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A Rabbit Model of Hormone-induced Early Avascular Necrosis of the Femoral Head

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Objective To establish an experimental model of early stage avascular necrosis of the femoral head (ANFH) caused by corticosteroid in adult rabbits and to observe the pathological changes with various imaging techniques. Methods ANFH was induced by a combination of hypersensitivity vasculitis caused by injection of horse serum and subsequent administration of a high dose of corticosteroid. The pathological changes were detected with digital radiography (DR), computed tomography (CT), magnetic resonance imaging (MRI), ink artery infusion angiography, hematoxylin–eosin staining, and immunohistochemistry. Results The imageological and pathological changes corresponded to the clinical characteristics of early stage ANFH. DR showed bilaterally increased bone density, an unclear epiphyseal line, and blurred texture of cancellous bone. CT showed spot-like low-density imaging of cancellous bone, thinner cortical bone, osteoporosis, and an unclear epiphyseal line. MRI showed bone marrow edema and spot-like high signals in T2-weighted imaging in cancellous bone. Ink artery infusion angiography showed fewer obstructed blood vessels in the femoral head. HE staining of pathological sections showed fewer trabeculae and thin bone, an increased proportion of empty osteocyte lacunae, decreased hematopoiesis, thrombosis, and fat cell hypertrophy. Immunohistochemistry showed attenuated expression of vascular endothelial growth factor in osteoblasts and chondrocytes, and on the inner membrane of blood vessels. Conclusion Experimental rabbit model of early stage ANFH caused by corticosteroid can be successfully established and provide the foundation for developing effective methods to treat early stage ANFH.

Key words: Avascular necrosis of the femoral head; Corticosteroid; Animal model

INTRODUCTION

Avascular necrosis of the femoral head (ANFH), frequently encountered in the clinical practice, is a progressive pathological process due to obstruction of blood circulation in the femoral head. Occurring most often in adults at the age of 20-50 years, ANFH has many possible causes and can result in in situ avascular necrosis and disability in a high proportion of patients. In most patients, ANFH involves both femoral heads. Without treatment, femoral heads will deform, even collapse, impairing the hip joint function and causing permanent disability.

ANFH is classified clinically into two major types: traumatic and non-traumatic. The former is seen usually with a fracture of the femoral neck and dislocation of the hip joint[1], whereas the latter results from overuse or abuse of hormones or alcoholism[2]. In recent years, hormone use has become the primary cause of ANFH, and over 57% of ANFH patients are due to hormone use or abuse. This increasing incidence has attracted the attention of researchers and clinicians.

Although ANFH can be treated conservatively or surgically, no single treatment achieves consistently good results. Early diagnosis and treatment are especially important to preserve the femoral heads. An animal model of early ANFH should help develop new therapeutic methods.

We used a combination of injection of horse serum and a large dose of corticosteroid to develop a hormone-induced rabbit model of early ANFH. We used several imaging and histopathology techniques to observe the progression of ANFH, including digital radiography (DR), computed tomography (CT), magnetic resonance imaging (MRI), ink artery infusion angiography, histopathological observation, and immunohistochemistry.
MATERIALS AND METHODS

Experimental Animals

Healthy adult New Zealand rabbits were provided by Experimental Animal Center of Nanfang Hospital, Guangzhou, China.

Agents

Horse serum was purchased from Hyclone Ltd. (USA). Prednisolone acetate was purchased from Pharmacia & Upjohn Company (Belgium). Dextran was purchased from Shanghai Fu Min Pharmaceutical Factory (China). Veterinary sumianxin II for injection was provided by Changchun Veterinary Institute of Military Medical Academy of Sciences (China). Atropine sulfate for injection was provided by Tianjin Pharmacy Group Xinzheng Co., Ltd. (China). A human vascular endothelial growth factor (VEGF) immunochemistry kit was purchased from Fujian Mai Xin Co., Ltd. (China).

Establishment of Rabbit Model of Early ANFH

Twenty healthy New Zealand rabbits (10 males, 10 females), weighting 2.6-3.2 kg (mean 3 kg) were assigned randomly into two groups (n=10). Horse serum (10 mL/kg) was injected through an ear vein. Two weeks later, 5 mL/kg of horse serum was injected in the same way once a day for 2 days. Two weeks later, 7.5 mg/kg of prednisolone acetate was injected into the abdomen twice a week for 2 weeks. When hormone was injected, 200,000 U of penicillin was injected into the buttock of each animal. In the control group, an equal amount of saline was injected into the buttock muscle. The animals were examined at the end of week 5 after the hormone injection.

DR

A mixture of 1.5 mL veterinary sumianxin II and 1 mL atropine sulfate was injected into the buttock. The first dose was 0.7-0.8 mL/kg and a half-dose was injected 30-40 minutes later. The animals were placed at the supine position, both hip joints were fixed, and a posteroanterior radiogram was taken. The radiation conditions were 75 KV, 250 mA, and 16 ms/4 mAs.

CT

The anesthetized animals were fixed on the operating board at the supine position and both hip joints were positioned laterally as symmetrical as possible. Transect scanning of the entire hip joint including the upper and lower edges was performed. The scanning parameters were 100 KV and 220 mA. The combination detector was 16 mm × 0.625 mm, the screw pitch was 0.625:1, the bed speed was 5.62 mm/r, the reconstruction layer was 1.25 mm thick, and the diameter of field of view (FOV) was 9.6 cm.

MRI

An orthogonal head coil was placed on the anesthetized rabbit with its center located on the hip joint. Fast spin echo was used. T2-weighted imaging (T2WI) (TR/TE 4500 ms/96 ms), T1-weighted imaging (T1WI) (TR/TE 550 ms/20 ms), and T2WI fat-suppression sequence (FS-T2WI) were collected twice at the coronal position. The layer thickness was 3 mm and the FOV was 100 mm × 100 mm.

Ink Artery Infusion Angiography

The anesthetized animal was injected with 10,000 IU heparin through an ear vein. The abdomen was opened to expose the abdominal aorta. A polyethylene tube was inserted in the opposite direction of the heart with a 12-gauge needle, through which about 3,000 mL saline was perfused into the artery to wash away the blood. Ink and dextran were mixed at the ratio of 7:3, and about 100 mL of the mixture was injected into the blood vessel until the skin of the bilateral crura and nails became uniformly black. The body was kept at 4°C overnight. The thighbones were removed the next day and fixed in 4% paraformaldehyde for 3 days. The bones were placed in phosphate-buffered saline containing 10% disodium ethylene diamine triacetic acid (Na2-EDTA) to demineralize the calcium, and the buffer was changed every 2 days until the sufficient demineralization was achieved by observing the surface color of the samples and detecting the bone hardness by needle stabbing. The samples were dehydrated stepwise and turned transparent by dimethylbenzene and sodium salicylate. The samples were cut into 50 μm-thick sections and the configuration of femoral head, blood distribution, and growth condition were observed under a stereomicroscope.

Histopathological Assay

After the animal was killed, both femoral heads including the metaphyses and thighbones were removed, observed by macrography, fixed, and demineralized as described above. The samples were dehydrated stepwise, turned transparent by dimethylbenzene, embedded in wax, and cut into thin layers. The layers were stained with hematoxylin-eosin and observed under a microscope. Changes in the periosteum, cartilage, trabeculae, and hematopoietic organization were observed. During the observation of the trabeculae, 10 fields were
chosen randomly and 50 bone lacunae were counted in each. The number of empty bone lacunae was also counted, and the proportion of empty bone lacunae was calculated.

**Immunohistochemistry Assay**

Expression of VEGF protein was determined in wax sections according to the manufacturer’s instructions.

**Statistical Analysis**

The data were analyzed using SPSS 13.0 software package.

**RESULTS**

**DR**

DR showed a clear edge, smooth surface, and a distinct epiphyseal line of the normal femoral head. Five weeks after hormone injection, the density of the bilateral femoral head increased, the epiphyseal line became blurred, and the bone texture was unclear. However, the joint gap was normal with no deformation of the femoral head (Fig. 1).

**CT**

Both of normal femoral heads were symmetrical, the joint surface was distinct, and the cortex was smooth and intact. No change was found in the texture of cancellous bone, and the epiphyseal line was clear. In contrast, CT showed that the femoral heads of the experimental animals had multiple spot-like low-density areas with structural changes in cancellous bone. The bone cortex was thinner, the epiphyseal line was blurred, and osteoporosis was evident (Fig. 2).

**MRI**

Both of normal femoral heads were symmetrical. In FS-T2WI, a low fat level signal was observed in the cortex of the femoral head. In the model group, MRI showed a larger articular cavity of the femoral head. The high level FS-T2WI signal at the metaphyses suggested an edema in the bone marrow. The cancellous bone of the femoral head appeared with irregular spot-like or line-like patterns in the high-level signal image (Fig. 3).

**Ink Artery Infusion Angiography**

Rich medullary cavities were found in capillaries of the normal femoral heads, forming a network. The vessels in the network were patent and small vessels were abundant. In contrast, fewer blood vessels were found in the model group, and the capillary network was sparse. The blood vessels appeared obstructed, and the shallow-layer vessels under the joint surface of the femoral heads were almost disappeared (Fig. 4).
**Histopathological Observations**

The periosteum of the normal femoral heads was smooth, and cartilage cells were in a tidy arrangement. The trabeculae were intact, and their arrangement was regular, compact, and full. Bone cells in the trabeculae were clearly visible with few empty bone lacunae. Osteoblasts were columnar, cuboidal or spindle-shaped and distributed along the trabeculae in strings. There were abundant medullary hematopoietic cells and small fat cells with a normal morphology. In contrast, the periosteum of the femoral heads in the model group was incomplete with cartilage cells partly shed. There were few thin trabeculae with a disordered texture, some trabeculae were broken into fragments. The nuclei of bone cells were condensed, and 23.7% of bone lacunae were empty (Table 1). Few spindle-shaped osteoblasts were distributed along the trabeculae. The medullary hematopoietic areas were poorly organized with fewer cells, a sparse capillary network and partly obstructed blood vessels. The larger fat cells were fused into bubbles (Fig. 5).

**TABLE 1**

| Group | n  | Empty Bone Lacunae (%) |
|-------|----|-------------------------|
| Control | 20 | 7.2±2.0                 |
| Model  | 20 | 23.7±4.25*              |

*Note. *P*<0.05 vs control group.

**Immunohistochemistry Assays**

In the normal group, VEGF expression was observed in trabeculae, medullary cavity, and plasma of the microvascular endothelial cells in the blood sinus under the cartilage. Some of the cartilage cells and the columnar or cuboidal osteoblasts around the trabeculae also expressed
VEGF. In the model group, weakly positive VEGF expression was observed only in blood vessels, cartilage cells, and osteoblasts. The lighter staining color indicated that the VEGF expression was lower in the model group than in the normal group (Fig. 6).

**DISCUSSION**

The rabbit model of hormone-induced ANFH has been limited to the early stage of ANFH. At present, no ideal model of late stage ANFH is available. Since ANFH is not reversible, it is important to develop new therapeutic agents for its early diagnosis and treatment.

Pure hormones have been used to induce ANFH with limited success. In our study, injection of serum in combination with corticosteroid hormone increased the successful rate for ANFH, showing that our model is consistent with the clinical disease progress of hormone-induced ANFH because a large dose of hormone was used to treat patients with underlying diseases, usually vasculitis. Systemic lupus erythematosus is associated with a high incidence rate of ANFH [4]. Saito *et al.* [5] showed that hormone treatment and vasculitis are the most likely cause of ANFH. Matsui *et al.* [6] induced vasculitis in animals by injecting horse serum and then a large amount of hormone, which produced typical osteonecrosis. Small arteries are the target organ of vasculitis, which is caused by the binding of immune complex to the blood vessel wall. Subsequent administration of hormone inhibits the synthesis of collagen and elastic protein [7], aggravating the contraction of the blood vessels with vasculitis, platelet aggregation, and necrosis of endothelial cells, and causing breakdown and obstruction of small arteries. The processes led eventually to avascular necrosis in the bone. While developing this model, we regulated the dose and injection time of horse serum and hormone. To avoid killing the animals with an excessive immune response caused by the two injections of a large dose (10 mL/kg) of horse serum, we halved the second dose of horse serum and injected it once a day for 2 days. To improve the success rate, we injected prednisolone acetate (7.5 mg/kg) four times instead of three times.

In clinical practice, DR is used most often to detect ANFH. However, DR is less sensitive than CT or MRI in detecting early changes in osteonecrosis lesions [8-9]. It was reported that MRI is the most accurate imaging method for diagnosing osteonecrosis, especially at its early stage with changes only in the bone marrow. The sensitivity of MRI in diagnosing ANFH is 88.8%–100%, and the specificity is 98%–100% [8-10]. However, CT is better than MRI for detecting a bone fracture under the joint cartilage [11]. In the present study, DR showed increased density of femoral heads, unclear epiphyseal line, and blurred texture of the cancellous bone. CT demonstrated spot-like low-density images of the cancellous bone, thinner bone cortex, unclear epiphyseal line, and osteoporosis. MRI revealed an edema and a spot-like high level T2WI signal in cancellous bone. These results are consistent with the image characteristics of early ANFH, which could be classified as I-II stage according to the clinical Ficat stage criteria [12].

The histopathological morphology in our model is consistent with the reported finding. The earliest and most obvious features are the significant decrease in the medullary hematopoietic organization, few cells and a sparse cell network, and a larger volume of fat cells. These data support that corticosteroid hormone plays a role in causing disorders of lipid metabolism and abnormal hypertrophy of medullary fat cells, which increase the pressure in bone cavity and medullary blood vessels, which in turn induce the pathological process of osteonecrosis [13]. In our model, 23.7% of bone lacunae were empty. Matsui *et al.* [6] classified the pathological stage of hormone-induced rabbit ANFH model into three grades scored 0-2. In grade 0, the trabeculae are intact and the bone...
marrow is normal with no necrosis. In grade 1, the bone marrow shows necrosis but dissolved bone cells and hypertrophic fat cells. In grade 2, both the bone marrow and trabeculae have necrosis. Beside the phenomenon in grade 1, there are increasing empty bone lacunae. Our model belongs to the pathological grade of 1-2.

During bone formation, VEGF secreted by osteoblasts functions synergistically with bone growth factor\textsuperscript{14}, while active hypertrophic cartilage cells in the epiphyseal growth plate express VEGF. This is important in regulating blood vessel infiltration during growth in the epiphyseal plate. In the rabbit model of early hormone-induced ANFH, the number of bone cells decreased, bone formation was attenuated, and the cartilage cells were in the resting stage. These changes occur at the same time when the hormone inhibits the cellular expression of VEGF\textsuperscript{16-17}. These events decrease VEGF secretion by cells in the femoral head. During hormone-induced ANFH, the decreased blood flow produces local hypoxia, thus stimulating VEGF expression. The decreased VEGF expression in our model also suggests a poor ability of necrotic tissue to repair itself.

In conclusion, our rabbit model combines horse serum and a large dose of corticosteroid to induce early-stage hormone-induced ANFH and can be used in further study of transplantation of transgenic self-bone marrow stromal cells for the treatment of early ANFH.

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