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ARTICLE

Human umbilical cord mesenchymal stem cells prevent glucocorticoid-induced osteonecrosis of the femoral head by promoting angiogenesis

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ABSTRACT

The impairment of angiogenesis is an outstanding pathogenic characteristic of glucocorticoid (GC)-induced osteonecrosis of the femoral head (ONFH). Human umbilical cord mesenchymal stem cells (hUC-MSCs) have been used in several diseases models, which were reported to be involved in the angiogenesis. However, whether hUC-MSCs suppress the GC-induced ONFH *via* promoting angiogenesis is still unclear. hUC-MSCs were isolated from the Wharton's jelly using the explant culture method. A GC-induced ONFH model was established *in vitro* and *in vivo*. The angiogenesis, proliferation and migration ability of HMECs were determined using the tube-forming, CCK-8, transwell and scratching assays *in vitro*. The protective role of hUC-MSCs in GC-induced ONFH was evaluated using micro-CT scanning and histological, immunohistochemical (IHC) and Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays *in vivo*. The results showed that hUC-MSCs treatment improved the tube-forming, proliferation and migration ability of HMECs *in vitro*. Moreover, hUC-MSCs treatment enhanced the integrity of trabecular bone of the femoral head, and the tube-forming ability *in vivo*. hUC-MSCs prevent the femoral head against necrosis and damage caused by GCs though promoting angiogenesis.

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KEYWORDS
hUC-MSCs; osteonecrosis of the femoral head; glucocorticoid; Necrosis; Microvascular

Introduction

Osteonecrosis of the femoral head (ONFH) is a destructive orthopedic disease that is a series of pathological changes and clinical symptoms including obstruction of the blood circulation in the femoral head of the necrotic site and the death of local bone cells, resulting in trabecular necrosis, and subsequent changes in the normal structure of the femoral head until local collapse occurs [1]. It is estimated that the total prevalence of non-traumatic osteonecrosis in China is 0.725%, and it tends to be younger [1,2]. Clinically, it is characterized by severe hip pain, claudication, and limited flexion and extension activities [1]. Especially, many previously difficult diseases have been alleviated or even cured due to the improvement of medical standards and the continuous improvement of diagnostic techniques; however, the toxic effect of some drugs involved in this longer course can eventually develop into osteonecrosis.

Glucocorticoid (GC) is extensively used to treat various diseases in clinic that is one of the most common causes of non-traumatic ONFH. Although the accurately pathogenic mechanisms of GC-induced ONFH remains further researches, the potential pathogenic mechanisms of GC-induced ONFH have been identified including destroyed blood supply to the femoral head [3], debilitated osteogenic ability [4] and osteoblast apoptosis [5]. Angiogenesis is the course of generating new vessels based on the existing vascular network, which has been demonstrated to play a vital role in proliferation and metastasis of a variety of tumors [6]. GCs have exhibited to directly damage endothelial cells, which causes hypercoagulability and abnormal microthrombosis in the femoral head necrosis area, thereby seriously reducing the blood supply to the trabecular bone [7]. Moreover, the overspend of GCs may lead to impairment and dysfunction of vascular endothelial cells [8]. Thus, improvement of angiogenesis might be one of underlying methods to prevent and early treat of GC-induced ONFH.

Mesenchymal stem cells (MSCs) are a member of the stem cell family, which can be expanded in large quantities *in vitro* and have the ability to differentiate into bone, cartilage, and fat. It has been reported to be used for the treatment of a variety of diseases, such as spinal cord injury, cerebral ischemia, and degeneration-induced tissue cell degeneration [9]. The most common sources of MSCs are bone marrow, however, bone marrow-derived MSCs decrease with age, and their differentiation ability is also reduced, which will cause certain damage to the human body when they are collected. Pleasantly, umbilical cord-derived MSCs (hUC-MSCs) can avoid the above shortcomings, and are of low immunogenicity and easily obtained material. Furthermore, hUC-MSCs have been used in several diseases models, which was reported to be associated with angiogenesis [10]. Therefore, in

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the present study, we explored the role of hUC-MSCs in the GC-induced ONFH. We found that hUC-MSCs prevent GC-induced ONFH by promoting angiogenesis.

Materials and methods

The separation of hUC-MSCs

The umbilical cord samples were from normal term cesarean section neonates. The mother and child with various infectious diseases and family genetic diseases were excluded, and informed consent was obtained from the mother and the family. This research has been approved by the IRB of the authors' affiliated institutions. The fresh umbilical cords were taken about 10 cm aseptically and washed away the residual blood with phosphate buffer saline (PBS) (YuanMu Biological Technology, Shanghai, China). After umbilical cords were cut into small pieces of 2–3 cm and rinsed with PBS again, they were cut longitudinally, removed the one umbilical vein and two umbilical arteries, and peeled off the Wharton's jelly. Subsequently, the Wharton's jelly was cut into small tissue pieces and cultured in MDS1601 culture medium (AKSO CELL) containing 10% foetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 1% penicillin-streptomycin (HyClone, Logan City, Utah, USA), and incubated in an incubator with 5% CO_2 at 37 °C. After 7 days, the Wharton's jelly tissues were removed and the culture medium was replaced for the first time. Afterwards, the medium was changed once every 2 days. Cells were passaged until the confluent of cells reached approximately 80%.

Flow cytometry assay

When the third-generation cells were 90% confluent, culture medium was discarded and PBS was used to wash cells 2–3 times. Subsequently, cells were digested with trypsin-EDTA solution, resuspended in PBS and count to 1×10^6/mL. Next, cell suspension was mixed with CD14-APC (MHCD1405), CD29-APC (17-0299-41), CD34-PE (CD34-581-04), CD44-FITC (MHCD4401), CD45-APC (MHCD4505), CD73-FITC (11-0739-42), CD90-APC (A14727), CD105-PE (MHCD10504) and HLA-DR-APC (MHLDR05) (Caltag, Thermo Fisher Scientific, Waltham, MA, USA) in the dark for 30 min. The expression of surface antigens was detected using flow cytometer (B&D SYSTEMS).

Experimental groups and drug administration *in vitro*

Human microvascular endothelial cell line-1 (HMEC-1) were purchased from Procell Life Science & Technology (#CL-0576), and maintained with complete growth medium for HMEC-1 (CM-0576, Procell) in an incubator with 5% CO_2 at 37 °C. HMEC-1 was inoculated into six-well plates and divided into three groups including control, DEX and DEX + hUC-MSCs groups. HMEC-1 in DEX group was treated with 10 μM dexamethasone (DEX; Solarbio, Beijing, China) for 48 h, while HMEC-1 in DEX + hUC-MSCs group was co-cultured with hUC-MSCs and then treated with 10 μM DEX for 48 h. HMEC-1 in control group was cultured with the same volume of PBS for 48 h.

Cell count kit-8 assay

The proliferation of HMEC-1 was determined using Cell Count Kit-8 (Biosharp, Anhui, China). HMEC-1 was seeded into 96-well plates with a density of 1×10^5/well and cultured for 24 h at 37 °C with 5% CO_2. After HMEC-1 was incubated with different treatments, CCK-8 solution was appended to the wells and hatched for further 2 h. Three duplications and a blank medium without cells as a control were set. Optical density (OD) values were recorded at 450 nm by a microplate reader (spectra max PLUS 384, Molecular Devices, California, USA).

Transwell assay

The migration of HMEC-1 was detected by transwell assay. 100 μL of cell suspension ($\sim 2 \times 10^4$ cells) was seeded into the upper chamber, while 600 μL of complete growth medium was placed into the lower chamber. Cells were cultured for 24 h with 5% CO_2 at 37 °C, and then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Solarbio). The membranes were photographed and counted using a microscope (DMI1, LEICA, Germany).

Wound scratch assay

HMEC-1 was seeded into 6-well plates with a density of 1×10^6/well and cultured at 37 °C with 5% CO_2. When the cells were conflated with 80–90%, a scratch was created in the cell layer using a 200-μL pipette tip. After cells were cultured with complete growth medium with different treatments for 24 h, results were obtained under a microscope (DMI1, LEICA).

Tube formation assay

The dissolved Matrigel (Solarbio) was plated into a 24-well plate with 200 μL/well and solidified in an incubator at 37 °C for 30 min. After HMEC-1 was incubated with different treatments, cells were collected and resuspended with complete growth medium. Then, approximately 3×10^4 cells were plated in the 24-well plate with solidified Matrigel and cultured for 8 h. The plates were determined using a microscope (DMI1, LEICA).

Animal model and experimental groups *in vivo*

18 healthy female Sprague–Dawley (SD) rats (eight weeks, 300 ± 20 g) were randomly divided into three groups ($n = 6$): control, model and hUC-MSCs groups. Rats in model and hUC-MSCs group were induced ONFH described as follows. Rats were intraperitoneally injected with lipopolysaccharide (LPS) (40 μg/kg/day) for two consecutive days, then intramuscularly injected methylprednisolone (40 mg/kg/day) from day 3 to day 7, and continuously fed about 12 weeks to establish the ONFH model. In addition, Rats in hUC-MSCs group were injected with 0.2 ml hUC-MSCs (about 1×10^6 cells) *via* the tail vein once a week for a total of 6 weeks, while rats in control and model group were injected with 0.2 ml of PBS *via* the tail vein. Rats were sacrificed six weeks after the last injection for subsequent analysis. This research has been approved by the IRB of the authors' affiliated institutions.

Micro-CT scanning

The femoral heads were isolated and fixed in formalin overnight. The femoral heads were analyzed using a SCANCO μCT 100 (SCANCO, Switzerland) according to the specification.

Histological and immunohistochemical (IHC) assays

The femoral heads were fixed, decalcified, embedded and cut into sections. 5 μm sections were stained with hematoxylin and eosin (H&E). In addition, the expression of collagen type I (COL 1)

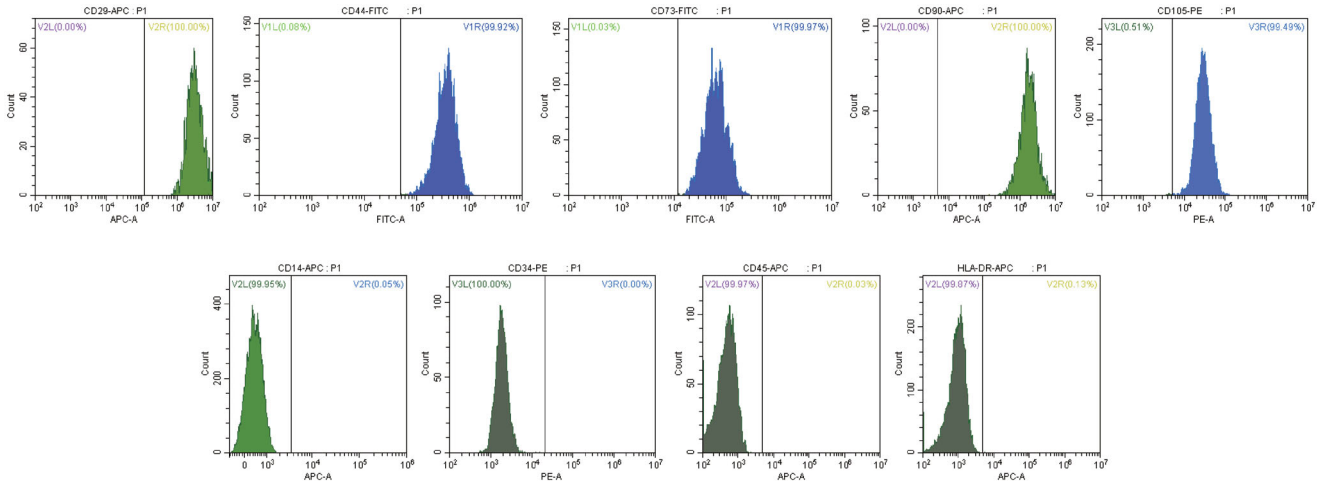

Figure 1. The separation and identification of hUC-MSCs. Surface markers of hUC-MSCs detected by flow cytometry.

(1:100, Rabbit polyclonal antibodies, bs-0578R, Bioss, Beijing, China) and vascular endothelial growth factor (VEGF) (ab69479) (1:100, Rabbit polyclonal antibodies, bs-1313R, Bioss) were analyzed using IHC assay. Pictures were obtained under a microscope (DMI1, LEICA).

qRT-PCR analysis

Total RNA from femoral heads was obtained using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was acquired by PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Then, qRT-PCR was executed using TB Green Premix Ex Taq II (Takara) in an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). β-actin was used as a house-keeping gene. The primer sequences used in the present study were as follows: Col-1 forward, 5′- AACT TTGCTTCCCAGATGTCC-3′ and reverse, 5′- AGCCTCGG TGTCCCTTCA-3′; VEGF forward, 5′- -GCGGGCTGCTGCAATG-3′and reverse, 5′- TGCAACGCGAGTCTGTGTTT-3′; β-actin forward, 5′-AGATCAAGATCATTGCTCCT-3′and reverse, 5′- TACTTCTGC TTGCTGATCCA-3′.

Statistical analysis

The data were showed as the means ± SD. Data with only two groups were analyzed by a Student's *t*-test, whereas differences among multiple groups were analyzed by one-way analysis of variance using the SPSS 22.0 statistical software (IBM, Armonk, NY, USA) followed by *Post Hoc* Bonferroni test. The differences were regarded as be statistically significant when $p < 0.05$.

Results

The separation and identification of hUC-MSCs

The Wharton's jelly was successfully separated from the umbilical cord tissue and observed with an inverted microscope after culturing. After 1–2 days of culture, the tissues began to adhere to the wall. After 7 days, some cells could be observed with fusiform and spindly morphology. About 14 days, the cells reached 80% confluent and formed circinate cell colonies. After passage 2, the cell growth ability was significantly enhanced, and the cells showed a uniform long spindle shape, resembling fibroblasts, and grew in a typical spiral arrangement. The morphology of the cells did not change significantly during the process of proliferation and passage. Flow cytometry analysis revealed that the passage three of hUC-MSCs had a prominent expression of CD29, CD44, CD73, CD90 and CD105, and almost did not express CD14, CD34, CD45 and HLA-DR. Taken together, these findings suggested that the hUC-MSCs was successfully obtained for subsequent assays (Figure 1).

hUC-MSCs improved migration ability and angiogenesis of HMECs *in vitro*

To explore the role of hUC-MSCs in migration ability and angiogenesis of HMECs-1, HMECs were co-cultured with or without hUC-MSCs, and then treated with 10 μM dexamethasone for 48 h. Analysis of CCK-8 data showed that 10 μM DEX significantly inhibited the proliferation of HMECs-1, which was observably inverted by hUC-MSCs treatment (Figure 2(A)). Also, hUC-MSCs treatment prominently rescued the inhibitory effect of DEX on the migration ability of HMECs-1 as exhibited an increase of the migrated cells numbers and migration distances according to transwell and scratching assays (Figure 2(B–E)). In addition, the results of tube formation assay revealed that DEX markedly suppressed the loop formation ability, total mesh area, total length and number of nodes, which was notably rescued by hUC-MSCs treatment (Figure 2(F,G)). Therefore, these results indicated that hUC-MSCs facilitated migration ability and angiogenesis of HMECs *in vitro*.

hUC-MSCs prevent the femoral head against necrosis *in vivo*

Rats were intraperitoneally injected with 40 μg/kg/day LPS for two consecutive days, and then intramuscularly injected 40 mg/kg/day methylprednisolone from day 3 to day 7, and then kept for 6 weeks to establish the ONFH model. To investigate the role of hUC-MSCs in the femoral head, the ONFH rat model were injected with or without 0.2 ml hUC-MSCs (1×10^6 cells) *via* the tail vein once a week for a total of 6 weeks. The result of micro-CT exhibited that rats in model group showed more bone mineral loss and cystic degeneration in the subchondral area of the femoral head, and sparser and thinner trabecular bone of the femoral head compared with control group, which was mitigated in hUC-MSCs group (Figure 3(A)). Furthermore, H&E staining revealed that femoral head chondrocytes were necrotic, and the columnar arrangement disappeared; the bone trabecula appeared uneven on the surface, local cracks appeared, and the number of empty bone lacunas increased; the cell composition in the bone marrow

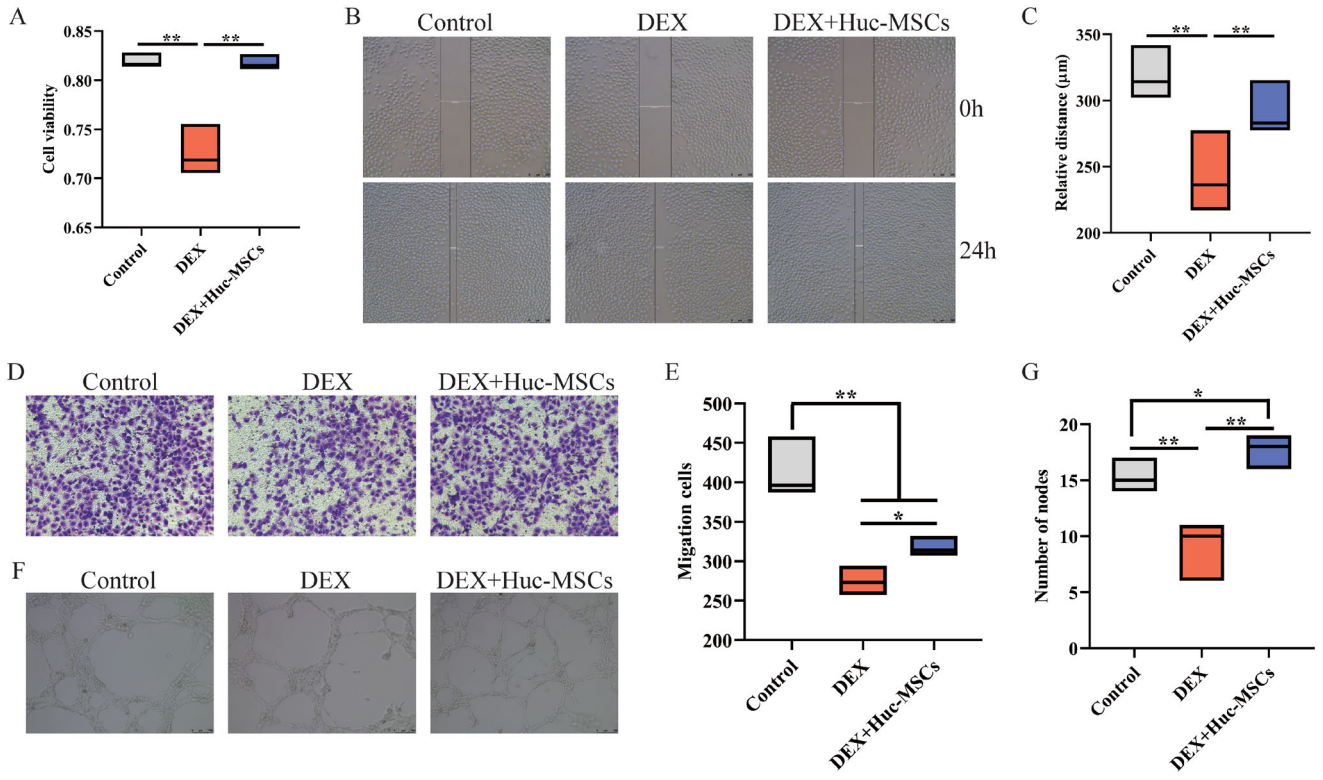

Figure 2. hUC-MSCs improved migration ability and angiogenesis of HMECs *in vitro*. (A) The proliferation of HUVECs was determined by CCK-8 assay. (B) The migration distances were detected by scratching assay. (C and E) The migrated cells numbers was analyzed by transwell assay. (F and G) The tube-forming ability of HMECs was determined by tube formation assay. The means ± SD of three independent samples were shown. $^{*}p < 0.05$ and $^{**}p < 0.01$.

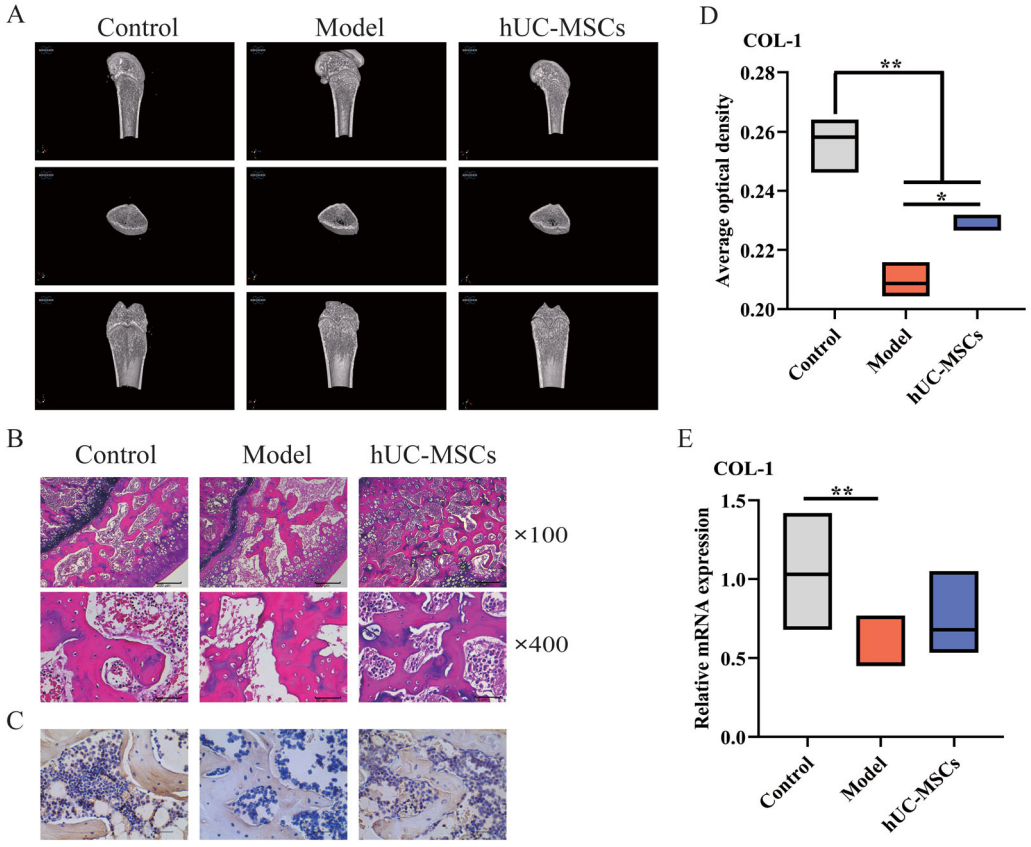

Figure 3. hUC-MSCs prevent the femoral head against necrosis in the rat ONFH model. (A) Images of micro-CT. (B) Histological analysis was determined by H&E staining. Scale bar: 50 μm. (C and D) The expression of COL 1 was detected by IHC assay. (E) The mRNA level of COL 1 was detected by qRT-PCR. The data were expressed after being normalized to β-actin. Scale bar: 200 μm. The means ± SD of six independent samples were shown. $^{*}p < 0.05$ and $^{**}p < 0.01$.

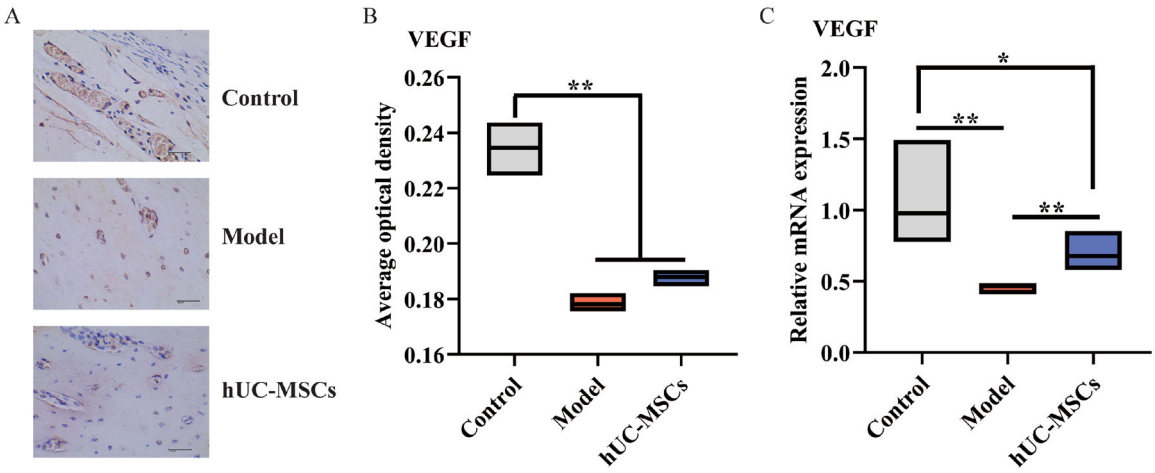

Figure 4. hUC-MSCs contributed to the vascular repairability in the rat ONFH model. (A and B) The expression of VEGF was analyzed by IHC staining. (C) The mRNA level of VEGF was detected by qRT-PCR. The data were expressed after being normalized to β-actin. Scale bar: 200 μm. The means ± SD of six independent samples were shown. $^{*}p < 0.05$ and $^{**}p < 0.01$.

cavity was reduced, and more cells were necrotic in model group. Nonetheless, all these pathological symptoms were ameliorated in hUC-MSCs group (Figure 3(B)). Additionally, IHC analysis (Figure 3(C,D)) exhibited that the expression of COL 1 was observably reduced and qRT-PCR assay (Figure 3(E)) also showed that COL 1 levels were notably diminished in a rat ONFH model, which was restored with hUC-MSCs injection. In brief, these results suggested that hUC-MSCs prevent the femoral head against necrosis in a rat ONFH model.

hUC-MSCs contributed to the vascular repairability in the rat ONFH model

Analysis of VEGF IHC staining (Figure 4(A,B)) and qRT-PCR resluts (Figure 4(C)) revealed that the expression and mRNA level of VEGF was significantly decreased in the rat ONFH model, while was rescued by hUC-MSCs injection, indicating that hUC-MSCs contributed to the vascular repairability in the rat ONFH model.

Discussion

GC-induced ONFH is a destructive orthopedic disease in which the improvement of angiogenesis and osteogenesis is significant for the prevention or treatment [11]. In this present study, we found that hUC-MSCs could enhance migration ability and angiogenesis of HMECs *in vitro*, and prevent the femoral head against necrosis and damage *in vivo*.

Explant culture and enzymatic digestion have been demonstrated to be mainly two methods for isolating MSCs from the UC. Compared with the methods of enzymatic digestion, the explant culture method took longer to yield MSCs, but the acquired MSCs were purer and have a higher proliferation rate [12]. In addition, it is reported that Wharton's jelly of UC is one of optimal choice for MSCs isolation due to its easy proliferation, multi-differentiation and non-tumorigenesis [13]. Therefore, MSCs that grew adherently and formed colonies in our study was isolated from Wharton's jelly using the explant culture method. Furthermore, MSCs isolated from Wharton's jelly of UC in our study were enrichment of CD29, CD44, CD73, CD90 and CD105, and lack of CD14, CD34, CD45 and HLA-DR expression according to the flow cytometry assay, which was consistent with the characteristics of high expression of MSCs matrix-related antigens and low expression of hematopoietic-related antigens [14]. Thus, hUC-MSCs were successfully obtained for our subsequent assays.

A lack of blood supply is the major pathogenic cause of GC-induced ONFH. Improvement of the blood supply of the femoral head is crucial for the precaution and treatment of GC-induced ONFH. A variety of MSCs have been used for angiogenesis in regenerating necrotic bone tissue, including MSCs derived from bone marrow [15], human adipose-derived MSCs [16], dental-pulp MSCs [17], peripheral blood-derived MSCs [18] and hUC-MSCs [19]. In the present study, the role of hUC-MSCs in angiogenesis of necrotic bone tissue was also investigated *in vitro*. hUC-MSCs not only enable to differentiate into various cell types including osteocytes, chondrocytes, myocyte, adipocytes and neuron-like cells [20,21], but also are easy to acquire and low immunogenicity. Moreover, hUC-MSCs have been reported to be used in a variety of diseases such as cerebral ischemia [22], nerve regeneration and myelination [23], and traumatic brain injury [24]. More importantly, hUC-MSCs could partly ameliorate the traumatic brain injury though regulating the angiogenesis [10]. Results in the present study showed that hUC-MSCs improved angiogenesis of HMECs as indicated that the tube-forming ability of HMECs was markedly enhanced with hUC-MSCs treatment. Additionally, the vascular plexus formation required the endothelial cells migratory ability [25]. The proliferation and migration ability of HUVECs were notably improved with hUC-MSCs treatment according to CCK-8, transwell and scratching assays. Therefore, hUC-MSCs could increase the proliferation and migration ability of HUVECs, thereby further ameliorating the angiogenesis of HUVECs.

In addition, the protective role of hUC-MSCs *in vivo* was also verified in the present study. Micro-CT is an important indicator for the analysis of the fine structure of the femoral head. Combined with the pathological results, hUC-MSCs treatment observably alleviated the deteriorations shown in ONFH model. Col I, the most affluent protein in the extracellular matrix of skin and bone, contributes to the tissue stability and regeneration [26]. It has been demonstrated that Col I promotes cell attachment, proliferation, and osteogenic differentiation. The native specific D-period structure of Col I plays a main in hemostasis and is also concerning few cellular behaviors. Also, the Col I level indicates the degree of the state of bone tissue. Thus, Col I generally is regarded as one of the bone formation markers. Our results revealed that the induced protein and mRNA expression of Col I could be prominently rescued with hUC-MSCs treatment, which

underpinned a recent report [27]. Therefore, these findings suggested that hUC-MSCs could protect the femoral head from necrosis *in vivo*. Moreover, VEGF can facilitate the proliferation and angiogenesis of endothelial cell [28], as well as promote the bone formation through the vascular permeability and network indirectly [29]. Thus, VEGF is a pro-angiogenic marker for the bone formation. Similarly, the protein and mRNA level of VEGF was notably decreased in ONFH model, which was observably reversed with hUC-MSCs treatment. Taken together, these results indicated that hUC-MSCs could prevent the femoral head against necrosis, and promote the angiogenesis *in vivo*.

Conclusion

In summary, *in vitro* and *in vivo* studies demonstrated that hUC-MSCs improved the migration ability and angiogenesis as well as prevent the femoral head against necrosis and damage in the GC-induced ONFH. We hope our study can lay a firm basic for the prevention and treatment of GC-induced ONFH.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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The study was approved by the Board and Ethics Committee of Weihai Central Hospital Affiliated to Qingdao University [No. 20211302 A]. All participants authorized the written informed consent. This work was supported by Key R&D Projects of Shandong Province [2019GSF107007].

Data availability statement

The datasets used or analyzed during the current study are available from the corresponding author.

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