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Research in the precaution of recombinant human erythropoietin to steroid-induced osteonecrosis of the rat femoral head

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Abstract

Objective: To elucidate the effects of recombinant human erythropoietin (rHuEPO) on steroid-induced osteonecrosis of the femoral head in rats.

Methods: Twenty-four adult Wistar rats were randomly divided into three groups of eight rats each. The rats in the positive control group were injected with dexamethasone at 1 mg/kg twice a week for 5 weeks. The rats in the negative control group were injected with sodium chloride alone. The rats in the experimental group were injected with dexamethasone at 1 mg/kg twice a week for 5 weeks and rHuEPO (500 u/d/kg) daily for 5 weeks. The femoral head on one side was examined by hematoxylin and eosin staining, and that on the other side was examined by CD31 staining of the capillaries.

Results: Hematoxylin and eosin staining in the positive control group showed that the bony trabeculae had become obviously narrow and sparse with discontinuity of the integrity. The integrity of the trabeculae was better in the experimental group than positive control group. The CD31 expression was lower in the positive control group than in the other two groups.

Conclusion: rHuEPO can effectively prevent osteocyte apoptosis, delaying or decreasing osteonecrosis of the femoral head.

Keywords

rHuEPO, steroid, osteonecrosis, femoral head

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Introduction

Steroid-induced avascular necrosis of the femoral head (SANFH) is characterized by damage to the blood supply of the femoral head. Regardless of the pathogenesis,

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SANFH leads to degeneration and necrosis of the subchondral bone, collapse of the femoral head, and eventual disease related to retrogressive damage of the hip joint.[1,2] With the wide clinical application of steroids, the incidence of SANFH-associated morbidity has been increasing in China each year. Common clinical characteristics of such morbidity include short onset (Short onset is equivalent to fast onset.It means that the disease is progressing fast), collapse of the femoral head, a poor prognosis, and a high disability rate. Consequently, SANFH is associated with great economic and psychological burdens on patients and society and has a severe impact on patients' quality of life. Research on prevention measures has great clinical and social significance. The final link in the pathogenesis of SANFH is mainly the insufficient blood supply to the femoral head, which is an obstacle to revascularization and leads to subsequent necrocytosis.[3] Therefore, the key to curing SANFH is to adopt effective means and then promote revascularization and new bone formation in the necrotic zone.

Erythropoietin (EPO) is a single-stranded acidoglycoprotein with a molecular weight of about 30 kDa and thermal stability at 80°C. Previous research indicates that EPO not only accelerates erythropoiesis but also exhibits a cytoprotective effect, especially by preventing apoptosis of endothelial cells, nerve cells, and myocardiocytes, and accelerates the generation of new vessels. Increased research of EPO and its receptor has in turn increased the number of reports on clinical treatment using EPO, and its applications are being continually expanded.[4] Because dexamethasone (Dex) is the most commonly used steroid in clinical practice, we chose it to establish an SANFH model. This animal study was performed to elucidate the preventive effect of recombinant human EPO (rHuEPO) on SANFH in rats.

Materials and methods

Establishment of rat SANFH

This study included 24 healthy adult Wistar rats (12 male, 12 female; weight of about 300 g). All were bred under the same conditions (fed a common pelleted diet and maintained under natural lighting with a temperature of $20°C \pm 2°C$ and humidity of about 50%). They were weighed 1 week after adaptive feeding and randomly divided into three groups of eight rats each (four male, four female) using a random number table: the positive control group, negative control group, and experimental group. The body weights of the rats were not significantly different among these three groups according to one-way analysis of variance. During the experiment, the animals were handled based on the Guidance Opinion about Kindly Treating Animals (Ningbo University) released by the Ministry of Science and Technology in 2006. The animal model was established as follows. Rats in the positive control group were injected with Dex at 1 mg/kg body weight in the rear leg muscle twice a week for 5 consecutive weeks. Rats in the negative control group were injected with an equivalent volume of normal saline in the rear leg muscle twice a week for 5 consecutive weeks. Rats in the experimental group were injected with Dex at 1 mg/kg body weight in the rear leg muscle twice a week for 5 consecutive weeks and intraperitoneally injected with rHuEPO at 500 u/d/kg body weight once daily for 5 consecutive weeks.

General observation

The status of the rats was observed every day, and data were collected on survival, appetite, activity level, weight change, excretion, superficial wounds and infection, and other factors. The rats were weighed every week. All rats were given 2 h of free activities outside the cage every day to increase their

activity range and make the model as realistic to daily life as possible.

Hematoxylin and eosin staining

All rats were killed by bloodletting under anesthesia after 5 weeks. Bilateral femoral head specimens were histopathologically stained with hematoxylin and eosin (HE) and immunohistochemically stained with CD31. The surrounding soft issue was carefully removed to determine whether the femoral head was smooth and complete and whether the color and form of the coronalis was normal. The resistance was also checked. The obtained specimens were fixed with formaldehyde solution (0.1 mol/L, pH = 7.4) with a 10% volume fraction at 4°C for 4 days, washed with phosphate-buffered saline, decalcified at 37.4°C in EDTA–Tris buffer solution with a 10% volume fraction, and decalcified at 37.4°C. The decalcification liquid was changed once a week. The surface of the bone specimen was observed, and its degree of decalcification was measured through a physical method. Get material after the complete decalcification. The specimen was the dehydrated in increasing gradients of ethyl alcohol. The xylene was handled transparently for 2 hours. The specimen was embedded in paraffin and observed by HE staining. An optical microscope was used to observe the bone tissue, marrow tissue, and bone trabecular structure.

Immunohistochemical CD31 staining

The specimen of the femoral head proper was cut horizontally along the coronal plane, immediately placed into 40 g/L paraformaldehyde stationary liquid, and then placed in a refrigerator at 4°C for 48 hours. It was commonly cut through general SP immunohistochemical paraffin, and immunohistochemical testing was performed to evaluate the expression of CD31 in the rat femoral head. CD31 positivity was indicated by the presence of yellow particles in the vascular endothelial cells. Under a microscopic camera, each immunohistochemical side was observed by randomly selecting three different views (×400). The count presents yellow positive expression vessel.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed with PASW Statistics for Windows 18.0 (SPSS Inc., Chicago, IL), and differences between groups were tested with the mean t value. A P value of < 0.05 indicates a significant difference, and $P < 0.01$ indicates a remarkably significant difference.

Results

General condition of the rats

The rats of each group remained in good condition throughout the study. Five weeks after beginning treatment, the hair on the hind limbs and backs of most rats in the positive control group and experimental group became sparse, and the activity level of some animals in the positive control group decreased. The other two groups showed no obvious abnormalities.

Observation of HE staining results

(1) Negative control group: The bony trabeculae in both male and female rats had an integral circular or oval-type arch structure and high connectivity without osteoclasts, narrowed bone trabeculae, or fractures (Figure 1). (2) Positive control group: The bony trabeculae of three male rats became sparse, narrow, and fractured; the connectivity was reduced, some arch structures disappeared, the form became irregular, and the partitions of the bony trabeculae were decreased. Many osteoclasts were present, and some nuclei had shrunken, dissolved, or disappeared. These lesions were

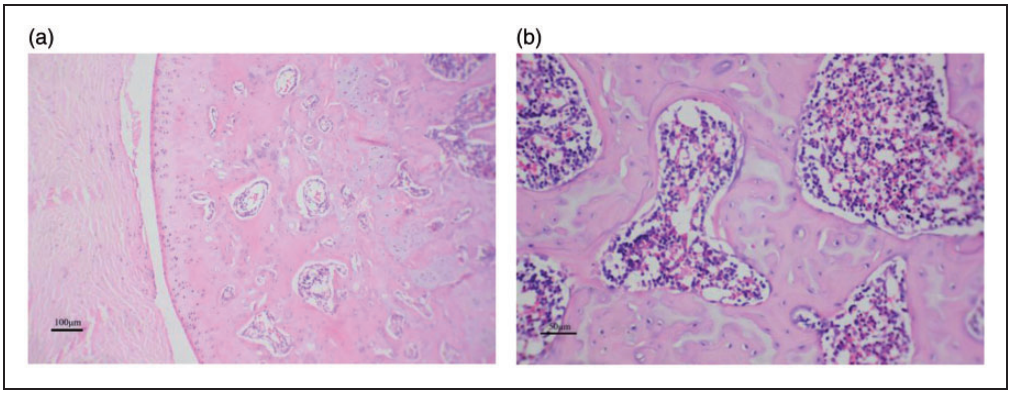

Figure 1. Histopathological observation of the femoral head with hematoxylin and eosin staining in the negative control group. (a) ×100. (b) ×250.

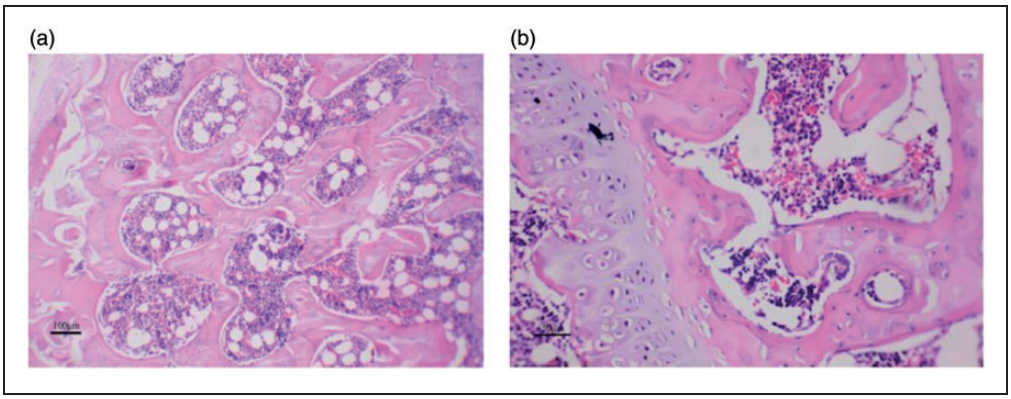

Figure 2. Histopathological observation of the femoral head with hematoxylin and eosin staining in the positive control group. (a) ×100. (b) ×250.

found in three of the female rats; two other rats showed different degrees of bone trabecular narrowing, decreased connectivity, and other lesions (Figure 2). (3) Experimental group: Compared with the positive control group, the lesions in both male and female rats in the experimental group were obviously alleviated. The bony trabeculae were relatively regular in form. The connectivity was markedly superior to that in the positive control group. Some bony trabeculae had become coarse. The fracture rate was obviously decreased. Connection and repair had occurred in the defects. Some of the structure resembled that of the normal group. The degree of partitioning of the bony trabeculae was relatively high, and many visible osteoblasts were present (Figure 3).

The bony trabeculae were complete and their arrangement was regular. The osteoblasts were clearly visible. The marrow was rich with hematopoietic cells, and relatively few fat cells were present. No osteoclasts

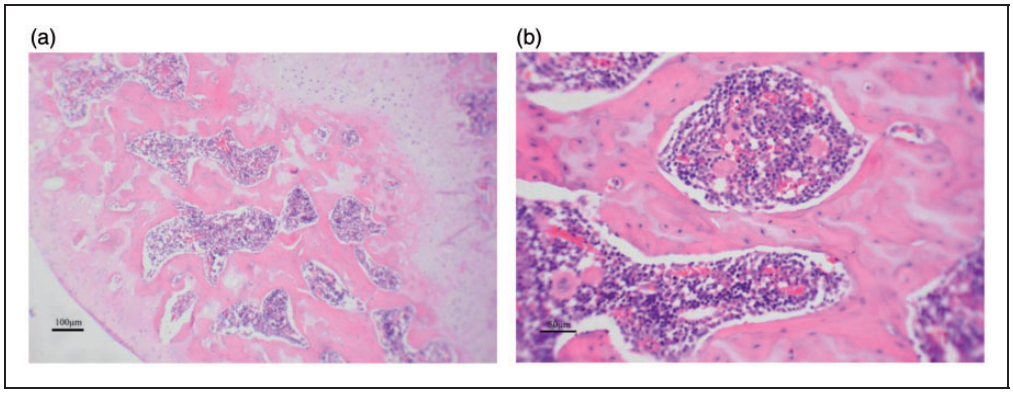

Figure 3. Histopathological observation of the femoral head with hematoxylin and eosin staining in the experimental group. (A) ×100. (B) ×250.

were observed, and the bony trabeculae became narrow and exhibited fractures.

The bony trabeculae were obviously sparse and broken and showed a chaotic, irregular morphology. Some of the arch structure had disappeared. The number of empty bone lacunae was increased. The degree of partitioning of the bony trabeculae was decreased, and many osteoclasts were present. Some visible bone nuclei had shrunken and dissolved, and some had disappeared.

The connectivity of the bony trabeculae was obviously better than that in the positive control group. Some trabeculae had become coarse, and the fracture rate was obviously decreased. Connectivity and repair had occurred in the defects. Some of the structure was close to that in the normal group. The degree of partitioning of the bony trabeculae was relatively high, and many osteoblasts were visible.

Immunohistochemical expression of CD31

Immunohistochemical staining for CD31 was clearly and selectively present in the endothelial cells. Positivity was indicated by the presence of yellow particles. The degree of vascular staining was evaluated by viewing of random areas under the microscope.

Table 1. CD31 staining count of the rat femoral head.

Group	Male	Female
Negative control group	26.00 ± 6.02	27.67 ± 7.99
Positive control group	12.72 ± 3.39*	14.11 ± 5.02*
Experimental group	$15.61 \pm 3.13^{\#}$	$18.94 \pm 4.22^{\#\#}$

Data are presented as mean ± standard deviation.
*$P < 0.01$ compared with negative control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with positive control group.

CD31 expression was lower in the positive control group than in the negative control group and experimental group (Table 1).

(1) Negative control group: CD31 expression was strong, and the blood supply of the femoral head was sufficient. The microcirculation was normal (Figure 4(a)). (2) Positive control group: CD31 staining was obviously decreased, and the light yellow color indicated that the microcirculation was insufficient. The blood supply to the femoral head was significantly decreased ($P < 0.001$) (Figure 4(b)). (3) Experimental group: The CD31 expression and the

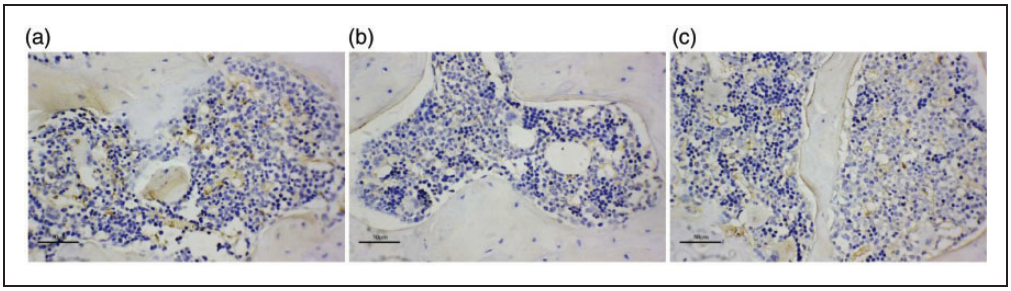

Figure 4. Immunohistochemical staining for CD31. (a) Negative control group (×400). CD31 staining was dense, indicating that the vasculature was rich and the microcirculation was good. (b) Positive control group (×400). CD31 staining was sparse, indicating that the blood supply was insufficient and the microcirculation was poor. (c) Experimental group (×400). Compared with the positive control group, CD31 expression was rich, indicating that the vasculature was increased and the microcirculation was relatively good.

blood supply were significantly higher than in the positive control group ($P < 0.05$) (Figure 4(c)).

Discussion

SANFH is a disease caused by improper use of steroids, which damage the blood supply of the femoral head, resulting in osteonecrosis of the bony trabeculae and marrow in some parts of the femoral head. Steroids and their metabolites act as strong inhibitors of vessel growth by inhibiting capillary growth and regeneration and promoting capillary degeneration. Zhang et al.[5] reported that steroids can induce apoptosis of microvascular endothelial cells, resulting in reduced numbers of capillaries. This may give rise to disorders of the microcirculation. When high-dose glucocorticoids are used for a long period of time, the microvascular density and thus blood perfusion of the femoral head decrease, which in turn decreases the blood flow. This lack of blood flow leads to hypoxia and fatty degeneration of bone cells. When the hypoxia and blood shortage reach a certain threshold, bone osteonecrosis will occur. Many treatment methods are currently available for protecting the femoral head. Core decompression is regarded as the most common technique for treatment of early-stage osteonecrosis of the femoral head. The aim of this technique is to reduce the internal pressure inside the femoral head and recover the normal blood circulation. The core drilling required for decompression helps to improve the blood circulation of the bone and alleviate marrow edema, thus preventing or postponing disease progression.

EPO is a glycoprotein that is mainly secreted from the kidney. It can stimulate the proliferation and differentiation of reticulocytes and endothelial cells; promote cell regeneration and vessel formation; resist inflammation, oxidation, and apoptosis; and accelerate vessel formation, cell proliferation, and cell protection. On one hand, EPO can increase red blood cell production to improve tissue oxygen perfusion, thereby affecting the processes of tissue reshaping and fibrosis in patients with chronic heart failure and renal dysfunction. On the other hand, EPO resists oxidation and apoptosis, decreases inflammatory symptoms, reduces cardiac and renal tissue injury, and promotes angiogenesis.[6] Some studies have revealed that EPO may improve cardiac function and reduce the hospitalization rate in patients with cardiac failure and

anemia.[7–10] Zheng et al.[11] used real-time polymerase chain reaction to detect EphB4 expression and gene expression related to osteoblasts by breeding a rat bone marrow-derived stromal cell line (ST2) in vitro after adding EPO. The calcium deposition was obviously higher in the EPO group than in the control group, and the expression of genes related to RUNX2, Col1, and ALP were correspondingly increased. These findings show that EPO plays an important role in promoting osteoblast differentiation and function.

Hu et al.[12] discovered that ischemia and reperfusion–induced renal dysfunction in rats was remarkably improved by EPO, which worked to inhibit p38MAPK, reduce the TNF-α level, and alleviate the inflammatory injury. These findings have implications for improvement in early renal function recovery after kidney transplantation. In their animal experiment, Ishii et al.[13] found that EPO pre-processing could reduce the cerebral infarction area and number of apoptotic nerve cells, thereby alleviating the damage induced by cerebral ischemia and reperfusion in rats. Ahn et al.[14] ligated the femoral artery to establish an animal model of acute artery embolism and studied the influence of EPO on blood perfusion in the lower limbs of rats. The experimental results indicated that EPO can accelerate vessel generation, improve blood flow, and effectively improve the circulation of the lower limbs. These findings provide insight and an experimental basis for clinical recovery of blood perfusion after acute artery embolism.

In the present study, we evaluated the effects of rHuEPO for treatment of SANFH by studying the mechanism of SANFH-associated morbidity and EPO-induced improvements in the microcirculation and apoptosis. Our main findings are as follows. The HE staining results in the positive control group showed that the bony trabeculae in five of the eight rats had become obviously sparse, narrow, and broken and exhibited a low connectivity rate. The remaining three rats showed different degrees of narrowing of the bony trabeculae and decreased connectivity rates. The establishment of this experimental model was successful. The histopathologic examination showed greater alleviation of the lesions in the experimental group than in the positive control group, the bony trabeculae were more regular in form, the degree of connectivity was obviously better than that in the positive control group, connectivity and recovery had occurred in the defects, and many osteoblasts were visible. These findings prove that rHuEPO can reduce bone cell apoptosis and thereby prevent the occurrence of osteonecrosis of the femoral head.[15] The femoral head CD31 staining count in the experimental group was 15.61 ± 3.13 (male) and 18.94 ± 4.22 (female), which was significantly higher than that in the positive control group [12.72 ± 3.39 (male) and 14.11 ± 5.02 (female), $P < 0.05$]. Thus, rHuEPO may increase the blood supply of the femoral head and help to prevent osteonecrosis by improving the microcirculation. The results of this experiment might lead to the use of rHuEPO to prevent the occurrence of femoral head necrosis in patients undergoing long-term treatment with steroids.

In summary, the pathogenesis of SANFH remains unclear. Apoptosis and tissue ischemia may be contributors. The use of rHuEPO in the early treatment period to prevent osteonecrosis of the femoral head has achieved a certain short-term curative effect. However, its long-term effect and preventive mechanism require further research and observation.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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