Basic Investigations

Effect of Chinese Drugs for Promoting Blood Circulation and Eliminating Blood Stasis on Vascular Endothelial Growth Factor Expression in Rabbits with Glucocorticoid-induced Ischemic Necrosis of Femoral Head

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Objective: To probe into the mechanism of Chinese drugs for promoting blood circulation and eliminating blood stasis in the prevention and treatment of glucocorticoid-induced ischemic necrosis of femoral head.

Methods: Thirty New Zealand adult white rabbits were randomly divided into a normal control group (n=5) and a model group (n=25). Hydroxyprednisone acetate was intramuscularly administered to the rabbits in the model group in a dosage of 7.5 mg/kg, twice per week for 6 weeks, to induce ischemic necrosis of femoral head and normal saline of the equal volume was intramuscularly administered to the rabbits in the normal control group, twice per week for 6 weeks. Then, the 5 rabbits from the normal control group and 5 rabbits selected randomly from the model group were sacrificed and the changes in histopathology and the expression of Vascular Endothelial Growth Factor (VEGF) were observed. The other 20 rabbits in the model group were randomly divided into the treatment group 1 and the treatment group 2, and the control group 1 and the control group 2, 5 rabbits in every group. Taohong Siwu Tang (桃红四物汤 Decoction of Four Drugs with Addition of Peach Kernel and Safflower) was orally administered to the rabbits in the treatment group 1 and the treatment group 2 in a dosage of 7 ml/kg, once daily and normal saline of the equal volume was orally administered to the rabbits in the control group 1 and the control group 2 once daily. After 10 weeks the rabbits in the treatment group 1 and the control group 1 were sacrificed and after 13 weeks the rabbits in the treatment group 2 and the control group 2 were sacrificed, and the expression of VEGF was detected in these rabbits.

Results: The expression of VEGF was significantly enhanced in rabbits of the model group as compared with the normal control group (P<0.01), and gradually reduced with the lapse of time. The expression of VEGF in the control groups was significantly reduced as compared with the treatment groups (P<0.001).

Conclusion: Chinese drugs for promoting blood circulation and eliminating blood stasis can improve the microcirculation of femoral head in rabbits with glucocorticoid-induced ischemic necrosis of femoral head by promoting the expression of VEGF.

It has been shown that Chinese drugs for promoting blood circulation and eliminating blood stasis can prevent and treat glucocorticoid-induced ischemic necrosis of femoral head. In this experiment, the Vascular Endothelial Growth Factor (VEGF) expression is observed to probe further into the mechanism of Chinese drugs for promoting blood circulation and eliminating blood stasis in the prevention and treatment of glucocorticoid-induced ischemic necrosis of femoral head.
Materials and Methods

Experimental animals
Thirty healthy adult New Zealand white rabbits weighing 2.0–2.5 kg, averaging (2.29±0.23) kg, without sex limit, were supplied by Zhejiang Animals Center.

Drugs: Taohong Siwu Tang (桃红四物汤 Decoction of Four Drugs with Addition of Peach Kernel and Safflower) consists of Dang Gui (当归 Radix Angelicae Sinensis), Chuan Xiong (川芎 Rhizoma Chuanxiong), Chi Shao (赤芍 Radix Paeoniae Rubra), Tao Ren (桃仁 Semen Persicae), Hong Hua (红花 Flos Carthami), and Sheng Di (生地 Radix Rehmanniae), and was prepared by the Pharmaceutical Center of the Second People’s Hospital Affiliated to Fujian College of Traditional Chinese Medicine. These Chinese drugs were decocted in water and 1ml of the decoction contained 0.75 g of crude drugs. The decoction was kept at 4℃ and taken out 12 hours before using to make it reach the room-temperature.

Reagents: Mouse monoclonal antibody against human VEGF (production No. MAB-0243, clone NO. JH 121) was purchased from Fuzhou Maixin Bio-technological Development Co., Ltd.

Grouping and developing of the model
Thirty New Zealand white rabbits were randomly divided into a normal control group (n=5) and a model group (n=25), and were fed for one week to adapt themselves to the environment. The models of necrosis of femoral head were developed by He Xijing’s method. Hydroxyprednisone acetate was intramuscularly administered to the rabbits in the model group at a dosage of 7.5 mg/kg, twice per week for 6 weeks to induce ischemic necrosis of femoral head and the rabbits in the control were given a similar injection of normal saline. The accurate weighing was carried out in per experiment. Penicillin in a dose of 160,000 units and streptomycin in a dose of 150,000 units were administered by the intramuscular injection in all the rabbits twice per week to prevent the infection. After 6 weeks, 5 rabbits from the control group and 5 rabbits selected randomly from the model group were sacrificed and the changes in histopathology and the expression of VEGF were observed. The other 20 rabbits in the model group were randomly divided into the treatment group 1 and the treatment group 2, and the control group 1 and the control group 2, 5 rabbits in every group.

Administration of the drugs
Taohong Siwu Tang (桃红四物汤 Decoction of Four Drugs with Addition of Peach Kernel and Safflower) was administered by the oral route using a gastric tube to the rabbits in the treatment group 1 and the treatment group 2 in a dosage of 7 ml/kg once daily and normal saline of equal volume was orally administered to the rabbits in the control group 1 and the control group 2 once daily. After 10 weeks the rabbits in the treatment group 1 and the control group 1 were sacrificed and after 13 weeks the rabbits in the treatment group 2 and the control group 2 were sacrificed, and the expression of VEGF was detected in these rabbits.

Histopathological observation
The one-side femoral head was removed, fixed for a week in 10 per cent formaldehyde, decalcified in 5 per cent nitric acid, dehydrated with a series of alcohol, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (HE). The bone trabecula, bone cells, marrow cavity, and morphological, structural and numerical changes of hematopoietic cells were observed by light microscope. The count of 50 bone lacunae per visual field under a higher-power microscope were performed in 10 random fields of vision and the percent of empty bone lacunae was calculated.

Immunohistochemical detection of VEGF
The one-side femoral head was removed, fixed in neutral formaldehyde, decalcified, dehydrated with gradient alcohols, embedded in paraffin, and cut into the sections. The deparaffinized and hydrated sections were washed with phosphate buffer solution (PBS), pH 7.4, three times, 3 min per time, maintained with EDTA, pH 9.0, and incubated in
water bath for 20 min. 50 µl of 3% hydrogen peroxide was added to per section, and the sections were incubated at room-temperature for 10 min, and washed with PBS three times, three minutes per time. After throwing off PBS, the each section was incubated with 50 µl of the primary antibody overnight at 4°C and then washed with PBS three times, three min per time. After throwing off PBS, the each section was incubated with 50 µl of polymer enhancer for 20 min at room-temperature. After washing with PBS and throwing off PBS, the each section was incubated with 50 µl of the secondary antibody for 30 min at room-temperature. 10 ml fresh 3-amino-9-ethyl-carbazole (AEC) was added to each section to develop color after washing with PBS and throwing off PBS. The sections were observed for 30 min under a microscope, with red positive coloration. The sections were washed with distilled water, counterstained with hematoxylin, differentiated with 0.1% hydrochloric acid, and then washed with tap water and PBS to turn blue. Lastly, sections were mounted with hydrophilic mounting medium. The sections without addition of the primary antibody were prepared as the negative controls. 7–10 sections from every group were selected per time, and a micrograph was taken for every section, which was analyzed using Motic Images Advanced 3.1 image-analysis software. The obtained grey scale was inversely proportional to the strength of VEGF expression.

Statistical processing
SPSS 11.0 software for Windows was adopted in statistical analysis and the differences between the two groups were compared by t-test.

RESULTS
Histopathological changes
In the normal control group, thicker cartilage layer, active osteogenesis, well ordered, compact plump subcartilaginous bone trabeculae, distinct osteocytes with larger nucleus located in the centre were seen in the sections of the femoral heads. Empty bone lacunae were occasionally found, accounting for 14.5±1.72 per cent of bone lacunae. Active proliferation of myeloid lacunae. Active proliferation of myeloid tissue and abundant hematopoietic cells were shown in the marrow cavity. In the model group, thinner cartilage layer, irregular and thin subcartilaginous bone trabeculae with wider gap, breaking of a part of bone trabeculae, and necrosis of most osteocytes in the bone trabeculae was seen in the sections of the femoral heads. Empty bone lacunae were obviously increased, accounting for 27.50±5.40 per cent of bone lacunae. The difference in count of empty bone lacunae between the normal control group and the model group was statistically very significant (P<0.001). The bone marrow was replaced almost completely by fat tissue and the lipocyte increased with bulked, some of them fusing into vesicular fat cells. Hematopoietic cells decreased.

Expression of VEGF
The mean grey scale was 90.39±4.31 in the model group and 97.58±0.56 in the normal control group with a very significant difference between the two groups (P<0.01), indicating that expression of VEGF in the model group was stronger than that in the normal control group. The mean grey scale of 117.68±7.04 in the control group 1 was significantly higher than that in the normal control group (P<0.001), which suggests that the expression of VEGF in the control group 1 was markedly weaker than that in the normal control group. The mean grey scale of 133.53±4.96 in the control group 2 was significantly higher than that in the normal control group (P<0.001), indicating that the expression of VEGF in the control group 2 was markedly weaker than that in the normal control group. However, the mean grey scale in the treatment group 1 was 98.06±0.65, and significantly lower than that in the control group 1 (P<0.001), indicating that expression of VEGF in the treatment group 1 was stronger than that in the control group 1; And the mean grey scale in the treatment group 2 was 97.55±0.74, and significantly lower than that in the control group 2 (P<0.001), indicating that expression of VEGF in the treatment group 2 was stronger than that in the control group 2. As compared to the model group,
VEGF expressions were significantly decreased ($P<0.001$) in the control group 1 (mean grey scale = $117.68\pm7.04$) and in the control group 2 (mean grey scale = $133.53\pm4.96$). The significant difference in the mean grey scale ($P<0.001$) was found between the control group 1 and the control group 2.

**DISCUSSION**

In this study, VEGF expression was found in the femoral head of the normal rabbit, and the VEGF expression was increased in the femoral head of the rabbit at early period of administration of glucocorticoid in high dosage, which indicate that the damage of blood vessels at early necrosis of the femoral head promotes VEGF secretion to repair the blood vessels. With the lapse of time, VEGF expression became weaker and weaker, even weaker than the normal state. Therefore, it is considered that glucocorticoid leads to unable in reproduction of blood vessels in the femoral head of ischmeic necrosis, decrease blood supply to femoral head, death of bone cells and then ischemic necrosis of the femoral head possibly by inhibiting VEGF expression.

It has been demonstrated in previous studies that the effect of the treatment principle of promoting blood circulation and eliminating blood stasis is superior in the prevention and treatment of glucocorticoid-induced ischmeic necrosis of the femoral head to those of eliminating dampness by diuresis and resolving phlegm, and reinforcing the kidney to strengthen bone. Chinese drugs for promoting blood circulation and eliminating blood stasis can exert the inhibitory effect on generating and developing of glucocorticoid-induced ischmeic necrosis of femoral head. In this study, the grey scale in the treatment groups after orally administration of Chinese drugs for promoting blood circulation and eliminating blood stasis for 4 and 7 weeks was similar to that of the normal state. Therefore, it is thought that Chinese drugs for promoting blood circulation and eliminating blood stasis exert therapeutic effect on glucocorticoid-induced ischmeic necrosis of femoral head by gradually antagonizing the attenuating of VEGF expression resulted from administration of glucocorticoid, so as to indirectly promote VEGF expression to facilitate the generation of the blood vessels and alleviate and improve ischemia of the osseous tissue, hence control of glucocorticoid-induced ischmeic necrosis of femoral head, rather than by directly enhancing VEGF expression.

**REFERENCES**

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(Translated by ZHOU Yong-sheng 周永生)