

Exosomes Derived from Umbilical Cord Mesenchymal Stem Cells Activate PTEN/AKT Pathway and Promote Repair of Damaged Endometrium

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Research Article

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Abstract

Background

Endometrial injury contributes to impaired endometrial receptivity, and is well recognized as a critical factor in implantation failure. Increasing evidence suggests that the therapeutic effects of mesenchymal stem cells (MSCs) mainly depend on their capacity to secrete paracrine factors and are mediated by MSC-derived exosomes (MSC-Exos). In this study, we aimed to explore the effects of human umbilical cord mesenchymal stem cell-derived exosomes (hUCMSC-Exos) on injured endometrium in the mouse endometrial damage model and the potential mechanisms for these effects.

Methods

All female mice were randomly divided into control group, saline group, and exosome group. To observe the distribution of exosomes *in vivo*, DiI-labeled hUCMSC-Exos were injected into the tail vein of endometrium-injured mice. HE staining was used to detect changes in endometrial thickness and number of glands. TUNEL staining was used to detect cells apoptosis. The expression of Ki67 and CD31 was examined by immunohistochemistry. Then, western blotting was used to measure the expression of Bcl-2, Bax, Cleaved Caspase-3, PTEN, AKT and p-AKT. The expression of VEGF and IGF-1 was detected by RT-PCR.

Results

hUCMSC-Exos was able to migrate to the damaged endometrium. After hUCMSC-Exos injection, the endometrial thickness and the number of glands were significantly increased ($P < 0.05$). Compared with the saline group, apoptosis was significantly reduced in the exosome group, and the expression of Ki67 and CD31 was significantly increased ($P < 0.05$). Besides, the expression of Bax, Cleaved Caspase-3 and PTEN was reduced as hUCMSC-Exos addition, and the Bcl-2 and p-Akt expression was increased. The expression of VEGF and IGF-1 was significantly upregulated in the exosome group compared to the saline group ($P < 0.05$).

Conclusions

hUCMSC-Exos ameliorated the damaged uterus, increased endometrial thickness, suppressed apoptosis and improved the cell proliferation in the mouse injured endometrium model. Furthermore, we discovered that hUCMSC-Exos could activate the PTEN/AKT signaling pathways and induce the overexpression of VEGF and IGF-1 *in vivo*.

Background

Endometrial injury is commonly caused by various uterine cavity surgery that induces intrauterine adhesions (IUA), fibrosis or thin endometrium. These disorders are characterized by low pregnancy rates, frequent miscarriages, and even infertility[1-3]. Currently, intrauterine implant positioning and high-dose

hormone therapy are used in tandem to avoid postoperative recurrence of IUAs and facilitate endometrial repair and regeneration [4, 5]. However, the conventional hormone replacement therapy for endometrium injury generally causes adverse side effects.

Mesenchymal stem cells (MSCs) are a class of adult stem cells with a capacity for self-replication and multi-directional differentiation, which are currently (the ability to self-replicate, multidirectional differentiation, and a wide range of sources that are) being investigated for tissue regeneration [6-8]. Extensive studies have demonstrated that MSCs can restore damaged endometrium, enhance fertility rate, reduce endometrial fibrosis, increase angiogenesis, and improve conception rates [9-11]. However, the application of MSC may have some disadvantages, such as tumorigenic potential, thrombosis and fever [12-14]. Animal studies indicate that MSCs can promote the growth of tumor cells in mice [12]. The adipose-derived MSCs (AD-MSCs) have pro-coagulant effect in mice, which may form thromboembolism [13]. In addition, in clinical trials of MSCs, some patients have experienced adverse effects such as fever and headache, but no serious adverse effects were found [14]. Considering that paracrine action is thought to be the primary mechanism of action of MSCs in tissue repair [15], some investigators have explored the role of MSC-secreted exosomes, expect that MSCs could be used in the treatment of uterine injury.

MSCs derived exosome (MSCs-Exo), 30–150 nm in diameter and contains active substances, acts in a paracrine manner for cell-free therapy in many diseases. Many studies have demonstrated MSC-Exos could coordinate the immune system and regulate inflammatory responses in damaged tissues [16, 17]. It showed that transferring MSC-Exos to the injured site could accelerate the re-epithelial formation, minimize scar width and promote collagen maturation [18]. AD-MSCs-derived exosomes (AD-MSCs-Exo) stimulated endometrial regeneration and collagen remodeling and enhanced the expression of integrin- β 3, LIF and VEGF [19]. Bone marrow mesenchymal stem cells derived exosomes (BMSC-Exos) could facilitate endometrial repair by the TGF- β 1/Smad signaling pathway [20]. Moreover, our previous study found that hUCMSC-Exos preserved human endometrial stromal cells hEndoSCs from apoptosis induced by mifepristone and played an active role *in vitro* repair of the weakened hEndoSCs through the PTEN/AKT signaling pathway [21]. However, mechanisms that coordinate hUCMSC-Exos *in vivo* endometrial injury treatment, remain uncertain. In our work, the effects of hUCMSC-Exos on injured endometrium in the mouse ethanol-induced endometrial damage model and the mechanisms of hUCMSC-Exos on the repair of endometrial injury *in vivo* were explored.

Materials And Methods

1. Experimental Animals

C57BL/6 female mice were obtained from the Shandong Experimental Animal Center and placed in the Animal Laboratory of Anhui Medical University. Four to five mice per cage were lodged in a room with a cycle time of 12 hours of light and 12 hours of darkness, and provided food and water ad libitum. All

animals were treated based on the protocol approved by the Institutional Animal Care and Use Committee of Anhui Medical University.

2. Establishment of the mouse endometrial injury model

Eight weeks old female mice were used to establish the endometrial injury model with 95% ethanol. More specifically, a longitudinal incision in the abdominal wall was made after administration of 4% chloral hydrate (0.1 mL/10 g) by intraperitoneal injection and the uterus was uncovered. In the single uterine horn, 0.1 ml ethanol was injected and held for 3 min, then being washed three times with phosphate buffer saline (PBS) solution. We disinfected the uterus and then reset it.

3. Isolation and characterization of hUCMSC-Exos

The hUCMSC-Exos were isolated from the medium containing no exosomes using an exosome extraction and purification kit (Umibio, Shanghai, China). Exosomes were characterized according to their size and surface marker expression by negative-staining electron microscopy and western blotting. Exosomes were placed into a formvar/carbon-coated grid, negatively stained with 3% aqueous phosphor-tungstic acid for 1 min, and examined by transmission electron microscopy (TEM, FEI, USA) at an accelerating voltage of 120 kV. The distribution of particle sizes for the hUCMSC-Exos was calculated by Nanoparticles Tracking Analysis (NTA, Zeta View PMX 110, Germany). Western blotting has established the typical markers of exosomes, such as CD63 (1: 1000, Abcam, UK), HSPA8 (1: 1000, Abcam, UK), TSG101 (1: 1000, Abcam, UK), PDCD6IP (1: 1000, Abcam, UK), and β -Actin (1: 1000, Abcam, UK).

4. Intervention of endometrial injury model with HUCMSC-Exos.

After the endometrial injury model was developed, the mice were randomly divided into two groups: the saline group and the exosome group. Exosomes (1.25 mg/ml, 300 μ L) were injected via tail vein injection in the exosome group and the saline group was injected with the same volume of saline. Some mice were killed and the bilateral uterine horns were removed for histological staining on Day 7 after transplantation. In comparison, the mice of control group undergoing no treatment are used as blank control.

5. Ex vivo fluorescence imaging of DIR-labeled exosomes in mice with endometrial injury.

As previously described, DIR was used to fluorescently mark the lipid bilayer of exosome [22]. The mice were transplanted with DIR-labelled exosomes after the endometrial injury model was set up. Images of ex vivo endometrial damage were collected after 6h, 12h or 24h after injection (FMT 2500X, PerkinElmer LifeSciences). The Spectrum was obtained with cooled charge-coupled device (CCD) camera with excitation wavelength at 750 nm and emission wavelength at 780 nm. The fluorescence of the undamaged uterine horn was described as the autofluorescence of the background tissue. The resulting images were analyzed with imaging software by subtracting the background tissue autofluorescence from the activatable fluorescence.

6. Hematoxylin-eosin (HE) staining

After fixation, dehydration and implantation, isolated uterine tissues in paraffin sections were prepared with a thickness of 4 μm and routine HE staining. The microscope was used to detect morphological variations in the uterus. The scraped endometrium fragments were fixed in 4 percent paraformaldehyde for 24 hours and then embedded in paraffin. Serial paraffin-embedded parts were collected, sequentially dewaxed for 20 min each in xylene I and xylene II, and rehydrated in a sequence of ethanol solutions with a decreasing concentration (100 percent for 10 min, 100 percent for 10 min, 95 percent for 5 min, 90 percent for 5 min, 80 percent for 5 min, and 70 percent for 5 min). The pieces were then rinsed in purified water (three times, 5 min each). As per the manufacturer's orders, the parts were stained with an H&E solution (Servicebio, China). Endometrial morphologic characteristics were observed after staining. And the number of uterine glands and the thickness of endometrium was counted based on five randomly chosen regions.

7. TUNEL staining

To investigate the effects of hUCMSC-Exos on uterus cell apoptosis induced by [ethanol](#), a TUNEL apoptosis assay kit (KeyGen BioTECH Co., Ltd., China) was used to detect endometrial cell apoptosis in each group after one week of exosomes injection according to the manufacturer's instructions. Apoptotic cells are stained with green fluorescence. Sections were observed and imaged using an optical microscope (LSM800, Zeiss, Germany). The intensity of apoptotic cells were analyzed using the image analysis software (ZEN, Zeiss, Germany).

8. Immunohistochemistry

The fixed uterine tissues with 4% paraformaldehyde were embedded in paraffin. Sections were fixed for 5 min in neutral buffered formalin, after which endogenous peroxidase activity was quenched by incubating in 3% hydrogen peroxide with methanol for 10 min. The antibody retrieval of each section was treated by microwave two times and washing three times with PBS. Subsequently, rabbit anti-Ki67(1:400; Cell Signaling, USA), rabbit anti-CD 31 (CD 31; 1:100; Cell Signaling, USA) was added and incubated at 4°C overnight. Secondary antibody (ZSGB-BIO, Beijing, China) was then added and incubated at 37°C for 30 min. Color was developed with DAB (Sigma Co., St. Louis, MO, USA) and counterstained with hematoxylin. The sections were observed under an optical microscope (Eclipse E200, Nikon Co., Tokyo, Japan) and photographed. The protein expressions were analyzed with the Image-pro Plus image analysis software (Media Cybernetics, MD, USA).

9. Western blot

The total protein was collected and the protein concentration was determined by BCA method. Proteins were isolated by 10 percent SDS-PAGE and transferred electrophoretically from the gels to the transport membranes of polyvinylidene difluoride (PVDF). The membranes were briefly washed in PBS-Tween and incubated at 4°C overnight with the antibodies Bcl-2 (1:1000, Cell Signaling, UK), Bax (1:1000, Cell

Signaling, UK), Cleaved Caspase-3 (1:1000, Cell Signaling, UK), PTEN (1:1000, Cell Signaling, USA), AKT (1:2000, Cell Signaling, USA) and p-AKT (1:2000, Cell Signaling, USA). By using a rotary shaker, the membranes were then washed 3 times in PBS-Tween. Horseradish peroxidase- (HRP-) conjugated anti-rabbit was incubated with the washed membranes for 1h. In compliance with the guidance of the manufacturer, the membranes were washed again and treated with an ECL detection kit (Biosharp, USA) to image the proteins recognized by the antibodies.

10. Quantitative real-time PCR (qRT-PCR)

Using Trizol Reagent (Ambion, Carlsbad, CA, United States), total RNA was extracted from each uterine according to the manufacturer's instructions. 1 µg of total RNA was subjected to reverse mRNA transcription using oligo dT as a primer and a total cDNA generation reverse transcription kit (ABclonal, Wuhan, China). RT real-time PCR with SYBR Premix Ex Tap™ (TaKaRa, China) and the ABI 7500 real-time PCR framework measured the expression levels of VEGF and IGF-1 mRNA. Gene amplification reaction conditions were 95 °C for 3 min, 95 °C for 5 s, and then 60 °C for 32s, respectively. In the NCBI database, the unique PCR primers were planned according to DNA sequences (Table 1). The level of expression of the gene was standardized to GAPDH and the reference gene. Each sample was detected in triplicate in each group. The procedure was repeated at least three times.

11. Statistical analyses

A statistical software SPSS 23.0 was used to analyze the data. Figures were indicated as mean ± standard error (SEM). Comparisons were made between two samples using the independent samples t-test. One-way analysis of variance (ANOVA) was used when the mean values of multiple groups differed significantly, and the LSD method was used for comparison between groups. The condition P value < 0.05 was considered to be statistically significant.

Results

1. Characterization of hUCMSC-Exos

Using the ExoQuick-TC reagent, we isolated exosomes from the hUCMSC culture medium and characterized the hUCMSC-Exos. TEM visualized the exosome morphology and scale (Fig.1A), and the diameters of isolated exosomes as spherical forms were 30-100nm. Particle size distribution at 20-300nm diameter was measured by NAT (Fig.1B). Western blot confirmed that hUCMSC-Exos expressed CD63, HSPA8, TSG101, and PDCD6IP, exosome-specific markers, but not the β-Actin negative marker autophagosome protein (Fig.1C). The concentration of exosome was determined at 1.25mg/ml by BCA assay.

2. Fluorescence imaging of DIR-labeled exosomes in mice with endometrial injury

To investigate the influence of endometrial injury on the migration of exosomes to the endometrium, hUCMSC-Exos was pre-labelled with DIR and transplanted into mice with endometrial injury. The right

horns of female mice were injured by ethanol, leaving the left horns untreated as self-controls. The mice were intravenously injected with DIR-labeled exosomes. Bilateral horns were collected at 6, 12 and 24 hour after injection, and fluorescent signals were detected through IVIS to determine exosomes recruitment. Fluorescence was only detected in injured horns after the exosomes transplantation, while no fluorescent signal was seen in control groups and undamaged horns. Some red-dotted fluorescent signals were found at 6h after exosome transplantation, and the fluorescent signals increased at 12h and 24h (Fig.2). The findings predominantly revealed that the transplanted hUCMSC-Exos were home to injury endometrium.

3. hUCMSC-Exos ameliorate the damaged uterus and increase endometrial thickness.

To evaluate the therapeutic efficacy of hUCMSC-Exos on endometrial injury, we assessed the morphological endometrial structure, the endometrial thickness and the number of gland (Fig.3). At Day 7 after transplantation, the endometrial structure of the control group seemed more complete, epithelial cells were arranged closely, and blood vessels and glands were clearly visible. In the saline group, the endometrium was poor and endometrial thinning was serious. The uterus recovered well in the exosome group, and the glands were obvious (Fig.3B). A significant difference in the endometrial thickness and the number of glands was compared between two groups (Fig.3C, D). These results indicated that hUCMSC-Exos promote the restoration of the damaged endometrium effectively.

4. hUCMSC-Exos suppresses apoptosis and improve the cell proliferation in mouse injured endometrium.

To evaluate the possible roles of hUCMSC-Exos on ethyl alcohol-induced uterus cells apoptosis, TUNEL assays were evaluated by immunofluorescence after hUCMSC-Exos injection in day 7 (Fig.4A). More apoptotic cells were identified in the endometrium of saline group than in that of the control group ($P < 0.05$). Compared with the saline group, significant difference in apoptosis was observed after hUCMSC-Exos injection ($P < 0.05$) (Fig.4B). To determine the proliferating cell populations after exosome therapy, Ki-67 expression of uterine tissue sections was performed by immunohistochemical staining (Fig.5A). In our mouse model, we found that the expression of Ki-67 in the saline group was significantly downregulated compared to the control group. In the exosome group, it was found that the expression of Ki-67 was significantly up-regulated (Fig.5B) compared to the model group. These results indicated that hUCMSC-Exos suppressed apoptosis and improved cell proliferation in mouse injured endometrium.

5. hUCMSC-Exos increase Angiogenesis of the endometrial damaged uterus.

To investigate the proangiogenic effect of hUCMSC-Exos *in vivo*, we detected the enhanced neovascularization of injured tissues at 7 days by histologic examination. The CD31 immunostaining revealed that microvascular density in the damaged uterus was significantly enhanced by administration of hUCMSC-Exos (Fig.6A). These results suggested that the therapeutic based on exosomes dramatically increased the endogenous angiogenesis in the ischemia tissues.

6. hUCMSC-Exos activates PTEN/AKT signaling pathways, alleviate in the endometrial damaged uterus.

To further investigate the mechanisms of hUCMSC-Exos on repairing endometrial injury *in vivo*, the expression of Bcl-2, Bax and Cleaved Caspase-3 protein of uterus from mice were detected by Western blot. The positive expression of Bax and Cleaved Caspase-3 increased after damaged uterus. On the contrary, the Bcl-2 expression was found to be significantly decreased. Whereas, the expression of Bax and Cleaved Caspase-3 was reduced as hUCMSC-Exos addition, and the Bcl-2 expression was increased. These findings reveal that hUCMSC-Exos enhance anti-apoptosis, and have a robust protective effect on the damaged uterus. To further investigate the mechanisms of hUCMSC-Exos on repairing endometrial injury *in vivo*, the expression of PTEN, Akt and p-Akt were tested. These results show that phosphorylated level of AKT increased notably after hUCMSC-Exos treatment. Furthermore, the VEGF, and IGF-1 expression of angiogenesis cytokines were detected in the endometria by qRT-PCR. Increased positive expression was found in hUCMSC-Exos transplantation group as expected. After hUCMSC-Exos were injected into the tail vein, there were significant differences in epidermal growth factor (VEGF) and insulin-like growth factor (IGF-1) between the two transplanted groups (Fig.7). In addition, when compared with the saline transplantation group, the expression levels of VEGF and IGF-1 were significantly up-regulated in the hUCMSC-Exos transplantation group ($P < 0.05$), as well as the related cytokines that promote cell proliferation and tissue repair.

Discussion

Cell therapy has been suggested as a new strategy to repair tissue injury. The therapeutic effects of MSC on damaged endometrium have been verified in animal models [9-11, 23]. However, there are some adverse reactions to the application of MSC [12-14]. Thus, it is necessary to investigate an alternative approach of MSC for the therapy of damaged uterus. It has been shown that MSC-Exos exhibit similar functions to MSCs, with the quality of low immunogenicity and do not produce tumors[24]. Previous studies have demonstrated that hUCMSCs can repair the injured tissue and protect hEndoSCs against apoptosis [21]. Here, our work demonstrated that the thickness of endometrium significantly increased and the survival of endometrial glandular epithelial cells increased after exosomes treatment in mouse model. Furthermore, the results of these studies indicate that hUCMSC-Exos protected the endometrial cells from alcohol-induced apoptosis, promoted microvascular regeneration, stimulated endometrium regeneration through the PTEN/AKT signaling pathway and activated the AKT to regulate Bcl-2 and Cleaved Caspase-3 expression.

We demonstrated that hUCMSC-Exos can migrate to the damaged endometrium. Our results showed that 24 hours after the administration of hUCMSC-Exos in the tail vein of mice, aggregated hUCMSC-Exos were observed at the site of endometrial damage. This suggests that hUCMSC-Exos can migrate to the area of tissue damage and exert therapeutic effects. This targeted migration function of exosomes not only enables targeted therapy, but also promises to be the next generation of drug delivery systems.

Currently, the mechanisms of endometrial damage repair remain uncertain. Previous studies suggest that it may be primarily related to endometrial epithelial regeneration, angiogenesis and inflammatory response [25]. Besides, it was demonstrated that hUCMSC-Exos could promote the proliferation and

migration of wound epithelial cells in a burn model [26]. Consistent with this, our results indicated that in mouse endometrial injury model, hUCMSC-Exos may repair the damaged endometrium by inhibiting apoptosis, increasing cell proliferation and promoting angiogenesis.

Our results showed that *in vivo* hUCMSC-Exos can also activate the PTEN/AKT pathway by inhibiting PTEN expression, which is consistent with our previous *in vitro* experimental findings [21]. Moreover, there is ample evidence that AKT can facilitate cell survival by specifically inhibiting pro-apoptotic signals such as pro-apoptotic regulators Bad and Forkhead family transcription factors. And AKT plays an important role in both physiological and pathological angiogenesis by affecting endothelial cells and angiogenic signal producing cells [27]. Besides, the pathophysiology of the endometrium in patients with thin endometrium was characterized by a significant increase in the uterine artery resistance index (RA-RI), a remarkable decrease in the area of the glandular epithelium, the number of blood vessels and the expression of angiogenic factor (VEGF) [28]. AKT activation of endothelial cells contributes to the expression and eventual secretion of VEGF and other angiogenic factors, thus promoting angiogenesis by both autocrine and paracrine signaling [27]. Our studies have shown hUCMSC-Exos could stimulate PTEN/AKT pathway and promote angiogenesis cytokines expression, such as VEGF and IGF-1.

In our mouse endometrial injury model, hUCMSC-Exos may promote cell proliferation and angiogenesis, inhibit apoptosis, and thus repair the damaged endometrium by activating the PTEN/AKT pathway. Our work showed that hUCMSC-Exos improved the reconstruction of endometrium through activation PTEN/AKT pathway which has a broad range of effects on restoration. In our future works, we will examine the mechanism of exosomes on the PTEN /AKT pathway in-depth in endometrial repair and the role of exosomes in endometrial receptivity. More experimental studies are required to estimate the basic mechanism of exosomes in the endometrial injury model and for the clinical application of exosomes.

Conclusion

Our analysis showed that hUCMSC-Exos could improve the repair of damaged endometrium and activate the PTEN/AKT signaling pathway in the mouse model. hUCMSC-Exos may up-regulate the expression of Bcl-2 and down-regulate the expression of Cleaved Caspase-3 and facilitate cell proliferation. Furthermore, our study suggested that hUCMSC-Exos had the potential to be a novel approach for the clinical investigation therapy of endometrial injury.

Abbreviations

MSCs: mesenchymal stem cells;

hUCMSC-Exos: human umbilical cord mesenchymal stem cell-derived exosomes;

IUA: intrauterine adhesions;

VEGF: vascular endothelial growth factor;

IGF-1: insulin-like growth factors-1;

CD31: cluster of differentiation 31;

HSPA8: Heat shock protein family A member 8;

TSG101: Tumor Susceptibility 101;

PDCD6IP: Programmed cell death 6-interacting protein;

hEndoSCs: human endometrial stromal cells;

IVIS: International Veterinary Information Service;

Declarations

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Contributions

WJ, LD, YC and ZW conceived and designed the study. WJ and LD conducted the experiments, interpreted the data, and prepared the manuscript. XX made the animal models. XJ and ZL performed the cell culture. All authors read and approved the final manuscript.

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Availability of data and materials:

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate:

This study was approved by the Hospital Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Consent for publication:

Not applicable.

Competing interests:

The authors declare that they have no competing interests

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Tables

Table 1 Primer sequences

Gene name	Primer name	Sequence (5'-3')
GAPDH	GAPDH-F	TGACCTCAACTACATGGTCTACA
	GAPDH-R	CTTCCCATTCTCGGCCTTG
VEGF	VEGF-F	GCCAGGGACGGAGAAGGAGTC
	VEGF-R	GCAGAACCACAGAGCGACAGC
IGF-1	IGF-1-F	GTGAGCCAAAGACACACCCA
	IGF-1-R	ACCTCTGATTTTCCGAGTTGC