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Regular paper

Celastrol enhances bone wound healing in rats

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Bone fracture is one of the most common injuries in the human musculoskeletal system. This study was performed to investigate the effects of celastrol on bone wound healing in rats. Bone wound models of Sprague-Dawley rats were treated with low (10 μg/kg) and high (100 μg/kg) celastrol for 14 days. Serum calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP) contents, bone mechanical properties, bone mineral density (BMD), and the levels of osteogenesis-related and inflammation-related proteins were assessed at the end of the experiments. Rats feeding with celastrol grew normally as control. Compared with model, celastrol administration significantly increased fracture strength, elastic load (0.12 *vs* 0.16 kg/m), bending energy (11.23 *vs* 14.23 n x mm), and BMD (0.49 *vs* 0.54 g/cm³), particularly at a high dose. Serum Ca (2.2 *vs* 2.7 mmol/L) and ALP (217.3 *vs* 245.8 IU/L) contents were significantly increased after a high dose celastrol administration, although P content did not change. Western blot analyses showed that OPG (0.72 *vs* 1.15) and COL-1 (0.20 *vs* 0.42) but not RUNX2 were upregulated significantly after celastrol administration, and IL-1α (0.82 *vs* 0.37), IL-6 (0.62 *vs* 0.28), MCP-1(0.68 *vs* 0.18), and VEGF (0.62 *vs* 0.42) were significantly downregulated, while IFN-γ was upregulated (0.29 *vs* 0.46). Our data demonstrate that celastrol effectively promotes the healing of bone wound in rats and may be further explored as a therapeutic agent to treat bone fracture.

Keywords: fracture, wound healing, biomechanical property, bone mineral density, osteogenic gene, inflammation-related genes

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Abbreviations: ANONA, analysis of variance; ALP, alkaline phosphatase; AP, Alkaline phosphatase; ARRIVE, animal research: reporting of In vivo experiments; BMD, bone mineral density; Ca, serum calcium; COL-1, collagen-1; DXA, dual-energy x-ray absorptiometry; E2, estradiol; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IFN, interferon; IL, interleukin; IGF-I, insulin-like growth factor I; IPA, radioimmune precipitation assay; MCP-1, chemoattractant protein-1; RANKL, nuclear factor kappa-B ligand; PTH, parathyroid hormone; RUNX2, runt-related transcription factor 2; SD, Sprague-Dawley; ROPG, osteoprotegerin; PBS, phosphate buffered saline; P, phosphorus; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

INTRODUCTION

Bone fracture is one of the most common injuries of the musculoskeletal system. The risk of suffering a bone fracture increases with age and certain diseases such as diabetes and post-menopausal osteoporosis (Caplan, 1987; Dimitriou *et al*., 2005). Bone fracture increases alarmins and pro-inflammatory cytokines in the blood and provokes macrophage infiltration and pro-inflammatory cytokine synthesis in the hippocampus (Einhorn, 1998). Fracture healing is a multistage repair process that involves complex, well-orchestrated steps triggered in response to tissue injury. The bone healing process is influenced by many factors that are capable of altering the metabolism of osteogenic cells, including mechanical stress, biochemical mediators, bioelectric and piezoelectric properties, and neural and endocrine influences (Vortkamp *et al*., 1998). Calcium and vitamin D are essential for maintaining bone integrity. Consequently, calcium and vitamin D supplementation represents a potential strategy for treating compromised fracture healing in osteoporotic patients (Fischer *et al*., 2018). On the other hand, antiosteoporotic medications including bisphosphonates, denosumab, calcitonin, estrogen, and raloxifene are often used to alleviate fragility fracture. However, outcomes with these medications have been controversial and further clinical studies are needed to fully understand their effects on fracture healing in order to simultaneously treat fragility fracture and underlying osteoporosis (Hegde *et al*., 2016).

Several strategies have been proposed to enhance fracture healing using either biophysical or biological means or in combination. Substantial research has been conducted to use these approaches to promote fracture healing, including intervention with parathyroid hormone (PTH) throughout all the phases, antisclerostin or anti-Dickkopf-related protein 1 antibodies in late endochondral phase or bone remodeling phase, and BMP-2 in inflammatory phase (Einhorn & Gerstenfeld, 2015). However, due to the complexity of fracture healing, a significant number of fractures are complicated as a consistence of impaired healing and non-union (Song *et al*., 2019). Although PTH is approved for the treatment of osteoporosis, and a number of animal studies have suggested that PTH could be beneficial in the treatment of fracture, more pharmacological treatments of bone fracture are still needed to stimulate bone healing (Ellegaard *et al*., 2010).

In traditional Chinese medicine fracture healing is considered as a process where blood stasis is removed, tissue regeneration and bone integration are enhanced. This process may be achieved by promoting blood circulation, transforming stasis, and promoting movement of qi" to relieve pain and reduce swelling. It also involves renewing the tendons and nourishing the liver and kidney to improve bone repair in patients by accelerating fracture healing (Tseng *et al*., 2018). *Celastrus aculeatus* Merr, widely distributed in East Asia, Australasia, Africa, and the Americas (Tong & Moudgil, 2007), has been used as herb medicine for many years and has been demonstrated to have the functions of removing dampness, reducing blood stasis, and disinfection. It is also used to attenuate pain, limb numbness, rheumatoid arthritis, and traumatic injury (Huang, 2001). The main

components of the roots and stems of C. *aculeatus* and its relatives are terpenoids, alkaloids, sterols, and flavonoids (Ding & Tong, 2014) and have been demonstrated to have wound healing activity (Aleem, 2021; Kulkarni *et al*., 2015). Celastrol is a triterpene, isolated from the barks of C. *aculeatus* and its relatives (Ni *et al*., 2014), and has been shown to have anti-inflammation, analgesia, anti-arthritis and anti-tumor activity in experimental animals (Lei *et al*., 2020; Tong & Moudgil, 2007; Yu *et al*., 2012). However, whether it has a beneficial effect on wound healing is largely unclear.

The aims of this study were to investigate the wound healing activity of celastrol and possible mechanisms underlying the activity.

MATERIALS AND METHODS

Animals

Eight-week-old female Sprague-Dawley (SD) rats, weighing 200±20 g, were purchased from the Laboratory Animal Center, Medical Science Academy, Beijing, China. Animals were housed under pathogen-free conditions and had access to standard rodent food and water ad libitum. The animal rooms were illuminated with a 12/12 hours day/night cycle at 25–26°C. Experiments were performed on the rats between 10 and 20 weeks of age. All animal experimental protocols were approved by the Animal Care and Use Committee of Kunming Children Hospital and were carried out in compliance with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Reagents and instruments

Celastrol (cat. no. C0869) was purchased from Aldrich-Sigma, USA. Serum calcium (Ca, cat. no. C004-3-1), phosphorus (P, cat. no. A126-1-1) and alkaline phosphatase (ALP, cat. no. A059-3-1) assay kits were purchased from CIBI, Nanjing, China. RIPA buffer (cat. no. P0013), horseradish peroxidase (HRP)-labeled goat anti-rat IgG (H + L) (cat. no. A0192, dilution 2000), rabbit polyclonal antibodies against collagen I (COL-1) (cat. no. AF1840, dilution 1500), osteoprotegerin (OPG) (cat. no. AF8205, dilution 1500), runt-related transcription factor 2 (RUNX2) (cat. no. AF7923, dilution 1100), interleukin 1 (IL-1) (cat. no. AF7218, dilution 1200), IL-6 (cat. no. AF0201, dilution 1000), tumor necrosis factor-α (TNF-α) (cat. no. AF8208, dilution 800), interferon gamma (IFN-γ) (cat. no. AF1138, dilution 1400), monocyte chemoattractant protein-1 (MCP-1) (cat. no AF7437, dilution 1500), vascular endothelial growth factor (VEGF) (cat. no. AF0312, dilution 1000), β-actin (cat. no. AF5003, dilution 1000), and BeyoECL Plus enhanced chemical luminance kit (cat. no. P0018S) were purchased from Beyotime, Beijing, China. Ultrasensitive chemiluminescence imaging system (ChemiDoc XRS plus) was a product of Bio-Rad, USA. Portable bone density machine was purchased from MeamMed, USA. Bone and wound strength tester (cat. no. 50420) was obtained from Stoelting Co. Wood Dale, IL, USA. Electromechanical universal testing machines (Instron 3400) was purchased from Instron, MA, USA.

Rat model of bone wound and treatment

Bone wound rat models were made as previously described (Bernhardsson *et al*., 2015). Briefly, the rats were anesthetized with 3% pentobarbital sodium (30 mg/kg) and subcutaneously injected with 7 mg oxytetracycline as infection prophylaxis. Analgesic (0.015 mg buprenorphine per rat) was given every 12 h over the following 48 h. Right hind legs were shaved and cleaned with chlorhexidine and a 5- to 6-mm longitudinal incision was made along the tibia, and a cut of 2 mm width and 1/3 depth of the tibia was transversely saw in the anterior-medial surface of the proximal metaphysis, about 5 mm from the growth plate. The same surgeon performed all surgeries. The wounds were sutured and disinfected with iodine and penicillin injection. Rats subjected to the same sawing procedure were used as sham.

Two days after modeling, the rats were randomly divided into three groups (n=6) to receive oral administration of 1 ml of celastrol (10 (low dose) or 100 (high dose) μg/kg dissolved in 0.1% DMSO + 1% Tween 80 + 99.9% water) or vehicle (model, 1 ml (0.1% DMSO + 1% Tween 80 + 99.9% water) daily for 14 consecutive days. 17 beta-estradiol (E2) (4 mg/kg daily) was administered in the same way as positive control (Chow *et al*., 1992). During the administration, the general conditions, the activity of injured limbs, and wound healing were recorded. The body weight at the end of the experiments was measured. 20 days after modelling surgery, animals were euthanized by CO_2 asphyxiation and tissues and blood were collected for subsequent analysis. The death after exposure to carbon dioxide was confirmed based on careful assessment of the rats for cardiac arrest.

Bone mineral density (BMD) determination

BMD of callus was measured on 1 cm callus tissue cut from the fracture end of the right femur using dual-energy X-ray absorptiometry (DXA). The scan was made in a 5. 0 mm × 5. 0 mm area using small object scanning mode (resolution 1.0 mm × 1. 0 mm at a scanning speed of 60 mm/s and scanning width of 2.0 cm). BMD was determined using Image-Proplus (v.5.1, Media Cybernetics, MD, USA).

Bone biomechanics determination

The fracture strength of callus was measured using a bone and wound strength tester according to the manufacturer's instructions. Bone biomechanical parameters were measured as previously described (Hao *et al*., 2015). The right femurs were tested using Instron 3400 electromechanical universal testing machines for biomechanical properties such as elastic load, fracture strength and bending energy. The measurements used an indenter of 1 mm in diameter with a loading speed of 0.01mm/s and the span of 15mm.

Enzyme linked immunosorbent assay (ELISA)

Serum Ca, P and ALP were detected in the blood collected at the end of the experiments using ELISA. At the end of the experiment, 5 ml of blood from the abdominal aorta was collected from each animal and centrifuged at 1000 × *g* for 10 min to obtain serum. Serum Ca, P and ALP were measured using commercial kits according to the supplier's instructions.

Western Blot analysis

Proteins were extracted from the tissues using the RIPA buffer. Extracted proteins were quantified using a BCA protein assay kit. About 50 μg proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were

Table 1. The body weight of rat bone wound models after administration of celastrol

Groups	No. animals	Body weight (g)		
		Before celastrol administration	After celastrol administration	Weight increase (%)
Sham	6	191.8±11.48	289.3±16.48	50.9
Model	6	180.6±10.48	286.2±15.30	58.5
Positive control	6	185.9±12.41	287.2±14.40	54.9
Celastrol (10μg/kg body weight)	6	184.9±13.41	286.2±13.40	54.8
Celastrol (100μg/kg body weight)	6	185.2±10.99	288.5±13.41	55.7

blocked with 0.5% non-fatty milk and were incubated with primary antibodies at the above specified dilution overnight at 4°C with gentle sharking. After being rinsed with PBS buffer and reacted to HRP-labeled secondary antibody at room temperature for 2 hours, immunoreactive bands were visualized using BeyoECL Plus according to the supplier's protocols. The signal intensity of visualized bands was measured using ChemiDoc XRS plus (Bio-Rad, USA) and the gray values of reactive bands were analyzed using ImageJ software (a Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin)). The band detected by anti-β-actin antibody was used as loading control to calculate relative protein levels.

Statistical analysis

All experiments were repeated three times independently in triplicated assays. Data was presented as mean ±S.D. and analyzed via one-way ANOVA or Student's *t*-test for difference among and between groups, respectively. $P<0.05$ was considered to be statistically significant.

RESULTS

Celastrol does not impact the growth and behaviors of rat

Celastrol was orally administered to the rats for two weeks after bone wound surgery. The conditions and growth of animals were monitored and examined during and after the surgery and treatment. All rats survived the surgery and had normal behavior and activities. The surgical sites showed no infection nor suppuration and healed well. Measurements showed that compared with the model, treatment with low or high dose of celastrol did not change the weight of animals at the end of the experimental period ($P>0.05$). In addition, the weight was also similar among sham, positive control (E2) (4 mg/kg daily) and models ($P>0.05$, Table 1).

Celastrol improves biomechanics parameters and BMD

At the end of the experiments, bone biomechanics parameters and density were determined to investigate the effect of celastrol on wound healing. The results showed that bone wound surgery (model) significantly reduced fracture strength, elastic load, bending energy and slightly but not statistically decreased bone density as compared to the sham operation (Table 2). After celastrol administration at high dose, fracture strength, elastic load, bending energy, and BMD were significantly increased as compared to the models, although these parameters were still significantly lower than those obtained in the sham (Table 2, $P<0.05$). At low dose, celastrol also improved fracture strength and elastic load significantly, but the improvements were not statistically significant for bending energy and BMD at a low dose (Table 2, $P>0.05$). Positive control (E2, 4 mg/kg daily) also significantly improved the biomechanics parameters but not the BMD (Table 2).

Celastrol increases serum ALP, P and Ca

P and Ca are important components in bone and ALP is a key enzyme related to the metabolism of bone minerals. To assess the effect of celastrol on metabolism related to bone restructure and development, serum ALP, P and Ca were assessed at the end of the experiments. Compared with sham, bone injury resulted in significant decreases in serum ALP, Ca and P levels in the models (Table 3). After feeding with celastrol, particularly with high dose celastrol, there were significant increases in

Table 2. Callus biomechanics parameters and Bone mineral density in rat bone wound model after administered with celastrol

Groups	No. animals	Fracture strength (kg/m)	Elastic load (n)	Bending energy (n x mm)	Bone mineral density (g/cm^{-3})
Sham	6	0.19±0.047	119.14±12.11	18.33±1.27	0.55±0.067a
Model	6	0.12±0.027a	89.12±10.17a	11.23±1.22a	0.49±0.077
Positive control	6	0.18±0.045b	118.54±12.12b	15.33±1.33b	0.52±0.069
Celastrol (10μg/kg body weight)	6	0.14±0.033b	102.13±10.13b	12.31±1.25a	0.51±0.047
Celastrol (100μg/kg body weight)	6	0.16±0.045b	106.72±9.12b	14.23±1.37b	0.54±0.057a

[a]Figure labelled with different superscripts are statistically different at $p<0.05$ by one-way ANONA.

Table 3. Serum alkaline phosphatase, calcium, phosphorus contents in rat bone wound models after administered with celastrol

Groups	No. animals	Alkaline phosphatase (IU/L)	Calcium (mmol/L)	Phosphorus (mmol/L)
Sham	6	243.4±21.53	2.6±0.40	3.2±0.50
Model	6	217.3±20.53[a]	2.2±0.42[a]	2.7±0.56 [a]
Positive control	6	238.8±20.33	2.4±0.44[a]	3.1±0.57
Celastrol (10μg/kg body weight)	6	228.2±18.51[a]	2.4±0.36[a]	2.8±0.67[a]
Celastrol (100μg/kg body weight)	6	245.8±18.53	2.7±0.49	2.9±0.54[a]

[a]Figure labelled with different superscripts are statistically different at $p<0.05$ by one-way ANONA.

ALP and Ca, although the content of P remained unchanged (Table 3). Low dose celastrol also tended to increase the levels of ALP and Ca, but the increases were not statistically significant (Table 3). Positive control (E2, 4 mg/kg daily) showed the results similar to those of high dose celastrol (Table 3).

Celastrol increases the production of osteogenic proteins

Since we found that celastrol improved bone healing, the synthesis of several osteogenesis-related proteins was profiled using Western blot analysis. The results showed that OPG and COL-1 levels were significantly increased at the end of treatment at both celastrol doses ($P<0.05$% or $P<0.01$%, Fig. 1) as compared to model and sham, while no significant changes in RUNX2 levels were observed among the treated (Fig. 1). In comparison with sham, bone wound also increased COL-1 but decreased OPG levels significantly (Fig. 1, $P<0.05$). On the other hand, E2 appeared to have no effect on RUNX2 and COL-1 or slightly negative effect on OPG production (Fig. 1).

Celastrol downregulates the levels of proinflammatory cytokines

Inflammation is a common post- wound reaction due to tissue damage. To examine if celastrol impacts inflammations, we investigated the levels of several pro-inflammatory cytokines, chemokine (MCP-1) and VEGF at the end of the experiments. As shown in Fig. 2, compared with model, the protein levels of IL-1α, IL-6, MCP-1, and VEGF were significantly lower after the animals were treated with high and low doses of celastrol except for VEGF which did not change significantly after a low dose treatment. On the other hand, the level of IFN-γ significantly increased at both doses. On the other hand, compared with sham, wounding significantly increased the levels of MCP-1, IL-1α, and VEGF but did not generate statistically significant differences in IFN-γ and IL-6 levels between the two groups (Fig. 2).

DISCUSSION

Although the healing and repair of bone wound and fractures may occur naturally due to the ability of human body to regrow and reconstruct, various treatments have been developed to facilitate the process to minimize the risk of infection, disability, and other complications that would compromise the quality of life of those affected. Celastrol is a pentacyclic triterpene present in the root bark, leaf, and stem of *C. aculeatus* and its relatives. Although it has been reported to be effective in treating chronic inflammation (Faust *et al.*, 2009) and various types of cancers (Kapoor, 2016; Lin *et al.*, 2015) its effect on bone wound healing is not well documented. Our experimental data showed that celastrol improves the healing of bone wound in rat models, resulting in improved biomechanical properties and BMD. It upregulates the synthesis of osteogenic proteins and downregulates the synthesis of proinflammatory cytokines. These findings support further investigation of this compound for therapeutic use in bone wound and fracture treatment.

Wound healing after a fracture occurs as a natural repair process where the body is mobilized to repair the injured site in a multiple-step ways, including tissue reparative phase and the remodeling phase (Giannoudis *et al.*, 2007; Phillips, 2005). Various drugs such as estrogen receptor modulators and bisphosphonates have been demonstrated to facilitate the processes (Hadji, 2012). In

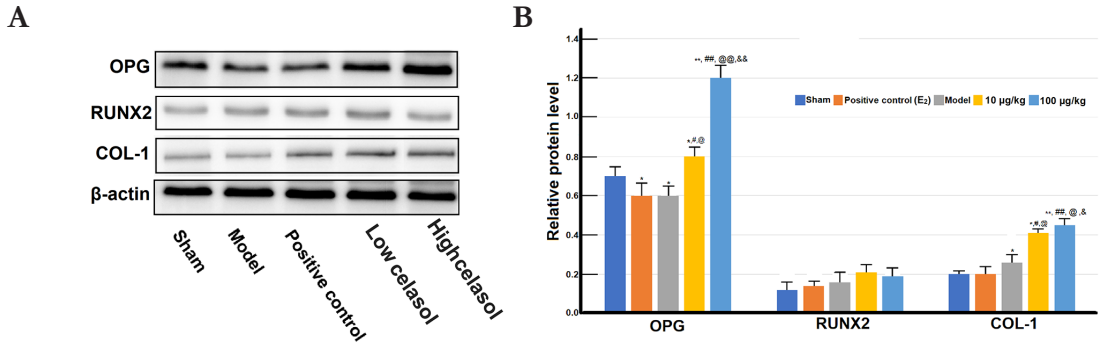

Figure 1. Level of osteogenesis-related proteins in bone wound healing rats after celastrol administration.
(**A**) left panel: representative Western blots of collagen I (COL-1), osteoprotegerin (OPG) and runt-related transcription factor 2 (RUNX2) from different treatments and (**B**) statistical analysis of relative protein levels. Experiments (6 animals per treatment) were independently repeated three times and three assays were performed for each treatment. β-actin was used as internal reference. $^{*,\#,@,\&}P<0.05$, $^{**,\#\#,@@,\&\&}P<0.01$, compared to sham, positive control, model and low dose celastrol, respectively.

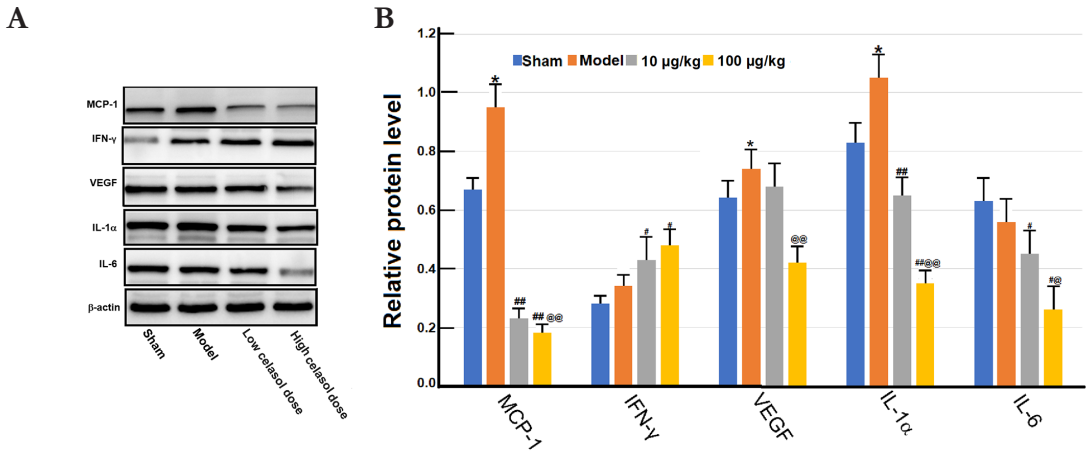

Figure 2. Level of pro-inflammatory cytokines and chemokines in bone wounding healing rats after celastrol administration. (**A**) representative Western blots of VEGF, MCP-1, IL-6, IFN-γ and IL-1α from different treatments and (**B**) statistical analysis of relative protein levels. Experiments (6 animals per treatment) were independently repeated three times and three assays were performed for each treatment. β-actin was used as internal reference. $^{*,\#,@}P<0.05$, $^{**,\#\#,@@}P<0.01$, compared to sham, model and low dose celastrol, respectively.

this study, we found that the bone wound rat models are tolerant to oral administration of up to 100 μg/kg of celastrol for 2 weeks without noticeable negative changes in body weight as compared to untreated model and sham, suggesting that celastrol at the doses used in this study is safe for the animal. Furthermore, we found that celastrol increases BMD and improves the bone biomechanical properties such as fracture strength, elastic load, bending energy in a dose-dependent manner, confirming that celastrol has a therapeutic effect on bone wound healing. Previously, crude extracts of this plant have been shown to have a beneficial effect on fracture injury (Huang, 2001), however, the active ingredients for this pharmaceutical activity have not been defined, although a number of chemical compounds have been identified, including celastrol.

To investigate the possible mechanisms underlying the therapeutic effect, we analyzed the serum components that are relevant to bone healing and reconstruction, and found that serum ALP, Ca and P content increases after celastrol treatment, implying that it is likely that some metabolism pathways are mobilized or activated to drive bone reconstruction. Ca and P are important bone minerals (Zhu & Prince, 2012) and increased serum Ca and P contents after celastrol treatment might result from an improved up-take from foods or reduced excretion from body of these elements. ALP plays a critical function in the formation of hard tissue and is often upregulated in mineralized tissue to increase the localization of inorganic phosphate and mineralization in bone (Vimalraj, 2020). Increased ALP may result from the increased osteoblastic activity in bone (Moss, 1987) and was previously observed in patients after cyclosporin treatments with increased bone formation (van Straalen *et al.*, 1991).

We further profiled the synthesis of osteogenesis-related proteins to gain molecular insight of modes of action of celastrol on bone wound repair. Our data showed that OPG and COL-1 are upregulated by the end of the experiments, while the levels of RUNX2 are similar to the untreated models, indicating that celastrol may regulate the levels of osteogenesis-related proteins to facilitate bone formation. OPG, a decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL), plays an important role in the synthesis of osteoclast and maintenance of bone homeostasis. Previous study showed that OPG reduces the resorption of osteoclasts on mineralized collagen scaffolds (Ren *et al.*, 2019) and increases osteogenesis through the activation of the wingless/integrated (Wnt)/β-catenin pathway (Gao *et al.*, 2019). It is also associated with bone strength of the hip (Samelson *et al.*, 2008). In animal models, OPG deficiency causes osteoporosis and transgenic mice overexpressing OPG lead to osteopetrosis (Bucay *et al.*, 1998; Simonet *et al.*, 1997). Treatment with recombinant OPG has been shown to improve structural and mechanical properties of bone in rodents and primates (Bateman *et al.*, 2000; Kostenuik *et al.*, 2001; Ominsky *et al.*, 2007). Collagens are the main bone matrix proteins and are commonly found in many connective tissues (Sroga & Vashishth, 2012). Previous study showed that in the differentiation of osteoblasts induced by IGF-I and TGF-β1 COL-1 was upregulated, which, in turn, lead to the development of osteoblasts and improved bone formation (Schmidmaier *et al.*, 2003). We also found that COL-1 level at the end of the experiments is significantly upregulated compared to untreated animals, suggesting that COL-1 may be involved in wound healing throughout the experimental period.

However, RUNX2, as an essential transcription factor in bone, remained relatively unchanged among the different treatments, although it is considered to be an inducer of osteoblast differentiation and promoter of chondrocyte maturation (Komori, 2018). Previously, RUNX2 was shown to be upregulated after the treatment with Ziyuglycoside I, an active compound isolated from the roots of *Sanguisorba officinalis* L with potential to treat bone diseases (Park *et al.*, 2021). In addition, *Leonurus sibiricus* L., a medicinal plant used in East Asia, Europe and the USA, is also demonstrated to promote the osteoblast differentiation of MC3T3-E1 cells and to upregulate the synthesis of RUNX2 (Kim *et al.*, 2019). The difference in RUNX2 levels in our study and previous studies may be due to different mode of action of the components, as well as the sampling time points. In our experiments, samples were taken at the end of the experiments, not allowing the detection of temporal variation in the protein synthesis over the healing process.

Another important process during wound healing is inflammation. While a necessary component of wound healing, it can alter processes that are associated with

successful tissue regeneration at the wound site (Hortensius & Harley, 2016). In this study, we also assessed the impact of celastrol on the levels of several proteins related to inflammation and the results showed that celastrol significantly reduces the production of pre-inflammatory cytokines and chemokine IL-1α, IL-6, MCP-1 and VEGF, indicating that it may exert anti-inflammatory activity by downregulating relevant proteins. Previously, celastrol was found to have an anti-inflammatory activity in corneal allograft (Li *et al*., 2021) and rheumatoid arthritis (Dudics *et al*., 2018). It decreases the level of IL-6 by blocking the NF-kappaB pathway in cancer cells (Yan *et al*., 2020) and attenuates the inflammatory response by inhibiting IL-1β in triple-negative breast cancer cells (You *et al*., 2021). Celastrol also reduces HIV-1 Tat-induced inflammatory responses via several mechanisms, including inhibiting NF-kappaB and AP-1 production and inducing heme oxygenase-1 synthesis in astrocytes (Youn *et al*., 2014). In rats with ischemia-reperfusion- induced renal injury, celastrol has been shown to decrease the levels of IL-1β and MCP-1, leading to amelioration of renal injury (Chu *et al*., 2014). Our data are in line with the earlier results and demonstrate that anti-inflammatory activity is an important aspect of celastrol's therapeutic effect. Angiogenesis is a critical aspect of wound healing. VEGF is one of the most potent pro-angiogenic factors in healing wounds (Nissen *et al*., 1998a). VEGF level increases as the results of wounds, such as surgery, particularly at the early wound stage (Nissen *et al*., 1998b). However, although VEGF level was upregulated following wounding in the rat models, it decreased after celastrol treatment, suggesting that VEGF might not participate in the wound healing process, or not until the end of the experiments. Since E_2 plays an unlikely role in wound healing-related inflammation, it is omitted from the analysis of the level of pro-inflammatory cytokines and chemokines as a positive control.

Although our data showed that celastrol is effective in promoting wounding healing, there are limitations in this study. The dose range of celastrol used was not big enough to optimize the treatment outcome, measurements for biomechanical properties and synthesis of proteins were not made at multiple-time points to allow the detection of temporal synthesis patterns. Furthermore, large animal and human studies are needed to verify the results observed in rats.

CONCLUSIONS

Our experimental data demonstrated that celastrol can facilitate bone wound healing, resulting in increased serum content of Ca, P and ALP and improved biomechanical properties. These therapeutic effects are likely achieved through the upregulation of osteogenesis-related protein synthesis and the downregulation of cytokines and chemokines prior to inflammation.

Declarations

Conflicts of interest. The authors declare no conflict of interest.

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