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Exosomes secreted by mice adipose-derived stem cells after low-level laser irradiation treatment reduce apoptosis of osteocyte induced by hypoxia

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Abstract. – OBJECTIVE: This study aimed to investigate the effect of exosomes released by adipose-derived stem cells after treatment with LLLI on osteocyte apoptosis under hypoxic-ischemic conditions

MATERIALS AND METHODS: Adipose-derived stem cells (ASCs) were extracted from specific pathogen-free (SPF) male C57 BL/6 mice aged 4-6 weeks old. The cells were identified, cultured and amplified. After treatment of low-level laser irradiation (LLLI) on ASCs for 24 h, exosomes released by stem cells were extracted. The bone cell line MLO-Y4 was culture, amplified and added with exosomes secreted by ASCs, followed by culture for 24 h under normal or hypoxic-ischemic conditions. The apoptosis was detected via flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Apoptosis-related proteins, Bcl-2 and Bax were detected via Western blotting.

RESULTS: Mouse ASCs were successfully isolated and identified, and they had the potentials of osteogenic differentiation and adipogenic differentiation. Exosomes secreted by mouse ASCs were successfully isolated and identified. Hypoxia ischemia can induce the apoptosis of MLO-Y4 cells. After treatment with LLLI, exosomes secreted by mouse ASCs can significantly inhibit the apoptosis of MLO-Y4 cells induced by hypoxia ischemia.

CONCLUSIONS: Hypoxia and ischemia synergistically promote the osteocyte apoptosis, and LLLI-treated ASC-derived exosomes can effectively inhibit the osteocyte apoptosis induced by hypoxic-ischemic environment, providing new ideas and methods for the treatment of osteoporosis.

Key Words:
Adipose-derived stem cells, Exosomes, LLLI, Osteocyte, Apoptosis.

Introduction

Osteoporosis (OP) is a kind of metabolic bone disease characterized by bone mass loss, abnormality in bone tissue microstructure and increased bone fragility, leading to deterioration of bone tissue. This disease can cause fractures when slight wound or low-energy trauma occurs, resulting in disability or even death[1,2]. Postmenopausal OP is one of the most common types, which often occurs in the elderly female population aged above 50 years old. After the concept of osteocyte apoptosis was introduced into the research field of OP first, the important role of abnormal osteocyte apoptosis in the occurrence of postmenopausal OP was gradually recognized by researchers, and osteocyte apoptosis became a research hotspot of OP[3,4]. Osteocytes are the most abundant cells in bone tissues. Studies have found that osteocytes are not only the passive occupants in bone tissues, but also the cells with many functions of coordinating the bone remodeling of osteoclasts and osteoblasts. In addition, osteocytes also play a role as endocrine cells[5,6].

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Adipose-derived stem cells (ASCs) are stromal cells extracted from adipose tissues, which are commonly used in regeneration and repair therapy of various tissues due to the advantages of convenient acquisition, small damage to donor site, large proportion of stem cells and strong cell proliferation capacity. Studies have shown that the transplanted stem cells promote the recruitment, proliferation and differentiation of surrounding cells mainly through paracrine or nutrition, rather than differentiation and replacement functions of stem cells, to regulate the tissue healing[7-9]. Extracellular vesicle (EV) is a key tool for cell-to-cell communication, which participates in the exchange among cells through the transport of proteins, bioactive phospholipids and nucleic acids. Exosomes are endocytosis-derived nano-vesicles of 30-130 nm in size, which can be released by a various types of cells and received by the target cells or reach the distant target with the biological fluid to regulate the physiological functions of recipient cells[10].

Low-level laser irradiation (LLLI) therapy is a kind of physical therapy that applies LLLI within the infrared to near-infrared wavelength of 630-1000 μm to lesion tissues or monolayer cells, causing the non-destructive and non-thermal biological reaction and achieving the purpose of treatment. Studies have shown that LLLI therapy can promote the bone repair, enhance the bone structure of perimenopausal rat model and increase the bone density, which can be used as one of the effective intervention measures of OP in middle-aged and elderly women[11,12]. This study aimed to investigate the effect of exosomes released by stem cells on osteocyte apoptosis under hypoxic-ischemic conditions after treatment of ASCs with LLLI, so as to provide new ideas and methods for the treatment of OP.

Materials and Methods

ASC Culture and Amplification

Specific pathogen-free (SPF) male C57 BL/6 mice aged 4-6 weeks were provided by the Animal Center of Kunming University of Technology. The subcutaneous adipose tissues in the inguinal region of mice were extracted, and the surface fascia and blood vessels were removed. The adipose tissues extracted were washed with phosphate-buffered saline (PBS) and cut into pastes, followed by digestion using 3 mg/mL type I Collagenase (Sigma-Aldrich, St. Louis, MO, USA) under constant temperature (37°C) for 40 min. The primary medium containing 10% fetal bovine serum (FBS) was added to terminate the digestion, followed by centrifugation at 1000 rpm for 5 min. Then, the supernatant was discarded, the primary medium containing 10% FBS was added for resuspension, and the mixture was filtered using the screen mesh. The mixture was inoculated into 25 cm^2 culture bottle and placed in an incubator with 5% CO_2 at 37°C for culture. After 24 h, the medium was replaced, and the non-adherent cells were removed. Afterwards, the medium was replaced once every 3 d, and cells were digested using 0.25% trypsin (Thermo Fisher Scientific, Waltham, MA, USA) when 80-90% cells were fused, followed by passage (1:3)[13]. This study was approved by the Animal Ethics Committee of the Kunming University of Technology.

MLO-Y4 Cell Culture

MLO-Y4 cells were inoculated into the type I collagen-coated culture dish and cultured in α-minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 2.5% FBS and 2.5% calf serum (CS). The medium was replaced every 3 d until 70-80% cells were fused, then cells were digested using 0.25% trypsin, followed by passage (1:3). The serum-free medium was used in ischemia-hypoxia group, and cells were cultured in an incubator with 1% O_2 and 5% CO_2 for 24 h.

Flow Cytometry

The second-generation ASCs were selected and 10^6 cells in each tube were resuspended in 100 μL phosphate buffered saline (PBS); flow antibodies [APC-CD44, APC-CD45 and fluorescein isothiocyanate (FITC)-CD31] (Invitrogen, Carlsbad, CA, USA) were added, respectively, for incubation for 3 min. The unlabeled antibodies were washed off using PBS, and 300 μL PBS were added to resuspend the cells; cells without antibody were used as negative controls. 100 μL MLO-Y4 cell suspension were placed in 5 mL flow tube, added with 5 μL Annexin V/FITC and 10 μL 20 μg/mL propidium iodide solution, mixed and incubated in a dark place at room temperature for 15 min. The mixture was detected using the Beckman flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA), and the results were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Colony-forming assay of mouse ASCs

The third-generation ASCs were selected and the medium was removed after routine culture for 14 d. Cells were washed twice with PBS, fixed with 4% paraformaldehyde solution for 15 min, stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, washed again with PBS and photographed. The colony with more than 50 cells was marked as one clone, and the ratio of clone number in each well to the number of inoculated cells indicated the colony-forming efficiency.

Detection of Multi-directional Differentiation Capacity of Mouse ASCs

Identification of osteogenic differentiation capacity of ASCs: the third-generation ASCs were selected and cultured in osteogenic induced solution (10^7 mol/L dexamethasone, 50 mg/L ascorbic acid, and 10 mmol/L β-glycerophosphate), and the solution was replaced every 3 d. After the cells were cultured for 21 d, they were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde solution for 15 min and stained with 0.5% alizarin red for 30 min.

Identification of adipogenic differentiation capacity of ASCs: the third-generation ASCs were selected, and cultured using adipogenic induced solution A (Cyagen, Suchow, Jiangsu, China). After induction for 3 d, solution A was replaced with adipogenic induced solution B. After 24 h, it was replaced with solution A again. After culture using solution A and B alternately for 16 d, cells were cultured continuously using solution B for 5 d, fixed for 30 min, and stained with oil red O for 30 min, followed by observation of the staining effect under microscope[14].

Extraction of Exosomes Released by Mouse ASCs

After centrifugation of FBS for 12 h, the supernatant was taken to prepare the exosome-free FBS. The fourth-generation mouse ASCs were selected and cultured using the medium containing 10% exosome-free FBS for 48 h; the supernatant was collected. The exosomes released by mouse ASCs were extracted via ultracentrifugation. The supernatant collected was centrifuged at 300×g and 4°C for 10 min, at 2000×g for 20 min to remove cell debris, at 10000×g for 40 min to remove large cell vesicles, and finally at 110000×g for 90 min. The supernatant was discarded, and the sediment was resuspended using the pre-cooled PBS. The exosomes were centrifuged again at 110000×g for 90 min, the supernatant was discarded, 200 μL pre-cooled PBS were added for resuspension, and stored at -80°C for standby application. In LLLI treatment group, ASCs were exposed to LLLI for 24 h and then the exosomes were extracted.

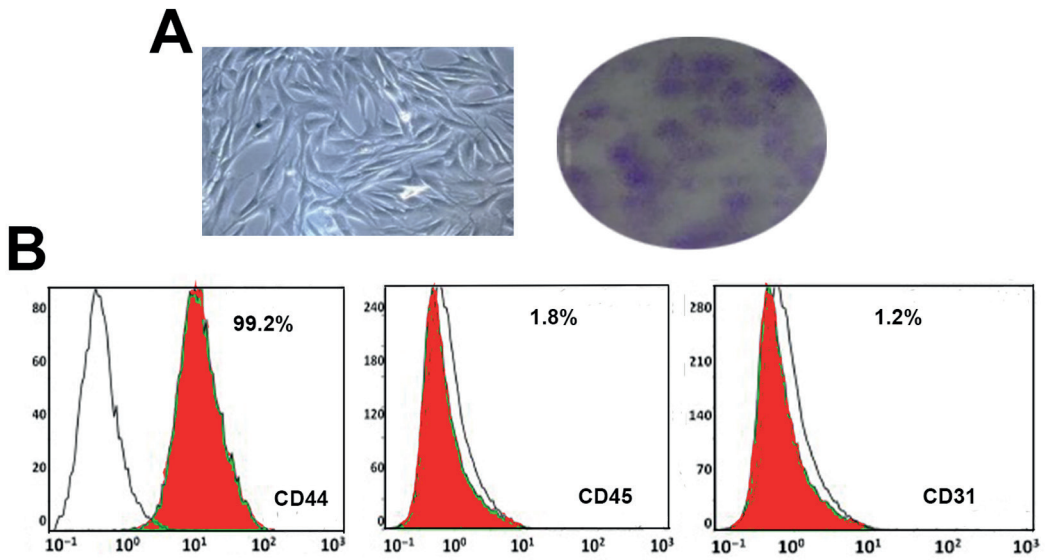

Figure 1. Identification of mice adipose-derived stem cell. ***A,*** The representative images of cell culture; ***B,*** Formation of adipose-derived stem cell clones after crystal violet staining; ***C,*** Identification of adipose-derived stem cell surface marker by flow cytometry.

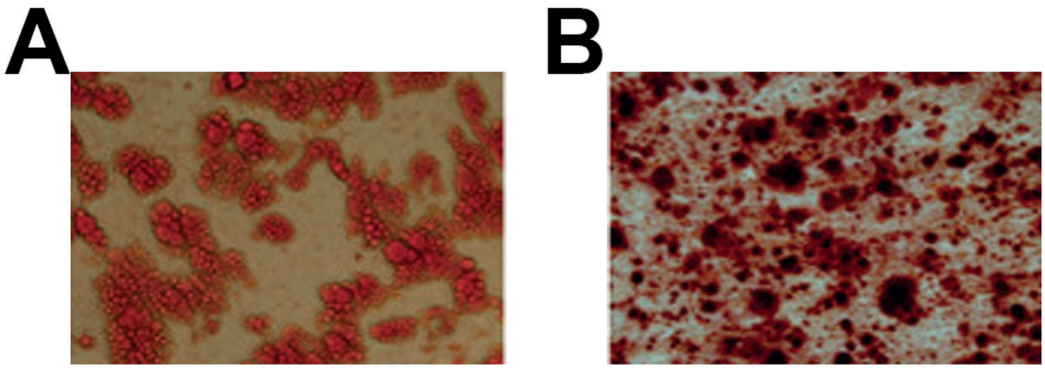

Figure 2. Identification of multi-directional differentiation capacity of adipose-derived stem cell. ***A,*** Oil red O in adipogenic induction of adipose-derived stem cell. ***B,*** Alizarin red staining in osteogenic induction of adipose-derived stem cell.

Morphologic Observation of Exosomes via Transmission Electron Microscope

10 μL isolated and purified exosomes were taken, added dropwise onto a 300-mesh copper grid and let stand at room temperature for 2 min. The excess liquid was gently aspirated from the edge with filter paper; exosomes were re-stained with 3% (w/v) phosphotungstic acid solution at room temperature for 1 min, dried, observed and photographed under transmission electron microscope.

Western Blotting

The ASCs, exosomes released by ASCs and osteocyte protein were taken; the protein concentration was detected using bicinchoninic acid (BCA) method. Proteins (40 μg/well) were loaded into 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by electrophoresis at 80 V for 30 min, electrophoresis at 120 V for 1 h, membrane transfer at 220 mA for 1 h, and sealing by 5% bull serum albumin (BSA) at room temperature for 1 h. The primary antibody was diluted using 5% bovine serum albumin (BSA), and incubated in a refrigerator at 4°C overnight. After proteins were washed with Tris buffered saline Tween (TBST), the secondary antibody (1:2000) was added for incubation at room temperature for 1 h, followed by color development with chemiluminescent solution.

Apoptosis Detection via Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-end Labeling (TUNEL)

MLO-Y4 cells in good growth status were selected and inoculated into a 48-well plate at a density of 5×10^4/mL. Cells were incubated for 24 h, and the supernatant was discarded after cells were adherent. Then, after cells were fixed with 4% paraformaldehyde for 1 h, they were stained according to the instructions of TUNEL apopto-

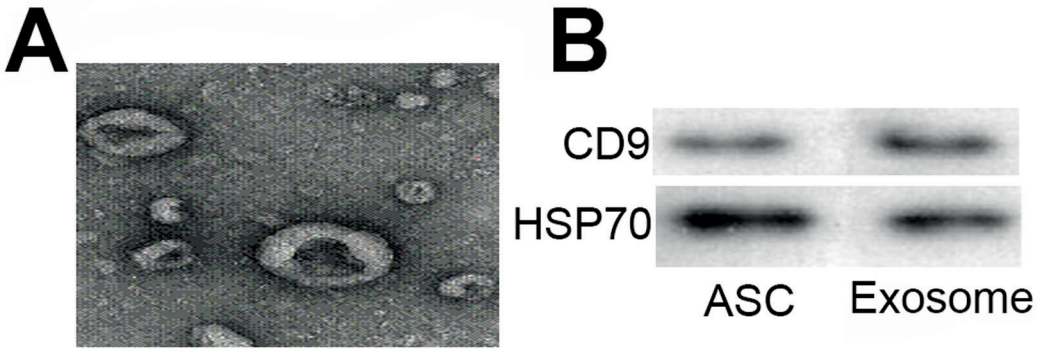

Figure 3. Identification of exosomes released adipose-derived stem cells. **A,** The morphology of ASC derived exosomes by transmission electron microscopic. **B,** Detection of CD9 and HSP70 protein expression in ASC and its released exosomes by Western blot.

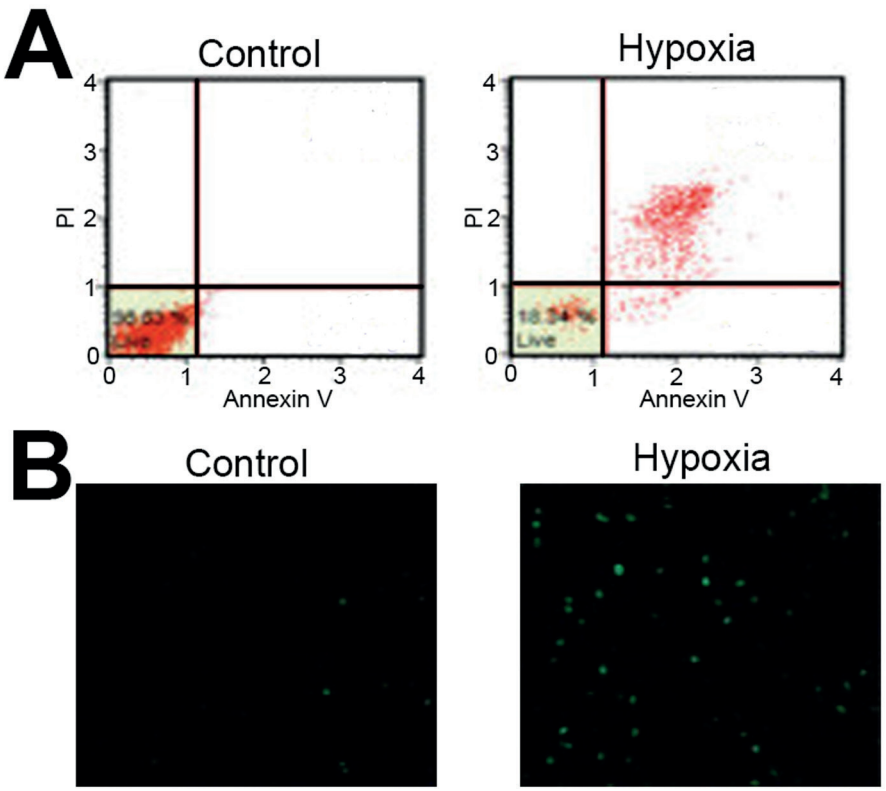

Figure 4. Hypoxia induced osteocyte apoptosis. ***A,*** Flow cytometry showed that hypoxia induced osteocyte apoptosis. ***B,*** Apoptosis of osteocyte induced by hypoxia by TUNEL.

sis detection kit (Sigma-Aldrich, St. Louis, MO, USA) and observed under inverted fluorescence microscope.

Statistical Analysis

Experimental data were analyzed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). *t*-test was used for the intergroup comparison, and analysis of variance (ANOVA) was used for the multi-sample comparison. Least Significant Difference (LSD) was served as its post hoc test. $p<0.05$ suggested that the difference was statistically significant.

Results

Identification of Mouse ASC Surface Markers

The primary-cultured ASCs grew in a colony-forming mode, and grew adhering to the wall in uniform morphology after subculture, showing long-spindle shapes. After culture for 14 d, obvious and scattered clonal colonies could be seen, indicating that the ASCs acquired had strong colony-forming and self-renewal abilities. The third-generation ASCs were selected and the surface markers (CD44, CD45 and CD31) were detected by flow cytometry. The results showed that the high-expression ASC mesenchyma-derived cells were marked as CD44 (positive rate was 99.2%), low-expression blood-derived cells were marked as CD45 (positive rate was 1.8%) and endothelial cells were marked as CD31 (positive rate was 1.2%), which met the phenotypic characteristics of ASCs.

Identification of Multi-Directional Differentiation Capacity of Mouse ASCs

After osteogenic induction of ASCs for 21 d, the stratified growth of cells and the formation of mineralized nodules could be seen; after alizarin red staining, a large number of red mineralized nodules in different sizes could be seen under microscope. After adipogenic induction of ASCs for 21 d, the cell volume was increased and the strings of vacuoles in different sizes could be seen in cells microscopically; after oil red O staining, red lipid droplets in different sizes could be ob-

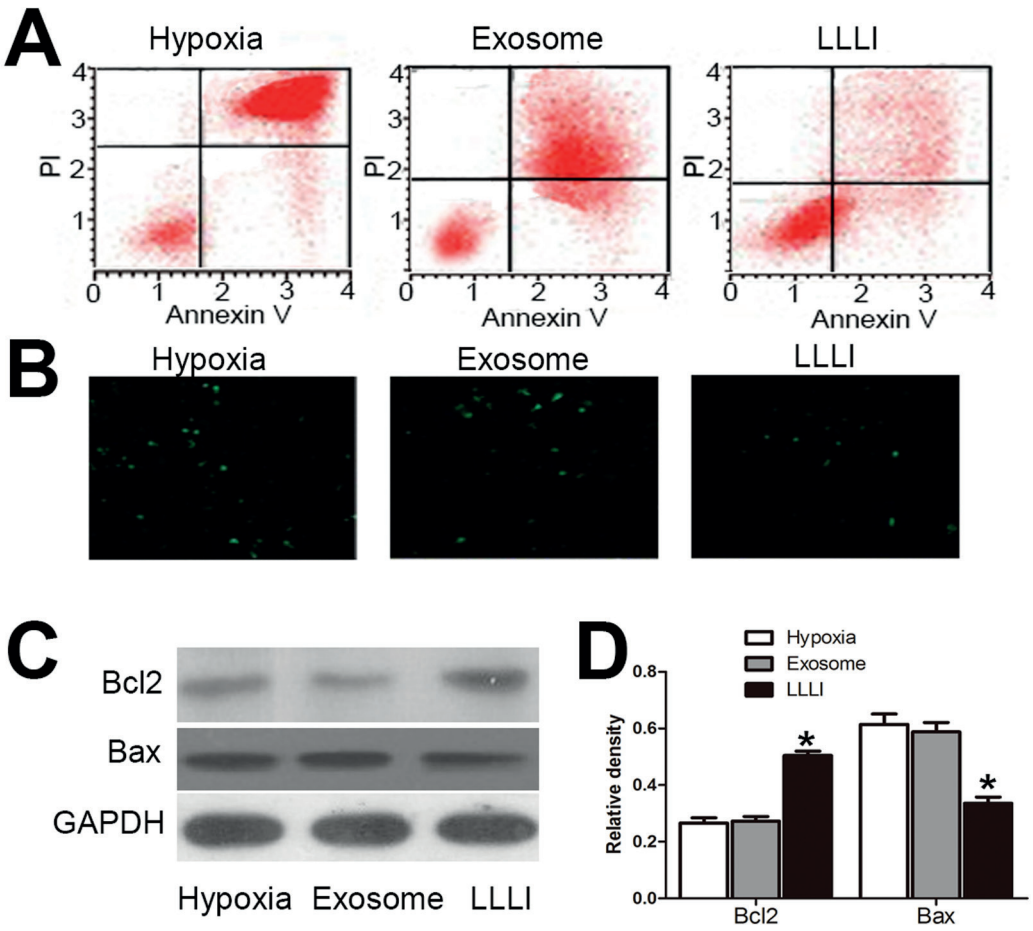

Figure 5. Exosomes released by ASCs inhibited osteocyte apoptosis after LLLI treatment. ***A,*** Flow cytometry showed the apoptosis rate of osteocytes. ***B,*** Apoptosis of osteocyte by TUNEL. ***C,*** Detection of apoptosis related protein expression by Western blot. ***D,*** Semi-quantitative analysis of apoptosis related protein. **p*<0.05 *vs.* Hypoxia group.

served in cells, indicating that the mouse ASCs extracted have a strong multi-directional differentiation capacity**,** meeting the characteristics of mesenchymal stem cells.

Morphology and Identification of Exosomes Released by Mouse ASCs

The exosomes released by mouse ASCs were observed under transmission electron microscope. The membranous vesicles in uniform size and consistent shape (circular or elliptic) with clear edge could be seen, and they were coated with bilayer lipid membrane. The diameter of vesicles was 30-130 nm, and some vesicles were fused. No cell debris or other subcellular organelles were observed under transmission electron microscope. After the protein concentration was detected using BCA method, the exosome marker protein [heat shock protein (HSP70)] was detected by Western blotting, and ASC was used as control group. The results showed that HSP70 and CD9 were expressed in ASC and exosomes, and the expression of CD9 in exosomes was higher than that in ASC.

Hypoxia-Induced Osteocyte Apoptosis

The apoptosis rate was detected by flow cytometry, and the results revealed that the number of apoptotic MLO-Y4 cells after serum-free treatment for 24 h under hypoxia was significantly increased compared with that in normoxia group. TUNEL assay further demonstrated that the number of apoptotic cells after serum-free treatment for 24 h under hypoxia was increased compared with that in normoxia group. The above results suggested that the apoptosis model was established successfully.

Exosomes Released by Mouse ASCs Inhibited Osteocyte Apoptosis After LLLI Treatment

The results of flow cytometry showed that the apoptosis rate of osteocytes was not significantly different from that in control group after ASC-derived exosomes were added into osteocytes cultured under hypoxia, and the osteocyte apoptosis could be significantly inhibited after LLLI-treated ASC-derived exosomes were added into osteocytes cultured under hypoxia. TUNEL assay further revealed that the osteocyte apoptosis could be significantly inhibited after LLLI-treated ASC-derived exosomes were added into osteocytes cultured under hypoxia. The results of Western blotting showed that the LLLI-treated ASC-derived exosomes could significantly increase the expression of anti-apoptotic protein, Bcl-2, and decrease the expression of pro-apoptotic protein, Bax; differences were statistically significant.

Discussion

ASCs are stromal cells extracted from adipose tissue that exhibit similar functions to bone marrow derived stem cells (BMSCs). However, compared with BMSCs, ASCs are characterized by convenient acquisition, small damage to donor site, large proportion of stem cells, strong cell proliferation capacity and high immunomodulation capacity. In recent years, many studies have used ASCs in bone tissue engineering to promote the bone regeneration and bone repair[15,16]. In this study, mouse ASCs grew adhering to the wall with strong self-renewal capacity. The positive rate of surface marker CD44 was more than 95% and the positive rate of CD45 was less than 2%, which was consistent with the characteristics of stem cell surface markers. It was confirmed via adipogenic and osteogenic differentiation that ASCs had a potential of multi-directional differentiation. The transplanted cells are mainly used as "seed" or "signal center" to promote the recruitment, proliferation and differentiation of surrounding cells mainly through paracrine or nutrition, rather than differentiation and replacement functions of mesenchymal stem cells (MSCs), to regulate the tissue healing[8,9]. At present, it has been gradually clear that in addition to the soluble factors secreted by cells, extracellular vesicles (EV) are a key tool for cell-to-cell communication. Among various subtypes of EV, the endosome-derived exosomes exhibit physiologic correlation. The exosomes are produced by the reverse foaming of multivesicular bodies in cells, merge with the cell membrane, and get out of cells via surface secretion, entering the vascular system or a variety of biological fluids. After that, exosomes are received by the target cells resident in the microenvironment or reach the distant target with the biological fluid. In addition to transport of characteristic proteins and characteristic lipids, the exosomes are coated with nucleic acids, mostly RNA (ribonucleic acid) with regulatory functions, to regulate the physiological function of recipient cells[17,18]. MSC-derived vesicles have superior performance, which is the typical feature of functional MSC. It is confirmed that the clinical application of MSC exosomes can effectively treat refractory graft-*vs.*-host disease[19]. Moreover, exosomes also have the functions of regulating neurite outgrowth[20], promoting the angiogenesis *in vitro* and *in vivo*, reducing myocardial ischemia/reperfusion injury[21,22], and repairing acute renal injury[23]. In this study, the exosomes released by mouse ASCs were successfully extracted and observed via transmission electron microscope. The results of Western blotting were consistent with the characteristics of exosomes.

Osteocytes are the most abundant cells in bone tissues, constituting the main mechanical receptor of bone tissues. Osteocytes can sense the changes in mechanical load and then regulate the functions of osteoblasts and osteoclasts. Studies have shown that OP and osteoarthritis lead to the osteocyte death, thus resulting in the increased bone fragility. Zahm et al[23] showed that osteocyte apoptosis will lead to the decreased mineralization capacity of osteocytes, increasing RANKL (Receptor Activator for Nuclear Factor-κ B Ligand) expression, thus promoting the osteoclast maturation and osteoclastic activity, producing the adverse conditions for bone regeneration. This suggests that the inhibition of osteocyte apoptosis has an important significance in the treatment of OP[24]. In this study, MLO-Y4 cells were cultured in hypoxic-ischemic environment for 24 h. The apoptosis was significantly increased, and the osteocyte apoptosis model was successfully established. Subsequently, LLLI-treated ASC-derived exosomes were added into osteocytes cultured under hypoxic-ischemic environment. It was found that LLLI-treated ASCs-derived exosomes could significantly inhibit the osteocyte apoptosis under hypoxic-ischemic conditions, suggesting that LLLI-treated ASC-derived exosomes contain

a variety of nutrients, which can resist the apoptosis caused by hypoxic-ischemic environment through regulating the physiological activity of osteocytes.

Conclusions

We showed that hypoxia and ischemia synergistically promote the osteocyte apoptosis, and LLLI-treated ASC-derived exosomes can effectively inhibit the osteocyte apoptosis induced by hypoxic-ischemic environment, providing new ideas and methods for the treatment of OP.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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