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# Curcumin Promotes Femoral Fracture Healing in a Rat Model by Activation of Autophagy

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**Background:** The aim of this study was to use a rat model of femoral fracture healing to study the effects of curcumin on cell autophagy, compared with treatment with 3-methyladenine (3-MA), an inhibitor of autophagy.

**Material/Methods:** Thirty-six Sprague-Dawley rats with right mid-femoral fracture were divided into three groups: the curcumin-treated group (N=12) (gavage with curcumin 400 mg/kg/day); the curcumin + 3-MA-treated group (gavage with curcumin 400 mg/kg/day + 3-MA 30 mg/kg/day); and the control group (N=12) (gavage normal saline). Each group underwent femoral bone imaging using anteroposterior X-ray and micro-computed tomography (CT) at two weeks and six weeks following bone fracture. All rats were euthanized at the end of the study. Histology of the bone was performed to compare bone healing. Immunofluorescence and immunohistochemical tissue staining and Western blots were performed, to compare the expression of autophagy-related proteins, Beclin-1 and LC3-II.

**Results:** Autophagy of rat femoral bone tissue was activated following fracture, increasing with time, reaching a peak at 24 hours. Imaging and histology showed that curcumin promoted the fracture healing in rats, which was reduced by treatment with 3-MA. Immunohistochemistry, immunofluorescence, and Western blot showed that curcumin treatment increased the expression of Beclin-1 and LC3-II, which were reduced by treatment with the autophagy inhibitor, 3-MA.

**Conclusions:** The findings of this study, in a rat model of femoral bone fracture healing, showed that curcumin promoted bone healing and autophagy, which were reduced by treatment with 3-MA, a known inhibitor of autophagy.

**MeSH Keywords:** **Autophagy • Curcumin • Fracture Healing**

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## Background

The process of bone fracture healing involves a series of complex and orderly regulated processes involving cellular changes and biochemical changes [1]. Bone tissue has a strong reparative capability, and the bone fracture healing can restore the structure and function of bone. However, although bone tissue has the capability for repair in different types of fracture, previous studies have shown that in between 5-10% of bone fractures, healing is poor or delayed or fracture nonunion may occur even after clinical treatment of fractures, leading to impaired patient rehabilitation and impaired quality of life [2,3].

Currently, several published studies have supported the role of specific molecular signaling pathways, the expression of proteins, and the regulation of key cytokines involved in fracture healing [4-6]. Although morphological studies on fracture healing have been well described, specific cellular and molecular mechanism of fracture healing remain unclear, with some views being that bone fracture healing is dominated by cell function [7].

Autophagy is a normal physiological process that involves an intracellular degradation system that delivers cytoplasmic constituents to the lysosome to maintain cell survival, balancing sources of energy at times of nutrient or physiological stress, in disease and trauma [8,9]. Some studies have confirmed the prominent role of autophagy in cancer [10], heart failure [11], hypertension [12], diabetes [13], and some strategies to treat disease through influencing the level of autophagy have been proposed. For bone tissue cells at the fracture site, autophagy plays an important role in the maintenance of bone cell survival in a stress environment, including bone fracture [14,15]. It has also been proposed that autophagy is closely related to the bone growth, bone resorption, bone remodeling, and bone tissue mineralization [14,15].

Curcumin, the active component of turmeric, is derived from the rhizomes of the ginger family (*Zingiberaceae*) and has a variety of pharmacological activities. The results of recently published *in vitro* experiments have shown that curcumin can inhibit osteoblast proliferation and function [16], and curcumin has a role in bone remodeling [17]. Recent studies have shown that curcumin can be used to treat disease by regulating cell autophagy [18].

The aim of this study was to use a Sprague-Dawley rat model of internal fixation of femoral fracture healing to study the effects of curcumin on cell autophagy, compared with treatment with 3-methyladenine (3-MA), an inhibitor of autophagy.

## Material and Methods

### Experimental animals

The model used in this study included 36 adult male Sprague-Dawley rats of more than 3 months of age, weighing between 230-270 gm, provided by the Experimental Animal Center. All rats had normal nutritional and behavioral status. All experimental procedures were in accordance with the Institutional Animal Care and Use Committee of Shihezi University, China.

### Establishment of the right middle femoral fracture rat model

After the rats were acclimatized to laboratory conditions and fed for one week, the right middle femoral fracture was performed. The rats received anesthesia in a supine position of an intraperitoneal injection of ketamine (0.19 gm/kg). The hair of the lateral right thigh was removed, the area of the thigh for each rat was disinfected three times, and each rat was placed on a sterile towel that had a hole for access of the right leg. A 3 cm transverse incision was made in the right lateral femur, the skin and subcutaneous tissue were removed, blunt dissection was used to separate the right *biceps femoris* muscle to expose the right femoral bone shaft, and the femur was sawed off with a metal-wire saw. A 1 cm in length longitudinal incision was made in the lateral patella of the right lower limb to expose and open the quadriceps tendon tissues. The patella was dislocated to expose the femoral intercondylar fossa. A size-20 hypodermic needle was inserted from the femoral intercondylar fossa to the femoral trochanter along the medullary space of the femoral shaft. A Kirschner wire with a diameter of 1.0 cm was inserted in the opposite direction, to align the fracture site. The proximal end of the Kirschner wire was inserted into the cortex of femoral trochanter, and the redundant Kirschner wire in the distal end was cut, and the tip was buried under the cortex of the femoral trochanter. Then the subcutaneous incision was sutured layer by layer; the skin incision was sutured before imaging examinations were performed.

### Experimental animal grouping and interventions

The 36 Sprague-Dawley rats with a right mid-femoral fracture were divided into three groups: the curcumin-treated group (N=12) (gavage with curcumin 400 mg/kg/day); the curcumin + 3-methyladenine (3-MA)-treated group (gavage with curcumin 400 mg/kg/day + 3-MA 30 mg/kg/day); and the control group (N=12) (gavage normal saline) [19,20].

### Qualitative analysis of X-ray examination of callus

Each group of Sprague-Dawley rats received an anteroposterior X-ray examination at two weeks and six weeks after bone

surgery under the same conditions. Callus formation and fracture healing were observed and recorded.

### Quantitative analysis of micro-computed tomography (CT) imaging of bone callus

Sprague-Dawley rats were euthanized with anhydrous ether, the soft tissues around the femur were removed, the callus was retained, the Kirschner wire was removed, and the fracture samples were collected. After formalin fixation for 24-48 hours, the samples were placed in the micro-CT machine, scanned along the long axis of the femoral samples and the region of interest, 10 mm around the fracture site, was selected for analysis. Finally, the three-dimensional reconstruction was performed for the fracture samples in the region of interest.

### Tissue fixation, processing, sectioning, and light microscopy

Bone callus tissue was fixed in neutral buffered formalin, processed and embedded in paraffin wax, and then sectioned at 4  $\mu\text{m}$  onto glass slides for histology. The sections were dewaxed, followed by gradient dehydration using 95%, 80%, and 70% ethanol, and then the sections were stained histochemically using hematoxylin and eosin (H&E), before light microscopic examination.

### Immunohistochemistry and immunofluorescence

De-waxed 4  $\mu\text{m}$  tissue sections of callus on glass slides underwent antigen retrieval, and the primary antibodies, including antibodies to Beclin-1 and LC3-II, were incubated on the sections. The secondary antibodies were conjugated to a chromogen for immunohistochemistry and light microscopy, and to an immunofluorescence-conjugated label for immunofluorescence and laser scanning confocal microscopy.

### Western blot analysis

The total protein was extracted from the fracture site according to the standard total protein extraction process, followed by protein quantification using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentrated gel and separation gel were prepared according to the protein molecular weight, the membrane transfer was performed after the gel electrophoresis, and the primary and secondary antibodies were incubated, followed by color development using a chemiluminescence kit with semi-quantitative analysis.

### Statistical analysis

Data obtained were analyzed using the SPSS version 18.0 statistical software. Data measurements were presented as the

mean  $\pm$  standard deviation (SD). A p-value of  $<0.05$  indicated statistical significance.

## Results

### Cell autophagy in healing bone was activated and changed with time

Immunofluorescence localization of the LC3-II protein showed expression in cells in the normal bone tissues and the bone callus following fracture or the rat femur. Increasing expression levels of LC3-II protein were found at each time point following fracture, and the expression of LC3-II protein at each time point was greater when compared with normal bone tissue. Comparison of LC3-II protein expression at each time point showed that the autophagy level increased gradually after fracture, reached a peak at 24 hours, and then decreased gradually after 24 hours. The autophagy level at seven days after bone fracture was significantly decreased compared with that at 24 hours, but the autophagy level was still greater when compared with normal bone tissue (Figure 1).

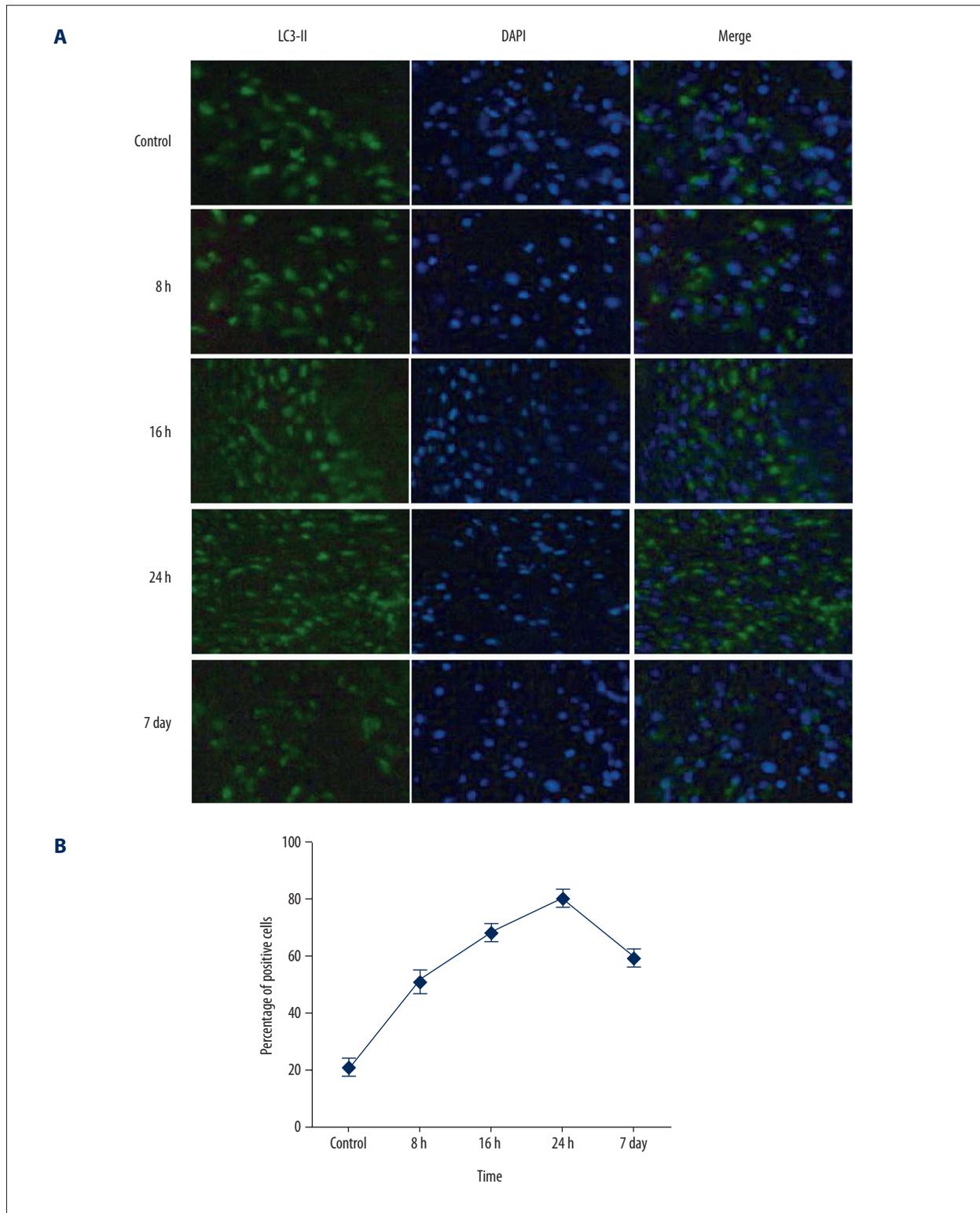
### Imaging showed that curcumin could promote fracture healing in the rat model

At six weeks following fracture in the Sprague-Dawley rat model, X-ray imaging showed that the Kirschner wire was fixed in place in all three groups studied, and the alignment of the fracture site was good. The callus at the fracture site of the curcumin-treated group (N=12) was thicker in the second week, and the fracture line was blurred on imaging, consistent with healing. The femoral bone callus of rats in the control group (N=12) showed continuous growth, and the fracture line was blurred. There was discontinuous callus formation in the curcumin + 3-methyladenine (3-MA)-treated group, but the fracture line was still clearly visible, indicating non-healing (Figure 2A).

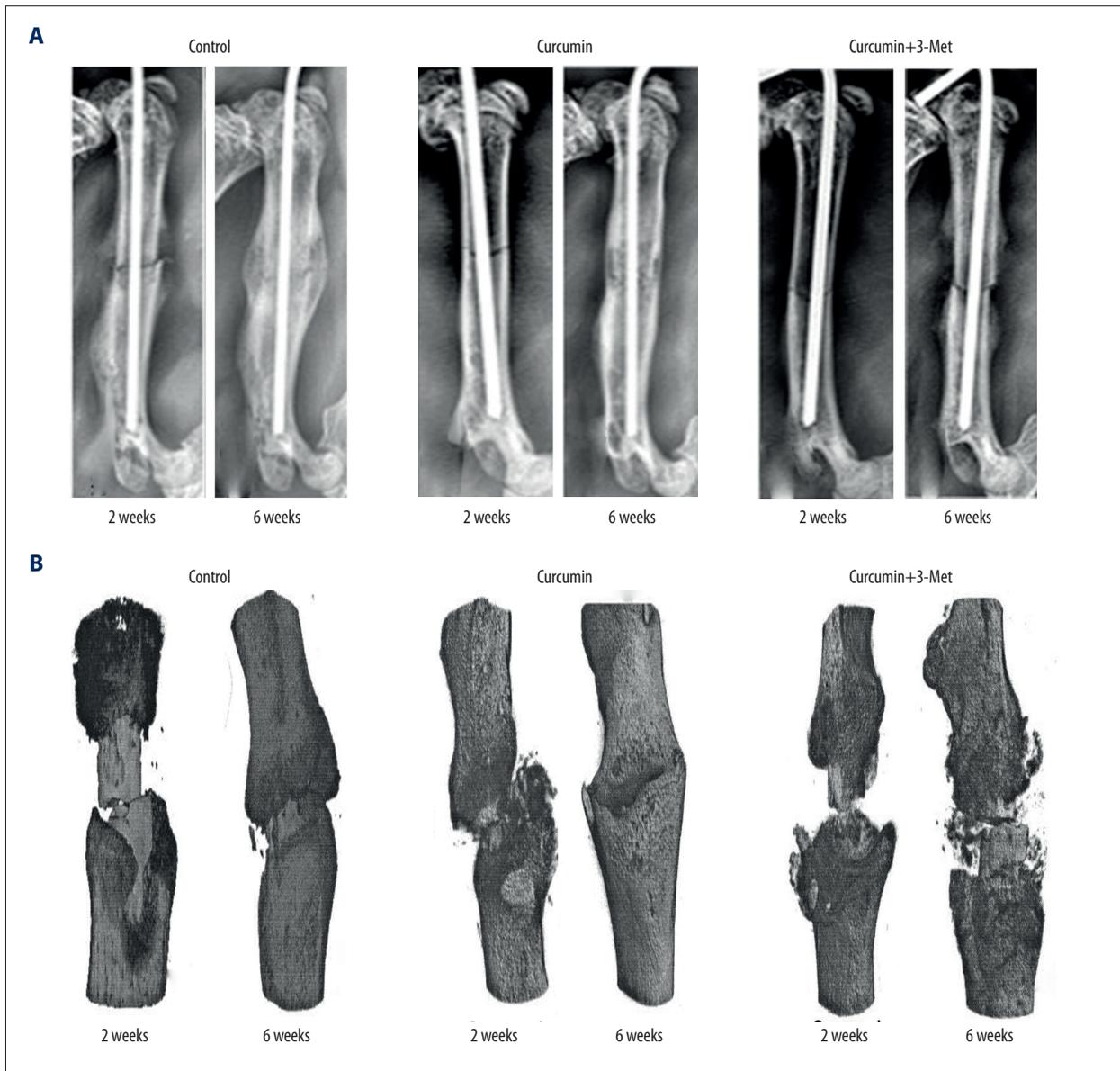
Micro-computed tomography (CT) showed that new bone formation occurred at the fracture site of the femoral shaft of rats in the curcumin-treated group, and the callus thickness was increased. Callus formation in the control group was increased and most bone tissues were associated with callus. Some new bone also developed at the fracture site. The size of the fracture site in the curcumin + 3-MA-treated group was increased compared with that before treatment (Figure 2B).

### Histological examination showed that curcumin treatment promoted the healing of the femoral fracture in the rat model

At six weeks after fracture modeling of Sprague-Dawley rats, the bone trabeculae in the curcumin-treated group were fused



**Figure 1.** Bone tissue cell autophagy in the rat model is activated after fracture and the autophagy levels change with time. **(A)** Representative immunofluorescence staining of bone tissue at different time points after femoral fracture. **(B)** Analysis of percentage of cells in rat tissues at the fracture site, stained for LC-III using immunofluorescence.



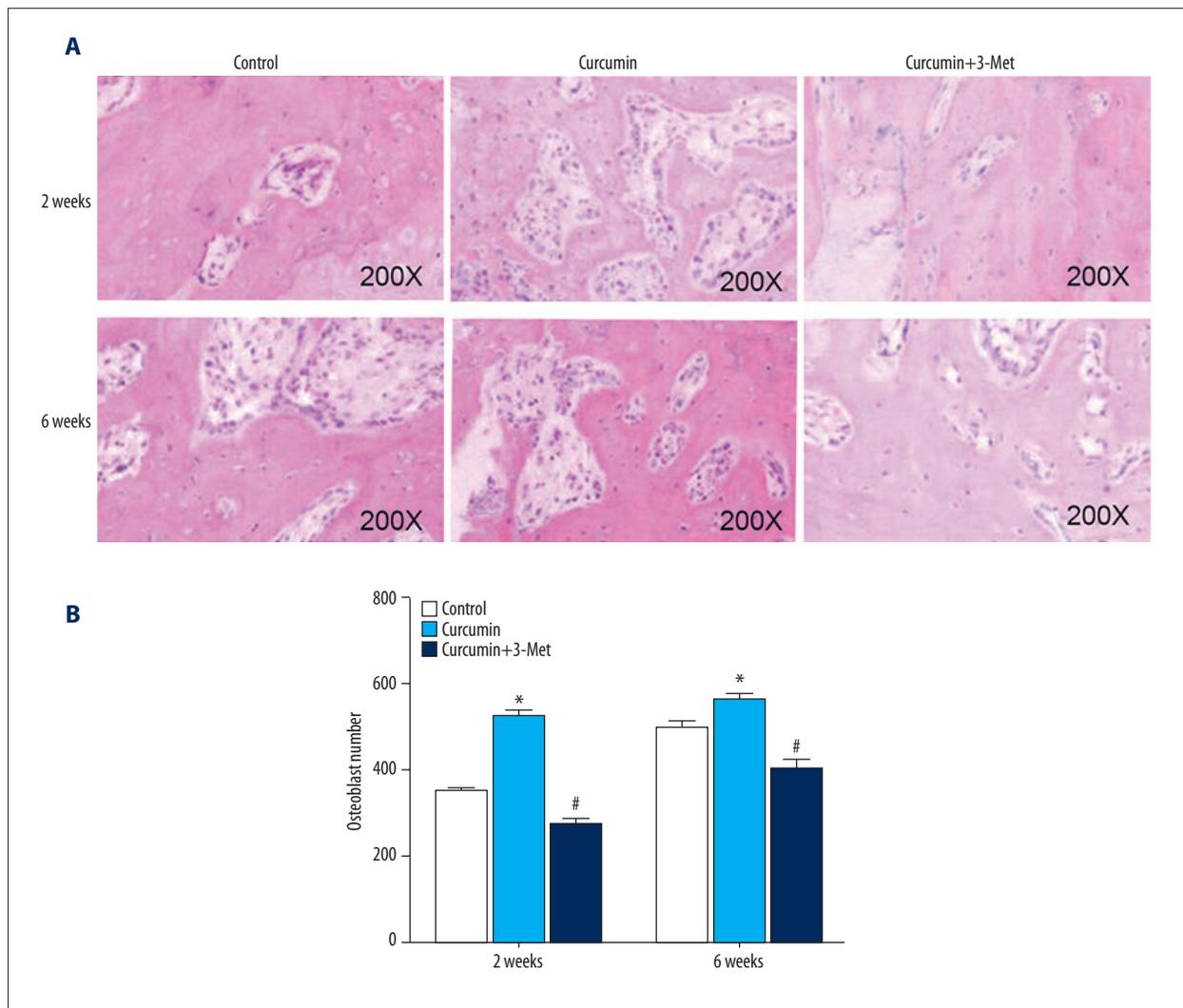
**Figure 2.** Imaging studies of rat femurs show that curcumin promotes the fracture healing in the rat model. **(A)** Representative X-ray images of the rat femur after fracture. **(B)** Representative micro-computed tomography (CT) images of rat femur after fracture.

with bone formation, and the mean number of osteoblasts counted was  $591.08 \pm 14.53$ . In comparison, the bone trabeculae in the control group were mildly fused, the trabecular space was wider with little bone formation, and the mean number of osteoblasts was  $536.36 \pm 17.42$ . The bone trabeculae in the curcumin + 3-MA-treated group were looser compared with that of the other two groups, the trabecular space was wider, but the mean number of osteoblasts was decreased at  $402.17 \pm 16.81$  (Figure 3).

### Curcumin activated autophagy in rat femurs following fracture

In the rat femur fracture model, the Beclin-1 protein was expressed in the new bone tissue cells (osteoblasts) of each group and was mainly distributed in the cell membrane and cytoplasm. The expression Beclin-1 protein in the curcumin-treated group was significantly increased compared with that in the curcumin + 3-MA-treated group and the control group.

Immunofluorescence of LC3-II protein showed that LC3-II protein was expressed in the cytoplasm with a diffuse distribution



**Figure 3.** Histological examination of the rat femur fracture site shows that curcumin promotes healing. **(A)** Representative photomicrographs of callus of rats at two weeks and six weeks. Hematoxylin and eosin (H&E). **(B)** The number of osteoblasts in femoral fracture callus of each group at two weeks and six weeks. \*  $p < 0.05$  vs. the control group (N=12); #  $p < 0.05$  vs. the curcumin-treated group (N=12).

after femur fracture The expression of LC3-II protein in the curcumin-treated group was significantly increased compared with that in the curcumin + 3-MA-treated group where expression was significantly decreased, and the control group.

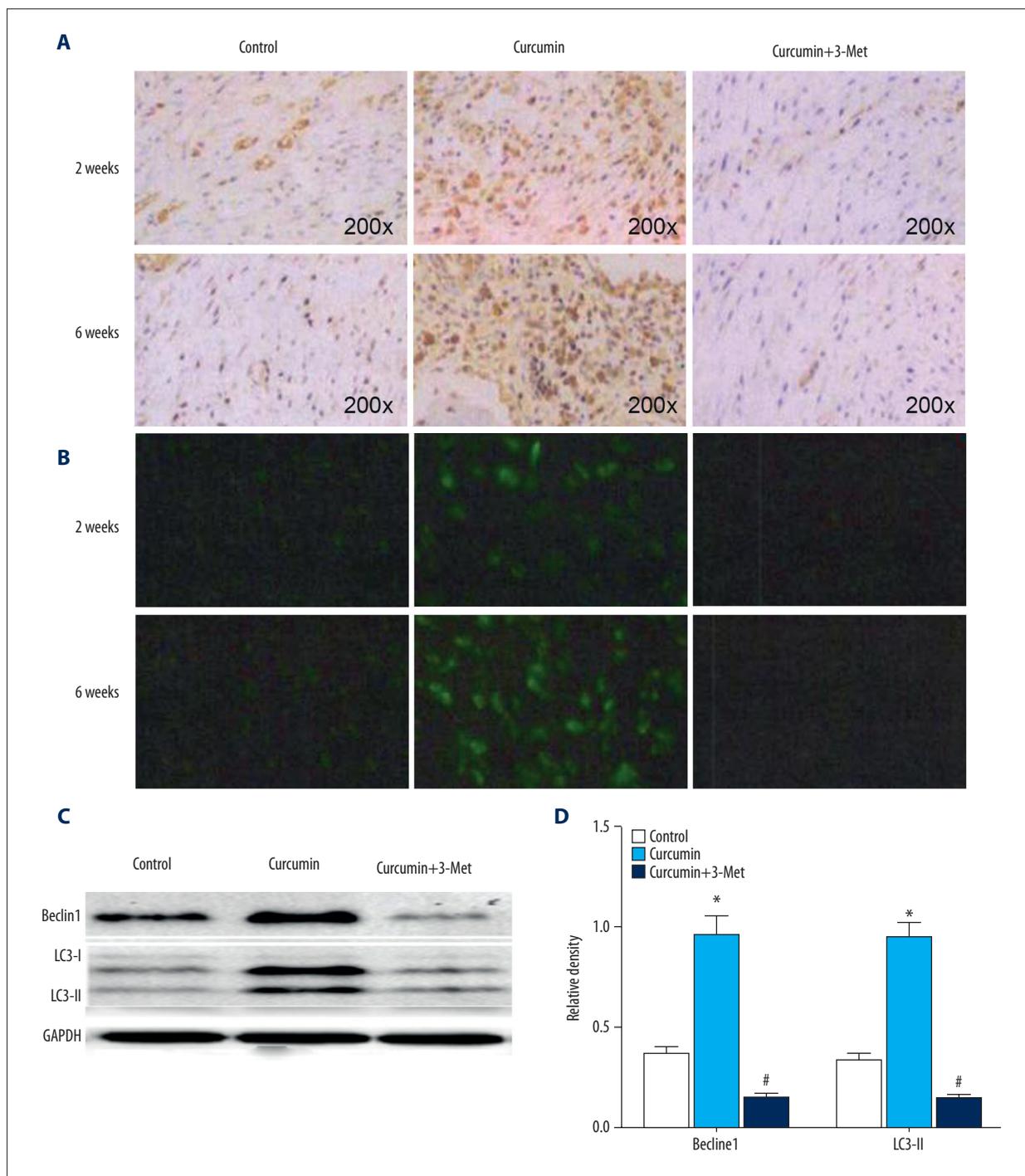
Western blot showed that the expressions of Beclin-1 and LC3-II in the curcumin-treated group were significantly increased compared with that in the curcumin + 3-MA-treated group and the control group (Figure 4).

## Discussion

The establishment of an animal model to study bone fracture healing is of value in determining the mechanism of fracture

healing and compounds that may affect the healing process. The use of large mammalian animal models, as opposed to rodent models, can be used to simulate the human fracture healing but can be time-consuming models requiring complex breeding environments. The use of rodents, as in the present study, can be used for large-sample experiments in a limited space due to the simple feeding and short breeding time. Therefore, for this study, the middle femoral shaft of Sprague-Dawley rats was selected to establish the fracture model and the model of internal fixation of middle femoral shaft fracture was successfully established.

In the present study, the changes in autophagy at the fracture site in the rat model changed over time, with the expression of the LC3-II protein, which began to increase at 8 hours



**Figure 4.** Curcumin activates autophagy in rats after fracture of the femur. **(A)** Representative photomicrographs of Beclin-1 immunohistochemical staining of each group at two weeks and six weeks. **(B)** Representative photomicrographs of LC3-II immunofluorescence staining of each group at two weeks and six weeks. **(C)** Western blot analysis shows the expression of Beclin-1 and LC3-II. **(D)** Semi-quantitative analysis of the expression of Beclin-1 and LC3-II. \*  $p < 0.05$  vs. the control group; #  $p < 0.05$  vs. the curcumin-treated group.

after the fracture, reaching a peak at 24 hours after fracture, and a downward trend by one week after fracture. Also, the LC3-II protein was also expressed in a certain number of cells in the control group, which indicated that cells during normal bone fracture healing have an autophagy level that is related to the maintenance of cell stability and cell growth. The findings of this study have shown that autophagy plays an important role in the process of fracture healing.

The aim of this study was to investigate the effects of curcumin on the process of bone healing, with imaging performed to detect the changes in bone specimens at two weeks and six weeks after bone fracture. The effects of curcumin on cell autophagy were compared with treatment with 3-methyladenine (3-MA), an inhibitor of autophagy. X-ray imaging showed that the earliest occurrence of callus and the time to loss of the fracture line on imaging (an indicator of fracture healing) in the curcumin-treated group was significantly reduced when compared with the untreated control group of rats. In addition to the X-ray image analysis, micro-computed tomography (CT) imaging was also performed for the fracture samples to obtain the more detailed images, which supported the findings of the X-ray imaging.

The process of fracture healing is affected by various factors with bone cell (osteoblast) activity and nutrient supply being two important factors [21,22]. To further evaluate the effect of curcumin on fracture healing, the osteoblasts were observed histologically. Improved fracture healing is related to a sufficient number of osteoblasts and osteoblast activity. Osteoblasts are cells that can be transported to the bone surface through the microvasculature, resulting in the production of bone matrix, with the transformation of osteoblasts to osteocytes [2,3]. The results of this study showed that the number of osteoblasts in the curcumin-treated group was greater than that of the control group, and the number of osteoblasts in the curcumin + 3-MA-treated group was significantly decreased compared with that in the control group.

Darcy et al. [23] reported the use of rapamycin to induce the autophagy level of cells and found that the differentiation of osteoblasts after induction was significantly increased. Liu et al. [24] demonstrated that the suppression of autophagy in osteoblasts by FIP200 deletion resulted in osteopenia in mice through the inhibition of osteoblast terminal differentiation and showed that bone mass was significantly decreased after the autophagy level was reduced. These previously published

studies have shown that different levels of autophagy can affect fracture healing through the differentiation, proliferation, and maturation of osteoblasts.

In the present study, 3-MA, an inhibitor of autophagy, is known to inhibit phosphatidylinositol 3-phosphate (PI3P), which is a regulator of membrane traffic, leading to the formation of autophagosomes [25]. X-ray results showed that the extinction time of fracture line of rats in the curcumin + 3-MA-treated group was later than that in the curcumin-treated group. Micro-CT three-dimensional reconstruction imaging suggested more intuitively that the curcumin-treated group is still superior to the curcumin + 3-MA-treated group in the process of bone healing. Histology showed that the number of osteoblasts in the curcumin + 3-MA-treated group was significantly lower than that of the curcumin-treated group. The above results suggested that autophagy plays an important role in the process of promoting fracture healing.

In support of the finding that autophagy is involved in promoting fracture healing, this study also investigated levels of autophagy-related proteins, Beclin-1 and LC3-II. In the curcumin-treated group, the expression of Beclin-1 and LC3-II were significantly increased compared with the control group and the curcumin + 3-MA-treated group, supporting the view that curcumin treatment activated the autophagy pathway at the fracture site. Following 3-MA treatment, the effect of curcumin autophagy activation was decreased, supporting the view that curcumin activates cell autophagy through the PI3P pathway, which can be blocked by 3-MA, as 3-MA is an inhibitor of PI3P protein. When the activity of PI3P is inhibited, the autophagy can be blocked. Future studies are required to further investigate the mechanisms of autophagy in bone fracture repair in detail, including the aspect the role of the PI3P signaling pathway.

## Conclusions

The findings of this study, in a rat model of femoral bone fracture healing, showed that curcumin promoted bone healing and autophagy, which were reduced by treatment with 3-methyladenine (3-MA), a known inhibitor of autophagy.

## Conflict of interest

None.

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