Bushenhuoxue formula inhibits osteoclastogenesis and bone resorption on rabbit steroid-related osteonecrosis of the femoral head by suppressing NF- κ B/NFATc1 pathway

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Abstract

Background: To investigate the effect and underlying mechanism of Bushenhuoxue formula (BSHXF) on steroid-related osteonecrosis of the femoral head (SONFH).

Methods: Seventy-five male New Zealand white rabbits were divided into three groups: control group, model group and BSHXF group. Rabbit SONFH was induced by methylprednisolone (MPS) combined with lipopolysaccharide (LPS). At week 2 and 6 post induction, the corresponding number of rabbits were sacrificed, and the femoral heads were harvested for tissue analyses, including histopathology, mechanical test of femoral heads, micro-CT, tartrate-resistant acid phosphatase (TRAP) staining, immunohistochemistry for p-P65 and co-staining of TRAP and alkaline phosphatase (ALP). Additionally, the serum TRACP5b level was measured using enzyme-linked immunosorbent assay (ELISA). Meanwhile, we also evaluated the effect of BSHXF treatment on osteoclastogenesis in vitro. The protein level of cathepsin K (CTSK), P65, p-P65 and NFATc1 in rabbit femoral heads and RAW264.7 cells were detected, respectively.

Results: At weeks 2 and 6 post induction, the elevated TRAP, p-P65 expression and reduced ALP expression were observed in the model group, with decreases in weight-bearing capacity of femoral heads and bone mass. After BSHXF treatment, the ratio of empty lacuna and the incidence of osteonecrosis in BSHXF group were markedly lower than that in model group. Micro-CT evaluation indicated that BSHXF has a preventive effect on bone loss in rabbit SONFH. In addition, BSHXF treatment increased weight-bearing capacity of femoral heads and reduced TRAP⁺ osteoclasts and serum TRACP5b level. Interestingly, CTSK, p-P65 and NFATc1 upregulation in necrotic femoral head could be reversed by BSHXF treatment, which also effectively inhibited RANKL-induced osteoclast differentiation and down-regulated CTSK, p-P65 and NFATc1 expression in vitro.

Conclusions: BSHXF could inhibit osteoclastogenesis and bone resorption on rabbit steroid-related osteonecrosis of the femoral head by suppressing NF- κ B/NFATc1 pathway.

Background

Steroid-related osteonecrosis of the femoral head (SONFH) usually occurs after high-dose steroid treatment for several diseases, such as severe acute respiratory syndrome (SARS), and systemic lupus erythematosus (SLE) [1–3]. As a degenerative and refractory bone disease, it ultimately leads to the collapse of subchondral bone and total hip joint replacement [4, 5]. Subchondral collapse of femoral head is strongly associated with changes in osteoclast and osteoblast activities [6–8], ie, a dominate bone resorption of necrotic bone without adequate repair of bone formation [9, 10]. Meanwhile, previous study reported that osteogenic process takes about 3 months to establish new bone with good mechanical properties, while the osteoclastic process only needs about 3 weeks to affect the bone structure of trabeculae [7]. Current treatments for ONFH focus on preventing the subchondral collapse, including core decompression with autologous bone graft [9] and/or implanted marrow cell therapy [11],

etc, which do not always prevent progress to subchondral collapse. Thus, if bone resorption associated with osteonecrosis can be inhibited or delayed until sufficient new bone with good mechanical properties has formed, it would appear that the collapse of the femoral head could be delayed or avoided. Although several clinical studies have reported satisfactory results in the treatment of ONFH with bisphosphonates [12, 13], there are also serious side effects in the jaw and femur regions [14, 15]. Therefore, exploration of novel and efficient agents that have therapeutic effects targeting the osteoclasts are urgently needed.

Osteoclast (Oc), derived from hematopoietic progenitor, accomplish bone resorption by secreting acid and proteases in the bone microenvironment and then dissolving the organic bone matrix [16]. Receptor activator of nuclear factor- κ B ligand (RANKL), a transmembrane ligand, interacts with its receptor RANK to activate a cascade of intracellular signaling pathways [17] and then induce osteoclasts differentiation. Meanwhile, these signaling cascades enable the expression of Oc function-related genes and proteins, including tartrate resistant acid phosphatase (TRAP) and cathepsin K (CTSK), thus eventually leading to the formation of mature osteoclasts [16, 18].

NF- κ B is a family of transcription factors, including the signaling proteins p65 (RelA), c-Rel, RelB, NF- κ B1 (p105-p50) and NF- κ B2 (p100-p52), which are involved in cell proliferation, apoptosis, inflammatory response, immune response [19]. NF- κ B activation, in response to RANKL or TNF, has been shown to activate the transcription factor c-Fos and T cell nuclear factor (NFATc1), both of which are critical for the induction of osteoclastogenesis [20, 21]. Several osteoporosis animal models have confirmed that activation of NF- κ B/NFATc1 signaling pathway can induce osteoclast differentiation and increased osteoclast activity [22, 23]. Meanwhile, another study showed that ONFH is related to the up-regulation of NF- κ B signaling pathway [24]. However, it has not been reported whether there is a correlation between NF- κ B/NFATc1 signaling pathway and osteoclast formation in ONFH.

Traditional Chinese medicine (TCM) has become a promising complementary and alternative medicine for ONFH with limited side effects [25]. According to the theories of TCM, sufficient essence of kidney and liver is the key to maintaining bone homeostasis and bone regeneration [26]. In addition, a previous study proved that the main pathogenesis of osteonecrosis of the femoral head is blood-stasis [27]. Bushenhuoxue formula (BSHXF), a tonifying kidney and promoting blood circulation decoction, have been shown to be effective in the treatment of SONFH in clinic in China, but the underlying mechanisms remain unclear.

Thus, we aimed to investigate BSHXF for the preventive effects in rabbit SONFH and to examine its possible mechanisms.

Material And Methods

Preparation of BSHXF

Ten kinds of herbal medicines in BSHXF were offered from the First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China). The extraction process described as follows contains two

parts: aqueous and ethanol extracts. In brief, eight of these herb medicines, *Rehmannia glutinosa* (Libosch), *Eucommia ulmoides* (Oliv.), *Aconitum carmichaeli* (Debx.), *Lycium barbarum* (L.), *Cornus officinalis* (Sieb.), *Carthamus tinctorius* (L.), *Glycyrrhiza uralensis* (Fisch.) and *Dioscorea oppositifolia* (Thunb.), were mixed in a ratio of 3:2:2:2:1:1:1:2 and then used for aqueous extraction. *Cinnamomum cassia* (Presl) and *Prunus persica* (Batsch.) were mixed in a ratio of 1:2 for ethanol extraction. At last, the two types of extracts were thoroughly mixed and concentrated to 2 g crude drug/ml and stored at -20 °C for further use. We have performed the high-performance liquid chromatography (HPLC) to identify the active ingredients of BSHXF. In our study, the major six constituents including loganin, amygdalin, pinoselin diglucoside, cinnamaldehyde, liquiritin and hydroxysafflor yellow A were identified [28].

Animals And Modeling

Seventy-five 28-week-old male New-Zealand white rabbits weighting 3 ~ 4 kg were obtained from the animal center of Zhejiang Chinese Medical University (SCXK 2015-0004). All rabbits were fed a standard diet and water ad libitum. Meanwhile, our studies were conducted in accordance with the guides established by the Committee on the Ethics of Animal Experiments of Zhejiang Chinese Medical University.

All rabbits were randomly divided into 3 groups: control (N = 30), model (rabbits with steroid-related ONFH, N = 30), BSHXF (rabbits with steroid-related ONFH treated with BSHXF, N = 15). The rabbit steroid-related osteonecrosis of the femoral head was induced according to previous study [29]. Briefly, 10 µg/kg body weight of lipopolysaccharide (LPS; Sigma, USA) was injected intravenously on day 0. Twenty-four hours after LPS injection, three intramuscular injections of 20 mg/kg body weight of methylprednisolone (MPS; Pfizer, USA) were given, at a time interval of 24 h.

Drug Administration

Rabbits in BSHXF group with oral BSHXF (2.38 g/kg body weight/day) for 6 consecutive weeks post induction. The dose for oral administration was determined according to the dosage conversion formula [30]. The same dose of 0.9% normal saline was fed to the rabbits of control and model group.

Biochemical Analysis

At week 2 and 6 post induction, blood sample was taken from the three groups, and the serum of samples was prepared by centrifugation at 12000 rpm and 4 °C for 5 minutes, and finally stored the serum at -80 °C. Using a TRACP5b assay kit (Nanjing JianchengBioengineering Institute, Nanjing, China) to quantify the serum TRACP5b, which is a serum marker of bone resorption.

Tissue Sample Preparation

At week 2 post induction, fifteen rabbits in the control and model groups were sacrificed, and the bilateral femora were obtained. At week 6 post induction, fifteen rabbits in each group were sacrificed, and the bilateral femora were also obtained.

The right femurs of rabbits were fixed in 4% paraformaldehyde (pH 7.4) for 3 days, and then scanned by a micro-CT equipment (Skyscan 1176, Bruker microCT N.V., Kontich, Belgium). Next, bone samples were decalcified with 10% EDTA solution for 30 days and embedded in paraffin. The 3- μ m-thick coronal sections were used for hematoxylin & eosin (H&E) staining, tartrate-resistant acid phosphatase (TRAP) staining, and immunohistochemistry. The left femurs of rabbits were used for western blots and mechanical test, respectively.

Histopathology

Osteonecrosis lesions in steroid-related rabbits were observed by histopathological examination. The evaluation criteria for osteonecrosis were based on the previous study by Yamamoto et al [31]. Osteonecrosis was determined when there were necrosis of medullary hematopoietic cells or fat cells, empty lacunae or pyknotic nuclei in osteocytes. Rabbit with one or more osteonecrosis lesions was considered to be osteonecrosis-positive one [32]. Meanwhile, the ratio of empty lacunae was calculated for each femoral head by H&E staining.

Micro-CT Analysis And Mechanical Test

The representative femoral head images of rabbits were obtained from a micro-CT equipment. NRecon, CTAn and CTVol were used to reconstruct and analyze parameters of images of the femoral head, respectively. We selected the subchondral bone of rabbit femoral head as the region of interest (red area, Fig. 2D). Ten consecutive images from the region of interest of rabbit femoral head were used for 3-D reconstruction and analysis. Then the trabecular bone volume per tissue volume (BV/TV), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp) were calculated.

At weeks 2 and 6 post induction, the femoral heads of three groups were tested for the weight-bearing capacity using the axial compression testing machine (EnduraTec TestBench™ system, Bose Corp., USA). The working principle of the axial compression testing machine was to apply a static load to the femoral head at a speed of 0.5 mm/min (schematic diagram, Fig. 2H). During the load deformation process of the femoral head, the appearance of the first mechanical turning point was considered as the weight-bearing capacity of the femoral head.

Immunohistochemistry

Co-staining for TRAP and alkaline phosphatase (ALP) was performed by a TRAP/ALP stain kit following the manufacturer's protocol (294-67001, Wako, Japan). The 3- μ m-thick coronal sections were treated with 0.3% hydrogen peroxide for 10 min to reduce endogenous peroxidase activity. The 3- μ m-thick coronal sections were incubated in 0.01 mol/L citrate buffer (pH 6.0) at 60 °C for 4 h antigen retrieval. Subsequently, the sections were treated with primary antibodies of p-P65 (diluted 1:200, LS-B652, LSBio) and incubated overnight at 4 °C. After washing, HRP-conjugated second goat anti-rabbit antibody (diluted 1:1000) was added for 20 min, and DAB was used for detecting positive staining while hematoxylin for

counterstaining. Finally, we calculated the number of positively stained cells in five consecutive sections of each rabbit.

Preparation Of BSHXF-mediated Serum

Twenty 8-week-old SD rats weighting 200 g were randomly divided into control group and BSHXF group. The rats of BSHXF group were fed with BSHXF (1 mg/10 g body weight) once a day for 7 days [30]; the same dose of 0.9% normal saline was fed to the rats of control group. The blood sample of two groups were taken from the abdominal aorta, and the serum of samples was prepared by centrifugation at 5000 rpm and 4 °C for 10 minutes, and finally stored the serum at -80 °C.

Trap Staining And Cell Proliferation Assay

In the experiments of in vitro and in vivo, tartrate-resistant acid phosphatase (TRAP) staining was performed to identify osteoclasts. The number of cells per mm² was used to calculate osteoclast formation in the experiments of in vivo. In the experiments of in vitro, RAW264.7 cells (5×10^3 cells/well) were seeded in 6-well plates and cultured in α -minimum essential medium containing 10% (v/v) FBS. The cells were divided into a control group and three treatment groups, which treated with BSHXF serum (0, 10%) or Bay 11-7082 (2 μ M, NF- κ B pathway inhibitor). The effective concentration of Bay 11-7082 (2 μ M) we used as described previously [33]. And Bay 11-7082 (#S2913) was purchased from Selleckchem (Houston, TX, USA). RANKL (50 ng/ml, PeproTech) was used to induce osteoclast formation in the three treatment groups. Six days later, the cells of all groups were fixed and stained with TRAP staining solution. Among them, cells with at least 3 nuclei were considered as osteoclasts

Using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) to test cell proliferation. RAW264.7 cells (5×10^3 cells/well) were seeded in 96-well plates and incubated in 37 °C, 5%CO₂ humidified atmosphere overnight. After removing the supernatant and washing with phosphate buffered saline, the cells were cultured in FBS-free medium containing different concentrations of BSHXF-mediated serum. Twenty-four hours later, 10 μ l CCK8 solution was added to each well and incubated for 3 h, then the absorbance values were measured at 490 nm.

Western Blot

The effects of BSHXF-mediated serum on NF- κ B pathways in RAW264.7 cells were evaluated by Western blotting. The RAW264.7 cells were seeded (5×10^3 cells/well) into 6-well plates and divided into 4 groups: a control group and 3 groups treated with BSHXF serum (0, 10%) or Bay 11-7082 (2 μ M). RAW264.7 cells were induced into osteoclasts by RANKL for 6 days. At day 7, all cells were lysed in lysis buffer to obtain total protein extracts and the expression levels of cathepsin K (CTSK), P65, p-P65, NFATc1 were determined. Proteins extracts were loaded on SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h and then incubated overnight at 4°C with primary antibody against CTSK (diluted 1:1000, ab188604, Abcam), P65 (diluted 1:1000, bs-0465R, Bioss), p-P65 (diluted 1:1000, LS-B652, LSBio), NFATc1 (diluted 1:1000, NB300-620, Novus Biologicals Inc.,) and

GAPDH (diluted 1:10000, G8795, Sigma-Aldrich), followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. Finally, the densities of the bands were visualized with an Image Quant LAS 4000 (EG, United States).

The protein expression levels of CTSK, P65, p-P65, NFATc1 in the rabbit femoral heads were also detected by western blot, and the steps are the same as above

Statistical analysis

All data were described as means $\pm$ standard deviation (SD). Fisher's exact probability test and independent-samples t-test and one-way ANOVA test were performed in the present research using SPSS software. A P value of less than 0.05 was considered significant.

Results

In rabbit steroid-related ONFH, the balance of bone resorption and bone formation has been disrupted at an early stage

H&E staining showed the osteonecrotic changes of each rabbit. The model group showed typical osteonecrotic changes at weeks 2 (early stage of rabbit SONFH) and 6 post induction, which was consistent with previous report [34], including bone marrow oedema, broken trabeculae, empty bone lacunae surrounded by necrotic marrow cells or fibrous connective tissue (Fig. 1A). Meanwhile, the osteonecrotic changes were more serious at the time-point of 6 weeks post induction. At weeks 2 and 6 post induction, the incidence of osteonecrosis in model group was 66.7% (10/15), 86.7% (13/15), respectively (Fig. 1B). Moreover, the ratio of empty lacunae at week 6 post induction was significantly higher than that at week 2 post induction (Fig. 1C). Micro-CT images of control group showed the subchondral bone of femoral heads was finely spread and integrated. However, the subchondral trabeculae of model group were damaged, and the damage was more serious as time goes on (Fig. 1D). Compared to the control group, BV/TV and Tb.Th were significantly lower in the model group, while Tb.Sp was higher (Fig. 1E, F, G). In addition, mechanical test at week 6 post induction showed the weight-bearing capacity of the femoral head of the model group was significantly lower than that of the control group (Fig. 1I), which can reflect the obvious bone loss in the necrotic femoral heads at this moment.

The result of double staining for TRAP and ALP showed that there was an increase in the expression of TRAP⁺ osteoclast (Oc) and a decrease in the expression of ALP⁺ osteoblast (Ob) in the model group (Fig. 1J). Additionally, the ratio of TRAP⁺ Oc/ALP⁺ Ob increased with the extension of time post induction (Fig. 1K). These results indicated that the balance of bone resorption and bone formation has been disrupted at an early stage in rabbit SONFH.

Upregulated NF- κ B pathway in necrotic femoral head is associated with osteoclastogenesis

To confirm the osteoclastogenesis, the sections of femoral head were stained with TRAP. As shown in Fig. 2A, compared to the control group, the model group showed more TRAP⁺ osteoclasts in the femoral heads. In addition, the number of osteoclasts at week 6 post induction was more than that at week 2 post induction (Fig. 2B). Similarly, changes in serum TRACP5b levels were consistent with histological changes (Fig. 2C).

To determine whether osteonecrotic changes are associated with NF- κ B signaling pathways, we performed immunohistochemical staining of p-P65 in the sections of rabbit femoral head. The sections of rabbit femoral head were positive for p-P65 by immunohistochemical staining, especially the necrotic femoral head sections (Fig. 2D). Meanwhile, the protein expression of p-P65 at weeks 2 and 6 post induction in model group were significantly higher than that in control group (Fig. 2E). The results indicated that high osteoclast activity in model group may be related to the excessive activation of NF- κ B signaling pathways.

BSHXF attenuates osteonecrotic changes and prevents bone loss in steroid-related ONFH in rabbits

At week 6 post induction, the effect of BSHXF treatment on SONFH were examined by H&E staining to observe the osteonecrotic changes of each rabbit. The BSHXF group compared to the model group showed fewer empty bone lacunae and relatively intact structure of medullary cavity (Fig. 3A). The incidence of osteonecrosis in BSHXF group was 33.3% (5/15), which was lower than 86.7% (13/15) in model group (Fig. 3B). Moreover, up-regulated ratio of empty lacunae observed in model group was reduced by BSHXF treatment (Fig. 3C).

The 3-D images of Micro-CT scanning indicated that the subchondral trabeculae of model group were severely damaged (Fig. 3D). Interestingly, BSHXF group showed a good protective effect on the subchondral trabeculae compared to the model group (Fig. 3D). Consistent with 3-D general view, BV/TV and Tb.Th were increased in the BSHXF group, while Tb.Sp was decreased (Fig. 3E, F and G). Meanwhile, mechanical test at week 6 post induction indicated that BSHXF treatment increased the weight-bearing capacity of femoral heads (Fig. 3H).

BSHXF inhibits osteoclastogenesis may through suppressing NF- κ B/NFATc1 pathway in steroid-related ONFH in rabbits

As shown in Fig. 4A and 4B, TRAP staining showed numerous TRAP⁺ osteoclasts in the femoral head of model group, which was significantly decreased after BSHXF treatment. In line with histological observations, the serum TRACP5b level were dramatically increased in model group. Treatment with BSHXF significantly reduced the serum TRACP5b level (Fig. 4C). Additionally, we also found that BSHXF significantly downregulated the expression of NFATc1, CTSK and p-P65 in femoral heads (Fig. 4D). All these results indicated that BSHXF had effect on suppression of osteoclastogenesis may through inhibiting NF- κ B/NFATc1 pathway.

BSHXF inhibits RANKL-induced osteoclast differentiation by suppressing NF- κ B/NFATc1 pathway in vitro

RANKL (50 ng/ml) induced RAW264.7 cells for 6 days, many multinucleated osteoclasts were generated, however, cells in the control group without the presence of RANKL did not observe osteoclast formation (Fig. 5A). Addition of BSHXF serum (10%) and Bay 11-7082 (2 μ M, acts as a positive control group) into the same cultures showed the similar results, both of which inhibited osteoclast formation as measured by TRAP staining (Fig. 5A). CCK8 tested the effect of the corresponding concentrations of BSHXF on RAW264.7. (Fig. 5B). Notably, osteoclasts in cultures that were treated with BSHXF not only reduced the number of osteoclasts, but also changed the morphology of osteoclasts, which contains fewer numbers of nuclei compared to osteoclasts in RANKL alone group (Fig. 5C and D). In addition, BSHXF treatment significantly reduced abundance of NFATc1, CTSK and p-P65 in RANKL-induced osteoclast differentiation (Fig. 5E). The results indicated that BSHXF had effect on suppression of osteoclastogenesis by inhibiting NF- κ B/NFATc1 pathway.

Discussion

Bone homeostasis is controlled by the dynamic balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation [35]. When bone homeostasis is disrupted, it can cause many orthopedic diseases, such as osteoporosis [36] and osteonecrosis [37–39]. Osteonecrosis of the femoral head is a complex disease that causes loss of bone microstructural integrity, which leads to the collapse of the femoral head and ultimately to the need for total hip replacement [40]. Recently, the imbalance in ONFH between osteoclastogenesis and osteogenesis has become a concern [37]. The pathological mechanism of collapses of the femoral head is that osteoclast-mediated bone resorption is stronger than osteoblast-mediated bone formation [7, 12, 41], which leads to bone loss. Therefore, inhibiting osteoclast activity and promoting osteoblast activity are the key to prevent collapse of femoral head. As the initiating factor of the collapse of femoral head, osteoclast activity should be inhibited as early as possible, which may delay or avoid the collapse of the femoral head.

For thousands of years, Chinese medicine has been widely utilized in China to treat bone-related diseases. Among them, BSHXF has been proven to reduce cartilage degeneration in mice osteoarthritis [28], and effectively treat SONFH in clinic in China. However, the underlying mechanism of BSHXF in the treatment of SONFH was unknown. In the present study, we found that BSHXF has the effect on suppression of osteoclastogenesis by suppressing NF- κ B/NFATc1 pathway in rabbits SONFH.

In a series of pathological examinations, we found that osteonecrotic lesions in SONFH rabbits showed bone marrow oedema, broken trabeculae, empty lacunae surrounded by necrotic marrow cells or fibrous connective tissue, which are the typical osteonecrotic characteristics of this disease. After BSHXF treatment, the osteonecrotic changes were improved and the incidence of osteonecrosis was also reduced. Micro-CT scanning results showed that BSHXF treatment can effectively preserve the bone mass of subchondral bone in the femoral head, which can be reflected from the data of bone volume (BV/TV), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). These results suggest that BSHXF could prevent bone loss in SONFH rabbits.

Furthermore, we evaluated the inhibitory effect of BSHXF on osteoclastogenesis in vitro and in vivo, respectively. The elevated TRAP expression and reduced ALP expression were observed in the model group at weeks 2 and 6 post induction, with decreases in weight-bearing capacity of femoral heads and bone mass. Our findings on animal models are not only consistent with previous reports [7, 8, 37], but for the first time demonstrated that the balance between bone resorption and bone formation has been disrupted during the early stage of rabbit SONFH. In vivo treatment with BSHXF, we found that BSHXF reduced TRAP⁺ osteoclasts and serum TRACP5b level and down-regulated the expression of CTSK, which may act by inhibiting NF- κ B/NFATc1 signaling pathways in rabbits with SONFH. In order to confirm the inhibitive effects of BSHXF on osteoclastogenesis, we used BSHXF-mediated serum to interfere with RANKL-induced osteoclast differentiation. By calculating the number of TRAP⁺ osteoclasts, we found that BSHXF-mediated serum can effectively inhibit osteoclast differentiation by affecting the activity of transcription factors in the NF- κ B/NFATc1 signaling pathways.

SONFH is closely related to the up-regulation of NF- κ B expression [24]. Meanwhile, activation of NF- κ B and transcription factor NFATc1 promotes osteoclast differentiation, which was reported in other bone diseases models [42, 43]. Interestingly, we found that the increase of osteoclasts in the necrotic femoral head was also accompanied by upregulation of NF- κ B and NFATc1 expression, which leads us to believe that the correlation also exists in SONFH. It is well known that NF- κ B acts as an initiator of NFATc1 induction during RANKL-induced osteoclastogenesis. Previous studies found that one hour after the interaction of RANKL with RANK, p50 and p65 (two components of NF- κ B) activate the NFATc1 promoter, indicating the close relationship between NF- κ B and NFATc1 [44, 45]. Another study demonstrated that the activity of NFATc1 was significantly inhibited after treatment with dehydroxymethylepoxyquinomicin (DHMEQ) (an NF- κ B inhibitor) [46]. As is shown in our study, both in vivo and in vitro evidence indicate that BSHXF could effectively inhibits osteoclastogenesis, which is achieved by inhibiting NF- κ B/NFATc1 signaling pathway.

Conclusions

In conclusion, our study revealed that BSHXF reduces abnormal osteoclastogenesis in SONFH by inhibiting NF- κ B/NFATc1 signaling pathway. Thus, BSHXF should be considered as a potential candidate drug for the treatment of SONFH.

Abbreviations

BSHXF: Bushenhuoxue formula; SONFH: steroid-related osteonecrosis of the femoral head; MPS: methylprednisolone; LPS: lipopolysaccharide; ALP: alkaline phosphatase; ELISA: enzyme-linked immunosorbent assay; CTSK: cathepsin K; SARS: severe acute respiratory syndrome; SLE: systemic lupus erythematosus; ONFH: osteonecrosis of the femoral head; Oc: osteoclast; RANKL: receptor activator of nuclear factor- κ B ligand; TRAP: tartrate resistant acid phosphatase; TCM: Traditional Chinese medicine; H&E: hematoxylin & eosin; DHMEQ: dehydroxymethylepoxyquinomicin.

Declarations

Ethics approval and consent to participate

All animals were handled according to the Council for International Organization of Medical Sciences on Animal Experimentation (World Health Organization, Geneva, Switzerland) and Zhejiang Chinese Medical University guidelines for laboratory animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Zhejiang Chinese Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated or analyzed during the current study are available from the corresponding author upon written request.

Competing interests

The authors declare that they have no conflicts of interest.

Authors' contributions

PZ and HSF performed the main experiments of this work. HHX, QM and WHY contributed to the materials acquisition and data analysis. CJX, ZYS and RX helped modify the data analysis. LF and QWG conducted the animal experiment. SFH and PEW designed this work. PZ wrote the paper. PJT and HTJ critically reviewed all content. All authors approved the version of the manuscript.

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Figures

Figure 1

In rabbit steroid-related ONFH, the balance of bone resorption and bone formation has been disrupted at an early stage (A) Histological features of rabbit femoral heads. The model group shows empty bone lacunae or pyknotic nuclei of osteocytes (black arrowheads), fibrous connective tissue (black arrows) alongside newly formed bone (blue arrows), broken trabeculae (purple arrow), and marrow oedema (green arrows) surrounded by necrotic marrow cells. In the control group, no sign of SONFH was observed. Scale

bar, 200µm. (B) The incidence of osteonecrosis at weeks 2, 6 after SONFH induction. (C) The empty bone lacunae ratio in the model and control groups. (D) Three-dimensional micro-CT image of the femoral heads subchondral bone (sagittal view) of rabbits. The red area indicates the subchondral bone. (E-G) Quantitative analysis of bone volume/total volume (BV/TV), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). (H) Mechanical test schematic diagram. (I) Weight-bearing capacity of femoral heads are tested at weeks 2, 6 after SONFH induction. (J) Co-staining of TRAP activity (blue arrowheads) and ALP-positive osteoblasts (green arrowheads) at weeks 2, 6 after SONFH induction. Scale bar, 150µm. (K) Quantitative analysis of TRAP+.Oc/ALP+.Ob at weeks 2, 6 after SONFH induction. The data are shown as the means $\pm$ SD; * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 2

Upregulated NF- κ B pathway in necrotic femoral head is associated with osteoclastogenesis (A) Changes of TRAP⁺ osteoclasts in rabbit femoral heads at weeks 2, 6 after SONFH induction. The model group shows numerous TRAP⁺ osteoclasts both in the subchondral bone and the medullary trabeculae (blue arrowheads) at weeks 2, 6 post-induction. Scale bar, 200 μ m. (B) Quantitative analysis of TRAP⁺ osteoclasts at weeks 2, 6 post induction. (C) Serum TRACP5b level in the model and control groups at the

two time points. (D) Immunohistochemistry of p-P65 in rabbit femoral heads in the model and control groups (green arrowheads). Scale bar, 200μm. (E) Positive cell ratio of p-P65 in rabbit femoral heads. The data are shown as the means ± SD; *P<0.05, **P<0.01.

Figure 3

BSHXF attenuates osteonecrotic changes and prevents bone loss in steroid-related ONFH in rabbits (A) Histological features of normal bone from normal rabbits, and osteonecrotic bone from rabbits with

SONFH with or without BSHXF treatment. The model group shows typical osteonecrotic changes, while the BSHXF group attenuates osteonecrotic changes, with fewer empty bone lacunae and necrotic marrow cells. Scale bar, 200μm. (B) The incidence of osteonecrosis in the model group and the BSHXF group at week 6 after SONFH induction. (C) The empty bone lacunae ratio in the three groups. (D) Three-dimensional micro-CT image of the femoral heads subchondral bone (sagittal view) of rabbits. (E-G) Quantitative analysis of bone volume/total volume (BV/TV), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th). (H) Weight-bearing capacity of femoral heads in the model group and the BSHXF group are tested at week 6 after SONFH induction. The data are shown as the means ± SD; *P<0.05, **P<0.01, ***P<0.001.

Figure 4

BSHXF inhibits osteoclastogenesis by suppressing NF-κB pathway in steroid-related ONFH in rabbits (A) Changes of TRAP+ osteoclasts in rabbit femoral heads of the three groups at week 6 after SONFH induction. The model group shows numerous TRAP+ osteoclasts both in the subchondral bone and the medullary trabeculae, however, BSHXF effectively inhibits osteoclastogenesis. Scale bar, 200μm. (B) Quantitative analysis of TRAP+ osteoclasts at week 6 post induction. (C) Serum TRACP5b level in the

control, model and BSHXF groups at week 6 post induction. (D)Western blotting result of NFATc1, P65, p-P65 and CTSK protein expression in rabbit femoral heads at week 6 post induction. The data are shown as the means $\pm$ SD; * $P\leq0.05$, ** $P\leq0.01$, **** $P\leq0.0001$.

Figure 5

BSHXF inhibits RANKL-induced osteoclastogenesis in vitro (A) Representative images of osteoclasts after treatment with BSHXF (10%) or Bay 11-7082 (2µM, acts as a positive control group) (magnification =

200×). (B) The effects of the indicated concentrations of BSHXF on RAW264.7 was tested by CCK8. Quantitative analysis shows the number of TRAP+ multinucleated cells (>3 nuclei) per well (C), and mean osteoclasts nuclei number (D). (E) Western blotting result of NFATc1, P65, p-P65 and CTSK protein expression at day 7 after stimulation by RANKL (50 ng/mL) with BSHXF serum (10%) or Bay 11-7082 (2μM). The data are shown as the means ± SD. *P<0.05, **P<0.01.