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Effect of Yang-Warming and Kidney-Tonifying Prescription on Expression of Osteogenic and Angiogenic Factors and H-Type Vascular Markers in Steroid-Induced Avascular Necrosis of Femoral Head in Rabbits

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Abstract

Objective: To investigate the effect of Yang-warming and Kidney-tonifying Prescription (YKP) on the treatment of steroid-induced avascular necrosis of the femoral head (SANFH) in rabbits. And to further explore whether its therapeutic mechanism is related to the expression of HIF-1*α* and VEGF (angiogenic factors), BMP2 and Osterix (osteogenic factor), CD31 (type H vascular marker) and MMP13 (bone destruction-related factor). **Methods:** Twenty-seven healthy male New Zealand white rabbits were divided into a normal group, model group, traditional Chinese medcine (TCM) group (clinical equivalent dose group of YKP), miR-130a inhibitor group and TCM + inhibitor group. The SANFH model was established by combining horse serum with methylprednisolone. After the model is successfully established, TCM group was given 6.44 g/kg·d YKP by gavage, and the miR-130a gene inhibitor group was intraperitoneally injected with 25 mg/kg miR-130a inhibitor, locked nucleic acid (LNA)-anti-miR-130a. TCM + inhibitor group was treated with YKP intragastrically and miR-130a inhibitor intraperitoneally. The rabbits in the normal group and the model group were intragastrically administered with normal saline 10 ml/d. Once a day for 4 weeks. The avascular necrosis was detected by HE staining. The contents of HIF-1*α*, VEGF, BMP2 and Osterix in rabbit tissues were detected by qRT-PCR kit, and the expression of CD31 and MMP13 was detected by immunofluorescence staining. **Results:** In the normal group, the surface of the cartilage layer of the femoral head was smooth, the bone trabeculae were intact and densely arranged, the cells of each layer were neatly arranged, the morphology of the bone cells, the

chondrocytes and the adipocytes were normal. In the model group, cartilage surfaces of the femoral head showed exfoliative cracks. The bone trabecular structure was loose and incomplete, chondrocytes, osteoblasts and bone marrow cells were significantly reduced, and the number of empty bone traps was significantly increased. In the TCM-treated group, more chondrocytes, thicker cartilage layer, and more regular bone trabeculae were detected as compared to model rabbits. In contrast, the cartilage layer was thinner, the destruction and fracture of bone trabeculae was more serious, chondrocytes and osteocytes were decreased as compared to model group. The expression of HIF-1*α*, VEGF, BMP2, and Osterix in the model group decreased significantly as compared to the normal group ($P < 0.05$), which was up-regulated in the TCM group ($P < 0.05$) and down-regulated in the miR-130a inhibitor group ($P < 0.05$). CD31 expression in the model group was significantly lower than that in the normal group, which was significantly increased in TCM group and decreased in miR-130a inhibitor group. MMP13 expression in the model group was significantly higher than that in the normal group, which was up-regulated by TCM treatment, and down-regulated by miR-130a inhibitor. **Conclusion:** YKP can regulate the expression of angiogenic-related factors (VEGF and HIF-*α*), osteogenic-related factors (BMP2 and Osterix), and H-type vascular marker CD31, resulting in increased expressions of VEGF, HIF-*α*, BMP2, and Osterix, which promote intra-femoral head revascularization. Meanwhile, YKP decreased the expression of bone-destruction-related factor MMP13, thus enhancing the ability of bone tissue to repair itself. Regulation of these molecules' expression may be one of the mechanisms of YKP in the treatment of hormonal femoral head necrosis.

Keywords

Steroid-Induced Avascular Necrosis of the Femoral Head, Yang-Warming and Kidney-Tonifying Prescription, HIF-1*α*, VEGF, BMP2, Osterix, CD31, MMP13

1. Introduction

Steroid-induced Aavascular Necrosis of Femoral Head (SANFH) is one of the most serious side effects of clinically applied hormones. The incidence of SANFH is about 40% in patients treated with glucocorticoids, which is about 20-fold higher than the incidence of SANFH in the normal population [1].

Modern medicine indicates that its occurrence may be related to intravascular coagulation, disorders of lipid metabolism, genetic polymorphisms, apoptosis, cellular autophagy [1]. Cell adhesion molecule 31 (CD31), osterix, vascular endothelial growth factor (VEGF), etc are its upstream regulated molecules [2]. This disease belongs to the category of "bone arthralgia", "bone erosion" and "bone atrophy" in TCM. Deficiency of kidney yang and stagnation of blood vessels is the pathogenesis of this disease. Firstly, steroids act on the body, resulting in blockade and congestion of meridians, qi and blood, stasis of marrow reser-

voir, as well as medullary depletion and resulting bone wilting; Secondly, long-term steroid use consume the yin essence of the injured kidney. Since the liver and the kidney have a common source, this results in liver yin loss and kidney essence consumption, the malnutrition of spleen and stomach and the reduced ability to promote blood flow, and finally blood stasis. And tonifying the kidney and strengthening the bone, promoting blood circulation and removing blood stasis are the basic treatment principle of this disease [3]. According to this therapeutic principle, Professor Wang Heming proposed Yang-warming and Kidney-tonifying Prescription (YKP), with the effects of warming and promoting blood circulation, tonifying liver and kidneys, and strengthening tendons and bones. A large number of clinical cases have shown that this prescription has a significant effect on steroid-induced avascular necrosis of the femoral head. Previous studies in this topic indicated that YKP can increase the expression of VEGF, thereby promoting angiogenesis and bone tissue repair in the femoral head. In addition, it can regulate the function of RANK/RANKL/OPG pathway, inhibit the excessive activation of osteoclasts, and promote the formation of new bone [4]. Therefore, the present study aimed to further investigate the efficacy of YKP in promoting blood circulation and removing blood stasis, and explore its effects on the expression of angiogenic factors hypoxia-inducible factor 1-alpha (HIF-1α) and VEGF, osteogenic factors bone morphogenetic protein 2 (BMP2) and Osterix, H-type vascular markers CD31, as well as the bone destruction related factor MMP13 in rabbits with steroid-induced avascular necrosis of femoral head.

2. Materials and Methods

2.1. Experiment Animals

Twenty-seven healthy male New Zealand white rabbits, weighing 2.74 - 3.45 kg, were provided by Chedun Laboratory Animal Improved Breeding Farm Co., Ltd., Songjiang District, Shanghai. Rabbits were fed with a standard strip diet, had a free access to diets, and were adaptively fed for 2 weeks before the start of the experiment. The feeding environment was a clean environment with constant temperature and humidity, the temperature was (22 ± 2)˚C, and the relative humidity was (55 ± 5)%.

2.2. Model Development

After 2 weeks of adaptive feeding, SANFH model was established using horse serum and methylprednisolone according to the literature [5]. Horse serum (10 ml/kg) was first injected intravenously through the ear margins of the rabbits. After an interval of 3 weeks, horse serum (6 ml/kg) was injected again. Then at another 2-week interval, methylprednisolone (45 mg/kg) was injected once daily for 3 consecutive days. The normal control group was injected with equal volume of saline during the same period.

At 4 weeks after hormone injection, three animals were randomly executed

and their bilateral femoral heads were removed, fixed in formalin solution, decalcified, and routinely stained with HE. The pathological changes of bone trabeculae, bone marrow hematopoietic tissue, osteoblasts, osteocytes and adipocytes in the proximal femoral bone tissue and bone marrow tissue were observed under light microscope. If more osteoblasts were observed with nuclei fixed and shifted, some nuclei were deeply stained, the proportion of normal osteoblasts in the trabeculae was reduced and the structure of the trabeculae was loose, no osteoblasts were visible in the bone traps, and the number of empty bone traps was significantly increased. All these suggested that this experiment was successful modeled.

2.3. Grouping Intervention

24 SANFH rabbits were divided into model group，TCM group and miR-130a inhibitor group. TCM group was given 6.44 g/kg·d YKP by gavage. The miR-130a gene inhibitor group was intraperitoneally injected with 25 mg/kg miR-130a inhibitor, locked nucleic acid (LNA)-anti-miR-130a. The rabbits in the normal group and the model group were intragastrically administered with normal saline 10 ml/d. They were treated once a day for 4 weeks.

2.4. HE Staining for Histopathological Changes of Femoral Head

All groups were executed by air embolization, and the femoral heads of the hind legs were taken under aseptic environment. The gross specimen of femoral head was taken by camera. The pathomorphological changes of the left femoral head were analyzed by HE staining after fixation, decalcification and embedding. The changes in morphology, structure and number of bone trabeculae, bone cells, medullary cavity and adipocytes were observed under light microscope. The number of empty bone traps was found and the ratio of empty bone traps in each of the three zones was calculated (empty bone trap ratio = number of empty bone traps/50 × 100%). The average value was taken as the ratio of empty bone traps in this specimen.

2.5. qRT-PCR Detection of HIF-1α, VEGF, BMP2, and Osterix Expression

First, RNA was extracted; the femoral tissues were placed in a mortar immediately with the addition of liquid nitrogen for grinding, ground into powder. Then RNA was extracted and RNA (2 μg) was reverse-transcribed according to our previous reports [6]. The sequences of the primers were showed in **Table 1**. RNA expression levels of the target genes were determined in triplicate.

2.6. Immunofluorescence Staining of CD31 and MMP13 Expression

Four sections from each group were taken for immunofluorescence staining and observed and photographed under a laser scanning confocal fluorescence

Table 1. Primer sequences.

Gene	Sequence (5'-3')
GAPDH	F: CATCAAGAAGGTGGTGAAGCA
	R: AGCATCGAAGGTAGAGGAGTG
HIF-1α	F: ATGACGACTTCCAGTTGCGG
	R: GGTTCTTGCCCCTGAGTCTG
VEGF	F: TGCGGATCAAACCTCACCAG
	R: CCGGGATTTCTTGCGCTTTC
BMP2	F: GAGACGACAGCGGTTTCCAT
	R: GGCTCGTGTTCTGATTCACC
Osterix	F: GCCCAGTGTCTACACCTCTC
	R: TGTCCCACCAAGGAGTAGGA

Note: F, Forward; R, Reverse; HIF-1α, hypoxia-inducible factor 1-alpha; VEGF, vascular endothelial growth factor; BMP2, morphogenetic protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

microscope (FluoViewTM FV1000). CD31 and MMP13 fluorescence staining was used to determine angiogenesis and destruction respectively.

2.7. Statistical Analysis

The data were analyzed using SPSS 25.0. The quantitative data that conformed to normal distribution were expressed as mean ± standard deviation ($\overline{x}$ ± S). One-way analysis of variance was used. The rank sum test for multiple independent sample comparisons was used if the normal distribution was not met. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Histopathological Changes of Femoral Head

In normal group, the gross specimen was intact and round, with hard bones, reddish articular cartilage, smooth and lustrous surface. The cartilage layer of femoral head had smooth surface, moderate thickness, intact trabecular structure, no osteonecrosis such as fracture or loss, and was arranged densely and regularly. The cells in each layer were neatly arranged and evenly distributed, the morphology of osteocytes was normal, the nuclei were located in the central position of the lacunae, a few scattered empty lacunae were occasionally observed, and chondrocytes were abundant. A large number of bone marrow cells were observed in the bone marrow cavity, and adipocytes were morphologically normal (**Figure 1**).

In model group, the femoral head was damaged or collapsed. The bone was thin and fragile. The cartilage surface was pale, rough and lusterless. Some of the cartilage surface of the femoral head showed stripping and cracks, and the carti-

lage layer was thinner than that of the normal group. The trabecular structure was loose and incomplete, slender and narrow, irregularly arranged, and even absent and broken. Chondrocytes and osteocytes were significantly decreased and the number of empty lacunae was significantly increased compared with the normal group. There were numerous hypertrophic adipocytes in the medullary cavity and hypocellularity in the bone marrow (Figure 2).

In inhibitor group, the plumpness and roundness further decreased. The roughness of bone surface was further increased. The cartilage layer of the femoral head was thinner, the trabecular destruction and fracture were more obvious as compared with the model group. Meanwhile, the chondrocytes, osteocytes and bone marrow cells were further reduced. And a large number of empty lacunae appeared (Figure 3).

In TCM group, the femoral head was repaired with less breakage, largely intact shape, and higher glossiness as compared with the model group. The morphology of each bone tissues was similar to that of the normal group. The chondrocytes increased and the cartilage layer thickened. The trabecular arrangement

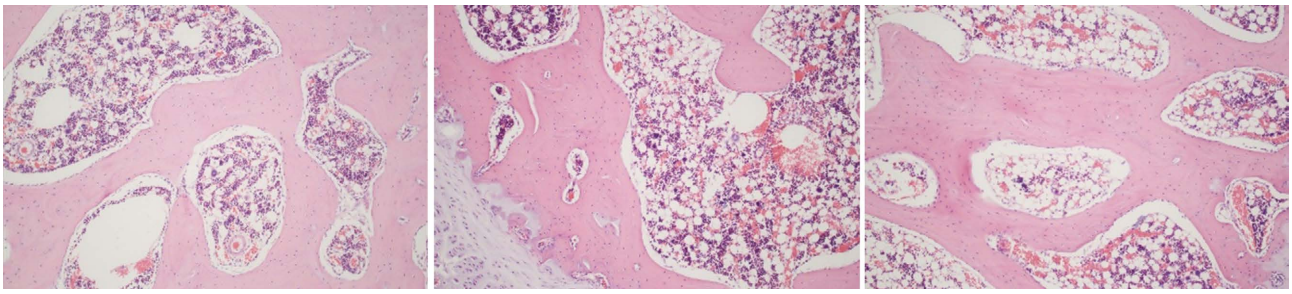

Figure 1. HE staining in the normal group 100×.

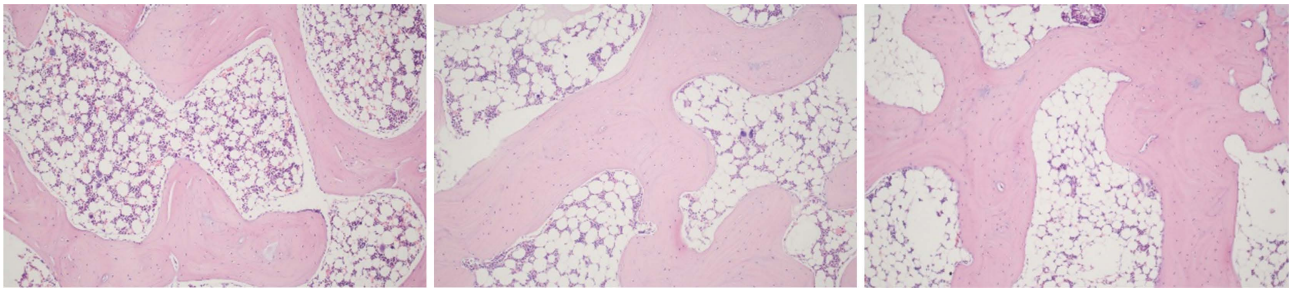

Figure 2. HE staining in the model group 100×.

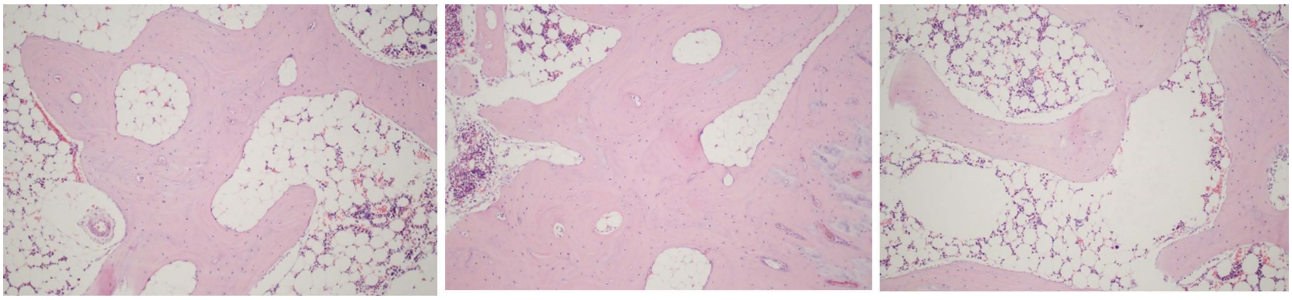

Figure 3. HE staining in the silenced group 100×.

tended to be regular, and the distribution of cells in each layer was more uniform. Occasionally, a few empty lacunae were observed (Figure 4).

Compared with the inhibited group, the cartilage surface of the femoral head was paler and rougher in inhibitor + TCM group. TCM partly improved the destruction of bone trabecula in the inhibition group, increase chondrocytes and osteocytes, and reduce empty bone lacunae.

Additionally, the empty lacuna rate in the model group was significantly increased as compared with the normal group (0.12 ± 0.03 *vs* 0.03 ± 0.01, $P < 0.05$). Whereas miR-130a inhibitor significantly increased the empty lacuna rate (0.19 ± 0.04) in SANFH rabbits, YKP treatment decreased the empty lacuna rate in SANFH rabbits (0.09 ± 0.03) and inmiR-130a inhibitor intervention group (0.14 ± 0.02).

3.2. RNA Expression of HIF-1*α*, VEGF, BMP2 and Osterix

As shown in Table 2, angiogenesis-related factors HIF-*α* and VEGF in the model group were significantly lower than those in the normal group ($P < 0.05$), which decreased more obviously in the inhibitor group than in the control group

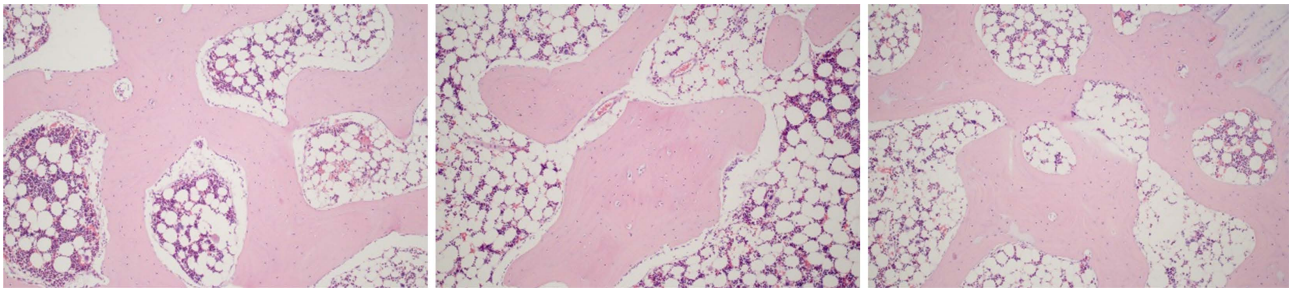

Figure 4. HE staining in TCM group 100×.

Table 2. RNA expression of HIF-1*α*, VEGF, BMP2, and Osterix expression analyzed by qRT-PCR.

	Angiogenesis-related factors		Osteogenesis-related factors	
	HIF-1*α* (pg/mL)	VEGF (pg/mL)	BMP2 (ng/mL)	Osterix (ng/mL)
Control	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.09
Model	0.31 ± 0.02*	0.31 ± 0.01*	0.36 ± 0.02*	0.42 ± 0.02*
Inhibitor	0.22 ± 0.02*#	0.23 ± 0.01*#	0.12 ± 0.01*#	0.22 ± 0.01*#
TCM	0.48 ± 0.03*#	0.66 ± 0.02*#	0.74 ± 0.02*#	0.75 ± 0.02*#
Inhibitor + TCM	0.37 ± 0.03&	0.44 ± 0.06&	0.53 ± 0.06&	0.65 ± 0.08&
F value	384.79	628.55	153.74	330.36
P value	0.00	0.00	0.00	0.00

n = 8 in each group. Compared with control group, *P < 0.05; compared with model group, #P < 0.05; compared with inhibitor group, &P < 0.05. qRT-PCR, real-time quantitative polymerase chain reaction; HIF-1*α*, hypoxia-inducible factor 1-alpha; VEGF, vascular endothelial growth factor; BMP2, morphogenetic protein 2.

and model group. HIF-α and VEGF in the TCM group were significantly higher than those in the model group ($P < 0.05$). The expression levels of osteogenesis-related factors genes BMP2 and osterix were significantly down-regulated in the model and inhibitor groups compared with the control group ($P < 0.05$). But these genes were significantly up-regulated in TCM group and inhibitor + TCM group as compared to model group ($P < 0.05$).

3.3. CD31 and MMP13 Fluorescence Intensity

CD31 is a H-type vascular marker and any single or clustered endothelial cells could be counted as a single vessel. The staining is green on fluorescence microscopy. Compared with that in the control group ($n = 6$), the number of microvessels in the model, inhibitor, and TCM groups was significantly lower ($P < 0.05$), especially in the inhibitor group (**Figure 5**). CD31 positive expression increased in TCM group compared to model group.TCM treatment improved the down-regulation of miR-130a inhibitor.

It has been shown that MMP13 is associated with bone destruction. The expression of MMP13 in the model group was significantly higher than that in the control group, the expression of MMP13 in the TCM group was significantly lower than that in the model group, and the expression of MMP13 in the inhibitor group was significantly higher than that in the normal and TCM groups (**Figure 6**). The fluorescence intensity of MMP13 in the inhibitor + TCM group was weaker than that in the inhibitor group.

4. Discussion

The steroid-induced avascular necrosis of femoral head is one of the most common and serious complications of clinical application of hormones, usually causing pain, deformity, and even disability. Modern medicine believes that the pathogenesis of hormonal osteonecrosis is closely related to reduced angiogenesis and increased bone destruction. Their treatments for osteonecrosis of the femoral head focus on fighting inflammation, which has certain limitations. Chinese medicine believes that deficiency of spleen and kidney, blood stasis and phlegm coagulation are the pathological products of hormonal osteonecrosis. They have better clinical results in the treatment of SANFH.

In this study, a rabbit ischemic femoral head necrosis model was developed using horse serum combined with methylprednisolone in New Zealand white rabbits. The results showed that the model group had depressed femoral head surface, inhomogeneous bone density, cystic changes in weight-bearing area, narrow joint gap, thinning cartilage, rough surface, enlarged subchondral fat cells, thinning bone trabeculae and atrophy of osteocytes. The above results indicated that the rabbit ischemic femoral head necrosis model was successfully constructed, and this model can be used for scientific research.

The evaluation of the efficacy of femoral head necrosis is based on neoangiogenesis and bone repair. Neoangiogenesis is closely related to HIF-1α, VEGF

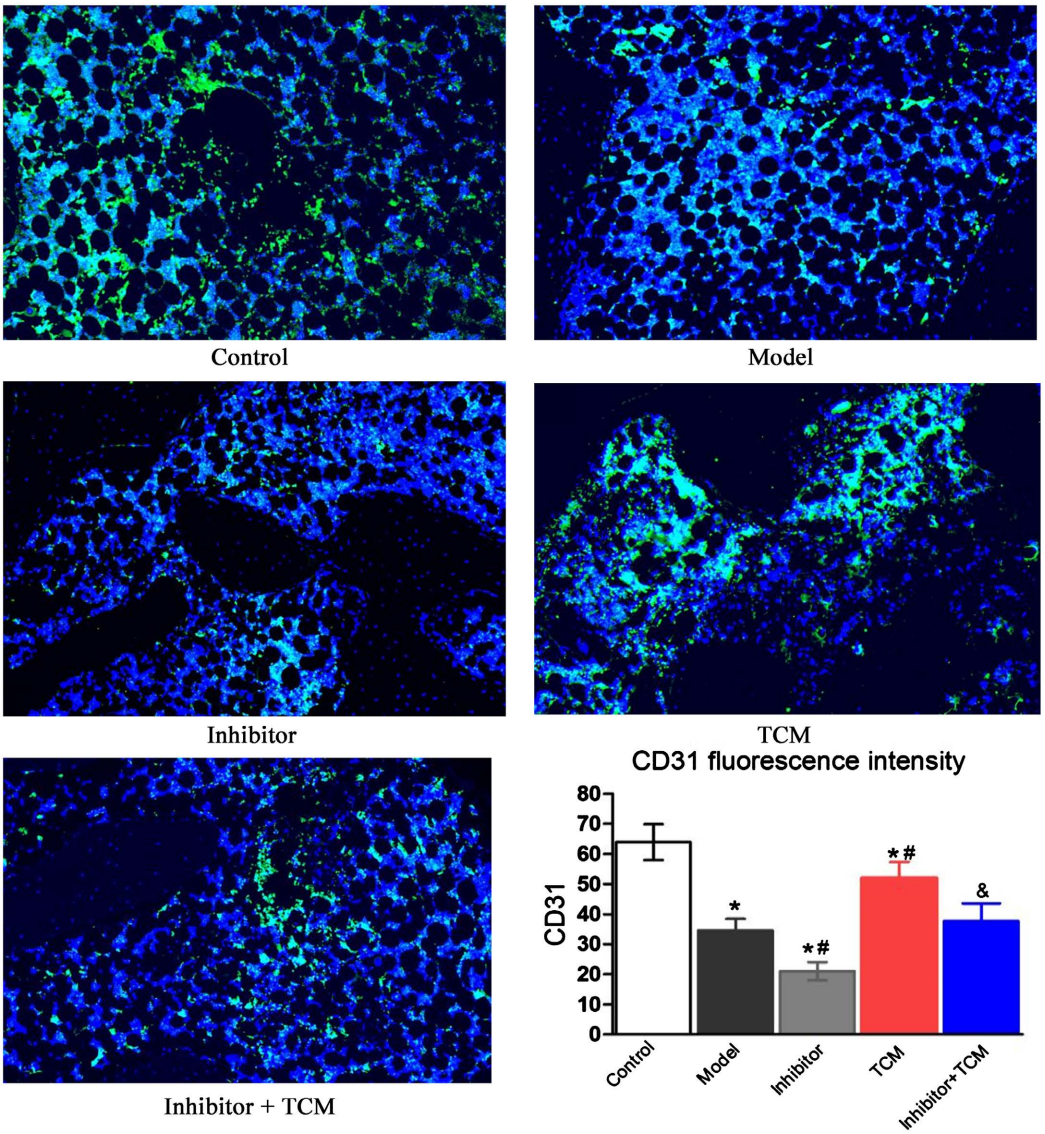

Figure 5. Effects of Yang-warming and Kidney-tonifying Prescription on CD31 expression in the steroid-induced avascular necrosis of the femoral head model. Blue represented the nucleus, and green staining represented CD31 molecule. Compared with control group, there were few green stained CD31 positive vessels in the model group, which was further reduced in the inhibitor group. CD31 positive expression increased in TCM group compared to model group. Compared with control group, $^{*}P < 0.05$; compared with model group, $^{\#}P < 0.05$; compared with inhibitor group, $^{\&}P < 0.05$· magnification, 100×.

and CD31. Bone repair is closely associated with BMP-2, Osterix and MMP13. miR-130a secretion can promote angiogenesis of bone marrow stromal cell [7]. Additionally, miR-130a can promotes osteogenesis by upregulating osterix expression [8]. Our data suggested that inhibition of miR-130a significantly increased empty lacunae and trabecular destruction. And the expression of HIF-1*α*, VEGF, and CD31 in the inhibited group was lower, while BMP-2 and Osterix were higher than those in the model group. Therefore, it is indicated that miR-130a has a role in promoting angiogenesis and osteogenesis in rabbit femoral head necrosis.

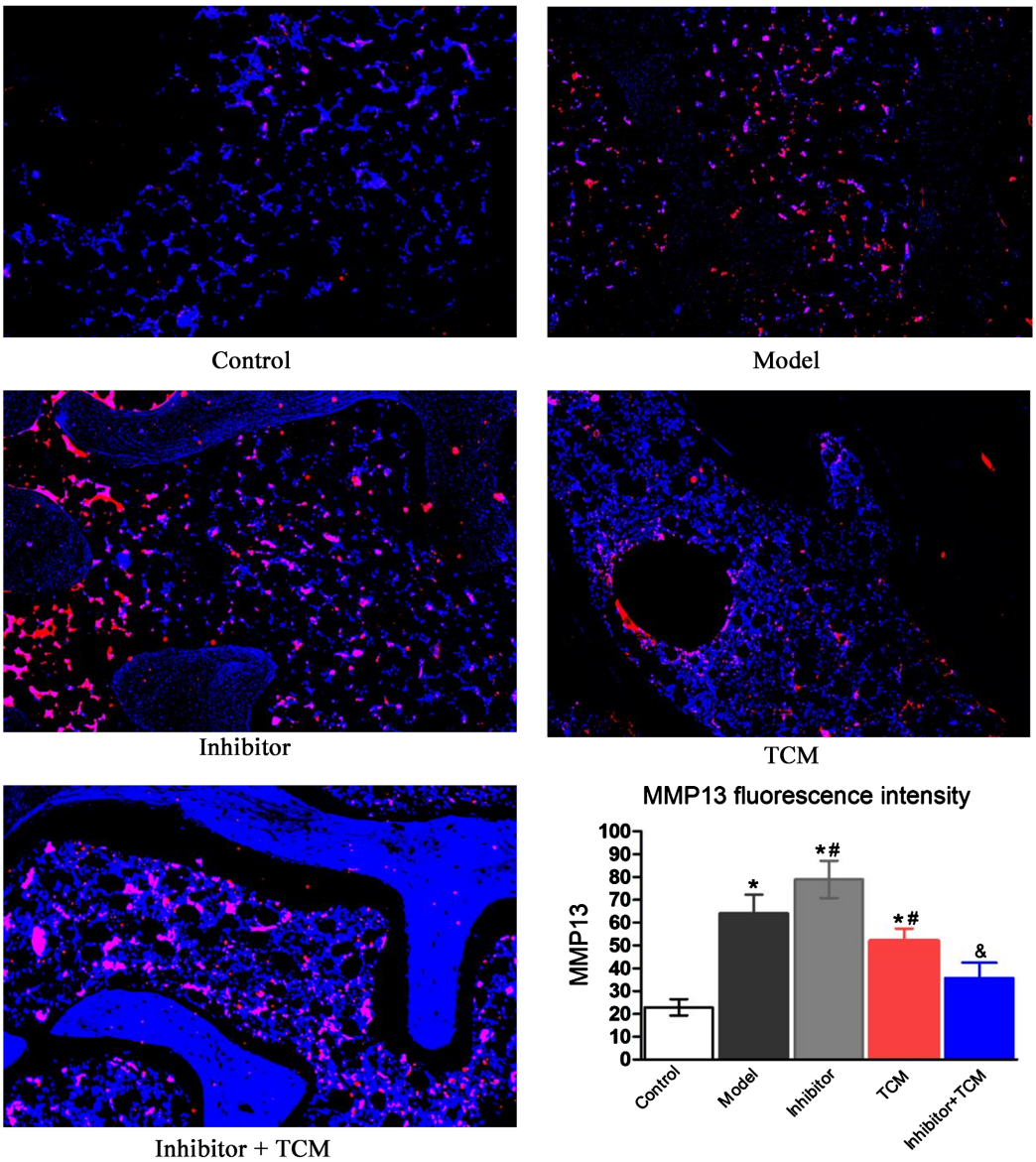

Figure 6. Effects of Yang-warming and Kidney-tonifying Prescription on MMP13 expression in the steroid-induced avascular necrosis of the femoral head model. Blue represented the nucleus, and red staining represented MMP13 molecule. Compared with control group, there were few green stained MMP13 positive vessels in the model group, which was further reduced in the inhibitor group. MMP13 positive expression increased in TCM group compared to model group. Compared with control group, $^{*}P < 0.05$; compared with model group, $^{\#}P < 0.05$; compared with inhibitor group, $^{\&}P < 0.05$. magnification, 100×.

According to the theory of Chinese medicine, kidney is the master of the bone and the production of marrow. SANFH, due to the long-term effect of hormones on the body, causes the yin and yang of the kidney to be injured, and the ability of produce bone and marrow decreasing, resulting in marrow impotence. In addition, the depletion of kidney essence causes Pi and Wei to lose nourishment, thus reducing the ability to promote blood flow, resulting in blood stagnation. And all these led to femoral head necrosis. In the formula of warming yang and tonifying kidney, Morinda officinalis has the effect of tonifying kidney yang,

strengthening muscles and bones, removing wind dampness, specifically treating kidney yang deficiency, mobilizing kidney yin and yang, and promoting bone marrow, which is the sovereign medicine. Herba Epimedii, Rhizoma Drynariae, and Cornu Glue all have the function of warming and tonifying the kidney yang, and can assist Morinda officinalis. They are official medicines. Salvia miltiorrhiza, Yujin and Panax notoginseng have the effect of promoting blood circulation and removing blood stasis. They are intended to dredge the blood vessels blocked by blood stasis, promote angiogenesis, nourish the necrotic femoral head, and reverse the course of disease. Astragalus membranaceus can replenish qi, promote yang and lift depression, and promote blood circulation when qi is flowing. It is an adjuvant to promote blood circulation and remove blood stasis. Achyranthes bidentata has the effect of tonifying the kidney and strengthening the bone, removing blood stasis, dredging the meridians, and guiding blood down. On the one hand, it can strengthen the dredging of the meridians, and on the other hand, it can make the effect reach the disease place directly. Licorice is used to mediate various medicines, relieve pain in an urgent manner, and is used together with Achyranthes bidentata. The whole prescription has the effect of warming yang and tonifying kidney, promoting blood circulation and removing blood stasis. This study showed that TCM treatment significantly decreased the empty lacunae rate, increased the expression of HIF-1*α*, VEGF, CD31, BMP2 and Osterix, and reduced MMP13 as compared to the model group and the inhibited group. Therefore, it is suggested that traditional Chinese medicine may promote angiogenesis and osteogenesis in rabbit femoral head necrosis by upregulating HIF-1*α*, VEGF, CD31, BMP2, Osterix, and downregulating MMP13.

In conclusion, Yang-warming and Kidney-tonifying Prescription can improve hormone-induced femoral head necrosis. And this process may be related to the upregulation of HIF-1*α*, VEGF, CD31 to promote angiogenesis, upregulation of BMP2 and Osterix to promote osteogenesis, downregulation of MMP13 to inhibit bone destruction. However, the mechanism of bone repair is very complex, and other mechanisms related to the repair of ischemic femoral head by YKP need to be investigated in depth, in order to provide a more adequate theoretical basis for its clinical application.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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