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Influences of hucMSC-exosomes on VEGF and BMP-2 expression in SNFH rats

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Abstract. – OBJECTIVE: To investigate the influences of human umbilical cord mesenchymal stem cell-derived exosomes (hucMSC-exosome) on steroid-induced necrosis of the femoral head (SNFH) and the expressions of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2) in rats.

PATIENTS AND METHODS: A total of 20 male Sprague-Dawley rats were randomly divided into SNFH group and SNFH + hucMSC-exosome group using a random number table. Prednisolone acetate (24.5 mg/kg) was injected twice a week to establish the rat model of SNFH, and hucMSC-exosome in a certain dose was additionally injected into the marrow cavity in SNFH + hucMSC-exosome group. After 3 weeks, the influences of hucMSC-exosome on the pathological changes and apoptosis of the femoral head in SNFH rats were detected via hematoxylin-eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. In addition, the expressions of cluster of differentiation 31 (CD31), VEGF, and BMP-2 in bone tissues in both groups were detected via immunohistochemical staining, and the messenger ribonucleic acid (mRNA) and protein expression levels of VEGF and BMP-2 in necrotic bone tissues in both groups were detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting.

RESULTS: The results of H&E staining revealed that the fibrous callus formation was good, the new trabecular structure was more obvious, the number of vacuum cleft declined, and there were fewer enlarged adipocytes in SNFH + hucMSC-exosome group compared with SNFH group. The results of TUNEL staining showed that the number of apoptotic cells in femoral head tissues was smaller in SNFH + hucMSC-exosome group ($p<0.05$). According to the results of immunohistochemistry, hucMSC-exosome could increase the expression of vascular endothelial marker CD31 in SNFH rats ($p<0.05$). Besides, the results of RT-PCR, immunostaining and Western blotting manifested that both the mRNA and protein levels of BMP-2 and VEGF in femoral head tissues were significantly increased in SNFH + hucMSC-exosome group ($p<0.05$).

CONCLUSIONS: HucMSC-exosome can improve SNFH in rats, whose mechanism may be related to the up-regulation of VEGF and BMP-2 by exosomes.

Key Words:
Mesenchymal stem cells, Exosomes, Steroid-induced necrosis of the femoral head.

Introduction

Since the 1960s, glucocorticoids have been applied in the clinical treatment of a variety of diseases, such as systemic lupus erythematosus, severe respiratory distress syndrome, and various complications after organ transplantation[1]. In recent years, with the widespread application of glucocorticoids in clinic, the incidence rate of steroid-induced necrosis of the femoral head (SNFH) has also become increasingly higher, and SNFH accounts for 51% in non-traumatic femoral head necrosis[2]. On the one hand, the reduced number of blood vessels in the lesion lowers the blood supply in local bone tissues and causes ischemia and hypoxia[3]. On the other hand, the impact of a large number of hormones leads to the increased apoptosis of osteoblasts[4]. These are all important causes of femoral head necrosis. However, there are a large number of genes and proteins involved in the occurrence and development of SNFH, and the mechanism of SNFH remains unclear yet, so clarifying the pathogenesis of SNFH is significant in the future prevention and treatment.

Mesenchymal stem cells (MSCs) are one of the main candidates for cell therapy, widely distributed in many organs in the human body, including the liver, bone marrow, placenta, amniotic fluid, and dental pulp[5]. MSCs are pluripotent, and the transplantation of adult MSCs has been proved to be able to improve myocardial infarction, kidney injury, liver fibrosis, and joint injury[6-8]. Currently, the most widely-used MSCs in clinical exper-

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iments are mainly from the bone marrow, adipose tissues, and umbilical cord[9]. Human umbilical cord mesenchymal stem cells (hucMSCs), similar to bone marrow-derived MSCs, have high self-renewal ability and low immunogenicity, and can be obtained non-invasively and cultured easily *in vitro*, making them superior to MSCs from other sources in cell transplantation therapy[10]. Currently, the effect of MSCs in tissue repair remains controversial. It is believed that such an effect, on the one hand, may be related to the trans-differentiation of MSCs, and, on the other hand, may be related to some cytokines produced in paracrine. During paracrine, exosomes produced by MSCs are considered as the main mechanism of tissue repair[11].

In this study, the rat model of SNFH was established, and hucMSC-exosome in a certain dose was injected into the marrow cavity of the necrotic femur. Then, the improvement of SNFH by exosomes was observed, and its potential mechanism was explored, so as to provide references for clinical prevention and treatment of SNFH in the future.

Patients and Methods

Experimental Grouping and Modeling

A total of 20 male Sprague-Dawley rats aged 12-14 weeks old and weighing (267.48±12.83) g were randomly divided into SNFH group (n=10) and SNFH + hucMSC-exosome group (n=10) using a random number table. There were no significant differences in the basic data, such as week age and body weight, between the two groups. All the above laboratory animals met the criteria for first-class animals issued by the Ministry of Health and were approved by the Animal Ethics Committee of our hospital. Prednisolone acetate (24.5 mg/kg) was intraperitoneally injected into rats in both groups to induce the SNFH model, and penicillin sodium was injected (40000 U/rat) twice a week to prevent infection.

Primary Culture of hucMSCs

The human umbilical cord was taken from the Obstetrics-Gynecology Department of our hospital and approved by the Ethics Committee of The General Hospital of Jinan Military. The primary hucMSCs were isolated and cultured strictly according to the method in the literature[12]. The residual blood in umbilical cord tissues was washed away with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) first and washed again with 70% ethanol solution. Then, the umbilical cord tissues were cut into small pieces (1-3 mm) and added with DMEM, followed by culture at 37°C. When 80% of cells were fused, they were digested with trypsin and subcultured.

Collection of Exosomes

The exosomes were collected strictly according to the method in the literature[13], and the specific steps are as follows: the supernatant of the medium was collected once every 2 d and placed into an EP tube, followed by centrifugation at 126000 × g and 4°C for 4 h. The precipitated cell debris was taken out, the supernatant was filtered with a filter (pore diameter: 0.22 mm) and the filtrate was collected into a new EP tube, followed by centrifugation at 126000 × g and 4°C for 2.5 h. The supernatant was discarded, and the precipitate on the EP tube wall was resuspended with alkaline phosphate to obtain exosomes. 100 μg exosomes and 100 μL HyStem-HP hydrogel were added into 50 μL phosphate-buffered saline (PBS), mixed evenly and injected into the marrow cavity of the necrotic femoral head.

Hematoxylin-Eosin (H&E) Staining

The bone tissues obtained in each group were placed in 10% formalin overnight, and then, they were dehydrated and embedded in paraffin. Then, all bone tissues were sliced into 5 μm-thick sections, fixed on a glass slide and baked dry, followed by staining. According to the instructions, the sections were soaked in xylene, ethanol in gradient concentration, and hematoxylin, respectively, and sealed with resin. After drying, the sections were observed and photographed under a light microscope to observe the pathological changes in bone tissues.

Immunofluorescence detection of related expression in tissues

The bone tissue sections were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times), and dehydrated with 100% ethanol, 95% ethanol, and 70% ethanol for 3 times, respectively. Then, the endogenous peroxidase activity was inhibited with hydrogen peroxide-methanol in a concentration of 3%. The tissues were sealed with goat serum for 1 h, incubated with the antibody (diluted at 1:200 with PBS) at 4°C overnight, and washed with PBS for 4 times on a shaker. Then, the fluorescein isothiocyanate (FITC) secondary antibody was added for incubation at 37°C for 1 h, and the nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). After that, 6 samples were randomly selected in each group, and 5 fields of view were random-

ly selected in each sample, followed by photography under the light microscope (200×).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

The bone tissue sections were baked in the oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times), and dehydrated with 100% ethanol, 95% ethanol, and 70% ethanol for 3 times, respectively. Then, the sections were incubated with protein kinase K for 0.5 h, washed with PBS, reacted with the terminal deoxynucleotidyl transferase (TdT) and luciferase-labeled dUTP at 37°C for 1 h, and incubated again with the horseradish peroxidase (HRP)-labeled specific antibody in an incubator at 37°C for 1 h. The nucleus was stained with hematoxylin, followed by photography and counting under a fluorescence microscope.

Detection of Expression of VEGF and BMP-2 Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA was extracted from femoral head tissues using the TRIzol method (Invitrogen, Carlsbad, CA, USA), the concentration and purity of RNA extracted were detected using an ultraviolet spectrophotometer, and the RNA with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. (2) The messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in the refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10 × Buffer, 2 μL cDNAs, 0.25 μL forward primers (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L), and 19 μL ddH_2O. The amplification system of RT-PCR was the same as above. (4) Calculation of CT value: the total cycle number was recorded when the fluorescence signal in each plate reached the threshold, and the expression levels of VEGF and BMP-2 in each group were calculated using the relative quantitative method. The primer sequences are shown in Table I.

Detection of Protein Expression Via Western Blotting

The bone tissues of rats in each group were fully ground in the lysis buffer, followed by ultrasonic lysis. Then, the lysis buffer was centrifuged, and the supernatant was taken and placed into the EP tube. The protein concentration was detected *via* ultraviolet spectrometry, and the protein samples were quantified to be the same concentration. The protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated with the primary antibody at 4°C overnight and then incubated again with the goat anti-rabbit secondary antibody in a dark place for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of protein to be detected was corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the data analysis. Measurement data were expressed as mean ± standard deviation, and the *t*-test was used for the comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Identification of hucMSC-Exosomes

First, hucMSC-exosomes extracted were identified under a transmission electron microscope.

Table I. Primer sequences in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GAGCTCAGCTCGCCTGGAGAAAC-3'
	Reverse	5'-TGCTGATCGTAGCCCTTTAGT-3'
VEGF	Forward	5'-ACTAGTCGATAGCTAGTCGAGCA-3'
	Reverse	5'-CCGATGCTACTAGCTAGCTAGC-3'
BMP-2	Forward	5'-TTGTGTTAGCTTAGCCCGATCGTA-3'
	Reverse	5'-CCGTCGTAAAAGCTAGTCGATC-3'

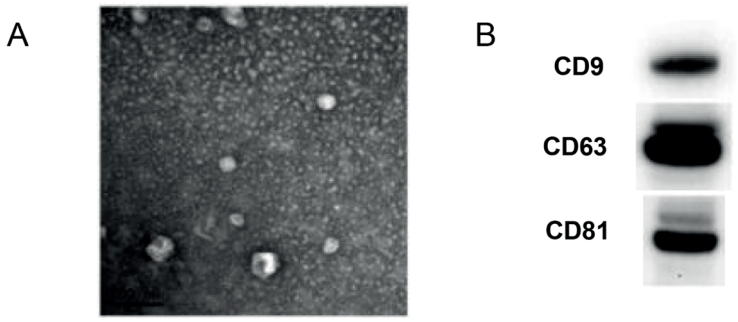

Figure 1. Identification of hucMSC-exosomes. ***A,*** Observation of exosomes under a transmission electron microscope (Magnification: 150,000×), ***B,*** expression of marker protein for exosomes.

As shown in Figure 1, the exosomes were in a cup or round shape, and the diameter of them was 50-80 μm. At the same time, the protein expression levels of specific markers (CD9, CD63, and CD81) for hucMSC-exosomes were detected *via* Western blotting, and the results revealed that the protein expression of all the three markers was positive.

Pathological Changes in Bone Tissues in Both Groups

It was found in the H&E staining of bone tissues in both groups that hucMSC-exosomes could effectively improve the SNFH-induced pathological injury of bone tissues. The specific manifestations are as follows: the number of vacuum cleft in bone tissues declined, the new trabecular structure was more obvious, the fibrous callus formation was good, and there were fewer enlarged adipocytes in SNFH + hucMSC-exosome group (Figure 2).

Apoptosis of Bone Tissues in Both Groups

At the same time, the influence of hucMSC-exosomes on apoptosis of necrotic bone tissues of SNFH rats was evaluated *via* TUNEL staining. As shown in Figure 3, the number of apoptotic cells in bone tissues was significantly smaller in SNFH + hucMSC-exosome group compared with that in SNFH group ($p<0.05$).

Angiogenesis in Bone Tissues in Both Groups

The reduced number of blood vessels at the lesion site is one of the important pathological

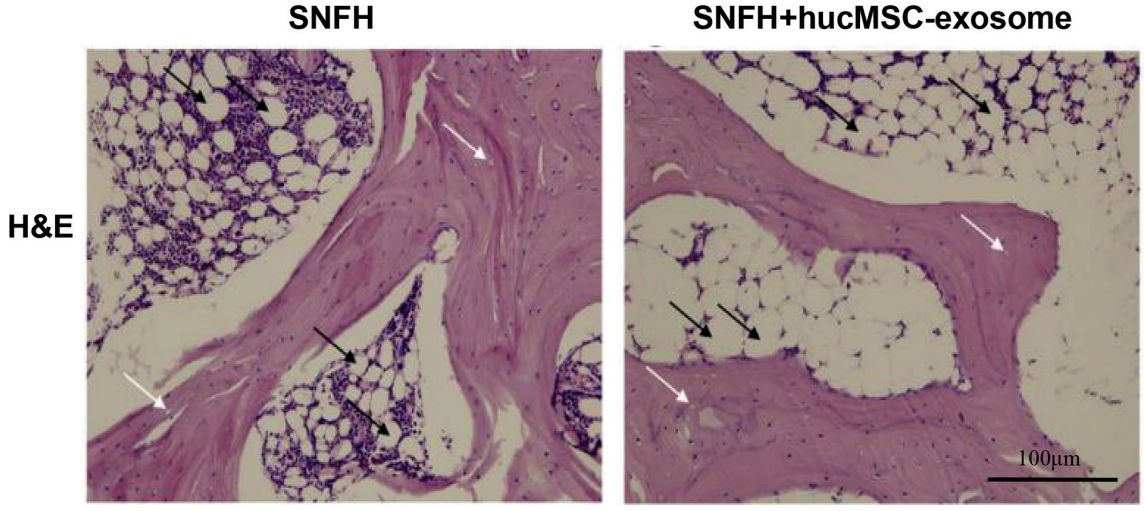

Figure 2. Pathological changes in bone tissues in both groups (Magnification: 100×). SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group.

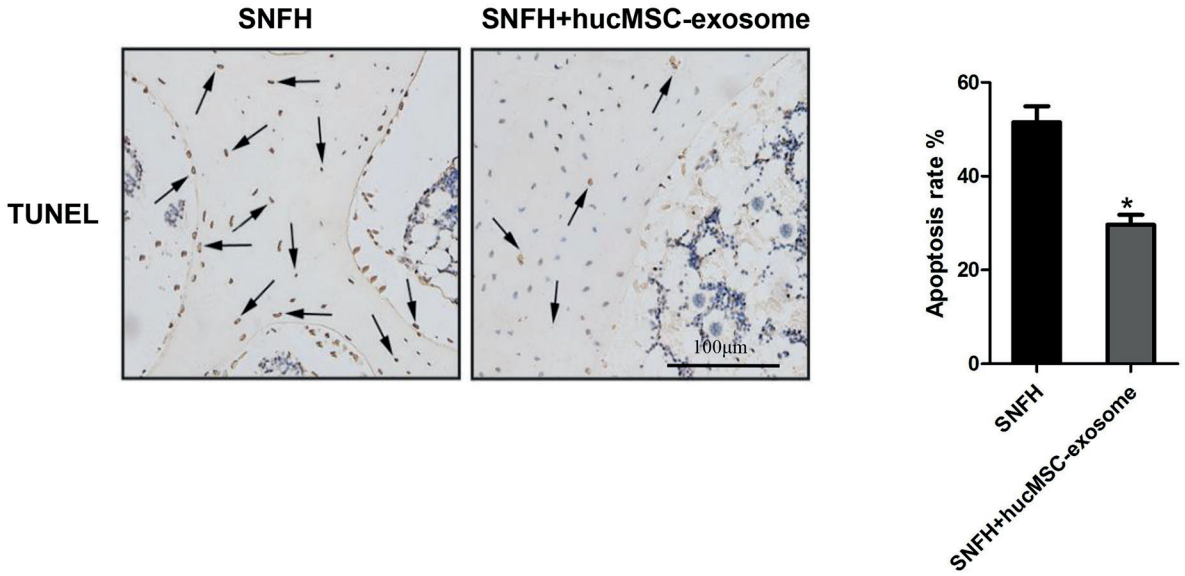

Figure 3. TUNEL staining of apoptosis of bone tissues in both groups (Magnification: 100×). SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group. *p<0.05 suggests a statistically significant difference compared with SNFH group.

changes in SNFH. Therefore, the expression of VEGF in bone tissues in both groups was detected *via* immunohistochemical staining. The results revealed that hucMSC-exosomes markedly promoted the expression of CD31 (an important marker for vascular endothelium) in necrotic bone tissues (Figure 4), indicating that hucMSC-exosomes can promote the angiogenesis in necrotic bone tissues of SNFH rats.

MRNA Levels of VEGF and BMP-2 in Bone Tissues in Both Groups

VEGF and BMP-2 are important indexes for evaluating the severity of SNFH, as well as important proteins for the occurrence and development of SNFH. The mRNA expression levels of the two proteins were detected *via* RT-PCR. It was found that the mRNA expression levels of BMP-2 and VEGF in bone tissues were significantly increased in SNFH + hucMSC-exosome group (p<0.05) (Figure 5).

Protein Expression Levels of VEGF and BMP-2 in Bone Tissues in Both Groups

Furthermore, the protein expression levels of VEGF and BMP-2 in lesion tissues in both groups were detected *via* Western blotting. As shown in Figure 6, hucMSC-exosomes could remarkably

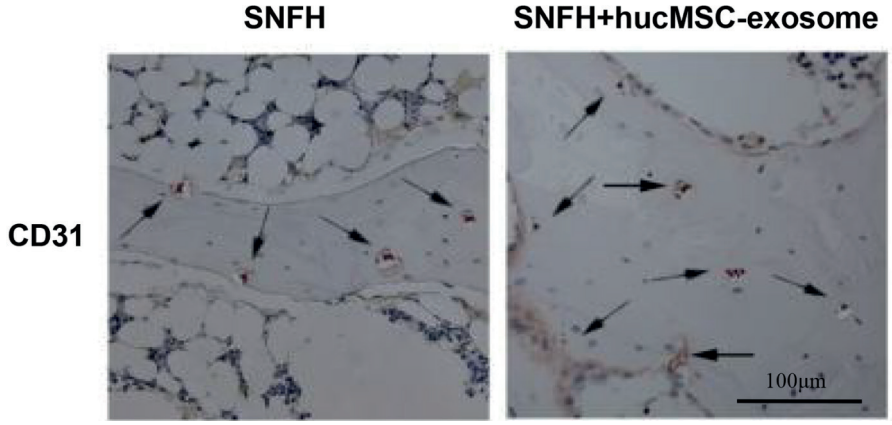

Figure 4. Angiogenesis in bone tissues in both groups (Magnification: 100×). SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group.

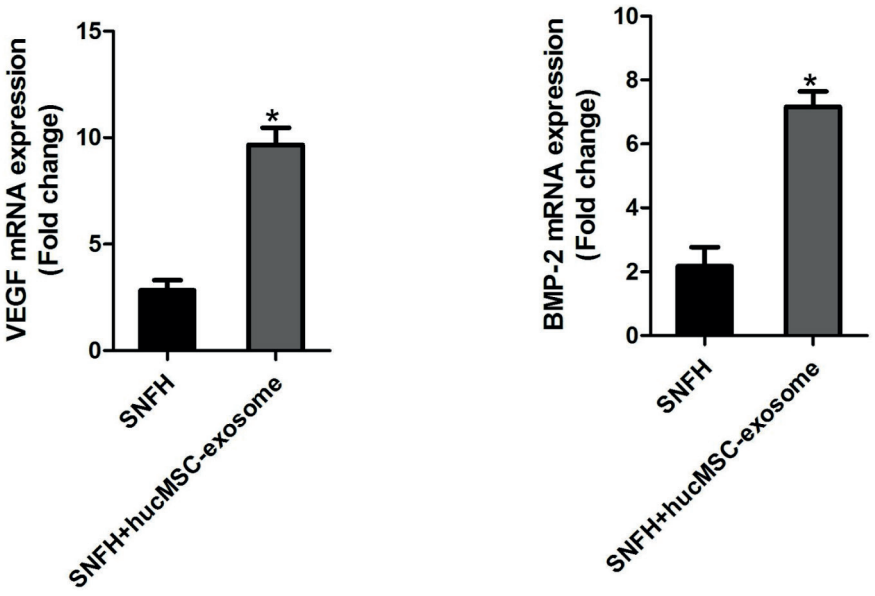

Figure 5. mRNA levels of VEGF and BMP-2 in bone tissues in both groups. SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group. *$p<0.05$ indicates a statistically significant difference compared with SNFH group.

up-regulate the protein expression levels of VEGF and BMP-2 in necrotic bone tissues ($p<0.05$).

Immunohistochemical Staining Results of VEGF and BMP-2 in Bone Tissues in Both Groups

Finally, the distribution and expression of VEGF and BMP-2 in bone tissues in both groups were confirmed again *via* immunohistochemical staining. The results proved that hucMSC-exosomes could promote the expression of VEGF and BMP-2 in bone tissues (Figure 7).

Discussion

SNFH is mainly characterized by femoral head collapse caused by impairment of femoral blood flow[14]. Steroids are commonly-used hormones, which are mainly used to improve the immunity and shorten the course of disease of patients[15]. The patients with systemic lupus erythematosus and inflammatory bowel disease and those receiving organ transplantation need to take steroids regularly[16]. Due to the extensive application of steroids, especially exogenous glucocorticoids, the incidence rate of SNFH increases year by year[17]. A larger number of studies are trying to reveal the important genes or proteins in the occurrence and development of SNFH currently, but the precise molecular mechanism of SNFH remains unclear, so the effective and precise prevention and control of SNFH are lacked in clinic. It is recognized currently that the pathogenesis of SNFH includes metabolic disorders and local factors affecting blood flow, such as thrombosis, increased intraosseous pressure and mechanical stress, and the combined effects of these factors may lead to osteonecrosis[18].

It is well known that stem cells are able to promote angiogenesis. Stem cell transplantation can facilitate endothelial cell growth, thereby promoting the growth and infiltration of surrounding tissue vessels[19]. MSCs can recruit pericytes and smooth muscle cells to promote the maturation of new vessels. The effect of stem cell transplantation in angiogenesis in the necrotic region of the femoral head has also been confirmed in recent studies. The transplantation of MSCs into bone tissues can accelerate the bone tissue repair in the lesion[20-21]. Tabatabaee et al[22] proved that the clinical outcome of patients with femoral head necrosis can be significantly improved *via* the transplantation of autologous bone marrow stem cells into the femoral marrow cavity. In fact, the direct transplantation of stem cells still has some limitations. For example, it is hard for stem cells to survive in the ischemic region. The clinical application of stem cells is also limited by the stem cell dedifferentiation, immune rejection,

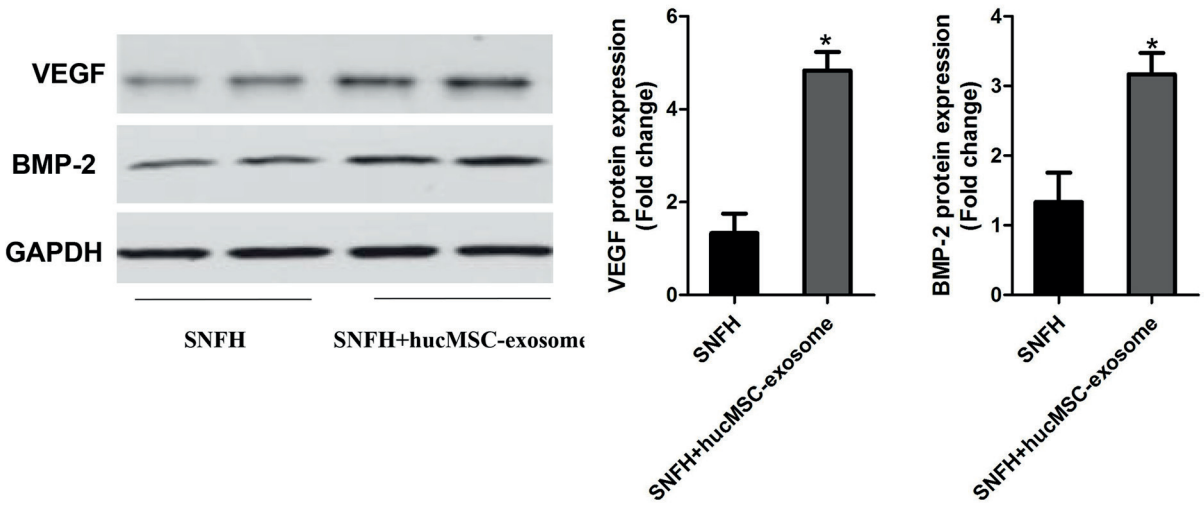

Figure 6. Protein expression levels of VEGF and BMP-2 in bone tissues in both groups. SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group. *$p<0.05$ indicates a statistically significant difference compared with SNFH group

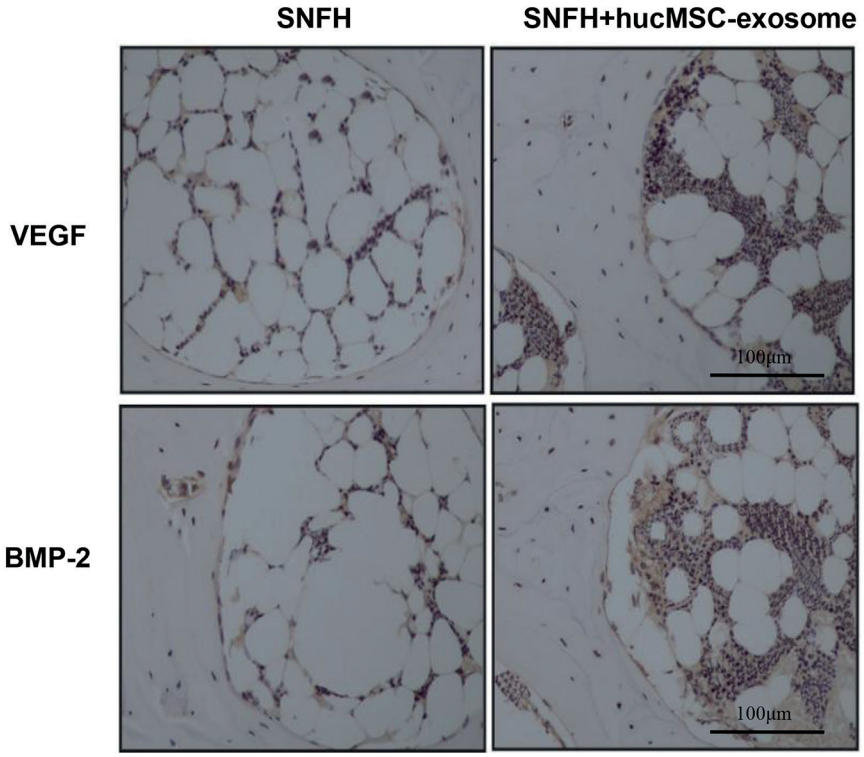

Figure 7. Immunohistochemical staining of VEGF and BMP-2 in bone tissues in both groups (Magnification: 40×). SNFH group: steroid-induced necrosis of femoral head group, SNFH + hucMSC-exosome: steroid-induced necrosis of femoral head + exosome treatment group.

genetic modification, and tumor formation[23]. According to recent studies, the therapeutic effect of stem cell transplantation is mainly mediated by its paracrine[24]. Among the active substances secreted by stem cells, exosomes have been proved to play an important role in tissue repair. Exosomes are nano-sized liposomes with 40-100 nm in diameter, which are sunken and formed in

multivesicular bodies *via* inner membrane and then released into the extracellular space *via* cytoplasm membrane fusion. These extracellular vesicles are involved in the transport of functional biochemical substances, such as cytokines, RNA, and proteins. The contents of exosomes are usually wrapped with the lipid membrane to prevent damage or degradation. The specific surface ligands of exosomes can bind to target cells, and transmit information or active substances to target cells to stimulate the production of specific biological functions[25]. Researches have demonstrated that the transplantation of stem cell-derived exosomes, similar to stem cell transplantation, plays a role of tissue repair[26]. In this study, the rat model of SNFH was established. The primary cells of human umbilical cord were isolated *in vitro* to obtain exosomes, and exosomes were injected into the necrotic marrow cavity of rats. The repair effect of hucMSC-exosomes on necrotic bone tissues in SNFH rats was observed. The results revealed that the bone tissue necrosis in hucMSC-exosome group was significantly improved compared with that in SNFH group, which was mainly manifested as the reduced apoptosis of bone tissues, increased level of trabecular reconstruction, increased angiogenesis in necrotic bone tissues and up-regulated mRNA and protein levels of VEGF and BMP-2. However, there were still some limitations in this study: the *in-vitro* experiments were not designed for verification, and the molecular mechanism of exosomes in affecting SNFH was not explored.

Conclusions

We revealed for the first time that hucMSC-exosomes can improve SNFH in rats through up-regulating the VEGF and BMP-2 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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