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ORIGINAL ARTICLE

Effects of Huogu Ⅰ Formula (活骨Ⅰ方) on Correlated Factors of Bone Regeneration in Chickens with Steroid-Induced Necrosis of Femoral Head*

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ABSTRACT **Objective**: To study the mechanism of Huogu Ⅰ Formula (活骨Ⅰ方) in treating osteonecrosis of femoral head. **Methods**: Forty-eight healthy female Leghorn chickens were randomly divided into control group, model group and Huogu Ⅰ group, and each group consisted of 16 chickens. At the meantime of model establishment, chickens of the Huogu Ⅰ group were administrated with decoction, while the model and control group with distilled water by gavage. At the 8th and 16th week after medication, blood samples were obtained for blood lipid detection while both sides of femoral head were harvested for the rest of examinations. Specifically, expressions of bone morphogenetic protein-2 (BMP2), transforming growth factor beta1 (TGFβ_1), Smad4 and Smad7 were evaluated by immunohistochemistry, while expression of osteoprotegerin/receptor activator of nuclear factor kappaB ligand (OPG/RANKL) mRNA was detected by in situ hybridization. **Results**: Compared with the control group, serum levels of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) in the model group rose significantly. Positive cell counting of BMP2, TGFβ_1, Smad4 and OPG in femoral head of the model group dropped prominently. Positive cell counting of Smad7 and RANKL increased dramatically. In contrast with the model group, levels of TC, TG and LDL-C in Huogu Ⅰ group reduced significantly. Positive cell counting of BMP2, TGFβ_1, Smad4 and OPG in femoral head of the Huogu Ⅰ group increased prominently. Indices of Smad7 and RANKL both decreased significantly. Especially at the 8th week, these variations were more significant. **Conclusion**: Huogu Ⅰ Formula is effective in promoting repair of necrotic femoral head by regulating the expressions of BMP2, TGFβ_1, Smads and OPG/RANKL of osteoclast in femoral head.

KEYWORDS osteonecrosis of femoral head, bone morphogenetic protein-2, transforming growth factor beta1, osteoprotegerin, receptor activator of nuclear factor kappaB, Smads

Avascular necrosis of femoral head is a common medical condition caused by various kinds of pathogenic factors that obstruct the blood supply of femoral head leading to necrosis of trabeculae and bone marrow in unfixed areas. In its histopathology, increased bone absorption as well as decreased bone repair was manifested. Thus, it is the major therapeutic method to inhibit the resorption activity and improve the regeneration activity of bone. The Huogu Ⅰ Formula (活骨Ⅰ方) is one of the most frequently used empirical recipes in treating early stage osteonecrosis of femoral head caused by steroid.[1] However, its mechanism is not fully understood yet. This study explores its effects and mechanism on preventing this disease by mainly focusing on the effects of Huogu Ⅰ Formula on bone morphogenetic protein-2 (BMP2), transforming growth factor beta1 (TGFβ_1) and its downstream protein—Smads, and osteoprotegerin/receptor activator of nuclear factor kappaB ligand (OPG/RANKL) in a chicken model of necrosis of femoral head.

METHODS

Animals

Healthy female Leghorn chickens whose weight ranged from 1.80 to 2.30 kg were provided by Beijing Merial Vital Laboratory Animal Technology Co., Ltd. [certification No. SCXK (Beijing) 2005-0002]. These animals were allowed to drink water and eat food freely in a secondary laboratory animal center. The

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surrounding temperature fluctuated from 20 to 25 ℃ and the lighting regimen was 12 h light to 12 h darkness.

Drugs and Reagents

The Huogu Ⅰ Formula (consisting of *Poria Cocos*, *Atractylodes Rhizome*, *Codonopsis Pilosula*, prepared *Pinellia tuber*, *Radix Paeonia Rubra*, *Ramulus Cinnamoni*, *Angelica Sinensis*, *Ligustici Chuanxiong* and prepared *Rhizome Rehmannia*) provided by Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, was decocted into solution which contained crude drug in 1 g/mL. Methyl prednisolone sondium succinate was purchased from Pharmacia & Upjohn Company of Belgium (batch No. MD0215). Rabbit anti-BMP2, TGFβ_1, Smad4 and Smad7 antibody and strept actividin-biotin complex (SABC) detection kit were purchased from Wuhan Boster Bio-engineering Co., Ltd., China. In situ hybridization kits for OPG and RANKL mRNA were purchased from Wuhan Boster Company, China. Total cholesterol (TC) assay kits, triglyceride (TG) assay kits, high-density lipoprotein cholesterol (HDL-C) assay kits and low-density lipoprotein cholesterol (LDL-C) assay kits were all provided by Beijing Baitai Clinical Reagent Ltd., Co., China.

Grouping and Administration

Forty-eight healthy Leghorn chickens were divided into 3 groups as control group, model group and Huogu Ⅰ group by a random number table. The method of establishing steroid-induced necrosis model of Wang, et al[(2)] was modified and adopted in this experiment. The chickens were injected with methylprednisolone sodium succinate at 5.2 mg/kg in chest muscle once a week. Meanwhile, chickens in the Huogu Ⅰ group were given decoction by gavage at the dose of 6 mL/(kg•d), which is a comparable dose used in some clinical regimens. And chickens in the control and model groups were given distilled water in the same volume and at the same frequency as their counterparts in Huogu Ⅰ group. All chickens were administrated with penicillin at 20,000 U/kg and streptomycin at 50 mg/kg twice a week by muscular injection to prevent inflammation. Then, chickens were sacrificed at the 8th and 16th week in batches and thereafter their femoral heads of both sides were harvested for further examination.

Blood Lipid Test

After 12-h food deprivation (without water deprivation), blood samples were obtained from brachial vein of the experimental animals. Sodium citrate based anticoagulants were added to the samples and then the serum was separated routinely. According to instructions on detection kits, levels of serum HDL-C and LDL-C were tested by direct method and levels of serum TC and TG were determined by enzymatic assay.

Histopathological Examination

After the chickens were sacrificed, femoral heads of both sides were harvested under enzyme-free condition and then were fixed in 4% paraformaldehyde solution which contained 0.1% diethyl pyrocarbonate (DEPC) for 48 to 72 h. After that, the femoral heads were decalcified in 12.5% ethylenediamine tetraacetic acid (EDTA), rinsed with running water, dehydrated in a graded ethanol series, routinely embedded in paraffin, sliced into sections and stained with haematoxylin and eosin (HE), and then they were examined under a light microscope to observe their pathological variations. In the course of choosing images after being stained by HE, weight-bearing area was set as center and then 5 vision fields were chosen successively from it and surrounding trabeculae. Next, the empty lacuna rate was calculated under objective lens (40×). At last, under 20× objective lens, MIS image analyzer (imported from USA) was applied to analyze tissue sections for fat area in medullary channel.

In Situ Hybridization Assay

The paraffin section was dewaxed and rehydrated. Then expression levels of OPG and RANKL mRNA were detected under instructions on their detection kits. With assistance of an objective lens in 40× magnification, 9 sites in cartilage, trabecula, and bone marrow (3 sites in each part) were selected from weight-bearing area centered around ligament of femoral head for examination. SPOT Ⅱ image collection system was applied to collect images while MIS image analyzer was adopted to analyze the tissue section. Three fields of vision were selected under a light microscope (400×) for positive cell counting.

Immunohistochemistry

The paraffin section was stained by immunohistochemistry as routine. Then SABC detection kit was employed to detect expression of BMP2, TGFβ_1, Smad4 and Smad7 in femoral heads. MIS system was utilized under a 400 magnified visual

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field to perform positive cell counting per unit area.

Statistical Analysis

All statistical analyses were performed by using SPSS 13.0. Quantitative data were manifested as mean ± standard deviation. One-way ANOVA was carried out for comparison among groups and *P*-value less than 0.05 was considered significantly different.

RESULTS

Influence on Blood Lipid Metabolism

Serum lipid indices of the 3 groups were listed in Table 1. Compared with the control group, the serum levels of TC, TG and LDL-C of model group increased dramatically while the serum level of HDL-C decreased significantly at the 8th week and 16th week after medication ($P<0.01$ or $P<0.05$). In contrast with the model group, Huogu Ⅰ group experienced notable decrease of serum levels of TC, TG and LDL-C at the 8th week and 16th week after medication ($P<0.01$ or $P<0.05$). In addition, although HDL-C level showed an elevating trend but it was of no statistical significance ($P>0.05$).

Table 1. Comparison of the Serum Levels of TC, TG, HDL-C and LDL-C (mmol/L, $\bar{x} \pm s$)

Group	*n*	Time (Week)	TC	TG	HDL-C	LDL-C
Control	8	8	3.8±0.6	4.2±0.6	1.8±0.3	1.9±0.5
	8	16	3.7±0.9	4.9±1.9	1.8±0.1	1.1±0.2
Model	8	8	5.6±0.5*	7.6±1.3*	1.2±0.1*	2.3±0.4*
	8	16	6.0±1.1**	9.1±0.8**	1.3±0.2**	1.7±0.3**
Huogu Ⅰ	8	8	3.5±0.4$^{\triangle\triangle}$	4.2±1.0$^{\triangle\triangle}$	1.8±0.6	1.5±0.5$^{\triangle}$
	8	16	3.6±0.7$^{\triangle}$	5.8±1.5$^{\triangle\triangle}$	1.5±0.3	1.4±0.2

Notes: $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with the model group; the same below

Influence on Femoral Head in Histomorphology

Eight weeks after steroid administration, the model group showed several histomorphological changes as compared with the control group: thin trabecula, increased number of empty lacuna and adipocyte proliferation and hypertrophia. At the 16th week, collapsed bone trabeculae, necrosis accompanied by hyperplasia in some regions, increased empty lacuna ($P<0.01$) were observed. Moreover, the fat area in marrow cavity was higher than that in the normal group ($P<0.01$). At the 8th and 16th week after treated by Huogu Ⅰ Formula, trabeculae were arranged in regular and compact shapes, large amount of osteoclast and few osteoclast distributed around them, and the empty lacuna rate and fat area in medullary channel were significantly lower than those in the model group ($P<0.05$ or $P<0.01$, Figure 1).

Influence on Expressions of OPG and RANKL mRNA

The expressions of OPG and RANKL positive staining were observed in chondrocye, osteocyte and hematopoietic cells in medullary channel of

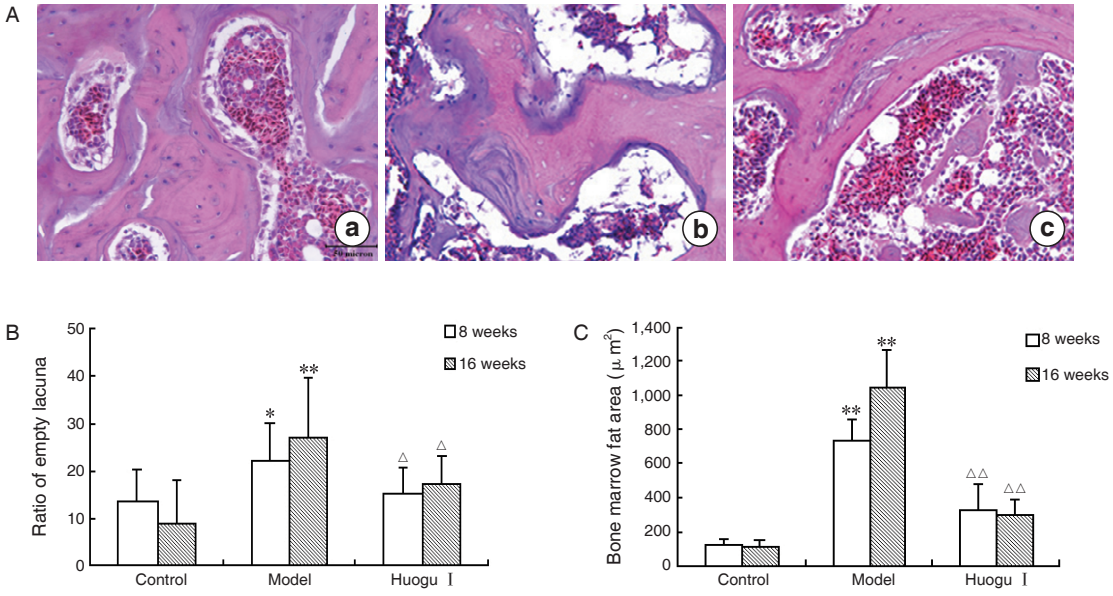

Figure 1. Morphological Observation on Femoral Head of Chickens

Notes: A: histopathologic changes of the femoral head at 16th week (HE staining, 400×); a: control group, b: model group, c: Huogu Ⅰ group. B and C: histograms show quantitative evaluation of empty lacuna and fat area in bone marrow, respectively

femoral head in the control group. At the 8th and 16th week after steroid administration, positive cell counting of OPG decreased significantly (P<0.01). Moreover, as time of steroid delivery went on, the index dropped more obviously. Meanwhile, the positive cell counting of RANKL rose prominently (P<0.01). Compared with the model group, positive cell counting of OPG in Huogu Ⅰ group increased dramatically after medication. At the 8th week, the elevation was significant (P<0.01) and even approximate to level of its counterpart in the control group. At the same time, positive cell counting of RANKL dropped prominently at 8th and 16th week (P<0.05 or P<0.01, Figure 2).

Influence on Expressions of BMP2 and TGF β_1

The expressions of BMP2 and TGF β_1 positive staining were observed in chondrocye and osteocyte of femoral head in the control group. At the 8th week, the expressions of BMP2 and TGF β_1 in the model group were significantly lower than that in the control group (P<0.05). Additionally, the difference was even more obvious at the 16th week (P<0.01). Compared with the model group, the expressions of BMP2 and TGF β_1 in Huogu Ⅰ group were significantly higher (P<0.05 or P<0.01). At the 8th week, the increasing trend of the two factors in Huogu Ⅰ group was drastic and even approximate to counterparts in the control group (Figure 3).

Influence on Expressions of Smad4 and Smad7

Positive brown staining of Smad4 and Smad7 were observed in chondrocyte, osteocyte and bone marrow cell of the control group. At the 8th week, positive cell counting of Smad4 decreased dramatically (P<0.05) while that index of Smad7 increased significantly (P<0.05). In addition, the variation was even more significant at the 16th week (P<0.01). Compared with the model group, positive cell counting of Smad4 of Huogu Ⅰ group increased notably (P<0.05) at the 8th week while that index of Smad7 decreased significantly (P<0.05). Similar changes were also observed at the 16th week, which were of no significant difference (P>0.05, Figure 4).

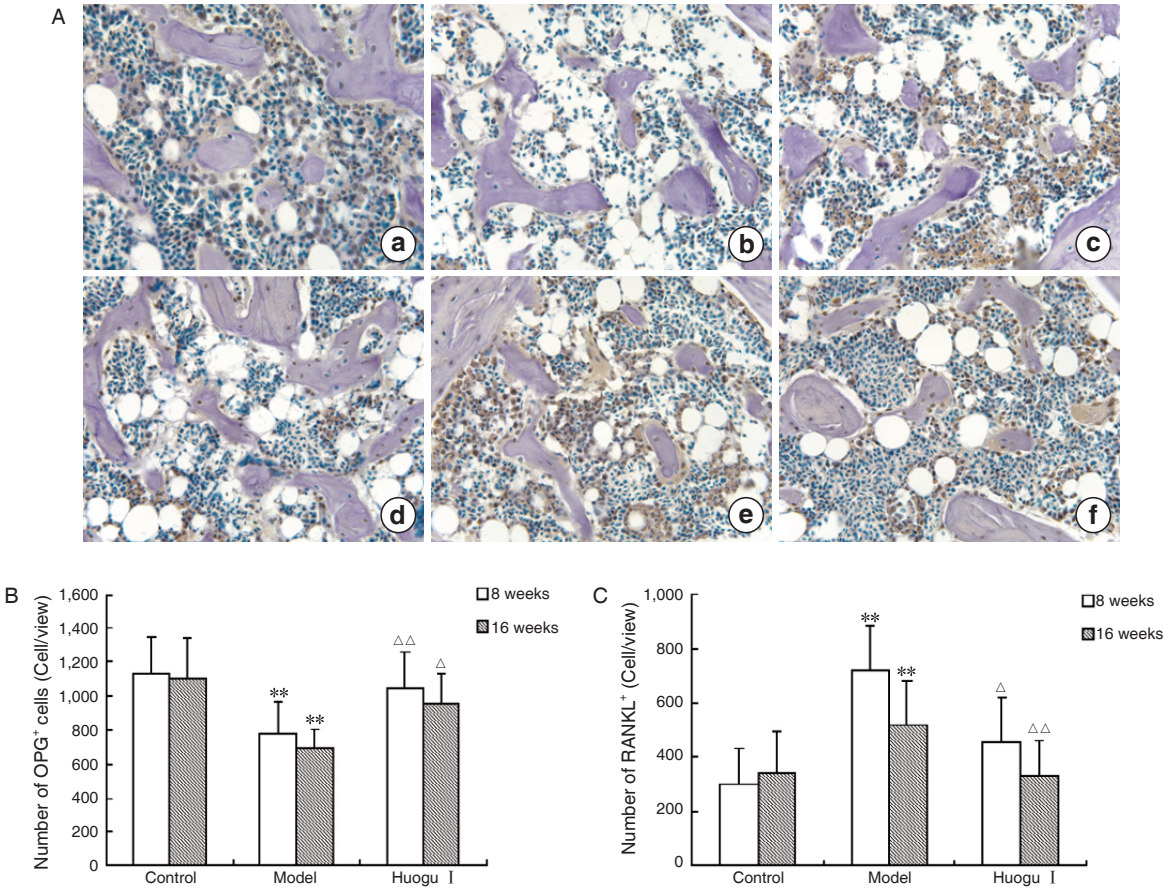

Figure 2. Expressions of OPG and RANKL mRNA in the Femoral Head of Chickens

Notes: A: in situ hybridization staining of OPG and RANKL mRNA in the femoral head (400×); a and d: control group; b and e: model group; c and f: Huogu Ⅰ group. B and C: histograms show quantitative evaluation of OPG^+ cells and $RANKL^+$ cells

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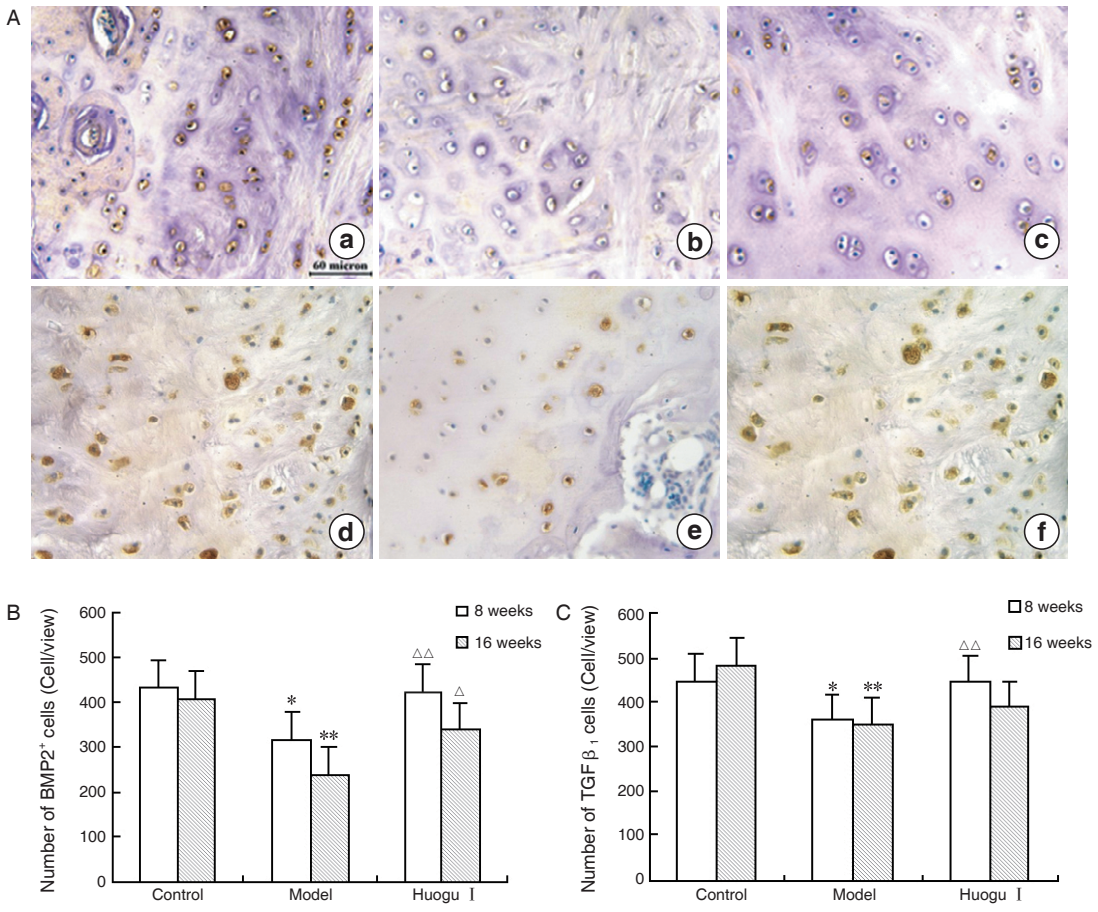

Figure 3. Expressions of BMP2 and TGF β_1 in the Femoral Head of Chickens

Notes: A: immunohistochemistry staining of BMP2 and TGF β_1 in the femoral head at 16th week (400×); a and d: control group; b and e: model group; c and f: HG Ⅰ group. B and C: histograms show quantitative evaluation of $BMP2^+$ cells and TGF $\beta 1^+$ cells, respectively

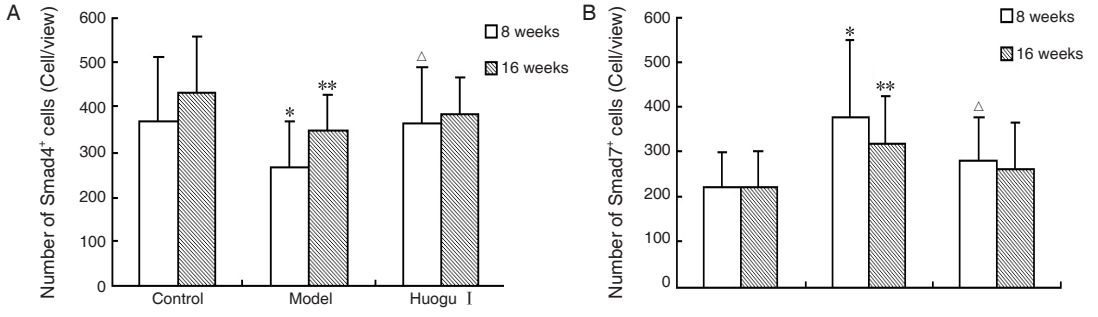

Figure 4. Expressions of Smad4 and Smad7 in the Femoral Head of Chickens

Notes: A and B: histograms show quantitative evaluation of $Smad4^+$ cells and $Smad7^+$ cells, respectively

DISCUSSION

Although the mechanism underlying osteonecrosis of femoral head caused by steroid remains poorly understood, explanations such as lipid metabolism disorder theory have received wide acceptance in medical community.[3] This theory holds that steroids are of effect in promoting lipolysis and suppressing lipogenesis and thus they increase the TC and TG levels in blood greatly and eventually lead to hyperlipidemia. The loading function and anatomic features of femoral head predisposes to block of fat emboli which contributes to microcirculation disorder and ischemic necrosis of osteocytes. Furthermore, steroid exerts its toxic effects directly on osteocytes leading to boosted bone destruction and suppressed bone repair and regeneration.[4-6] Thus, regulating blood lipid metabolism to promote bone repair has been the major therapeutic method in dealing with steroid-induced osteonecrosis of femoral head.

Song JN[7] held that elevated plasma lipids and lipoprotein disorders were known as invisible phlegm in Chinese medicine and it was the biochemical foundation of turbid phlegm in blood. Based on the theory that early stage of glucocortitoid-induced osteonecrosis was in syndrome pattern of intermingled phlegm and blood stasis, we applied Huogu Ⅰ Formula in clinic to control this disease in its early stage and received favorable curative effects.[8-10] According to our study, after delivery of steroid, serum levels of TC, TG and LDL-C all increased significantly. Pathological examination found the enlarged fat area in medullary channel of femoral head, karyopyknosis and necrosis of osteocytes in chickens of model group, which is in accordance with discoveries of Vande-Berg and co-workers.[11] After being medicated by Huogu Ⅰ Formula, several improvements were found as below: area of fat and number of empty lacuna decreased dramatically; affluent osteoblasts were observed around the trabecula; necrosis focal was replaced by regenerating tissue; serum levels of TC, TG and LDL-C decreased and condition of hyperlipidemia was improved. And it tallies with the result of previous studies—*Rhizoma Chuanxiong* is effective in improving microcirculation and reducing the blood viscosity;[12] *Atractylodes Macrocephala Koidz*, *Angelica Sinensis*, and prepared *Pinellia Tuber* possess similar effects and may improve the condition of hyperlipidemia and prevent fat from accumulating in marrow cavity.[13,14]

In the course of bone repair, osteogenic factors such as BMP2 and TGFβ_1 play a vital role in regulating the osteogenic activity. BMPs and TGFβ_1 both belong to TGFβ family whose downstream intracellular signal transduction is mediated by Smads—the unique intracellular protein kinase substrate of TGFβ receptor.[15] Smad4 is the co-Smads and the intracellular signal transductions of all factors of TGFβ superfamily depend on Smad4's mediation for translocation to nuclear.[16] Smad7, an I-Smad, binds to the receptor in competition with R-Smads, leading to blockage of intracellular signal transductions of TGFβ. Apart from enhancement of bone formation, the inhibition of bone resorbing activity also weighs heavily in bone repair. Osteoclast plays a crucial role in bone destruction and its differentiation is mainly regulated by OPG/RANKL/RANK system.[17] RANKL binds to osteoclast precursor or RANK which is on the surface of osteoclast to promote its generation and activation. OPG competitively binds to RANKL and thus blocks its binding to RANK, resulting in suppressed generation and activation of osteoclast. In addition, it also boosts the apoptosis of osteoclast and inhibits apoptosis of osteoblast.[18-20]

Our study shows that, at the 8th and 16th week after administrated with steroid, decreased expression of OPG, BMP2, TGFβ_1 and Smad4 and enhanced expression of RANKL and Smad7 were observed in femoral head of experiment chickens. This result is in consistence with previous studies involving observation on patients with steroid-induced necrosis of femoral head in clinic.[21] And it suggests that steroid suppresses bone regeneration and promotes the differentiation of osteoclast. Huogu Ⅰ Formula can partially obstruct or reverse the pathological change which means it notably promotes osteogenic ability as well as suppresses bone-resorptive activity. According to previous reports, it is known that tetramethylpyrazine, the major ingredient of this prescription, is effective in up-regulating expression of OPG and down-regulating levels of RANK and RANKL.[22] *Angelica Sinensis* possesses effects of up-regulating expressions of BMP2, cbfal, insulin-like growth factors (IGF), and other related genes of osteoblast.[23] We believe that, on one hand, Huogu Ⅰ Formula acts on osteoblast and bone marrow stromal cell to enhance the expression of related genes of osteogenesis. On the other hand, it regulates the lipid metabolism and improves the condition of abnormal blood rheology, leading to the promotion of blood supply of femoral head. Consequently, it maintains the stasis of microenvironment of osteoblast and bone marrow stromal cell.

To sum up, we believe that Huogu Ⅰ Formula is of preventative effect against steroid-induced necrosis of femoral head. It is possible that this formula exerts its effect through regulating blood lipid level, improving the condition of hyperlipidemia and consequently modifying the pathological state of this disease. Meanwhile, it enhances the synthesis and secretion of OPG, inhibits the expression of RANKL. Furthermore, it up-regulates the expressions of TGFβ_1, BMP2 and Smad4 and down-regulates the expression of Smad7, which contributes to the promotion of bone regeneration. In this study, only two signal pathways of OPG/RANKL and BMP2/ TGFβ_1 in experiment birds are grossly observed. Thus, further studies are still needed to find out the influence of Huogu Ⅰ Formula on these signal molecules of the two signal

pathways mentioned above.

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