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Original Article

Alterations of sympathetic nerve fibers in avascular necrosis of femoral head

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Abstract: Objectives: Avascular necrosis of the femoral head (ANFH) was mainly due to alterations of bone vascularity. And noradrenaline (NA), as the neurotransmitter of the sympathetic nervous system (SNS), leads to the vasoconstriction by activating its α-Receptor. This study was to explore the nerve fiber density of the femoral head in the rabbit model of ANFH. Methods: Twenty New Zealand white rabbits were used in this study. The rabbit model of ANFH was established by the injection of methylprednisolone acetate. The nerve fiber density and distribution in the femoral head was determined using an Olympus BH2 microscope. Results: Significant fewer sympathetic nerve fibers was found in the ANFH intertrochanteric bone samples (P = 0.036) with osteonecrosis. The number of sympathetic nerve fibers was compared between the two groups. And less sympathetic nerve fibers were found in later stage ANFH samples in comparison with those of early stages. Conclusions: ANFH might be preceded by an inflammatory reaction, and an inflammatory response might lead to arthritic changes in tissue samples, which in turn reduces the number of sympathetic nerve fibers.

Keywords: Avascular necrosis of the femoral head, sympathetic nerve fibers

Introduction

Avascular necrosis of the femoral head (ANFH) is a progressive nonbacterial localized bone disease in adulthood, and always accompanied with destruction of the femoral head and severe secondary osteoarthritis [1, 2]. Like all osteonecrosis, it is usually located near joints and affects the convex joint partner [3].

ANFH usually affects people aged between 30 and 50 years [4-7]. And the evidence linked the disease closely to problems in bone blood flow: all the pathologies that altered the circulation like trauma, polycythemia, diabetes mellitus, smoking, steroids, and others [8-11]. Although blood flow is in the focus of the etiologic considerations, sympathetic nerve fibers that regulate blood flow have never been carried out from ANFH. Manipulation of the sympathetic nervous system can elicit clear effects on bone remodeling [12, 13]. The recent findings of sympathetic inhibition of bone formation added the importance of nerve fibers in bone [14]. Importantly, a previous study reported there is a loss of sympathetic nerve fibers in the border zone adjacent to the necrotic area in patients with ANFH [15]. However, it is unknown whether it is a reason for developing ANFH or just an inflammatory following necrosis.

Therefore, it seems necessary to explore the role of sympathetic nerve fiber in developing ANFH. This study aims to compare the density of the sympathetic nerve fiber according to the state of ANFH of the femoral head in the rabbit model of ANFH.

Materials and methods

Animal

Twenty adult New Zealand white rabbits were used in the study. Animals were housed in separate cages in an air-conditioned room. The rabbits were free access to drink water, and fed a commercial rabbit diet. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research. The protocol was approved by the Animal Care and Use Committee of Qilu Hospital.

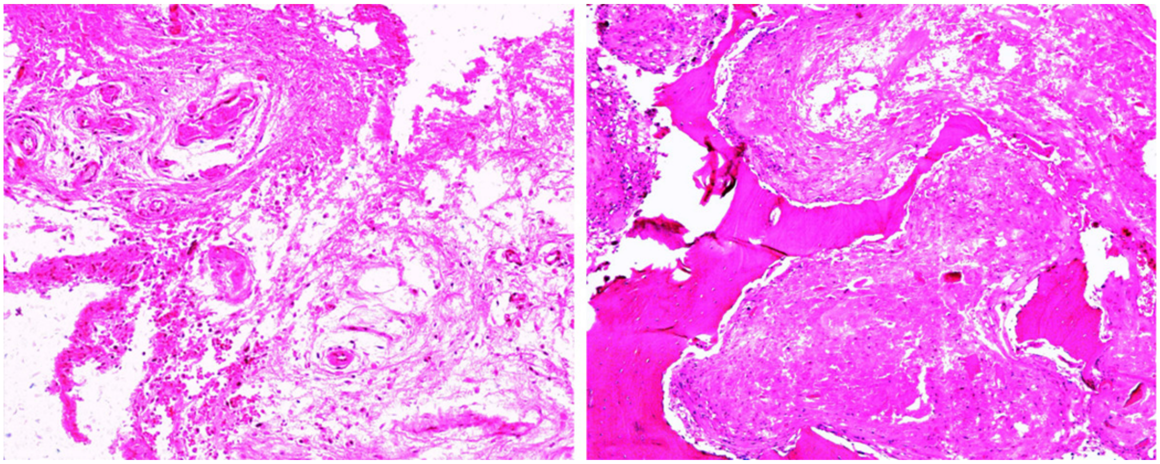

Figure 1. Histopathological examination of osteonecrosis.

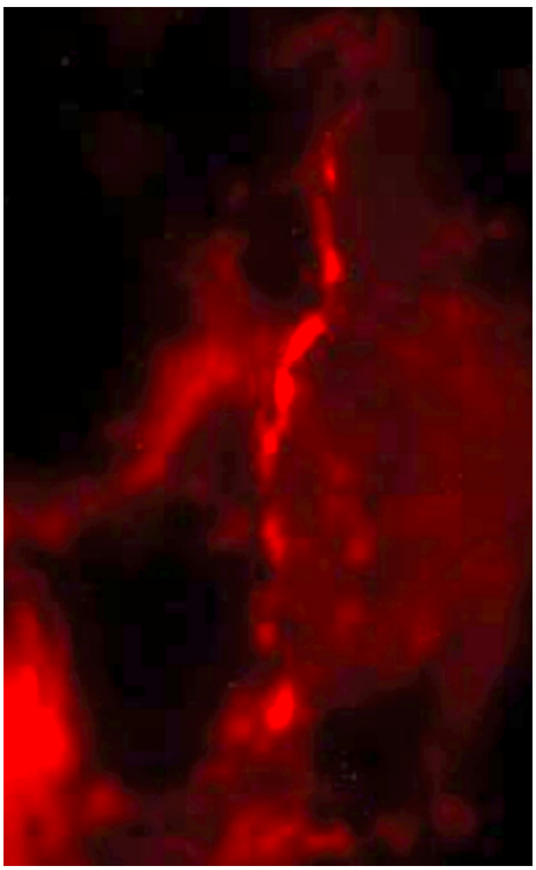

Figure 2. Immunoflurorescence evaluation of sympathetic nerve fibers.

ANFH model

The steroid-induced ANFH rabbit model was built as described previously [16]. A 20 mg/kg body weight dose of methylprednisolone acetate (MPSL; Upjohn, Tokyo, Japan) was administered once into the right gluteus medius muscle of all the rabbits in ANFH group to induce ANFH. The rabbits were sacrificed and tissue samples were prepared four weeks after the administration of MPSL. The rabbit model of OA were built by performing Cranial Cruciate Ligament Transection (CCLT) in the left femorotibial joint of all rabbits in OA group as described previously [17], while the right joint was left intact.

Sample preparation

After the rabbits were sacrificed, the samples of femur bone, periosteum, femoral head, synovium and ligamentum capitis femoris were collected from both ANFH and OA group. The samples were placed in phosphate buffered saline (PBS) supplemented with 4% formaldehyde (Merck, Denmark) for 12-24 fixed hours. Then, samples were incubated in PBS containing 20% sucrose for 12-24 hours. The next day, the samples were embedded in OCT compound (Tissue Tek; Sakura Finetec), and snap-frozen in liquid nitrogen at -80°C.

Evaluation of ON

Bone samples from the femoral head were cut along the coronal plane, and were histopathologically examined for the presence of osteonecrosis. The evaluation was blindly assessed by three pathologists based on the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by the surrounding bone marrow cell necrosis. Only bone marrow cell necrosis showing tissue debris consisting of both the hemato-

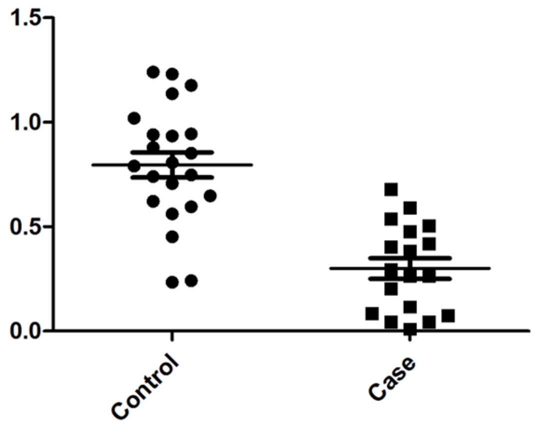

Figure 3. Significant fewer sympathetic nerve fibers was found in the ANFH intertrochanteric bone samples ($P = 0.036$) with osteonecrosis.

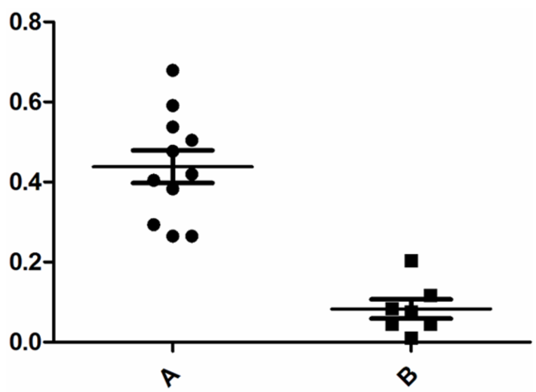

Figure 4. Significant less sympathetic nerve fibers were found in those of early stages in comparison with the late stages ($P < 0.0001$).

poietic cell necrosis and fat cell necrosis in which no bone trabecula was included was assessed as osteonecrosis. The presence of either granulation tissue, fibrosis: or appositional bone formation against necrotic tissue was also carefully determined in regard to the repair process. Then the samples were dived into two groups according to the existence of osteonecrosis.

Immunohistochemical evaluation

The frozen samples were sectioned at 8 μm in a cryostat microtome, and the frozen sections were put onto the Superfrost Plus slides (Menzel-glasses, Braunschweig, Germany). Subsequently, samples were incubated for 50 min in a blocking solution consisting of 10% bovine serum albumin (BSA), 10% chicken serum, 10% FCS (Sigma, Deisenhofen, Germany) and then pipetted on. Such a block solution was used to prevent nonspecific binding of antibodies directed against tyrosine hydroxylase in sympathetic nerve fibers. Thus, a nonspecific background staining can be avoided. The samples were washed with PBS, and then were incubated at 4°C overnight in rabbit polyclonal anti-TH (Chemicon, AB152) at a dilution 1:250 in PBS with containing 0.3% Triton and 10% goat serum. By the antibody binding to the enzyme, and thus it marked the sympathetic nerve fibers. The nerve fiber density was determined using an Olympus BH2 microscope. From each sample, 17 fields of view were counted. The nerve fibers were counted and the mean of these structures is converted to the area of 1 mm^2. A nerve fiber was considered positive when a beaded structure was represented with at least three members, or a length of 50 microns could be measured using a small scale within each visual field.

Statistical analysis

The data were represented by box blots. The calculations were done using Sigma Plot (version 9.0). Comparisons between groups of data were performed by using a Student's t-test or Mann-Whitney test. A P-value < 0.05 was considered to indicate statistical significance. Data were analyzed with the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL).

Results

Osteonecrosis was happened in 45.0% samples according to the histopathological examination (**Figure 1**). Therefore the control group contains 22 samples without osteonecrosis and the case group contains 18 samples with osteonecrosis.

Sympathetic nerve fibers were immunohistochemical evaluated successfully (**Figure 2**). Significant fewer sympathetic nerve fibers was found in the ANFH intertrochanteric bone samples ($P = 0.036$) with osteonecrosis, shown in **Figure 3**. Additionally, according to the pathologic evaluation, the 18 samples of osteonecrosis were divided into two groups, group A with 11 samples of early stage of osteonecrosis, and group B with 7 samples of later stage. The number of sympathetic nerve fibers was compared between the two groups. And less sympathetic nerve fibers were found in group B in comparison with those of early stages (**Figure 4**).

Discussion

At present, the cause of ANFH is considered as an alteration of the blood flow situation of the bone [18]. Atsumi et al. examined the vascular situation in ANFH patients at different time points by angiography. They found there was no radiological necrosist onset if the vessels in the capsule of the upper femoral neck missed completely. But these were just responsible for the vascularization of the greater part of the femoral head, as Sevitt et al. has already described [19]. And an angiographic study of the medial femoral circumflex artery and its outlets was carried out in the same patients as soon as the radiological signs of necrosis were seen, and they also found the increased retinacular vessels. However, the pathologies processes and finally become necrosis. It seemed the retinacular was interrupted around the necrotic tissue, so that the disease spread despite the endogenous vascularisation. However, no femoral head perfusion was presented sufficiency [20]. According to the results of this study, the sympathetic nerve fibers were lower around the femoral head; it seems the sympathetic nerve fibers play an important role in the pathologies of ANFH.

In recent years, it was delineated that sympathetic nerve fibers are often lost in inflammatory lesions such as rheumatoid arthritis, Charcot foot, Crohn's disease, and others [21-24]. This loss was interpreted as a proinflammatory stimulus via α-adrenergic pathways (which lead to strong vasoconstriction of small arterial vessels). In this study, a significant difference in fiber density was found in the femur bone, which is located around the necrotic tissue. A possible explanation for this is that the body tries to respond to the disease. ANFH is demonstrably a slowly progressive degeneration, which may take 80 months from initial diagnosis by MRI up to the onset of symptoms [11]. So, the organism has time to react to these pathologies and to counteract. As the blood flow seems to be defective, the sympathetic nerve fibers located around the necrotic tissue reduced so as to improve the microcirculation by dilating the blood vessels.

Previous studies have already demonstrated that the reduction of sympathetic nerve fibers was a response to the inflammatory, it seems the origin of the ANFH is an inflammatory action [25, 26]. Straub et al. demonstrated that the number of sympathetic nerve fibers reduced in synovial fluid in regarding with rheumatic joints [25]. In the immediate environment of an inflammatory process, sensory nerve fibers are stimulated, as well as the sympathetic nervous system, which is stimulated by the hypothalamus-pituitary axis. There is also an increase of endogenous cortisol, adrenaline, noradrenaline and adenosine with acute inflammatory reactions. However, if the inflammation persists over a long period, such as in rheumatoid arthritis, the body becomes adapt. The hypothalamus and the pituitary will not active permanently, on the contrary, the endogenous steroid hormone and catecholamine reduce [25]. This reduction leads to an anti-inflammatory synergistic effect. For example, the serum levels of both cortisol and norepinephrine are low in rheumatoid arthritis patients, whereas the levels of IL-6 and TNF increased, suggesting a generalized inflammatory response [27]. The inflammatory response of the bone also explained why scintigraphy could demonstrate the osteonecrosis of early stages [20]. There is a higher metabolic activity in the inflamed tissues, which enrich the administered nucleotides, so that the corresponding areas are visible scintigraphically. As a result, the inflammation precedes made the scintigraphy possible to show the necrosis several months before the X-ray [20].

Drescher et al. has already managed to prove that the vessels reaction in necrotic tissue was changed under the influence of corticosteroids on the vasoconstrictors noradrenaline and endothelin-1 sensitivity [28, 29]. This poses the question whether the increased sensitivity is due to the influence of steroids, or even to the disease? It could be explained that the reduction of sympathetic nerve fibers might be a repair attempt of the organism. It comes to hypoperfusion in the activation of an increased sensitivity of vessels to vasoconstrictors, and the body automatically tries to counter this lack effect by reducing the density of sympathetic nerve fibers. Here, it is considered almost certain that the tissue is hypersensitive to sympathetic activity.

Since groin pain is the most important complaint of the ANFH patients, it seems the number of sympathetic nerve fibers increases. Findings on Sudeck's Atrophy (Reflex Sympathetic Dystrophy Syndrome) indicated that pain could be maintained by the sympathetic nervous system through a direct stimulation of nociceptive C fibers. Thus, the pain perception can be enhanced by injection of norepinephrine in patients with Sudeck's atrophy [30]. In this way, ANFH may also share the pathogenesis of algodystrophy [18].

In summary, necrosis might be preceded by an inflammatory reaction, and an inflammatory response might lead to arthritic changes in tissue samples, which in turn reduced number of sympathetic nerve fibers.

Disclosure conflict of interest

None.

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