

**WENYANGBUSHEN-INDUCED-EXPRESSION OF VEGF, OPG, RANK, AND RANKL IN RABBITS WITH STEROID-INDUCED FEMORAL HEAD AVASCULAR NECROSIS**

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This study was aimed to investigate the effects of Wenyangbushen formula on the mRNA and protein expression of VEGF, OPG, RANK, and RANKL in the steroid-induced avascular necrosis of the rabbit femoral head (SANFH) and to further explore the potential mechanism of this formula on the treatment of SANFH. One hundred and thirty six New Zealand rabbits were randomly divided into 5 groups (n=24 rabbits in each group): normal group, model group, Traditional Chinese Medicine (TCM) Wenyangbushen decoction at low, moderate and high dose group. The normal group and positive control group were intragastrically (i.g.) administered with saline. The TCM group was treated with Wenyangbushen decoction at the indicated dosage. After treated for eight weeks, the mRNA and protein expression of VEGF, OPG, RANK, and RANKL in the femoral head tissues was determined by RT-PCR and Western blotting, respectively. Wenyangbushen decoction treatment can effectively promote bone cells, osteoblasts and chondrocytes growth, as well as prevent the cell apoptosis in SANFH. The mRNA and protein expression of OPG and VEGF was increased while the levels of RANK and RANKL were reduced in the necrotic tissue of model group compared with those in the normal rabbits. And Wenyangbushen treatment prevented those changes, as manifested by up-regulation of VEGF and OPG, while down-regulation of RANK and RANKL levels in a dose-dependent manner. Wenyangbushen Formula treatment can alleviate necrosis of femoral head induced by steroid. It can promote bone cells, osteoblasts and chondrocytes growth, as well as prevent the cell apoptosis. Meanwhile, it

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up-regulates OPG and VEGF while inhibits RANK and RANKL expression. This may be one of the mechanisms for the effective treatment of SANFH.

*Key words:* Steroid-induced avascular Necrosis of the Femoral Head, Wenyangbushen Formula, VEGF, OPG-RANK-RANKL Signaling pathways

## INTRODUCTION

SANFH (Steroid-induced Avascular Necrosis of Femoral Head, SANFH) is a pathological process of femoral bone degeneration induced by steroid, which can cause the death of the bone dynamic components (Beckmann *et al.*, 2014). Statistical studies show that the hormone plays critical roles in non-traumatic osteonecrosis (SHENG *et al.*, 2013; BEKLER *et al.*, 2007). So far, the etiology and pathogenesis of steroid-induced osteonecrosis has not been fully elucidated. Emerging data has indicated that various mechanisms are involved, including: stasis bone intraosseous hypertension due to venous stasis, osteoporosis, fat embolism, fatty degeneration and necrosis of bone cells, microvascular coagulation with intravenous vasoactive factors, arterial vascular damage due to the accumulation of immune complexes, apoptosis, bone marrow stromal stem cell adipogenic differentiation, and gene polymorphism (STEFFEN *et al.*, 2010; TONG *et al.*, 2011). Vascular endothelial growth factor (vascular endothelial growth factor, VEGF) is a glycoprotein characterized by promoting angiogenesis and osteogenic differentiation (VAROGA *et al.*, 2009; LCC *et al.*, 1999; WANG *et al.*, 2010). Osteoprotegerin (osteoprotegerin, OPG) is a new member of the TNF receptor family. It can inhibit osteoclast differentiation and increase bone density function (SIMONET *et al.*, 1997). Alternatively, NF- $\kappa$ B activation factor receptor ligands (receptor activator of nuclear factor- $\kappa$ B ligand, RANKL) can induce pre-osteoclast differentiation into functional osteoclasts and dose-dependently activate osteoclast maturation (SHIOTANI *et al.*, 2002). And it is reported RANKL is the only cytokine that can induce osteoclast differentiation and development. Receptor activator of NF- $\kappa$ B factor (receptor activator of nuclear factor- $\kappa$ B, RANK), in combination with RANKL, can induce the osteoclast precursors to differentiate, survive, integrate, and inhibit the apoptosis of osteoclast cell (BOYCE *et al.*, 2007). The RANKL/RANK/OPG pathway plays critical roles in the regulating osteoclast differentiation and bone resorption. Wenyangbushen formula is a cipher prescription from Orthopedics Department of the Second People's Hospital Affiliated to Fujian Traditional Chinese Medicine University. Compelling evidence shows that Wenyangbushen Decoction is efficacious in treating SANFH. Therefore, this study was designed to investigate whether Wenyangbushen formula treatment could ameliorate SANFH via VEGF and RANKL/RANK/OPG pathways.

## MATERIALS AND METHODS

### *Preparation of Wenyangbushen formula*

Wenyangbushen formula was made up by the following components: Morinda 15 g, Epimedium 9 g, Drynaria 9 g, Deerhorn Gelatin 6 g, Salvia miltiorrhiza 9 g, Radix curcumae 9 g, Panax pseudoginseng 3 g, Astragalus 15 g, Achyranthes 9 g, licorice 3 g. And this preparation was processed into decoction containing crude drug 2.5 g/ml by the Second People's Hospital of Fujian Province.

### *Experimental animals and design*

All of the procedures and protocols were approved by the *Animal Care Committee of Fujian Medical University* and followed the guidelines of *Animal Management Rules of the Chinese Ministry of Health* (Document No. 55, 2001). 136 New Zealand rabbits were obtained from Department of Laboratory Animal Science, Beijing University. All animals were kept under standardized lighting conditions (12 h light-dark cycle) and temperature ( $22\pm 2^{\circ}\text{C}$ ). Normal diet and mineral water were administered ad libitum. They were randomly divided into 5 groups (n=24 rabbits in each group): normal group, model group, Traditional Chinese Medicine (TCM) Wenyangbushen decoction group at low (6.44g/kg·d), moderate (9.66g/kg·d) and high (12.88g/kg·d) dosage. After treated with Wenyangbushen decoction for eight weeks, the femoral head tissues were collected.

### *Model establishment*

The SANFH model was replicated using horse serum combined with methylprednisolone according to WANG WEI *et al* (HOFSTAETTER *et al.*, 2009). Briefly, the rabbits were administered with 10ml/kg horse serum via ear vein. Three weeks later, 6 ml/kg horse serum followed. After 2 weeks, methylprednisolone was intraperitoneally injected at the dose of 45mg/kg, once a day for 3 consecutive days. Meanwhile, penicillin at 100,000 units per rabbits was administered, once a day for 7 days to prevent infection. Animals injected with saline served as controls. 4 weeks later, the animal model was established.

### *Histological analysis of the femoral head tissues*

After the establishment of the animal model, 2 rabbits in normal control group and 4 rabbits in model group were randomly selected. Their femoral head were collected, fixed with 4% paraformaldehyde for 48 h (PH7.4) and decalcified with 10% EDTA-Tris buffer. Paraffin sections at 4  $\mu\text{m}$  were stained with hematoxylin and eosin (HE). The pathological changes of femoral head were observed under the optical microscope. The successful animal model was characterized with the reduction of bony trabeculae, the disappearance of bone cell in lacunae and the increase of empty bone lacuna.

### *Reverse transcription-polymerase chain reaction (RT-PCR) for VEGF, OPG, RANK and RANKL mRNA expression*

The mRNA levels of VEGF, OPG, RANK and RANKL in femoral head tissue were determined using RT-PCR method. Total RNA isolated from VSMCs was treated with DNase I at  $37^{\circ}\text{C}$  for 30 min before reverse transcription was performed using a high capacity cDNA archive kit (TaKaRa, Japan). The sequences of primers were indicated in Table 1. The cDNA samples were amplified in a DNA thermal cycler under the following conditions: the mixture was annealed at  $57^{\circ}\text{C}$  (30 seconds), extended at  $72^{\circ}\text{C}$  (45 seconds), and denatured at  $94^{\circ}\text{C}$  (30 seconds) for 30 cycles. This was followed by a final extension at  $72^{\circ}\text{C}$  (10 min) to ensure complete product extension. PCR products were resolved on a 1.5% agarose gel and analyzed on White/Ultraviolet transilluminator digital science and analysis system. The expression levels of target genes were determined in triplicate.

Table 1. Primers for RT-PCR analysis

Gene	forward sequence ( 5' to 3' )	reverse sequence ( 5' to 3' )
VEGF	CGTTTCCTTCCTCATCTCCTC	GCTTGTTCCCTCCTTCTTGCTC
OPG	ACAATGAACAAGTGGCTGTGCTG	CGGTTTCTGGGTCATAATGCAAG
RANK	TTCAGGTTTGCTGTTCCCTACA A	CGCCGTTTTATCCTCTCTACAC
RANKL	GCAGCATCGCTCTGTTCTCTGTA	GCATGAGTCAGGTAGTGCTTCTGTG
$\beta$ -actin	AGACCACCTTCAACTCGATCAT	ACTCGTCATACTCCTGCTTGCT

VEGF: Vascular endothelial growth factor; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor kappa  $\beta$ ; RANKL: RANK ligand

#### *Western Blotting for VEGF, OPG, RANK and RANKL protein expression*

Right femoral head tissue was collected and lysed in RIPA buffer ( 50 mM Tris-Cl , pH 8.0, 150 mM NaCl , 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100  $\mu$ g/ml phenylmethyl- sulfonyl fluoride , 2  $\mu$ g/ml aprotinin ) . The suspension was incubated on ice and then centrifuged (14 000 g, 10 minutes, 4°C). The soluble fraction was stored at -80°C. Protein concentrations were measured by Bradford assay with bovine serum albumin as a standard. 100  $\mu$ g protein supernatant was used for electrophoresis. Primary antibodies were used at the indicated dilutions as follows: VEGF (1:500, Alomone), OPG (1:400, Abcam), RANK (1:300, Abcam), RANKL (1:650, Abcam).  $\beta$ -actin (Santa Cruz, 1:1000) was served as loading control. The intensity of the bands was quantified by densitometry. Blots were representative of at least three experiments.

#### *Statistical analysis*

All the values are expressed as the mean  $\pm$  SE unless otherwise indicated. The group comparisons were performed with the Student t test (2-sample test) or analysis of variance. The Mann-Whitney U test was used if the variance was not normally distributed. A P-value of 0.05 was accepted as significant. The statistical analysis was performed using SPSS 17.0 software.

## RESULTS

### *Histology analysis by hematoxylin and eosin (HE) staining*

#### *Normal group*

The cartilage thickness of femoral head layer was normal. The cartilage surface was smooth, lined with dense and regular bony trabeculae. The bone structure was integral and cells in each layer were evenly distributed. Cartilage cells were arranged regularly. Bone cells with normal morphology were clearly visible, located in the central position of bone lacuna, occasionally scattered empty lacunae was observed. Bone marrow cavity was rich in bone marrow cells. Meanwhile fewer fat cells with normal morphology were noted (Figure 1).

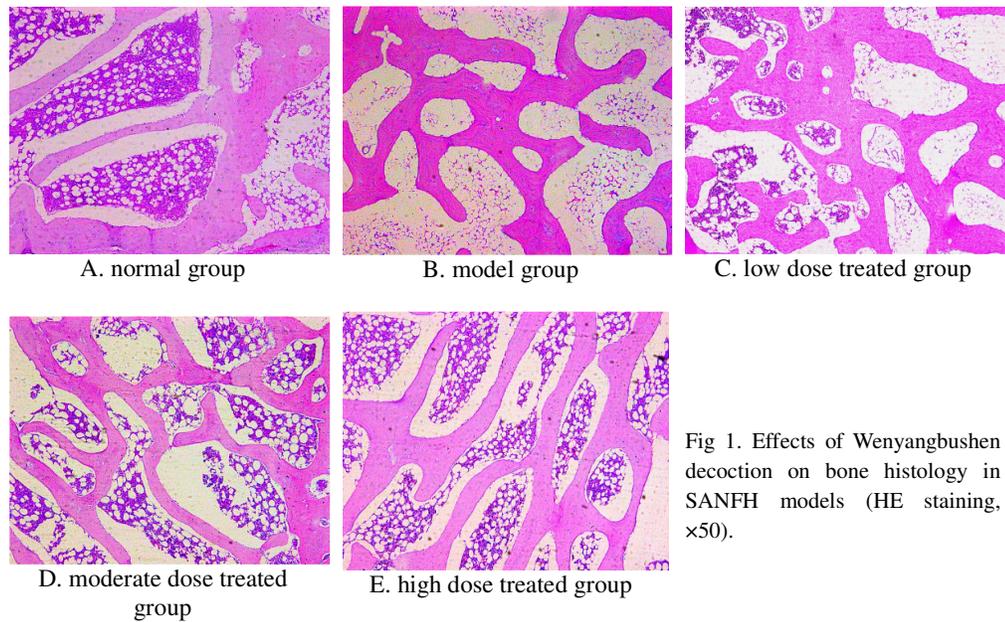


Fig 1. Effects of Wenyangbushen decoction on bone histology in SANFH models (HE staining,  $\times 50$ ).

#### *Model group*

The cartilage of femoral head layer was thinner than that of the normal group. Parts of the cartilage surface appeared exfoliation and cracks. The bony trabeculae were loose, irregular, slender and sparse, and even broken. All kinds of cells were scattered and sparse distributed. Chondrocytes reduced substantially and often clustered together. The number of bone cells dropped and empty lacunae were observed. Adipose cells increased, hypertrophied, degenerated and become necrotic. Additionally, the number of marrow cells decreased sharply. Wenyangbushen decoction treatment alleviated those pathological changes in varying degrees.

Alternatively, the number of empty lacunae in the normal group and the model group was counted under the light microscope. The ratio of empty lacunae in the normal group and model group was  $9.5\% \pm 1.152\%$  and  $21\% \pm 1.078\%$ , respectively. The difference between the two groups was statistically significant ( $P < 0.01$ ).

#### *Characteristics of bone cell ultrastructure manifested by electron microscope*

##### *Control group*

The bone cells in normal group were mostly elongated-oval shaped, located in bone lacunae and plumped. There were many prominentia on the cell surface. The nuclear heterochromatin was uniform. The mitochondria and rough endoplasmic reticulum was well-developed. The glycogen granules were rich in the cytoplasm. A small amount of lysosomes could be observed. The peripheral collagen fibers were densely distributed and their composition was regular and uniform.

### *Model group*

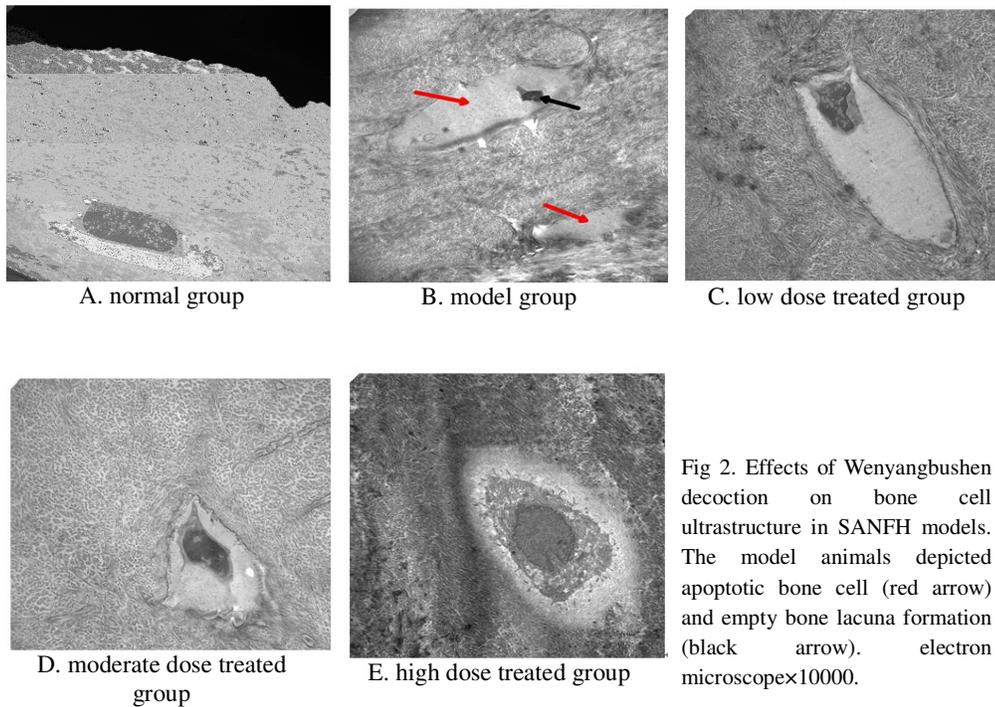
The bone cells exhibited karyopyknosis, chromatin aggregation and even nuclear fragmentation. The bone cells were squeezed aside by the large lipid droplets. The bone cells showed various degrees of necrosis, apoptosis, and empty bone lacuna formation. The collagen fibers were thick and disorganized.

### *Low dose TCM treated group*

The bone cells increased relative to the model group. The arrangement of rough endoplasmic reticulum, mitochondria and collagen fibers were improved compared with model group.

### *TCM at the moderate and high dose group*

The bone cells further increased, the organelles were well-organized, abundant in mitochondria and rough endoplasmic reticulum. The distribution of collagen fibers were dense and well arranged (Figure 2).



### *Wenyangbushen decoction up-regulated VEGF, OPG while down-regulated RANK, RANKL mRNA expression in SANFH animal models*

As compared with normal control rabbits, the mRNA levels of VEGF and OPG were decreased, while RANK and RANKL were increased in SANFH model group. Wenyangbushen

treatment prevented those changes, as manifested by upregulation of VEGF and OPG, while downregulation of RANK and RANKL mRNA levels in a concentration-dependent manner. In the presence of high dose Wenyangbushen decoction, the molecules of VEGF and OPG were increased by 45.5%, 42.1%, and the levels of RANK and RANKL were attenuated by 29.4%, 26.7% respectively, compared to those in model group (Fig 3).

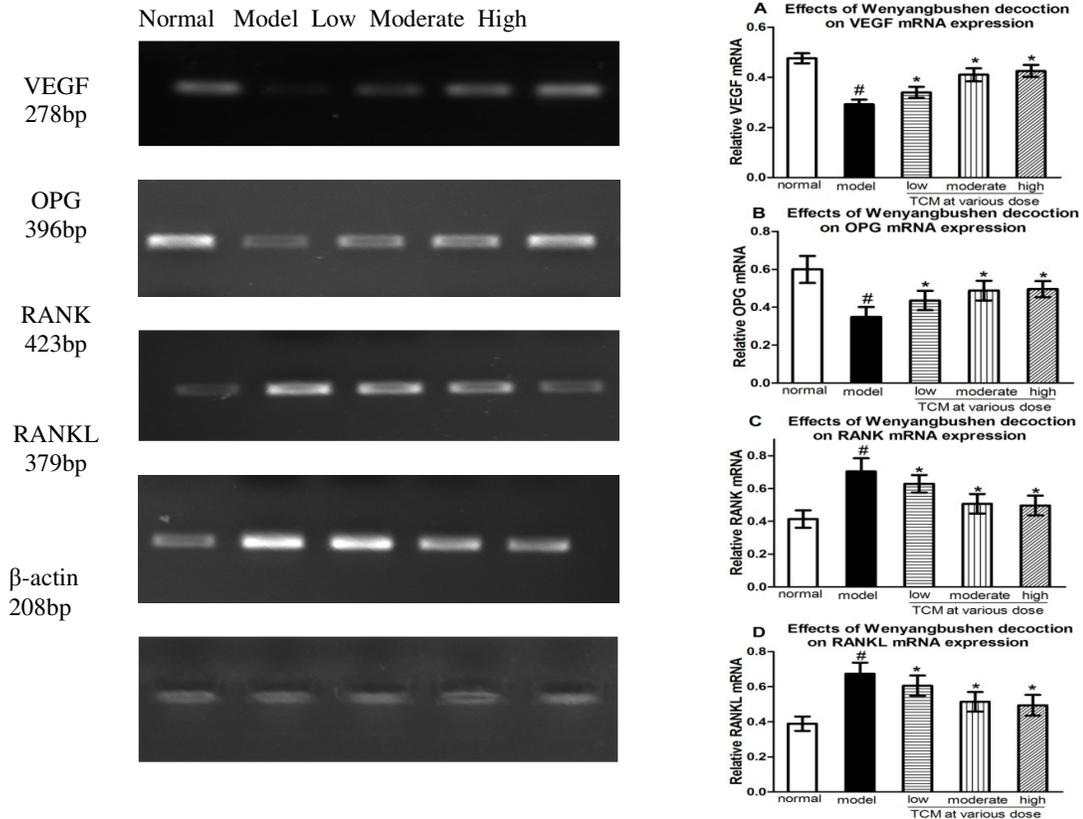


Fig 3. Effects of Wenyangbushen decoction on the expression of VEGF, OPG, RANK and RANKL mRNA in SANFH animals. Their expression was determined by RT-PCR.  $\beta$ -actin served as endogenous control. Results represented as mean  $\pm$  SE of 3 independent experiments in triplicate. <sup>#</sup>  $P < 0.05$  versus normal control, <sup>\*</sup>  $P < 0.05$  versus model rabbits. RT-PCR: reverse transcription-polymerase chain reaction; TCM: Traditional Chinese Medicine. VEGF: Vascular endothelial growth factor; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor-kappa B; RANKL: Receptor activator of nuclear factor-kappa B ligand.

*Effects of Wenyangbushen decoction on VEGF, OPG, RANK and RANKL protein expression in SANFH models*

Consistent with the mRNA expression, VEGF and OPG protein expression was also reduced while RANK and RANKL increased in SANFH group (Fig 4). The protein expressions of VEGF and OPG were markedly enhanced while RANK and RANKL was inhibited by Wenyangbushen treatment in dose-dependent manner. In comparison with SANFH model counterparts, high-dosage Wenyangbushen treatment increased 1.43-fold for VEGF, 1.48-fold for OPG. Meanwhile, protein level of RANK and RANKL expression was depressed by 32.9% and 44.3%, respectively.

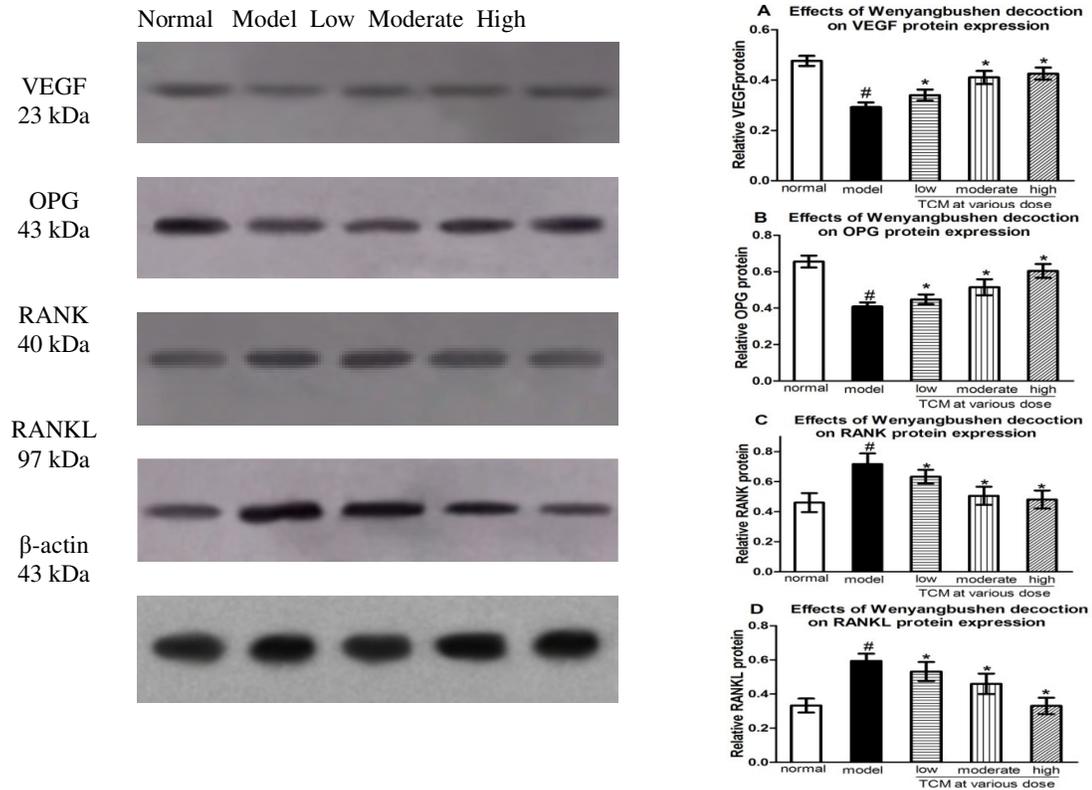


Fig 4. Effects of Wenyangbushen decoction on the protein expression of VEGF, OPG, RANK and RANKL in SANFH animals. Their expression was determined by western blotting.  $\beta$ -actin served as loading control. Results represented as mean  $\pm$  SE of 3 independent experiments in triplicate. <sup>#</sup>  $P < 0.05$  versus normal control, <sup>\*</sup>  $P < 0.05$  versus model rabbits. TCM: Traditional Chinese Medicine. VEGF: Vascular endothelial growth factor; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor- $\kappa$ B; RANKL: Receptor activator of nuclear factor- $\kappa$ B ligand.

## DISCUSSION

The pathogenesis of steroid induced femoral head necrosis is complex and is still elusive. Emerging data suggested SANFH was a multifactorial disease, which was mainly associated with bone structure destruction, cell necrosis and vascular stasis. Therefore, the fundamental way to the treatment of SANFH is to promote the formation of bone and regeneration of vessels. However, how to manage effectively these two points is a top priority. According to the Chinese medicine theory of "the kidney governs the bones and engenders marrow, the kidney stones the essence while the essence produces marrow", we established the fundamental principle of Warming Yang and Tonifying Shen. Our previous research indicated that Wenyangbushen decoction could promote the differentiation of bone marrow stromal cells, chondrocytes proliferation and ultimately prevent the development of SANFH (CHEN *et al.*, 2011).

In the process of bone formation and absorption, the molecules of RANKL, RANK, and OPG work jointly to regulate the differentiation of osteoblast and osteoclast. When various stimulating factors act on osteoblasts or stromal cells to expression RANKL on their surface, they identify specifically the osteoclast precursors and bind RANK receptor on the cell membrane. In the presence of M-CSF, the RANK-RANKL signal can be transduced into the cells, and then stimulate the maturation of osteoclast precursors into osteoclasts (KIM *et al.*, 2006; WITTRANT *et al.*, 2004). Meanwhile, actin ring in the mature osteoclasts is developed and activated via RANK-RANKL pathway, which can promote bone resorption. Alternatively, OPG can inhibit competitively the binding of RANKL and RANK and inhibit the biological effects of RANK-RANKL.

OPG also binds RANKL/RANK to form the trimer, which can direct inhibit the function of RANKL/RANK. RANKL or RANK deficient mice displayed abnormal bone resorption and serious bone sclerosis. OPG deficient mice developed osteoporosis after birth, which could be alleviated by the recombinant OPG. Therefore, RANKL/RANK/OPG is a key loop regulator of osteoblast and osteoclast differentiation as well as bone resorption (WITTRANT *et al.*, 2004; MANDELIN *et al.*, 2003). Our data suggested that the expression of RANK and RANKL was significantly increased while OPG was decreased in the femoral head tissues of model group. Wenyangbushen decoction can reverse those effects by inhibiting the expression of RANK and RANKL, while upregulating OPG expression, thereby inhibits osteoclast differentiation and function, as well as promotes osteoblast activity.

The expression of VEGF in bone tissue is affected by hormones. The decreased VEGF can lead to the reduction of angiogenesis in bone tissue, the lack of blood supply to the femoral head, and eventually the femoral head necrosis. It is reported that VEGF gene promoted angiogenesis, and further stimulated bone growth in SANFH (WANG *et al.*, 2010; LEE *et al.*, 2012). Similarly, our result suggests that the expression of VEGF in the model group is significantly decreased. Additionally, we find that its expression is substantially increased by Wenyangbushen decoction treatment, which indicating VEGF plays critical roles in SANFH.

## CONCLUSION

Our present study indicates that Wenyangbushen decoction can effectively promote bone cells, osteoblasts and chondrocytes growth, as well as prevent the cell apoptosis. And the mechanism may be related to its inhibiting the expression of RANK, RANKL, while promoting the expression of OPG, VEGF in SANFH.

## ACKNOWLEDGMENTS

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**WENYANGBUSHEN-INDUKOVANA-EXPRESIJA VEGF, OPG, RANK I RANKL  
KOD KUNIĆA SA STEROID-INDUKOVANOM AVASKULARNOM NEKROZOM  
FEMORALNE GLAVE**

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Izvod

Vršena su ispitivanja efekata Wenyangbushen formule na mRNK i ekspresiju proteina f VEGF, OPG, RANK i RANKL kod steroid – indukovane avaskularne femoralne nekroze kod kunića (SANFH) i mogućnosti korišćenja potencijala mehanizma ove formule na tretman SANFH. Prikazani su rezultati različitih tretmana pet grupa kunića . Posle tretmana u toku osam nedelja ekspresija mRNK i proteina VEGF, OPG, RANK i RANKL u tkivu femoralne glave je određivana metodom RT-PCR za mRNK i Western blotting metodom za ekspresiju proteina. Dobijeni rezultati ukazuju da Wenyangbushen može efektivno da poboljša ćelije kostiju, osteoblasta i rast hondrocita kao i prevenciju apoptoze ćelija kod SANFH. Ekspresija mRNK i ekspresija proteina OPG i VEGF je povećana dok su nivo RANK i RANKL bili redukovani u nekrotičnom tkivu model grupe u poređenju sa tkivom normalnih kunića. Wenyangbushen formula tretiranja može da ublaži nekrozu femoralne glave indukovane steroidima.

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