Experimental study on the expression of VEGF and BMP-2 in steroid-induced osteonecrosis of the femoral head

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Abstract: Objective: To study the expression and significance of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2) gene in steroid-induced osteonecrosis of femoral head. **Methods:** 30 healthy adult New Zealand white rabbits are randomly divided into model group (20) and control group(10), the model group will be used to make necrosis model of femoral head, by the aid of methods including immunohistochemistry and ELISA, we can study the changes and characteristics of VEGF and BMP-2 gene, thus comparing with the control group. **Results:** in the 8th week, the VEGF and BMP-2 gene expression level in the femoral head of the model group is lower than the control group, so is the expression intensity of gene positive, area percent and vacant osseous lacuna percentage, in this sense, the difference is of statistical significance($P<0.05$). **Conclusion:** In the produced exogenous necrosis model of rabbit femoral head, the VEGF and BMP-2 gene expression in the femoral head is restrained, which shows the VEGF and BMP-2 gene expression plays an important role in the pathogenesis of femoral head necrosis.

[Sun Naikun Rui Gang, Liu Fu-Ann. **Experimental study on the expression of VEGF and BMP-2 in steroid-induced osteonecrosis of the femoral head.** *Life Sci J* 2013;10(1):2853-2857] (ISSN:1097-8135). http://www.lifesciencesite.com. 344

Key words: steroid induced osteonecrosis of the femoral head; rabbit; model; vascular endothelial growth factor; bone morphogenetic protein

Steroid-induced avascular necrosis of femoral head (SANFH) refers to a pathologic process in which the active ingredients(osteocyte, medullae, hematopoietic cell and lipocyte) of femoral head dies due to the large doses of hormones [1], it is a frequently encountered disease and commonly encountered disease, with high disability. The exact pathogenesis is not fully understood. The rabbit is the most commonly used animal in the study of femoral head necrosis. The endotoxin (1ipo polysaccharide, LPS) plus hormone (methylprednisolone, MPS) produced FHN model is currently reported a FHN model [2-3] more akin to human. This paper, through the study of onfh, vegf and BMP-2 gene expression characteristics in the femoral head necrosis, discusses the causes to affect the bone repair after the necrosis of femoral head, and the intrinsic link between local pathological changes and the pathogenesis.

1 Materials and methods

1.1 Materials: Animal: Healthy adult New Zealand white rabbits, weighing 2.5 to 3.5 kg, half male and half female, are provided by the Experimental Animal Center of Kunming Medical College, with production license number: SCXK (Yunnan) 2005-0008, animal use permit No.: SYXK (Yunnan) 2005-0004. The experimental rabbits are fed and administered in Experimental Animal Center of Kunming Medical College, fed with pellets (provided by the Center) during the feeding period, capable of free access to water, and single-caged. The horse serum is produced by Hyclone company; rednisolone acetate injection: 5ml: 0.125g, provided by Zhejiang Xianju Pharmaceutical Co., Ltd., with batch number: 090316.

1.2 Methods

1.2.1 Grouping and modeling:

The said rabbits are expected to be weighed accurately after fed for 2 weeks, then randomly divided into model group(20) and the control group(10),the two groups of rabbits, through t inspection, see a weight and number difference subjecting to non-statistical significance ($P> O.05$). As for the model group, the steroid-induced avascular necrosis of femoral head is prepared; as for the control group, the rabbits will be normally fed. The model group(20) will be modeled in accordance with relevant requirements, each rabbit will be subjected the horse serum 10 ml / kg, which is injected via rabbit ear, with an interval of 2 weeks, then subjected to IP intraperitoneal injection of horse serum once every 2 days, with a dose of 5 ml / kg dose. After that, prednisone acetate will be injected thrice every 3 days at a dose of 7. 5 mg / kg. During the modeling period, the intramuscular injection of penicillin will be applied for preventing infection. Two were killed when the modelling with hormone injected runs for 5 weeks, then observing the situation of modelling. 8 weeks later, it is expected to measure the VEGF and BMP-2 level in the serum of remaining animal.

1.2.2 Methods:

8 weeks later after the modelling, take the blood at ear central artery of the two groups of animals, 2ml of each rabbit, then centrifuged at 15 000r/min for 10minutes, taking the supernatant and storing at -20 ℃. After that, use ELISA to measure the VEGF and BMP-2 concentration in the serum. Then use aeroembolism method to kill animals, take out the bilateral femoral heads, one is quickly placed in the liquid nitrogen container for PCR detection, the other is dissected along the coronal plane, then use 2O-times 4% paraformaldehyde fluid to fix for 24 hours before the decalcification; the decalcifying fluid is replced once a week, observe surface color of the specimen and use physical method to determine the extent of decalcification. Conventional dehydration embedded sections, then subjecting to immunohistochemical detection. Under the optical microscope, observe the change of organizational structure in the bone and bone marrow of femoral head as well as the change of bloodvessel endotheliocyte inside the osseous tissue.

1.3 Observing indicators:

histopathology. After HE staining, under the optical microscope, observe bone trabecula, osteocyte cavum medullare and the changes of morphology, structure and number of hematopoietic - cell. Under the high power lens, select 10 visual fields, count 50 osseous lacuna for each field, then calculate the percentage of vacant osseous lacuna. Use IMAGE-EX automatic digital image analysis system to observe the slice, then apply the IPP5.1, Smart-scape2002 biological image analysis software to determine the percentage of average positive staining area and average gray value, the former representing the size of distribution area, the latter representing the content per unit.

1.4 Statistical treatment:

The data is mainly collected by the Opticon own software. Use SPSS 13.0 version statistical software, use single factor analysis of variance to compare the data between the groups, use the mean plusminus sd to represent ($X \pm S$), use $P<0.05$ as the difference, which is of statistically significance.

2 Results

2.1 histopathological performance:

The femoral head HE staining of the control group shows that: smooth periosteum, cartilage cells arranged in neat rows, the full trabecular bone arranged in alignment, osteocyte in trabecular bone being visible, rare empty lacunae, rich hematopoietic cell in the medullae, rich hemopoietic tissue in the marrow cavity, normal morphology of lipocyte with a relatively small number. HE staining of the model group shows that: incomplete periosteum, cartilage being partially loss, sparse and thinning bone trabecula with disorder structure and fragmentation, the osteocyte lacunae of trabecular bone being vacant and sparse, osteocyte karyopyknosis; bloodvessel embolism is visible, hemopoietic tissue inside the marrow cavity is reduced significantly, cell number and reticulation is normal and sparse, the volume of lipocyte aggrandizes, some melt into bubble shape.

2.2 VEGF, BMP-2 measured values and the percentage of vacant osseous lacuna in the Serum of the two groups:

Tthose in model group are lower significantly than the control group, the difference is of statistically significance ($P<0.05$). See Table 1.

Table 1 comparison of VEGF, BMP - 2 measured value and the percentage of vacant osseous lacuna between the two groups' serums (X±S)

Group	VEGF(pg/ml)	BMP-2	percentage of vacant osseous lacuna (%)
Model group	11.25±1.71	2.62±1.47	31.45±2.87
Control group	24.05±3.46	7.03±1.82	11.33±1.73
t value	8.496	8.973	9.041
P value	0.004	0.004	0.005

2.3 Average grey scale value and area percent of VEGF, BMP - 2 positive expression of the two groups: The model group has a lower positive expression intensity and area percent compared with the control group, the difference is of statistical significance ($P<0.05$). For details see Table 2.

Table 2 Average grey value and area percent of VEGF, BMP - 2 positive expression of the two groups

Group	VEGF positive expression		BMP-2 positive expression	
	Ave. grey value (%)	area percent(%)	Ave. grey value (%)	area percent(%)
Model group	51.08±6.23	6.90±1.74	52.77±6.41	6.32±1.54
Control group	77.54±8.89	21.15±3.36	78.05±8.91	20.01±2.87
t value	-11.083	8.933	-12.375	9.472
p value	0.007	0.005	0.006	0.005

3 Discussion

Necrosis of femoral head, also known as avascular necrosis of the femoral head, divided into two categories of traumatic and non-traumatic, of which the non-traumatic is mostly caused by the use of hormone use [4]. the root causes for steroid induced osteonecrosis of the femoral head is the failure in blood supply, and the development of the course of disease will result in further failure of blood supply, thus aggravating femoral head necrosis, which is a process of vicious cycle. Pathogenesis mainly includes several doctrines as follows [5-6]: ① fat embolism doctrine: hormones cause fat embolism of vessel under the femoral head cartilage and the avascular necrosis of the femoral head. ② osteoporosis doctrine: hormone can cause osteoporosis, resulting in trabecular bone fractures, thus oppressing the vessel under the cartilage and causing ischemia and necrosis. ③ microvascular injury doctrine: hormone can cause blood hypercoagulability, elevates prostaglandin, resulting in microvascular inflammatory disease, thus resulting in avascular hypoxia-induced necrosis. ④ intraosseous high pressure and venous stasis doctrine: When intraosseous pressure rises, it oppresses sinusoidal capillaries, increases peripheral resistance, in this way, resulting in intraosseous venous stasis and the reduction of blood flow in femoral head; the anoxia caused by the microcirculatory disturbance of femoral head also results in oozing and swelling of of intramedullary tissue, aggravating the intramedullary high pressure, thus forming a vicious cycle, causing compartment syndrome, eventually leading to hypoxia and necrosis of femoral head. Some studies found [7] that stromal cells of bone marrow, under the function of hormones, are differentiated into lipocyte, and such genic expression promoting the differentiation of lipocyte will be enhanced with the increase of hormone concentration and the prolonging time of operation, meanwhile reducing its differentiation toward osteoblast. This study found the steatosis, necrosis of osteocyte in the model group, there are fat granules inside the cytoplast; the obvious pimelosis is found inside bone matrix, mesenchyme is full of fat granule, the lipocyte increases and aggrandizes, which shows that the fat metabolism chaos under functions of hormones is an important pathologic process for the femoral head necrosis. Boss et al [8] believes that adipose tissue accumulates in the bone marrow cavity, bone intrinsic pressure rises, intramedullary vascular is compressed, venous return is suffocated even blocked; bone intrinsic pressure further rises, thus forming a vicious cycle, eventually leading to arterial hypoperfusion and avascular necrosis of femoral head.

The generation of vessel is vital for the upgrowth, reconstruction and restitution of osseous tissue[7]. The femoral head usually repairs from the sequestrum edge and the coupling section of periphery living tissue, behaving as bloodvessel regeneration, new skeleton formation and sequestrum assimilation, while the vessel regeneration and osseous rebirth needs the participation and function of manifold growthfactor, for example, the VEGF and BMP - 2 is currently a vital in-depth studied growthfactor. VEGF is the angiogenesis factor at maximum efficiency, capable of exerting powerful functions like promoting endootheliosis and angiogenesis; at the same time, VEGF has such functions as chemotaxis and differentiation for the osteoblast, thus promoting skeleton formation. VEGF not only plays an important role in the osseous occurrence through its angiogenesis function, but also, through promoting the bone transition, restrains the death of chondrocyte and osteoblast which may affect the osteogenesis. Fan Yueguan et al [9] in his study using LPS (50 ug / kg weight) plus MPS's FHN animal model for hybridization in situ found that at 6th week, the expression of VEGF mRNA around the vessel wall is stronger, in the process of time, however, the expression tapers, at 10th week, reaching the lowest value, at 14th week, slightly increasing but still at a low value. Yang [10] in his research found that VEGF, in the osteogenesis activity, is capable of promoting hyperplasia of local bloodvessel and osteogenesis, many bone growthfactors, such as TGF - 13, BMP, through the osteoblast's VEGF expression, plays an important role in the skeleton formation and bone repair. VEGF may have a bridging action to connect the correlation between osteogenesis and angiogenesis. Bone morphogenetie protein-2 (BMP-2) is a sort of growthfactor to promote the osteocyte proliferation, maturity and new bone formation, its main osteogenesis is to induce mesenchymal cell to be differentiated into osteoblast and chondroblast, its inducement process is

involved with the entochondrostosis; BMP-2 also has a function to promote the repair of articular cartilage. Xue Yuansuo et al [11] established the rabbit femoral head necrosis model, used the methods including immunohistochemical and numerator hybridization in situ to observe the change of BMP - 2 in the course of femoral head necrosis, as a result, the BMP - 2 level in the necrosis bone trabecula and myeloid tissue drops significantly, BMP-2 can not be found in the osteocyte, the differentiation of medullae stromacell toward osteoblast is reduced, and the VEGF and BMP - 2 may interact and promote the bone repair. Kim et al [12] found that VEGF, through promoting the hyperplasia of local bloodvessel and the differentiation of osteoblast, participates in osteogenesis activity, but other bone growthfactors like TGF - 13, BMP and so on, through advancing the VEGF expression of osteoblast, plays an important role in the formation and repair of bone, these bone growthfactors may stimulate VEGF expression in vivo, and the VEGF insufficiency may result in the closing of bloodvessel cavity and bloodvessel degeneration. BMP - 2, in the rabbit cornea, can not induce the angiogenesis, but capable of inducing angiopoiesis through stimulating VEGF.

The experimental findings confirm that: in model group, VEGF and BMP-2 expression is significantly weakened compared with the control group, which shows when femoral head necrosis occurs, the reduction of local VEGF and BMP-2 expression is the important reason leading to local inadequate repair. Therefore, this study suggests that: in patients suffering from hormone-type femoral head necrosis, as the hormone restrains the local VEGF and BMP-2 expression of femoral head, while the VEGF and BMP-2 expression intensity directly affects the ability for angiopoiesis and new bone regeneration of necrotic area of femoral head, thus weakening the ability of bone regeneration and bone repair, when the necrosis bone under the cartilage is filled with granulation tissue, if no new bone timely repairs, which may result in cartilage fracture and thus causing the collapse of the femoral head. If there are ways to antagonize this inhibitory effect of the hormone, thus contributing to the expression of these cell factors, which is likely to contribute to the repair of the femoral head necrosis and delay the femoral head necrosis pathological process. Currently, in animal experiments and clinic, it has reported to apply VEGF and BMP-2 in the treatment of femoral head necrosis, and achieve a certain effect[13-14], but due the pathogenesis of ONFH has not yet completely clear, we can not clearly identify the exact mechanism and metabolic pathways which these cell factors do in the human body, as well as the impact which in vivo microenvironment impose over them.

In summary, the pathogenesis of steroid-induced osteonecrosis of the femoral head is still a hotspot and difficulty in research. At present, the research in the field of femoral head necrosis gradually run in the following two trends: First, with the progressive development of researches in the fields of molecular biology, stem cell and cell death, we were able to explore from a more micro level the essence of femoral head necrosis, provide molecular-level evidence for different kinds of doctrines, even to identify the intrinsic link between them; second, sensitivity of individual to the hormone is more and more concerned, more and more polymorphic gene is found to have relativity to the incidence of femoral head necrosis. Its significance lies not only in revealing its pathogenesis, but also in screening potential high-risk crowd, in this way, we can take precautions in advance to ensure at maximum degree the therapeutic effect of the hormone as a drug to the crowd, at the same time, avoid the harm of steroid induced osteonecrosis of the femoral head to high-risk crowd.

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1/11/2013