HXJGC+3-MA group(× 200).

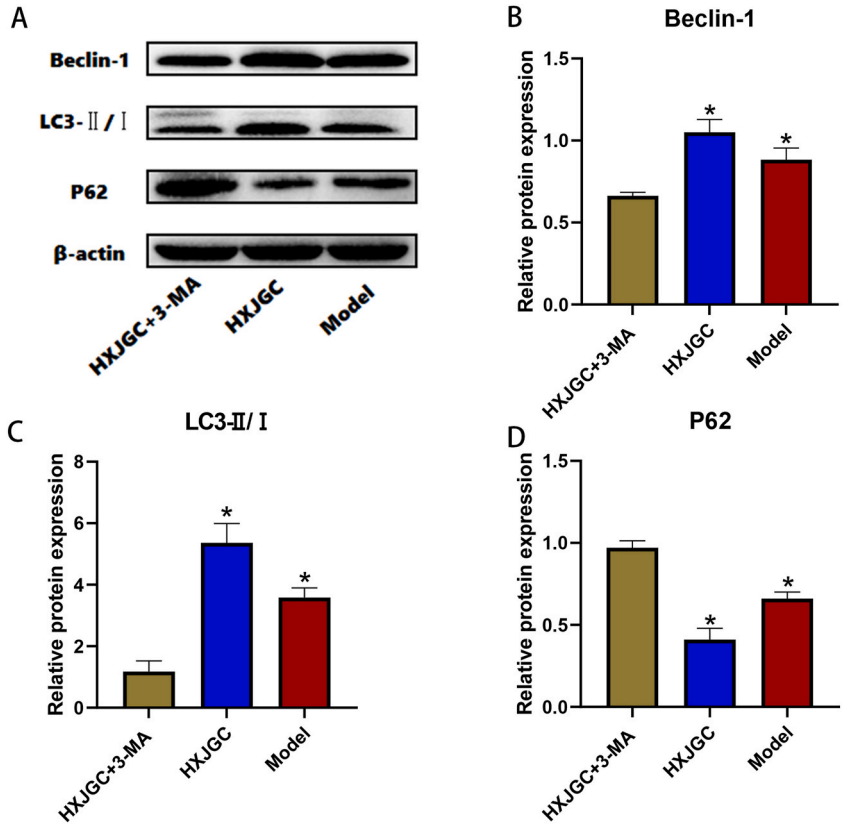

Fig. 7. Results of Beclin-1、LC3II/I and p62 protein expression in rabbit bone tissue of each group (A)WB strip (B–D) Protein expression histogram. *$P < 0.05$ * vs HXJGC+3-MA group.

osteogenic differentiation [22]. Another study showed that activation of the mTOR/ULK1 signaling pathway can induce autophagy and promote osteoblast differentiation, maturation, and biomineralization [23–25]. In this study, it was found that after taking HXJGC for 4W, the expression of PI3K/Akt/mTOR signaling pathway activation factor was inhibited, and the ratio of autophagy related factor

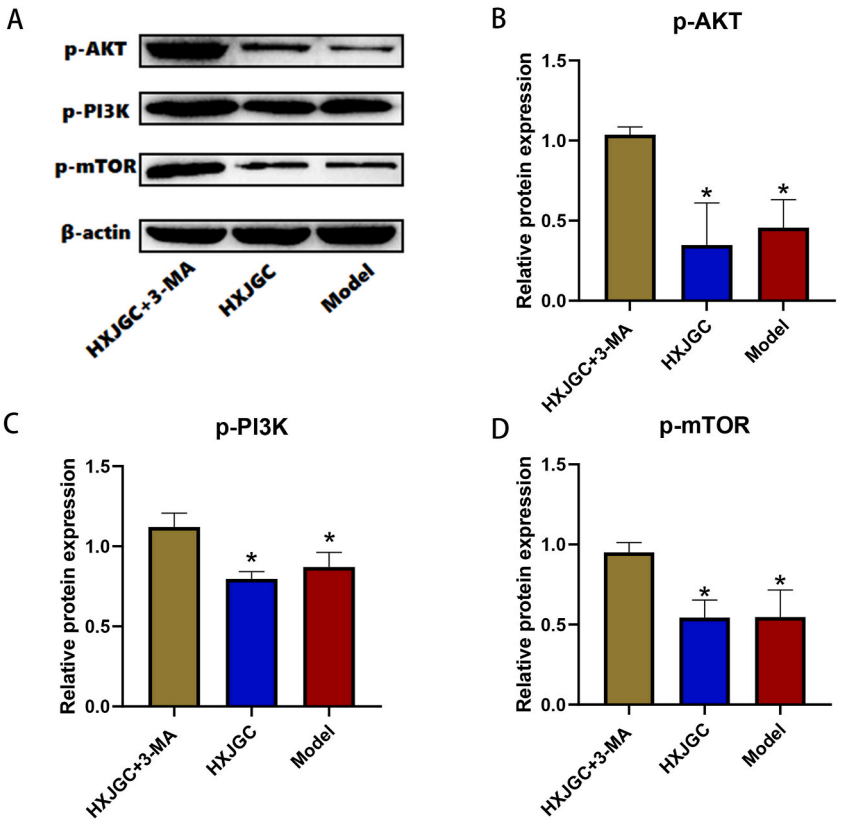

Fig. 8. Results of the phosphorylation level of PI3K/AKT/mTOR protein in rabbit bone tissue of each group (A)WB strip (B–D) Protein expression histogram. *$p < 0.05$ * vs HXJGC+3-MA group.

LC3-II/I was significantly induced to increase. Furthermore, some limitations are present in this study. The number of samples collected in our study was limited, and future validation will require a larger sample size.

In summary, Huexuejigu compound capsules can regulate the expression of the PI3K/Akt/mTOR signaling pathway, provide therapeutic intervention for rabbits with tibial fractures, promote the differentiation and maturation of osteoblasts at the broken end of fractures, accelerate the recovery of fractures, and achieve the purpose of treating this disease.

Ethics declarations

This study was reviewed and approved by [fExperimental Animal Ethics Committee of Guizhou University of Chinese Medicine] with the approval number: [20230148], dated [2023,10,30,].

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Data availability statement

All data are included in the study and in the Supplemental Materials.

CRediT authorship contribution statement

Yingjie Wu: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Mingxiang Fan:** Visualization, Formal analysis, Data curation. **Shixiang Tan:** Writing – original draft, Data curation, Conceptualization. **Qiucheng Guo:** Writing – original draft, Data curation, Conceptualization. **Hegui Xu:** Writing – review & editing, Methodology,

Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Y. Tang, Y. Mo, D. Xin, et al., Regulation of osteoblast autophagy based on PI3K/AKT/mTOR signaling pathway study on the effect of β-ecdysterone on fracture healing, J. Orthop. Surg. Res. 16 (1) (2021 Dec 19) 719. https://pubmed.ncbi.nlm.nih.gov/34924000/.

[2] B. Mi, Y. Xiong, L. Lu, et al., Macrophage-mediated fracture healing: unraveling molecular mechanisms and therapeutic implications using hydrogel-based interventions, Biomaterials 305 (2024 Mar) 122461. https://pubmed.ncbi.nlm.nih.gov/38171119/.

[3] C.L. Ekegren, E.R. Edwards, R. De Steiger, et al., Incidence, costs and predictors of non-union, delayed union and mal-union following long bone fracture, Int J Environ Res Public Health 15 (12) (2018 Dec 13) 2845. https://pubmed.ncbi.nlm.nih.gov/30551632/.

[4] Shen Zewei, Wei Yushrimp, Yu Canqing, et al., Descriptive analysis of the hospitalization rate of fracture in adults from 10 regions in China, Chin. J. Epidemiol. 42 (5) (2019) 771–779. https://qikan.cqvip.com/Qikan/Article/Detail?id=7104891120&from=Qikan_Search_Index.

[5] H.M. Shen, P. Codogno, Autophagic cell death: loch Ness monster or endangered species? Autophagy 7 (5) (2011 May) 457–465. https://pubmed.ncbi.nlm.nih.gov/21150268/.

[6] D.J. Klionsky, G. Petroni, R.K. Amaravadi, et al., Autophagy in major human diseases, EMBO J. 40 (19) (2021 Oct 1) e108863. https://pubmed.ncbi.nlm.nih.gov/34459017/.

[7] X. Yang, T. Jiang, Y. Wang, et al., The role and mechanism of SIRT1 in resveratrol-regulated osteoblast autophagy in osteoporosis rats, Sci. Rep. 9 (1) (2019 Dec 5) 18424. https://pubmed.ncbi.nlm.nih.gov/31804494/.

[8] S. Wang, Z. Deng, Y. Ma, et al., The role of autophagy and mitophagy in bone metabolic disorders, Int. J. Biol. Sci. 16 (14) (2020 Jul 30) 2675–2691. https://pubmed.ncbi.nlm.nih.gov/32792864/.

[9] G. Yuan, Z. Lian, Q. Liu, et al., Phosphatidyl inositol 3-kinase (PI3K)-mTOR inhibitor PKI-402 inhibits breast cancer induced osteolysis, Cancer Lett. 443 (2019 Feb 28) 135–144. https://pubmed.ncbi.nlm.nih.gov/30540926/.

[10] D. Tewari, P. Patni, A. Bishayee, et al., Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: a novel therapeutic strategy, Semin. Cancer Biol. 80 (2022 May) 1–17. https://pubmed.ncbi.nlm.nih.gov/31866476/.

[11] B. Zhao, Q. Peng, E.H.L. Poon, et al., Leonurine promotes the osteoblast differentiation of rat BMSCs by activation of autophagy via the PI3K/Akt/mTOR pathway, Front. Bioeng. Biotechnol. 9 (2021 Feb 23) 615191. https://pubmed.ncbi.nlm.nih.gov/33708763/.

[12] Xu Hegui, Wang Yi, Li Yuxiong, et al., Effect of Huoxue Jiegu compound capsule on rabbits with closed lumbar spine fracture based on PI3K/Akt/mTOR signaling pathway, Chinese Journal of Gerontology 42 (16) (2022) 4015–4018. https://kns.cnki.net/kcms2/article/abstract?v=_6cC4UgRj8TzJ6Mke2nXf1IWq9nxKKiqsXTc0_LWHMunIjr0K46P1QivK98kqGtg6dNCxq2BWAaDLsI5NaSnDdAK9jsw9AeMJ7inD61hvfCcEMmoTKzVhpOyIZXIKyvN-Ve0OKlw3Ks=&uniplatform=NZKPT&flag=copy.

[13] Xu Hegui, Dongxin Tang, Liu Yang, et al., Effect of Huoxuejigu Capsule on the expression of bFGF, TGF-β and BMP-2 in rabbit fracture healing, Chinese Journal of Orthopaedic Surgery 27 (20) (2019) 1900–1903. https://kns.cnki.net/kcms2/article/abstract?v=_6cC4UgRj8Tc8CMSRwgAR4LDFsmRI-hJ2a9mRG7f2pXsx4VWdPNfPJ4xGJPgUlQ3z4ZqhrHmLCbiW4ozd4OUIEM9j7WM-UohvuZ7O0clrBAv_8zIeH_HJRFgf6ebXJ18FKcN2wjVV1c=&uniplatform=NZKPT&flag=copy.

[14] F.A. Alhasson, D.A. Kareem, N.A. Shehan, et al., Effect of eggshell nanoparticles on healing bone fracture, Arch Razi Inst 77 (3) (2022 Jun 30) 1173–1180. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9759247/.

[15] I. Dumic-Cule, M. Peric, L. Kucko, et al., Bone morphogenetic proteins in fracture repair, Int. Orthop. 42 (11) (2018 Nov) 2619–2626. https://pubmed.ncbi.nlm.nih.gov/30219967/.

[16] A. Belal, H. Elkady, A.A. Al-Karmalawy, et al., Discovery of some heterocyclic molecules as bone morphogenetic protein 2 (BMP-2)-Inducible kinase inhibitors: virtual screening, ADME properties, and molecular docking simulations, Molecules 27 (17) (2022 Aug 30) 5571. https://pubmed.ncbi.nlm.nih.gov/36080338/.

[17] C.D. Lopez, J.M. Bekisz, C. Corciulo, et al., Local delivery of adenosine receptor agonists to promote bone regeneration and defect healing, Adv. Drug Deliv. Rev. 146 (2019 Jun) 240–247. https://pubmed.ncbi.nlm.nih.gov/29913176/.

[18] A. Mediero, T. Wilder, L. Shah, et al., Adenosine A2A receptor (A2AR) stimulation modulates expression of semaphorins 4D and 3A, regulators of bone homeostasis, FASEB J 32 (7) (2018 Jul) 3487–3501. https://pubmed.ncbi.nlm.nih.gov/29394106/.

[19] V. Fischer, M. Haffner-Luntzer, Interaction between bone and immune cells: implications for postmenopausal osteoporosis, Semin. Cell Dev. Biol. 123 (2022 Mar) 14–21. https://pubmed.ncbi.nlm.nih.gov/34024716/.

[20] M. Li, N. Yang, L. Hao, et al., Melatonin inhibits the ferroptosis pathway in rat bone marrow mesenchymal stem cells by activating the PI3K/AKT/mTOR signaling Axis to attenuate steroid-induced osteoporosis, Oxid. Med. Cell. Longev. 18 (2022 Aug) 8223737, 2022, https://pubmed.ncbi.nlm.nih.gov/36035224/.

[21] L. Yi, T. Zhong, Y. Huang, et al., Triiodothyronine promotes the osteoblast formation by activating autophagy, Biophys. Chem. 267 (2020 Dec) 106483. https://pubmed.ncbi.nlm.nih.gov/33010728/.

[22] J.M. Pan, L.G. Wu, J.W. Cai, et al., Dexamethasone suppresses osteogenesis of osteoblast via the PI3K/Akt signaling pathway in vitro and in vivo, J. Recept. Signal Transduct. Res. 39 (1) (2019 Feb) 80–86. https://pubmed.ncbi.nlm.nih.gov/31210570/.

[23] W. Ruolan, C. Liangjiao, S. Longquan, The mTOR/ULK1 signaling pathway mediates the autophagy-promoting and osteogenic effects of dicalcium silicate nanoparticles, J Nanobiotechnology 18 (1) (2020 Aug 31) 119. https://pubmed.ncbi.nlm.nih.gov/32867795/.

[24] X. Li, K.B. Yang, W. Chen, et al., CUL3 (cullin 3)-mediated ubiquitination and degradation of BECN1 (beclin 1) inhibit autophagy and promote tumor progression, Autophagy 17 (12) (2021 Dec) 4323–4340. https://pubmed.ncbi.nlm.nih.gov/33977871/.

[25] J. Zhou, Y. Jiang, H. Chen, et al., Tanshinone I attenuates the malignant biological properties of ovarian cancer by inducing apoptosis and autophagy via the inactivation of PI3K/AKT/mTOR pathway, Cell Prolif. 53 (2) (2020 Feb) e12739. https://pubmed.ncbi.nlm.nih.gov/31820522/.