Journal of Clinical and Nursing Research, 2024, Volume 8, Issue 11
http://ojs.bbwpublisher.com/index.php/JCNR
Online ISSN: 2208-3693
Print ISSN: 2208-3685

Umbilical Cord Mesenchymal Stem Cell-Derived Exosomes Inhibit the Proliferation and Invasion of Uterine Fibroblast Cells

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Abstract: *Objective:* To investigate the effects of exosomes derived from human umbilical cord mesenchymal stem cells (hUC-MSC) on the proliferation and invasion capacities of uterine fibroblast cells. *Methods:* Exosomes were isolated from the hUC-MSC culture medium via ultracentrifugation. The morphology, particle size, and surface markers of the hUC-MSC-derived exosomes (hUC-MSC-exo) were characterized using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting. The impact of the exosomes on uterine fibroblast proliferation and invasion was assessed using the CCK-8 proliferation assay and Transwell invasion assays. *Results:* The exosomes exhibited a typical bilayer membrane structure with a diameter of 100–150 nm, and their average particle size was approximately 130 nm. The zeta potential was around -33 mV. Specific exosome markers, including CD9, TSG101, and CD63, were prominently expressed. Functionally, hUC-MSC-exo significantly inhibited the proliferation and invasion of uterine fibroblast cells. *Conclusion:* This study reveals the inhibitory effects of hUC-MSC-derived exosomes on uterine fibroblast proliferation and invasion, highlighting their potential therapeutic value. These findings provide new insights into the mechanisms underlying uterine scarring and suggest novel approaches for pharmacological treatment.

Keywords: Umbilical cord mesenchymal stem cells; Exosome; Uterine fibroblast cells; Proliferation; Invasion

Online publication: November 28, 2024

1. Introduction

The uterus is a vital organ in the female reproductive system, essential for pregnancy and menstruation [1]. Uterine scarring, often a consequence of surgeries such as cesarean sections or myomectomies, results in fibrotic lesions within the myometrium [2]. This condition can impair uterine function, increasing the risk of

uterine rupture in subsequent pregnancies and, in severe cases, posing significant risks to maternal and fetal health [2]. Uterine fibroblast cells are pivotal in maintaining uterine tissue homeostasis, facilitating tissue repair, and supporting regeneration [3]. In addition to their role in endometrial reconstruction and repair, uterine fibroblast cells are key participants in myometrial fibrotic processes and scar formation [4]. Following uterine surgeries, delivery-related injuries, or ectopic pregnancies, the proliferative and invasive behavior of uterine fibroblast cells can become dysregulated, contributing to uterine scar development, which in turn affects fertility and uterine functionality [4]. Current treatment strategies for uterine scarring are often inadequate; surgical interventions have high recurrence rates, and hormone-based therapies can lead to adverse side effects with prolonged use [5]. Hence, identifying effective interventions to enhance UF function, promote normal uterine recovery, and reduce scarring is critically important.

In recent years, human umbilical cord mesenchymal stem cells (hUC-MSC) have garnered attention in regenerative medicine due to their potent differentiation abilities and immunomodulatory properties [6,7]. However, clinical applications of hUC-MSC face challenges, including issues with transportation, maintaining biological activity, and quantifying active therapeutic components [8]. Exosomes, one of the paracrine products secreted by stem cells, offer a promising alternative. These extracellular vesicles (EVs), with diameters from 10 to 100 nm, are found in nearly all eukaryotic biological fluids and are rich in proteins, mRNA, miRNA, and other biomolecules [9]. Exosomes exhibit similar functions to stem cells but with lower immunogenicity and no oncogenic risk [9]. In fibrotic diseases, hUC-MSC has been shown to modulate the local microenvironment and promote tissue repair through exosome secretion [10]. Exosomes derived from hUC-MSC (hUC-MSC-exo) have demonstrated anti-fibrotic effects in models of liver fibrosis and other pathologies [10]. However, the specific effects of hUC-MSC-exo on uterine fibroblast cells remain unclear.

This study aims to investigate the regulatory effects of hUC-MSC-exo on the proliferation and invasion of uterine fibroblast cells. Exosomes were isolated from the hUC-MSC culture medium using ultracentrifugation, and their morphology, particle size, and surface markers were characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting. Subsequently, the effects of the exosomes on UF proliferation and invasion were evaluated using the CCK-8 assay and Transwell invasion assay. The findings of this study provide experimental evidence supporting the development of novel exosome-based therapeutic strategies for treating uterine scarring and offer insights into potential applications of stem cell-derived exosomes in gynecological diseases.

2. Methods

2.1. Culture of human uterine fibroblast cells and human umbilical cord mesenchymal stem cells (hUC-MSC)

Human uterine fibroblast cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. Human umbilical cord mesenchymal stem cells (hUC-MSC) were obtained from Beijing Hanshi United Biotechnology Co., LTD (Beijing, China). These cells are known for their expression of various stem cell-specific markers and their strong proliferative and differentiation capabilities. hUC-MSC were cultured in F12 medium (Hyclone) with 10% FBS and 1% penicillin/streptomycin.

2.2. Isolation of exosomes from hUC-MSC

Exosomes were isolated from hUC-MSC at passages 4–6. When the cells reached approximately 80% confluence, the culture medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS). The medium was replaced with serum-free medium, and the cells were incubated for an additional 48 hours at 37°C in a humidified atmosphere with 5% CO_2. The conditioned medium was collected and subjected to a series of centrifugation steps. Initially, the medium was centrifuged at 300 × g for 10 minutes to remove cells, followed by centrifugation at 2,000 × g for 10 minutes to eliminate dead cells. The supernatant was further centrifuged at 10,000 × g for 30 minutes to remove cell debris. Finally, the supernatant was subjected to ultracentrifugation (Beckman) at 100,000 × g for 70 minutes to obtain a crude exosome pellet. The exosome pellet was resuspended in PBS and centrifuged again at 100,000 × g for 70 minutes to purify the hUC-MSC-derived exosomes (hUC-MSC-exo).

2.3. Nanoparticle tracking analysis (NTA) of exosomes

The size distribution and concentration of exosomes were measured using ZetaView 8.04.02 software (Particle Metrix, Meerbusch, Germany). Exosome samples were diluted appropriately in 1X PBS buffer, and particle size and concentration were analyzed across 11 positions using nanoparticle tracking analysis (NTA). The ZetaView system was calibrated using 110 nm polystyrene particles, and the temperature during measurements was maintained between 23°C and 30°C.

2.4. Morphological identification of exosomes

Exosomes from hUC-MSC were resuspended in 100 μL of 2% paraformaldehyde (PFA) for fixation. A 5 μL droplet of the suspension was applied to a copper grid covered with Formvar and a carbon film. The grid was incubated with 50 μL of 1% glutaraldehyde for 5 minutes to enhance fixation, followed by a 2-minute wash in 100 μL of deionized water. The grid was then stained with 50 μL of uranyl oxalate solution for 5 minutes and treated with 50 μL of methylcellulose solution for 10 minutes. Excess liquid was removed with filter paper, and the grid was air-dried for 10 minutes before examination under a transmission electron microscope (TEM) at 80 kV.

2.5. Detection of exosome-specific protein markers

Exosome-specific surface markers were detected using Western blot analysis. Exosomal proteins were extracted from hUC-MSC-exo, and their concentrations were quantified with a BCA protein assay kit. After adding the loading buffer, the samples were boiled for 5 minutes to denature the proteins. The proteins were separated by SDS-PAGE, transferred onto a membrane via semi-dry transfer, and blocked with 5% non-fat milk for 2 hours. The membrane was incubated overnight at 4°C with primary antibodies (abcam) against CD9, CD63, and TSG101. After three 10-minute washes with TBST buffer, the membrane was incubated with secondary antibodies for 1 hour at room temperature. Following additional TBST washes, chemiluminescence detection was performed, and the results were imaged using a chemiluminescence system.

2.6. Cell Proliferation Assay

Uterine fibroblast cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After cell adhesion, 100 μL of hUC-MSC-exo (10 μg/mL) or an equal volume of PBS (control) was added to each well. At 24, 48, and 72 hours post-treatment, 10 μL of CCK-8 reagent was added to each well, and the plates were incubated at

37°C in a 5% CO_2 incubator for 2 hours. The optical density (OD) at 450 nm was measured using a microplate reader to assess cell viability and proliferation.

2.7. Cell invasion assay

A Transwell invasion assay was used to evaluate the effect of hUC-MSC-exo on the invasive capacity of uterine fibroblast cells. hUC-MSC-exo (10 μg/mL) or an equal volume of PBS (control) was added to each well. Uterine fibroblast cells were resuspended in serum-free DMEM and seeded at a density of 5×10^4 cells per well in the upper chamber of a Transwell insert with an 8 μm pore size (Corning). The lower chamber was filled with 600 μL of DMEM containing 10% FBS as a chemoattractant. After 72 hours of incubation, the cells that invaded through the membrane were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet for 15 minutes. Cells on the upper side of the membrane were carefully removed, and the stained invasive cells were visualized and counted under a microscope.

2.8. Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 7.0 software. Differences between groups were compared using a *t*-test, with a *P*-value of less than 0.05 considered statistically significant.

3. Results

3.1. Identification of hUC-MSC-exo

hUC-MSC-exo were successfully isolated using ultracentrifugation. Nanoparticle tracking analysis (NTA) confirmed the size distribution of the exosomes, showing an average diameter of approximately 130 nm (**Figure 1A**). The zeta potential measurement indicated a value of approximately -33 mV (**Figure 1B**), suggesting the stability of the exosomes in suspension. Transmission electron microscopy (TEM) revealed the morphology of the exosomes, showing a characteristic bilayer membrane structure with diameters ranging from 100 to 150 nm (**Figure 1C**). Furthermore, Western blot analysis demonstrated the clear expression of exosome-specific markers CD9, TSG101, and CD63 in the isolated samples, confirming the successful extraction of hUC-MSC-exo (**Figure 1D**).

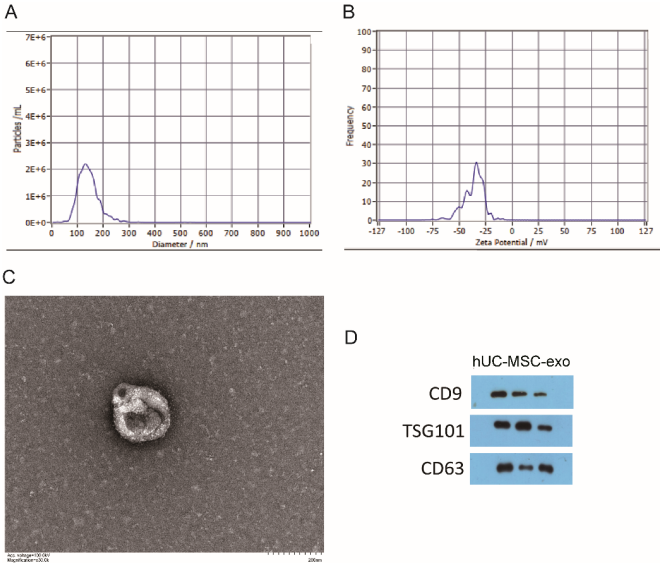

Figure 1. Identification of hUC-MSC-exo. **(A)** Particle size of hUC-MSC-exo. **(B)** The zeta potential value of hUC-MSC-exo. **(C)** Morphology of hUC-MSC-exo (scale bar = 200 nm). **(D)** Exosome-specific markers (CD9, TSG101, and CD63) of hUC-MSC-exo

3.2. hUC-MSC-exo inhibits uterine fibroblast cell proliferation

The effect of hUC-MSC-exo on uterine fibroblast cell proliferation was assessed using the CCK-8 assay. The results indicated that, compared to the Control group, where the optical density (OD) values for uterine fibroblast cells were 1.08 ± 0.06, 1.42 ± 0.07, and 1.80 ± 0.07 at 24, 48, and 72 hours respectively, the hUC-MSC-exo-treated group showed OD values of 0.90 ± 0.05, 1.22 ± 0.08, and 1.49 ± 0.07 at the same time points. This demonstrates a significant inhibition of cell proliferation in the hUC-MSC-exo group compared to the Control group. The reduction in cell proliferation was observed in a time-dependent manner, with statistical significance ($P < 0.05$) for all time points (**Figure 2**).

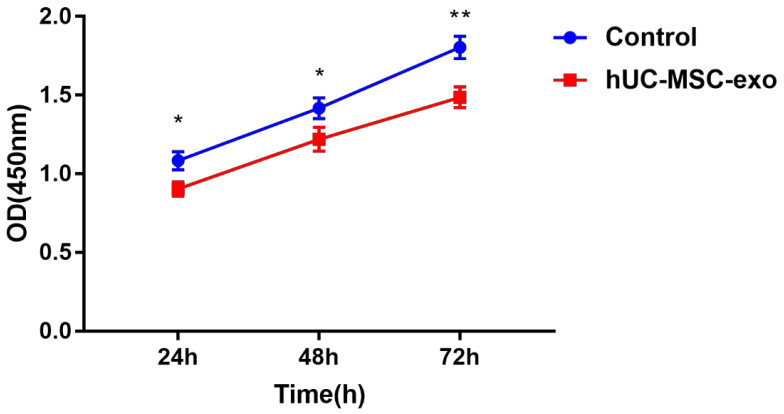

Figure 2. The effect of exosomes on the proliferation of uterine fibroblast cells was detected by CCK-8 assay. *$P < 0.05$, **$P < 0.01$

3.3. hUC-MSC-exo inhibits uterine fibroblast cell invasion

The impact of hUC-MSC-exo on uterine fibroblast cell invasion was evaluated using the Transwell invasion assay. The results demonstrated a significant reduction in the number of cells that penetrated the Matrigel matrix in the hUC-MSC-exo-treated group compared to the Control group (**Figure 3**). This finding indicates that hUC-MSC-exo exerts a marked inhibitory effect on the invasive capability of uterine fibroblast cells.

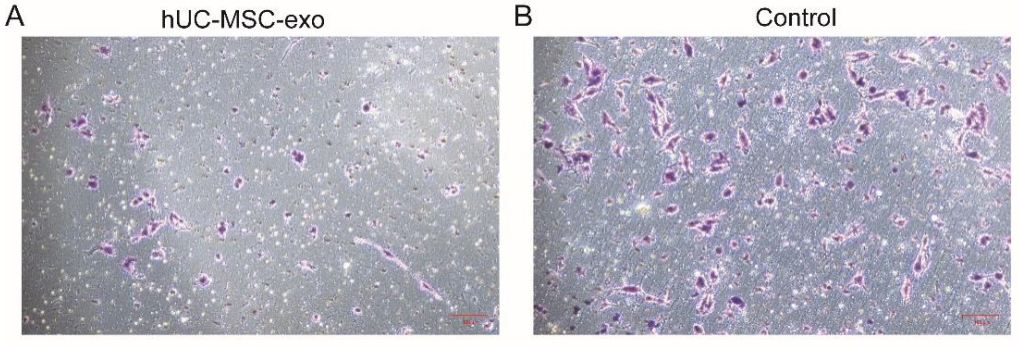

Figure 3. The effect of hUC-MSC-exo on the invasion ability of uterine fibroblast cells detected by Transwell assay. **(A)** The hUC-MSC-exo group; **(B)** Control group

4. Discussion

Recent advances in stem cell research have significantly impacted regenerative medicine and disease treatment. Human umbilical cord mesenchymal stem cells (hUC-MSC), known for their high proliferation capacity and multi-lineage differentiation potential, have garnered considerable attention [6,7]. hUC-MSCs not only show promising prospects in tissue repair and regeneration but are also widely used in clinical and basic research due to their abundant source and relatively non-invasive collection process [6,7]. Recent studies have revealed that hUC-MSCs play a crucial role in intercellular communication through the release of exosomes [11]. These exosomes are rich in biomolecules, such as proteins, lipids, and nucleic acids, which can modulate the physiological and pathological states of recipient cells, thereby influencing disease progression and tissue repair [12,13]. This study aimed to investigate the effects of exosomes derived from hUC-MSCs on the proliferation and invasion of uterine fibroblast cells. Our findings indicate that hUC-MSC-derived exosomes can significantly inhibit the proliferation and invasion of uterine fibroblast cells at certain concentrations, suggesting their potential therapeutic role in uterine scar repair and endometrial regeneration. This provides new insights into exploring uterine fibrosis and related diseases.

Uterine fibroblast cells, the principal stromal cells in uterine tissue, play a significant role in normal uterine function and pathological conditions such as a scarred uterus [14]. The aberrant proliferation of fibroblasts is often closely associated with the development of these pathological states. Our study demonstrates that the proliferation rate of uterine fibroblast cells treated with exosomes is significantly lower compared to the control group, indicating that exosomes derived from hUC-MSCs effectively inhibit the proliferation of uterine fibroblast cells. This highlights their potential in regulating cell proliferation. Exosomes may influence cell proliferation by releasing various bioactive molecules, such as proteins, miRNAs, and mRNAs, which affect the expression of cell cycle-related proteins. The inhibitory effect of hUC-MSC-exo on uterine fibroblast proliferation may involve multiple mechanisms. First, miRNAs within the exosomes are considered important regulators of cell proliferation. For example, miR-21 and miR-146a have been shown to inhibit the expression of cell cycle-related proteins, thereby slowing cell proliferation [15,16]. miR-21 directly affects the cell cycle by downregulating p53 target genes such as p21 and CDK4 [17], while miR-146a indirectly regulates the cell cycle and proliferation by inhibiting key components of the NF-κB signaling pathway [18]. Additionally, protein factors within exosomes, such as transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10), may also play critical roles. TGF-β inhibits cell cycle protein expression, preventing cells from entering the S phase and thus slowing proliferation [19], while IL-10 affects cell proliferation indirectly through immune and inflammatory responses [19]. These findings support our results and further confirm the multifaceted mechanisms of exosomes in cell proliferation regulation.

We further investigated the impact of hUC-MSC-exo on the invasion ability of uterine fibroblast cells using scratch assays. The results showed that the number of uterine fibroblast cells penetrating the membrane pores was significantly lower in the exosome-treated group compared to the control group, indicating that hUC-MSC-exo effectively inhibits the invasion ability of uterine fibroblast cells. Invasiveness is a key characteristic of uterine fibroblast cells in the progression of uterine diseases [14]. The mechanisms by which hUC-MSC-exo inhibits uterine fibroblast invasion may involve various bioactive molecules carried by the exosomes, including miRNAs, proteins, and lipids. These molecules regulate multiple signaling pathways that affect the adhesion, migration, and invasion of uterine fibroblast cells. Exosomes derived from mesenchymal stem cells have shown broad potential in inhibiting cell invasion. For example, miRNA-133b mediated by hUC-MSC-exo restricts

SGK1, promoting the proliferation, migration, and invasion of preeclampsia trophoblasts [20]. hUC-MSC-exo has also been shown to inhibit the proliferation, migration, and invasion of colorectal cancer cells through miR-3940-5p/miR-22-3p/miR-16-5p [21]. These studies provide theoretical support for our findings regarding the suppression of uterine fibroblast invasion by hUC-MSC-exo.

However, this study has some limitations. First, the experiments were conducted using in vitro cell models, and the actual effects of exosomes have yet to be validated in animal models or clinical patients. Second, although we examined some signaling pathways related to cell proliferation and invasion, other bioactive substances within the exosomes (such as miRNAs and proteins) may also be involved in the regulatory process, and these specific mechanisms need further investigation. Additionally, this study focused solely on the effects of exosomes on uterine fibroblast cells; future research should extend to other uterine-related cell types, such as endometrial epithelial cells and endothelial cells, to comprehensively evaluate the potential role of exosomes in uterine repair.

5. Conclusion

This study reveals that exosomes derived from hUC-MSCs significantly inhibit the proliferation and invasion of uterine fibroblast cells, providing a new perspective on the potential applications of exosomes in treating scarred uterine diseases. Future research will help further explore the mechanisms of exosome action and their clinical applications, offering new approaches and methods for treating scarred uterine conditions.

Funding

This study was supported by the Medical Research Project of Foshan Health Bureau (20220093), Project of Foshan Science and Technology (2220001003766), and Foshan Key Fields of Science and Technology (2020001005861).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

Conceptualization: Hai Tang, Zhengping Liu
Methodology: Hai Tang, Zhengping Liu
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Writing – original draft: Hai Tang
Writing – review & editing: all authors

Disclosure statement

The authors declare no conflict of interest.

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