Protective Effect of Curcumin on Bone Trauma in a Rat Model via Expansion of Myeloid Derived Suppressor Cells

Futian Zhang, Fu Liu, Shaofen Yu, Guihong Zhang, Jie Li, Xinjun Sun

Background: Bone fracture, a common injury to bones leads to various biophysiological changes and pathological responses in the body. The current study investigated curcumin for treatment of bone fracture in a rat model of bone trauma, and evaluated the related mechanism.

Material/Methods: The rats were separated randomly into 3 groups; sham, model, and curcumin treatment groups. The fracture rat model was established by transverse osteotomy in the right femur bone at the mid-shaft. The osteoblast count was determined using hematoxylin and eosin staining. Vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen (PCNA) expression were measured by western blotting.

Results: The rpS6-phosphorylation was suppressed and light chain 3 (LC3II) expression elevated in the curcumin treated group of the fracture rat model. In the curcumin-treated group, mineralization of fracture calluses was markedly higher on day 14 of fracture. The formation of osteoblasts was observed at a greater rate in the curcumin treated group compared to the model rat group. Treatment of rats with curcumin significantly (P<0.05) promoted expression of PCNA and VEGF. The decrease in CD11b+/Gr-1+ cell expansion in rats with bone trauma was alleviated significantly by curcumin treatment. A marked increase in arginase-1 expression in rats with bone trauma was caused by curcumin treatment.

Conclusions: In summary, curcumin activates autophagy and inhibits mTOR activation in bone tissues of rats with trauma. The curcumin promoted myeloid-derived suppressor cell (MDSC) proliferation and increased expansion of MDSCs in a rat model of trauma. Therefore, curcumin may have beneficial effect in patients with bone trauma and should be evaluated further for development of treatment.

MeSH Keywords: Autophagy • Barotrauma • Chemoradiotherapy, Adjuvant

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/924724
Background

Bone fracture, a common injury to bones, leads to various biophysiological changes and pathological responses in the body [1]. These responses initiate healing of fractures but are also accompanied by damage to tissues. The development of devices for internal fixation and the use of newly discovered biological materials have greatly improved the healing process but in ~10% of the cases nonunion or delayed union has been reported [2]. Therefore, to improve patient morbidity and minimize expenditure due to bone fractures novel therapies need to be discovered for enhancement of healing fractures [3]. Autophagy plays vital role in destroying the non-functional and damaged macromolecules besides regulating the cellular energy and nutrients [4,5]. The failure of autophagy leads to abnormal gene expression at a cellular level thereby causing the death of cells [6]. Additionally, autophagy failure is associated with the neuronal degeneration, premature death, development of abnormal skeleton and cardiomyopathies [7–9]. The autophagy activation is primarily indicated by enhanced expression of light chain 3 (LC3-II) at the cellular level [10]. An important autophagy suppressor is the mammalian target of rapamycin (mTOR) which acts as upstream effector of proteins linked to autophagy [11]. The mTOR complex is regulated by many pathways like phosphatidylinositol-3-kinase (PI-3K)/Akt and AMP-activated protein kinases [11]. The dysfunction of mTOR is associated with the development of inflammatory disorders, cardiac diseases, diabetes, etc. [12]. Thus, the mTOR pathway is believed to be potential target for treatment of multiple disorders [12]. Inhibition of ribosomal protein S6 activation which is an mTOR complex-1 downstream factor by chemotherapeutic agents leads to autophagy activation [13,14].

Curcumin has been used for a very long time in traditional medicine because of several pharmacological properties [15]. Multiple properties of curcumin include its role as an anti-inflammatory, antioxidant, and anti-fibrotic agent [15–17]. Additionally, studies have shown tumor growth inhibitory effect and myocardial injury protecting property of curcumin [15–17]. Although studies on the use of curcumin in bone trauma treatment are limited, reports suggest that curcumin may have a positive effect in bone remodeling [18]. The current study investigated curcumin for bone trauma treatment in a rat model and evaluated the related mechanism.

Material and Methods

Animals grouping and treatment

Thirty adult male Sprague Dawley rats (6 to 8 weeks old; weight; 245 to 265 g) were obtained from the Animal Center of Hubei Medical University (Shiyian, China). The rats were maintained at constant temperature of 25°C and 45% to 50% humidity in the laboratory. All rats were provided food and water ad libitum and acclimatized for 1 week to the laboratory environment prior to starting the actual experiment.

Bone trauma and treatment

The rats were separated randomly into sham, model bone trauma, and model curcumin treatment groups. The fracture rat model was established by transverse osteotomy in the right femur bone at the mid-shaft. Surgical procedures on rats were conducted after intraperitoneal injection of ketamine (50 mg/kg) and xylazine hydrochloride (10 mg/kg). The lower limb on right side of rats after shaving was disinfected by providone-iodine to carry out surgery under sterilized conditions. An incision was made carefully to expose the femurs and an oscillating microsaw was used for inducing transverse femur shaft fracture. Then intramedullary fixation was made using a 1-mm Kirschner wire and standard procedure was followed to close subcutaneous tissues and the skin. Curcumin was injected into rats in the treatment group at 8 mg/kg doses in dimethyl sulfoxide (DMSO) daily for 10 days from the day of fracture via intraperitoneal administration route. The rats in the untreated group received normal saline at equal doses. The approval for conducting the study was obtained from Animal Care and Use Committee National Institute of Health, China. The experimental procedures on rats were carried out according to guidelines from the Institutional Animal Care Committee. Five rats from each group were sacrificed on day 14, 28, and 42 of fracture via intraperitoneal administration route. The right femur bone was exposed to perform middle transverse fracture, using a wire saw, then the fracture was stabilized by Kirschner wire [19].

Osteoblast count analyses

The right femur bone of rats was harvested and subsequently treated for 24 hours with 4% formalin for fixation. The decalcification of bones was performed by treatment with 10% ethylenediaminetetraacetic acid (EDTA) solution for 1 month and then the bones were embedded in paraffin. The 3–5 µm sections of decalcified bones were cut longitudinally, stained with hematoxylin and eosin (H&E) and analyzed for osteoblast count in each callus section.

Immunohistochemistry

The samples embedded in paraffin were deparaffinized on treatment with xylene substitute Pro-Par Clearant and subsequently subjected to rehydration in ethanol and water. The samples were treated with sodium citrate buffer (10 mM) and heated for 2 minutes at 88°C in a microwave oven. After cooling the slides at room temperature, the samples were washed...
with phosphate-buffered saline (PBS) and then blocked for 25 minutes with 5% serum. The samples were incubated with anti-p-rpS6 primary antibodies (1: 100; Abcam) at 4°C for overnight. The sections after PBS washing were subjected to incubation for 1 hour with biotinylated goat anti-mouse secondary antibodies (1: 100; Abcam) at 37°C. The samples were incubated for 25 minutes with Vectastain ABC-alkaline phosphatase, washed in PBS, and incubated again with alkaline phosphatase for 20 minutes.

**Flow cytometric analysis**

The cells after harvesting were put into FACS medium containing 1x-PBS along with BSA (0.1%) and sodium azide (0.1%). Then cells were dyed with FITC-labeled anti-mouse CD11b, and PE-labeled GR1 in accordance with the suppliers’ instructions. The FACScan flow cytometer (BD Biosciences) was used for analysis of the stained cells.

**Statistical analysis**

The data expressed are the mean of ± standard deviation (SD) of 3 experiments conducted independently. The statistically significant differences between 2 groups were evaluated using Student’s t-test and for multiple group comparisons one-way analysis of variance (ANOVA) and Bonferroni multiple comparisons test were used. The values at P >0.05 were considered statistically significant.

**Results**

**Curcumin suppressed rpS6-phosphorylation and promoted LC3-II expression in rat fracture model**

The changes in rpS6-phosphorylation in the rats after bone fracture were determined for analysis of mTOR pathway activation (Figure 1). The rpS6-phosphorylation showed a marked reduction in the bone tissues of rats with fracture on treatment with curcumin on day 14, 28, and 42 post fractures. The phosphorylation of rpS6 was found to be markedly higher in the untreated group of fracture rat model. In curcumin treated group of the fracture rat model, the expression of LC3-II was found to be markedly elevated compared to untreated rats (Figure 2). The elevated LC3-II expression in curcumin treated rat bone tissues indicated activation of autophagy.

**Formation of callus by curcumin**

Mineralization of the calluses in rats was analyzed on day 14, 28, and 42 of fracture using radiography (Figure 3). The mineralization was not observed in rat calluses of untreated group on day 14 of fracture. However, density of mineralization increased on day 28 of fracture in the calluses in untreated group. The calluses in untreated group were remarkably small in size on day 42 of fracture which showed significant re-sorption. In curcumin-treated group mineralization of fracture calluses was observed on day 14 of fracture. Compared to untreated group calluses in curcumin treated rats were much larger in size on day 42 of the fracture.
Curcumin increased the number of osteoblasts in the rat callus

The changes in osteoblast proliferation in rats on day 14, 28, and 42 after fracture by curcumin treatment were analyzed in the callus sections (Figure 4). The formation of osteoblasts was observed at greater rate in curcumin treated group compared to model rat group. In model rats, the formation of osteoblasts was significantly ($P<0.02$) lower compared to the sham group.

The PCNA and VEGF levels increased in cells by curcumin treatment

The PCNA and VEGF expressions play vital roles in osteoblast proliferation and healing of fractures. The changes in PCNA and VEGF expression by curcumin were analyzed in the rats using western blot assays (Figure 5). In the model group PCNA and VEGF expression was much lower compared to the sham group of rats. Treatment of rats with curcumin significantly ($P<0.05$) promoted expressions of PCNA and VEGF on day 14 after fracture.
Curcumin alleviated trauma-induced reduction of CD11b+/Gr-1+ cell expansion in trauma rat model

In rats with bone trauma, the CD11b+/Gr-1+ cell expansion to spleen, bone marrow, and blood was significantly decreased (Figure 6). The decrease in CD11b+/Gr-1+ cell expansion in rats with bone trauma was alleviated significantly by curcumin treatment. In 8 mg/kg curcumin treated rats the trauma-mediated decrease in CD11b+/Gr-1+ cell expansion was completely alleviated.

Curcumin increased expression of arginase-1 in Gr-1+ cells of trauma rat model

Western blotting was employed to assess expression of arginase-1 in Gr-1+ cells of rats with bone trauma following curcumin treatment (Figure 7). A marked increase in arginase-1 expression in rats with bone trauma was caused by curcumin treatment.

Discussion

The rpS6-phosphorylation integrates translation of proteins with the process of cellular proliferation and growth [19].
inhibition of rpS6-phosphorylation indicates suppression of mTOR pathway [19]. The formation of bones and their degeneration is affected by the activation of autophagy [20,21]. It is reported that autophagy suppression and activation of mTORC1 are involved in the bone loss and other osteoarthritis diseases induced by ageing [22,23]. However, an increase in autophagy and deactivation of mTORC1 have been found to extend the life span of people [24,25]. In the present study, curcumin treatment of the trauma rat model effectively suppressed rpS6-phosphorylation in the bone tissues. The curcumin mediated rpS6-phosphorylation inhibition in the rat bone tissues indicated inhibition of the mTOR pathway.
treated bone trauma rats, the autophagosomal marker LC3-II expression was markedly promoted compared to untreated rats. Therefore, in the bone trauma rat model, the treatment with curcumin regulated the mTOR pathway and the activation of autophagy.

Bone repair is characterized by formation of calluses and subsequent mineralization. The present study showed rapid formation of calluses and mineralization in the rat model of bone trauma on treatment with curcumin. The VEGF expression has been shown to promote osteocyte survival, extracellular matrix (ECM) calcification, formation of new bone tissues and blood vessels [26]. The osteoprogenitor cells are influenced directly by VEGF thereby increasing the differentiation of osteoblasts and promoting the bone mineralization [27]. The content of PCNA has been shown to be promoted markedly by mTOR.

Figure 6. Effect of curcumin on expansion of CD11b+/Gr1+ cells. The rats with bone trauma were treated with curcumin or left untreated. The expansion of CD11b+/Gr1+ cells to (A) spleen, (B) bone marrow, and (C) blood was detected by flow cytometry. * P<0.5 and ** P<0.02 versus model (control).

Figure 7. Effect of curcumin on expression of arginase-1 in Gr-1+ cells. The rats were treated with curcumin immediately following trauma. (A) The expression of arginase-1 was measured by western blotting. (B) Quantification of western blotting data. * P<0.05 and ** P<0.02 versus model (control).
pathway inhibition in the calluses of a rat bone trauma model [28]. The present study demonstrated that curcumin treatment in a bone trauma rat model markedly enhanced the levels of VEGF and PCNA expression in bone tissues. Thus, curcumin improved repair of bone fracture in rats by promoting the expression of VEGF and PCNA. The present study demonstrated that curcumin prevents RU486 induced suppression of myeloid-derived suppressor cell (MDSC) expansion to spleen, peripheral blood, and in bone marrow in our trauma rat model. It has been reported that MDSC aggregation is promoted during trauma in various organs including the spleen [29,30]. The data from our present study showed a marked increase in MDSCs in the bone trauma rat model on treatment with curcumin. In an immunosuppressed rat model induced by endotoxin administration, MDSC proliferation was increased by glucocorticoid treatment [31]. In our present study, bone trauma suppressed MDSC proliferation which was evident by Gr-1+ cell accumulation in peripheral blood, spleen, as well as in bone of rats with trauma. However, the suppression in CD11b+/Gr-1+ cell expansion by trauma in rats with bone trauma was alleviated significantly by curcumin treatment. This suggests that curcumin prevents inhibition of MDSC proliferation induced by bone trauma. T-cell functioning is reduced by MDSCs via promotion of arginase-1 expression which leads to arginine depletion in the immune microenvironment [32,33]. The expansion of MDSCs is influenced by many other trauma related molecules such as PGE2 and S100A9/8 [34,35]. These factors are also believed to increase expansion of MDSCs in the trauma patients. Many studies for making clear the bone trauma mechanism is being continuously conducted [36,37]. The present study evaluated changes in arginase-1 expression by curcumin and trauma in a rat model of trauma. Our study data showed that bone trauma caused no significant changes in expression of arginase-1 in rats. However, arginase-1 expression in curcumin-treated rat model of trauma was markedly upregulated compared to the untreated group. Although our present study demonstrated that curcumin exhibits beneficial effect on bone fracture healing, more studies should be designed to confirm the effect using biochemical tests and micro computed tomography (CT) evaluation techniques.

Conclusions

Our study found that curcumin activated autophagy and inhibited mTOR activation in bone tissues of rats with bone trauma. The curcumin promoted MDSC proliferation and increased expansion of MDSCs in the rat model of trauma. Therefore, curcumin might have a beneficial effect in patients with bone trauma and should be evaluated further for development of treatment.

References:

1. Yang G, Duan X, Lin D et al: Rapamycin-induced autophagy activity promotes bone fracture healing in rats. Exp Ther Med, 2015; 10: 1327–33
2. Einhorn T: Enhancement of fracture-healing. J Bone Joint Surg Am, 1995; 77: 940–56
3. O’Neill KR, Stutz CM, Mignemi NA et al: Micro-computed tomography assessment of the progression of fracture healing in mice. Bone, 2012; 50: 1357–67
4. Mizushima N, Levine B, Cuervo AM, Klionsky DJ: Autophagy fights disease through cellular self-digestion. Nature, 2008; 451: 1069–75
5. Levine B, Kroemer G: Autophagy in the pathogenesis of disease. Cell, 2008; 132: 27–42
6. Mathew R, Karp CM, Beaudoin B et al: Autophagy suppresses tumorigenesis through elimination of p62. Cell, 2009; 137: 1062–75
7. Hara T, Nakamura K, Matsui M et al: Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature, 2006; 441: 885–89
8. Komatsu M, Waguri S, Ueno T et al: Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol, 2005; 169: 425–34
9. Shibata M, Lu T, Furuya T et al: Regulation of intracellular accumulation of P62/SQSTM1 in mice. J Biol Chem, 2006; 281: 14474–85
10. Su IC, Tseng PH, Hsu CY et al: RXF1-dependent activation of SHP-1 induces autophagy by a novel obatoclax derivative in hepatocellular carcinoma cells. Oncotarget, 2014; 5: 4909–19
11. Wullschlegler S, Loewith R, Hall MN: TOR signaling in growth and metabolism. Cell, 2006; 124: 471–84
12. Dann SG, Selvarey A, Thomas G: mTOR complex 1-S6K1 signalling: At the crossroads of obesity, diabetes and cancer. Trends Mol Med, 2007; 13: 252–59
13. Shigemitsu K, Tsujishita Y, Hara K et al: Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. J Biol Chem, 1999; 274: 10598–65
14. Sabers CJ, Martin MM, Brunn GJ et al: Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J Biol Chem, 1995; 270: 815–22
15. Qian Y, Zhong P, Liang D et al: A newly designed curcumin analog Y20 mitigates cardiac injury via anti inflammatory and anti oxidant actions in obese rats. PLoS One, 2015; 10: e0120215
16. Patel PB, Thakkar VR, Patel JS: Cellular effect of curcumin and citral combination on breast cancer cells: Induction of apoptosis and cell cycle arrest. J Breast Cancer, 2015; 18: 225–34
17. Jang EM, Choi MS, Jung UI et al: Beneficial effects of curcumin on hyperlipidemia and insulin resistance in high fat fed hamsters. Metabolism, 2008; 57: 1576–83
18. Selim S, Bahattin KA, Alaaddin N: Effect of curcumin on bone healing: An experimental study in a rat model of femur fracture. Injury, 2019; 11: 1915–20
19. Zoncu R, Efeyan A, Sabatini DM: mTOR: From growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol, 2011; 12: 21–35
20. Lin NY, Stefanica A, Distler JH: Autophagy: A key pathway of TNF-induced inflammatory bone loss. Autophagy, 2013; 9: 1253–55
21. Zhang L, Guo YF, Liu YZ et al: Pathway-based genome-wide association analysis identified the importance of regulation-of-autophagy pathway for inflammatory bone loss. J Bone Miner Res, 2010; 25: 1572–80
22. Chen K, Yang YH, Jiang SD, Jiang LS: Decreased activity of osteocyte autophagy with aging may contribute to the bone loss in senile population. Histochem Cell Biol, 2014; 142: 285–95
23. Stanfel MN, Shamieh LS, Kaeberlein M, Kennedy BK: The TOR pathway comes of age. Biochim Biophys Acta, 2009; 1790: 1067–74
24. Sclarrett S, Volpe M, Sadoshima J: Mammalian target of rapamycin signaling in cardiac physiology and disease. Circ Res, 2014; 114: 549–64
25. Mirzaei H, Longo VD: Acetyl-CoA synthetase is a conserved regulator of autophagy and life span. Cell Metab, 2014; 19: 555–57
26. Yu X, Guo Y, Kang Q, Luo C: Effects and mechanisms of mechanical stress on secondary fracture healing. Front Biosci (Landmark Ed), 2013; 18: 1344–48
27. Keramaris NC, Calori GM, Nikolaou VS et al: Fracture vascularity and bone healing: A systematic review of the role of VEGF. Injury, 2008; 39(Suppl. 2): S45–57
28. Morrow PW, Tung HY, Hemmings HC Jr: Rapamycin causes activation of protein phosphatase-2A1 and nuclear translocation of PCNA in CD4+ T cells. Biochem Biophys Res Commun, 2004; 323: 645–51
29. Barrera G, Landoni V, Martire-Greco D et al: Model of polymicrobial peritonitis that induces the proinflammatory and immunosuppressive phases of sepsis. Infect Immun, 2011; 79: 1280–88
30. Schwacha MG, Thobe BM, Daniel T, Hubbard WJ: Impact of thermal injury on wound infiltration and the dermal inflammatory response. J Surg Res, 2010; 158: 112–20
31. Rearte B, Maglioco A, Balboa L et al: Mifepristone (RU486) restores hormonal and T cell-mediated immune response in endotoxin immunosuppressed mice. Clin Exp Immunol, 2010; 162: 568–77
32. Makarenkova VP, Bansal V, Matta BM et al: CD11b+Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. J Immunol, 2006; 176: 2085–94
33. Bryk JA, Popovic PI, Zenati MS et al: Nature of myeloid cells expressing arginase 1 in peripheral blood after trauma. J Trauma, 2010; 68: 843–52
34. Ichikawa M, Williams R, Wang L et al: S100A8/A9 activate key genes and signaling pathways in colon tumor progression. Mol Cancer Res, 2011; 9: 133–48
35. Cheng P, Corzo CA, Luetteke N et al: Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. J Exp Med, 2008; 205: 2235–49
36. Jianping Z, Fengxue Y, Xiaolin X et al: Dynamic evaluation of orthodontically-induced tooth movement, root resorption, and alveolar bone remodeling in rats by in vivo micro-computed tomography. Med Sci Monit, 2018; 24: 8306–14
37. Wan J, Feng J, Li F et al: Therapeutic advantages of internal fixation with Kirshner wire and bone grafting via limited tarsal sinus incision approach for displaced intra-articular calcaneal fractures of children. Med Sci Monit, 2018; 24: 7862–68