

Original Article

An experimental study of transfection of a VEGF plasmid carried by an ultrasound microbubble contrast agent in rats with steroid-induced osteonecrosis of femoral head

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Abstract: Background: Long-term or intermittent use of large amounts of steroids can cause osteonecrosis of the femoral head, which leads to degeneration and fracture. Vascular endothelial growth factor (VEGF) promotes endothelial proliferation and angiogenesis. Many studies have shown that high exogenous VEGF expression can promote angiogenesis and improve local microcirculation of the femoral head. Ultrasound microbubble contrast agents can be used as a drug or gene carrier and can directly deliver drugs or genes to target organs and tissues. Some researchers have used ultrasound microbubble contrast agents to mediate VEGF gene transfection in animal models of ischemic diseases, such as myocardial ischemia, cerebral ischemic diseases, and skeletal muscle ischemia, and have found that it can significantly increase the amount of VEGF and improve microcirculation. Purposes: In this study, the transfection of a vascular endothelial growth factor (VEGF) plasmid carried by an ultrasound microbubble contrast agent in rats with steroid-induced osteonecrosis of the femoral head was examined. Methods: Thirty-two specific pathogen-free Sprague-Dawley rats were randomly divided into four groups: control group (C), glucocorticoid group (G), glucocorticoid + plasmid group (G + P), and glucocorticoid + plasmid + microbubble group (G + P + MB). Rats were sacrificed at 7 days after transfection. The mRNA and protein expression levels of VEGF in the femoral head were detected by qPCR and western blotting. Results: VEGF mRNA and protein levels were significantly higher in the G + P + MB group than in the G group ($P < 0.01$), C group ($P < 0.01$), and G + P group ($P < 0.05$). Conclusions: According to these results, the transfection of a VEGF plasmid carried by an ultrasound microbubble contrast agent was feasible for steroid-induced osteonecrosis of the femoral head, and this method may be useful for the treatment of various diseases that may benefit from exogenous gene transfection.

Keywords: Ultrasound microbubble contrast agent, VEGF plasmid, steroid-induced osteonecrosis of the femoral head, growth factor, gene transfection

Introduction

Long-term or intermittent use of large amounts of steroids can cause osteonecrosis of the femoral head, which leads to degeneration and fracture. Vascular endothelial growth factor (VEGF) is a potent vascular growth factor. It promotes endothelial proliferation and angiogenesis. Many studies have shown that high exogenous VEGF expression can promote angiogenesis and improve local microcirculation of the femoral head [1-4]. Ultrasound microbubble contrast agents can be used as a drug or gene carrier and can directly deliver drugs or genes to target organs and tissues [5, 6]. Some

researchers have used ultrasound microbubble contrast agents to mediate VEGF gene transfection in animal models of ischemic diseases [7-9], such as myocardial ischemia, cerebral ischemic diseases, and skeletal muscle ischemia, and have found that it can significantly increase the amount of VEGF and improve microcirculation.

In this study, a VEGF plasmid was constructed and transfected into the femoral head of rats with steroid-induced osteonecrosis of the femoral head using an ultrasound microbubble contrast agent to explore whether the method could enhance the expression of VEGF.

VEGF transfected by ultrasound microbubble

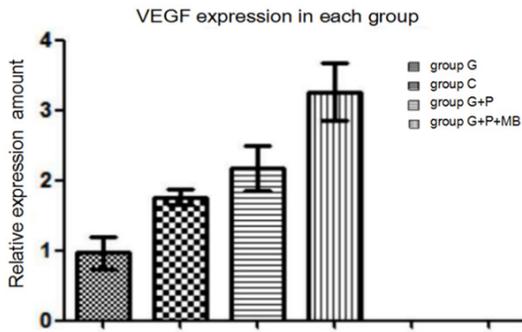


Figure 1. VEGF expression in each group. Note: Comparison between Group G and other groups, $P < 0.01$, Comparison between Group G + P + MB and Group G + P, $P < 0.05$.

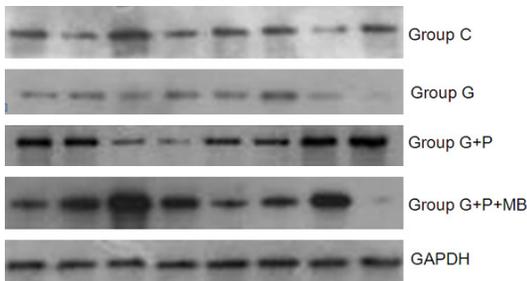


Figure 2. VEGF protein expression in each group.

Materials and methods

Experimental animals

A total of 32 specific pathogen-free Sprague-Dawley rats were provided by Jinan Pengyue Animal Breeding Inc. (License No. SCXK (Lu) 20140007). Both males and females were used and subjected to adaptive feeding for 1 week.

Experimental reagents and equipment

The ultrasound microbubble contrast agent (lyophilized powder) (SonoVue®; License No. J20130045) was obtained from Shanghai Bracco Sine Pharmaceutical Corp. Ltd. (Shanghai, China). An RT-PCR Kit (TaKaRa, Shiga, Japan) and CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) were obtained. The following primary antibodies were used: anti-VEGF (sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH-HRP (KC5G5; Shanghai KangChen, Shanghai, China). The secondary antibodies were Goat anti-rabbit IgG (H&L)-HRP (AP307P; Millipore,

Billerica, MA, USA) and Goat anti-Mouse IgG (H + L)-HRP, (BS12478; Bioworld, Irving, TX, USA). Other materials included 10 × RIPA Buffer (#9806S; CST, Danvers, MA, USA), Protease Inhibitor Cocktail Set (#539131; CALBIOCHEM, San Diego, CA, USA), BCA Protein Assay Kit (#23225; Pierce, Rockford, IL, USA), and Bis Gel Mixture, 1.5 mol/L Tris (pH 8.8), 1.0 mol/L Tris (pH 6.8), 10% SDS, 10% AP, TEMED, 10 × electrophoresis buffer, 5 × transfer buffer, PBST, ECL chemiluminescence substrate, developer, and fixing solution (all purchased from Redland Biotech, Guangzhou, China). The diagnostic ultrasound system was provided by the Imaging Department of the Qilu Medical University.

Preparation of the plasmid and microbubble suspension

The plasmid with immunofluorescent protein and VEGF (pIRES2-VEGF-EGFP) was constructed and amplified by Miaoling Bio (Wuhan, China). One part of pIRES2-VEGF-EGFP was directly diluted with saline and mixed gently. It was maintained at 25°C for 5-10 minutes, gently shaken several times, and placed on ice for use. The other part of pIRES2-VEGF-EGFP was gently mixed with a microbubble suspension in normal saline, maintained at room temperature for 5-10 min, and gently shaken several times to allow plasmids to thoroughly contact the microbubbles. In the 1-mL suspension, there was 68 μL of the microbubble contrast agent and 200 μg of immunofluorescent protein plasmid [10]. The suspension was placed on ice.

Groups and model construction

Thirty-two rats were randomly divided into the following four groups: control group (C, $n = 8$), glucocorticoid group (G, $n = 8$), glucocorticoid + plasmid (G + P, $n = 8$), and glucocorticoid + microbubble + plasmid group (G + P + MB, $n = 8$). Rats in the G group, G + P group, and G + P + MB group received intraperitoneal injections of 24.5 mg/kg prednisolone acetate twice a week for 12 continuous weeks, and animals were forced to take a standing position. After the successful establishment of the model, animals received the following treatments: Group C, 0.5 mL/kg saline was administered by intravenous tail injection; Group G, 0.5 mL/kg saline was administered by intravenous tail injection; Group G + P, 0.5 mL/kg saline with pIRES2-

Table 1. VEGF protein expression in each group (mean \pm SD)

Group	A_{VEGF}/A_{GAPDH} Ratio
Group C	1.15 \pm 0.56
Group G	0.72 \pm 0.16
Group G + P	1.48 \pm 0.23
Group G + P + MB	1.87 \pm 0.41

Note: Comparison between Group G and other groups, $P < 0.01$, Comparison between Group G + P + MB and Group G + P, $P < 0.05$.

VEGF-EGFP was administered by intravenous tail injection; Group G + P + MB, 0.5 mg/mL pIRES2-VEGF-EGFP microbubble suspension was injected via the tail vein and 3 MHz ultrasound was applied to the femoral head for 5 minutes. Samples were collected at 7 days after transfection.

Detection of VEGF mRNA in the femoral head by qPCR

Primers were designed according to the mRNA sequence in GenBank. Primers for VEGF were as follows: upstream, 5'-GGGAGCAGAAAGC-CATGAA-3', downstream, 3'-AGATGTCCACCA-GGGTCTCA-5'. ACTB was used as the internal control, and the primers were as follows: upstream, 5'-TCAGCAAGCAGGAGTACGATG-3', downstream, 3'-GTGTA AACGCAGCTCAGTAACA-5'. Primers were synthesized by Hongtu Biotechnology Co., Guangzhou, China.

Detection of VEGF protein by western blotting

Femoral heads were collected. Total protein was extracted, quantified, and separated by SDS-PAGE, followed by transfer, incubation with the primary antibody, and washing steps. GAPDH was used as the internal control. Images were collected using Uvpgrab-it Image software, and the ratio of A_{VEGF}/A_{GAPDH} was estimated as the relative amount of VEGF protein.

Statistical analysis

Data were analyzed using GraphPad Prism 5 and SPSS 18.0. The quantitative data are presented as mean \pm standard deviation (SD). Means of groups were compared using Student-Newman-Keuls Multiple Comparison Test (SNK test), and $P < 0.05$ was considered statistically significant.

Results

VEGF mRNA expression levels were compared among groups after normalization against ACTB (Figure 1). VEGF expression in the Group G was significantly different ($P < 0.01$) from that of other groups, and the expression of VEGF in Group G + P was significantly different from that in Group G + P + MB ($P < 0.05$), indicating that the microbubble contrast agent can increase the transfection of the VEGF plasmid.

Based on Western blotting, VEGF protein expression was significantly lower in Group G than in other groups ($P < 0.01$). The expression in Group G + P + MB was the highest (Figure 2 and Table 1).

Discussion

Long-term or intermittent use of steroids can cause osteonecrosis of the femoral head. Femoral head necrosis is caused by ischemia and hypoxia. VEGF is a potent vascular growth factor, and has strong functions in promoting endothelial cell proliferation and angiogenesis. Our previous studies have shown that the expression of VEGF increases at an early stage after the establishment of an animal model of steroid-induced osteonecrosis of the femoral head [11, 12], suggesting that at the early stage, the femoral head with osteonecrosis itself exhibits a repair response. However, over time, this expression gradually weakens. Our results further verified these findings.

Ultrasound microbubble contrast agents can be used as drugs or gene carriers; they can directly deliver drugs or genes to target organs or tissues. Some scholars [13] have used ultrasound microbubble contrast agents to carry immunofluorescent protein plasmids (pIRES-EGFP) to rabbit bone tissue and found that this method is safe and feasible while Effective may also be appropriate. In this study, transfection of VEGF in the femoral head of rats with steroid-induced osteonecrosis with an ultrasound microbubble contrast agent led to a significant increase in VEGF expression at the mRNA and protein levels in the femoral head relative to the expression observed in the glucocorticoid group ($P < 0.01$). In addition, our results indicated that in the femoral head of the rat model with the injection of only the plasmid, VEGF expression increased, but to a significant-

ly lesser extent than that observed in the ultrasound microbubble contrast agent group ($P < 0.05$).

The results of this study indicated that the ultrasound microbubble contrast agent can mediate the efficient transfection of VEGF in the femoral head, thereby providing experimental data for the development of gene therapy approaches for osteonecrosis of the femoral head.

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Disclosure of conflict of interest

None.

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