eISSN 2005-5447
International Journal of Stem Cells Vol. 16, No. 3, 2023 https://doi.org/10.15283/ijsc21177

REVIEW ARTICLE

Extracellular Vesicles Derived from Mesenchymal Stem Cells as Cell-Free Therapy for Intrauterine Adhesion

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Intrauterine adhesion (IUA) can occur after trauma to the basal layer of the endometrium, contributing to severe complications in females, such as infertility and amenorrhea. To date, the proposed therapeutic strategies are targeted to relieve IUA, such as hysteroscopic adhesiolysis, Foley catheter balloon, and hyaluronic acid injection have been applied in the clinic. However, these approaches showed limited effects in alleviating endometrial fibrosis and thin endometrium. Mesenchymal stem cells (MSCs) can offer the potential for endometrium regeneration owing to reduce inflammation and release growth factors. On this basis, MSCs have been proposed as promising methods to treat intrauterine adhesion. However, due to the drawbacks of cell therapy, the possible therapeutic use of extracellular vesicles released by stem cells is raising increasing interest. The paracrine effect, mediated by MSCs derived extracellular vehicles (MSC-EVs), has recently been suggested as a mechanism for their therapeutic properties. Here, we summarizes the main pathological mechanisms involved in intrauterine adhesion, the biogenesis and characteristics of extracellular vesicles, explaining how these vesicles could provide new opportunities for MSCs.

Keywords: Intrauterine adhesion, Mesenchymal stem cells, Extracellular vesicles, Endometrium

Introduction

Intrauterine adhesion, also known as Asherman syndrome, is characterized partial or complete obliteration of the uterine cavity by adherence of the uterine walls (1). Patients with IUA may present with a high rate of infertility, recurrent miscarriage, abnormal menstruation and cyclic pain (2, 3). IUA can be induced by various risk factors including miscarriage curettage, postpartum curettage, gynecologic surgeries, and infection, among which miscarriage curettage was the dominating predisposing factor accounting for 66.7% (4, 5). However, the true prevalence of IUA is difficult to determine, as a large number of patients are asymptomatic (6).

To date, hysteroscopic adhesiolysis has been considered as the first choice for the treatment of IUA patients. Other adjuvant therapies postoperative including hormones, intrauterine devices, hyaluronic acid gel and intrauterine balloons also have achieved certain effects (7-10). But in severe intrauterine adhesion, the pregnancy rate was still unsatisfactory due to the failure of functional endometrial regeneration was related to adverse pregnancy outcomes and high rate of recurrent adhesion (11). Therefore, it is crucial to find appropriate therapeutic strategies to overcome existing problems.

Over the last years, stem cell therapies have been initiated as a new attempt to repair and regenerate injured

Received: October 4, 2021, Accepted: November 17, 2021,
Published online: June 30, 2023
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tissues through their self-renewal, differentiation abilities and immunoregulatory capabilities. Among them, MSCs are particularly gaining attention due to their easy isolation and wide sources. Recently, MSCs have been extensively used in preclinical animal models and already successfully used in several clinical applications for therapy of IUA. However, the risks of iatrogenic tumor formation, cellular rejection and infusional toxicity in MSC transplantation remain unresolved. Accumulating evidences suggest that the therapeutic efficacy of MSCs therapy may be mediated largely to their paracrine action by releasing EVs, rather than the engraftment of MSCs at the site of injury. Hence, using MSC-derived EVs as a cell-free therapy tool has become a promising alternative strategy for the treatment of IUA. In this review, we will discuss the formation of IUA, MSCs-EVs and the role of MSCs-EVs in the treatment of IUA.

The Formation of IUA

Endometrium is a highly regenerative tissue that undergoes cyclical phases of differentiation, proliferation and shedding under the control of fluctuations in circulating ovarian hormones (12). IUA is primarily caused by the destruction of the endometrium, which lead to the proliferation of fibrous connective tissues and excessive deposition of extracellular matrix. Endometrial fibrosis is considered as a crucial pathological characteristic of IUA, and it is regulated by a variety of cellular and molecular mechanisms throughout the process.

Transforming growth factor-β (TGF-β) served as a key driver in endometrial fibrosis (13). The mechanism of the TGF-β1/Smad signaling pathway was associated with the activation of the myofibroblasts, overproduction of extracellular matrix (ECM) by inducing transcription of fibrotic factors such as α-SMA, collagen I, fibronectin and tissue inhibitor of matrix metal-loproteinases (TIMP) (14). Additionally, evidence has accumulated showing that the NF-κB, Hippo or Wnt signaling pathway could form a complex signaling network with TGF-β signaling pathway to mediate endometrial fibrosis (15-17). Moreover, Ai et al. (18) found that lncRNA TUG1 promotes endometrial fibrosis and inflammation by sponging miR-590-5p to regulate Fasl in intrauterine adhesions. Matrix metalloproteinase 9 (MMP9) is also considered to be an important protein for the degradation of ECM, which can be downregulated through the PI3K/AKT signaling pathway, leading to accumulation of ECM and proliferation of collagen fibers on the damaged endometrium (19).

Moreover, under pathological conditions, epithelial-mesenchymal transition (EMT) of endometrial epithelial cells (EECs) also connected to the development of endometrial fibrosis (20). To date, little was known about the molecular mechanism in the pathogenesis of endometrial fibrosis. High mobility group AT-hook 2 (HMGA2) was regarded as an important regulator in EMT processes in some fibrotic diseases, such as pulmonary fibrosis, lens fibrosis and renal fibrosis. Similarly, in IUA patients and animal models, Song et al. (21) also observed the overexpression of HMGA2 directly mediated EMT. They further found that HMGA2 expression can be directly inhabited by let-7d to protect EEC from EMT. In addition, Circular RNAs also play an important role in the pathogenesis of endometrium fibrosis by inducing EMT. Studies demonstrated that upregulation of circPTPN12 in EEC of fibrotic endometrium negatively correlated with the expression and activity of miR21-5p, which in turn lead to upregulation of DNp63a to promote the EMT (22). Guo et al. (23) further found endometrial stromal cells (ESCs) can be transdifferentiated into a myofibroblast phenotype through the TGF-β/Samd signal pathway, resulting in the failure of the endometrium to regenerate normally.

Currently, various findings have identified the periodic regeneration of human endometrium is mediated to some extent by potential progenitors and stem cells located in the basal layer (24, 25). Once the basal layer of the endometrium is severely damaged due to the iatrogenic trauma and infection, the deficiency and inactivity of stem cell will cause the endometrial repair disorders and lead to IUA. Min et al. (26) demonstrated that there are fewer endometrial MSCs and the migration and invasion abilities of the MSCs decreased in the IUA patients, which indirectly lead to the cell differentiation, angiogenic and immunosuppressive abilities of patients with IUA were significantly decreased compared with those of healthy women.

Characterization and the Paracrine Action of MSCs

MSCs are multipotent stem cells that can be successfully isolated from a variety of adult tissues including the bone marrow, adipose tissue, umbilical cord blood, placenta and dental pulp and even the spleen, liver, kidney, brain, lung, thymus, and pancreas (27-29). The International Society for Cellular therapy (ISCT) in 2005 has established standards for defining MSCs. First, MSCs must be plastic-adherent in standard culture conditions. Second, MSCs must express CD105, CD73, and CD90 and lack expression of CD14, CD19, CD31, CD34, CD45, CD79 al-

pha and human leukocyte antigen-DR (HLA-DR) surface markers. Third, MSCs must differentiate into adipocytes, osteocytes, and chondroblasts *in vitro* differentiating conditions (30).

MSCs possess the capacity to differentiate into multiple cell types and undergo self-renewal (31). To date, MSCs have been intensively investigated as a cell-based therapy or in combination with biomaterial scaffolds in a tissue engineering approach to facilitate endometrial regeneration in clinical and animal studies. Researchers initially hypothesized that the repair function of MSCs was directly attributable to migration and homing to the damaged site, subsequently differentiate into functional cells. However, studies gradually found that the transplanted cells neither differentiate into resident injured cells nor for survival long time enough to assist the tissue replacement process (32, 33). Paracrine secretion was first identified by Gnecchi and colleagues in 2006. They reported evident improvement in ventricular function following injection of conditioned medium collected from modified MSCs into rat models of myocardial infarction (34). Instead, the paracrine effectors-EVs secreted by MSCs, contributes to the immunosuppressive, anti-apoptotic, anti-fibrotic, angiogenic and anti-inflammatory effects via transferring information with damaged cells (35).

Challenges for Use of MSCs Therapy and the Advantages of MSC-EVs

Transplanting MSCs was widely regarded as an ideal therapeutic strategy as much *vivo* and *vitro* research is being conducted regarding their use as a treatment for uterine repair and regeneration. However, there are many concerning issue in MSC clinical applications, one of which is the safety of MSCs. Røsland et al. (36) have observed spontaneous malignant transformation of MSCs in long term *vitro* culture, while opposite outcomes were stated by other investigators (36-38). In addition, due to the genomic and phenotypic instability of MSCs, it may serve as precursor cells for solid and hematological malignancies. And excess chemokines and growth factors produced by MSCs can directly act on receptors on the surface of cancer cells, thereby regulating tumor growth (39-42). Moreover, the therapeutic effect of MSCs was greatly weakened via intravenous injection, which was mainly owing to only a few MSCs access the target organ through the capillaries, and the rest will be blocked. Notably, a literature review of 844 procedures found one case of pulmonary embolism as possibly related to MSCs (43). Furthermore, hypoxic, inflammatory activated and low pH microenvironments at the damaged site lead to adverse conditions for stem cell survival, which further limits the effect of MSCs based therapy (32, 44). Besides, prior to clinical applications, MSC needs to be stored in an appropriate vehicle media to maintain cell viability. Unfortunately, it is considerably difficult to choose a standardized cell storage medium due to insufficient research (45).

Compared to the original MSCs, MSC-EVs would be safer for intravenous administration to patients and the risk of tumor formation would be much lower, as they are avoid of uncontrolled cell differentiation. Moreover, EVs are nano-sized particles, so there is no risk of embolism. Further, MSC-EVs are more stable to store and maintain bioactivity for a long time (46). Meanwhile, MSC-EVs do not carry cell surface major histocompatibility complex (MHCI and MHCII) proteins and thereby avoid the risk of immunological rejection. Therefore, MSC-EVs are going to be a novel and promising strategy for IUA.

Overview of Extracellular Vesicles

Extracellular vesicles are commonly defined as a heterogeneous group of particles that released from cells and are enclosed by a lipid bilayer. It is widely known as an important mode of intercellular communication by exchanging proteins, lipids and genetic material between cells in both physiological and pathological conditions (47).

EVs can be classified into three types based on their mode of release and size: exosomes, microvesicles (MVs) and apoptotic bodies (48). In general, exosomes have a cup-shaped morphology with a size range between 30 and 200 nm in diameter (49). The biogenesis of exosomes firstly involves the inward budding of the endosomal membrane to form multivesicular bodies (MVBs), which fuse with the plasma membrane and release contents limited in vesicles into the extracellular space (50).

Microvesicles, unlike exosomes, are directly generated and released through budding and fission of the plasma membrane and range in size from 50 to 2,000 nm (48). Apoptotic bodies, tend to larger than exosomes and MVs, are between 1,000 nm～6 μm in size (51). The formation of apoptotic bodies is a result of cell disassembly during programmed cell death. In the final stage of apoptosis, cells can divide into variable numbers of apoptotic bodies containing a wide variety of cellular components (52).

During their formation, extracellular vesicles incorporate a specific subpopulation of bioactive molecules from their cell of origin, including proteins, nucleic acids and lipids, which can be delivered to target cells by fusion with plasma membrane. As protein separation and detection tech-

nology have developed, thousands of vesicular proteins are identified, such as heat shock proteins (HSP70 and HSP90), cytoskeletal proteins (actins, cofilin-1, ezrin/radixin/moesin, profilin-1, and tubulins), tetraspanins (CD9, CD63, CD81 and CD82) and signal transduction–involved proteins and vesicle trafficking-related proteins (TSG101, ALIX, and RAB proteins,syntenin-1) etc (53, 54). Apart from proteins, the nucleic acids of NVs including mRNA, microRNA and DNA, also serve as important biomarkers and therapeutics for a variety of diseases (55-57). Additionally, EVs are enriched with a variety of lipid compounds containing hosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), lysophosphatidic acid, ceramide, cholesterol and sphingomyelin, which can be specifically associated with different cell sources of EVs (56).

Isolation and Characterization of MSC-EVs

Research in the field of EVs has grown rapidly in the last few years, and there is a plethora of techniques for the isolation based on their size, density, and immunoaffinity, among which ultracentrifugation remains the most widely used isolation method so far (58). This technique uses serial and differentiated centrifugation steps to sediment cells and large cellular debris, and finally ultracentrifugation at 100,000～200,000×g to isolate EVs (59). However, it is not suitable for large-scale clinical application due to its disadvantages such as long processing times, low EVs yield and limited purity. Density gradient ultracentrifugation can markedly reduce the contamination of EV isolates by adding iodixanol or sucrose of different densities to the sample before ultracentrifugation (60). It should be noted that the extra stages in EV purification not only increase the purity of the target EV, but also further decrease their quantity. Beside, other alternative methods like size exclusion chromatography (SEC), ultrafiltration (UCF), and immuno-based capturing (IBC) and precipitation with reagents have been shown to isolate EVs with much lesser time and more efficiently than ultracentrifugation (61). Currently, the combination of two or more isolation methods are increasingly being developed to achieve better throughput capacity of EV isolation, for example, ultrafiltration combined with size exclusion chromatography has been proven to preserve functional characteristics, while mediating isolation of EVs at high yield (62, 63).

In order to precisely use EVs in medical and pharmaceutical fields, simple and direct methods are needed to determine their size and concentration in biological fluids. The most common technique is the Nanoparticle Tracking Analysis (NTA), which can tracks fluctuations of the light scattered of individual vesicles based on their Brownian motion using a light microscope (64, 65). By combining NTA with fluorescence measurement, the vesicles can be labeled with specific cell tracker conjugated quantum dots, allowing their phenotype to be determined (66). However, this method is only accurate detection EVs of size 30～1,000 nm, similar sizes of contaminants could confound the results of EVs quantification and other sizes EVs may be lost in vain. Dynamic light scattering (DLS), similar to NAT, can detect NVs based on the Brownian motion, but it was inaccurate for polydisperse samples because the detection element collects scattered light from all particles at the same time (67). Scanning/Transmission electron microscopy (SEM/TEM) is a technique which widely used to display morphology and structure of EVs (68). An important consideration when using SEM/TEM is the sample preparation may induce changes the morphology and cause damages to EVs. To overcome this obstacles, cryo-EM is being applied for EV analysis under liquid nitrogen (69). Atomic force microscopy (AFM) technique includes its ability to measure samples in their native conditions with minimized sample preparation and obtain a real 3D image of surface topography recorded with very high resolution (70). To provide more comprehensive information about EV molecular characteristics, various techniques for protein-based and RNA-based molecular profiling of EVs have emerged over the years. Spectrophotometers, RiboGreen assay, qRT-PCR, Next-generation sequencing (NGS/RNA-seq), NanoString and microarrays can be effectively used for the quantification of nucleic acids (71). In addition, for protein, Western blotting and enzyme-linked immunosorbent assay (ELISA) are conventional immunoaffinity-based techniques representing targeted methods. Other techniques, such as reagent-based bicinchoninic acid (BCA), Bradford (Coomassie dye) assays or fluorescent reagent-based fluorometric assays, microfluidic approaches and electrochemical detection provides other promising mechanism for protein detection in EVs (70, 72).

Extracellular Vesicles in IUA

The potential of MSC-derived EV in IUA treatment has been extensively studied (Table 1). Zhang et al. (73) successfully extracted EVs from human menstrual blood derived stromal cells (MenSCs-sEVs) and verified that these EVs effectively and safely restored the impaired endometrium in the IUA rat model. Furthermore, treatment with MenSCs-sEVs increased BMP7 levels and activated the

Table 1. Potential benefits of extracellular vesicles as therapeutic agents in IUA

Donor cell	Animal	Time period	Dose of Evs	Outcomes	Mechanism	Reference
Umbilical cord mesenchymal stem cells	Albino rats	8 weeks	100 μg	• Restored endometrial glands in the uterine sections • Decreased collagen fiber deposition in the endometrium	Downregulated TNF-α, TGF-β, IL-1, IL-6, RUNX2, and collagen-I	(80)
Umbilical cord-derived mesenchymal stem cells	SD rats	8 weeks	100 μl	• Excellent neovascularization • Reduced fibrosis formation and promoted collagen remodeling • Unregulated ERα positive cells and PR positive areas	16 miRNA, especially miR-223-3p induced the polarization of macrophages to the M2 phenotype	(79)
Rat uterus derived mesenchymal stem cells	Wistar albino rats	8 weeks	25 μg	• Supported the formation of blood vessels	Upreguleated CD31 and VEGFR-1 decreased the MMP-2, MMP-9 and TIMP-2 expressions	(81)
Bone marrow mesenchymal stem cells	SD rats	14 days	None	• Repress endometrial fibrosis and promote functional recovery	miR-340 in the exosomes reduced the upregulated expression of Collagen I1α, α-SMA and TGF-βR1	(74)
Bone marrow mesenchymal stem cells	Mice	None	4.1E+9 particles/ml	• Promote cell proliferation and cell migration *in vitro* • Repair damaged endometrium	Overexpress miR-29a to reduce α-SMA, Collagen I, SMAD2, and SMAD3	(76)
Adipose-derived mesenchymal stem cells	SD rats	8 weeks	100 μg	• Endometrial regeneration • Improves endometrial receptivity and fertility	Upregulate the expression of VEGF integrin and LIF	(77)
Adipose-derived mesenchymal stem cells	SD rats	None	5 μg	• Inhabit endometrial fibrosis	lncRNA-MIAT in Exosomes regulate miR-150-5p	(78)
Menstrual blood-derived stromal cells	SD rats	18 days	4.25×10^{8} particle/ml	• Recovered the morphology and promoted the proliferation of endometrial cells • Restore endometrial receptivity and improve the fertility • Restored the thickness of endometrium, gland numbers and vascular	Inhibit TGFβ1/SMAD3 mediated endometrial fibrosis by upregulating BMP7 expression and SMAD1/5/8 and ERK1/2 phosphorylation	(73)
Adipose-derived mesenchymal stem cells	SD rats	None	None	• Facilitate the regeneration of the endometrium • Enhanced endometrial receptivity • Promoted neovascularization • Anti-infective • Anti-fibrotic activity	Upregulated HOXA-1, LIF, Integrin β3, IGF-1, VEGF and bFGF	(85)

SD: Sprague Dawley, TNF: tumor necrosis factor, TGF: transforming growth factor, IL: interleukin, RUNX: runt-related transcription factor, VEGFR: vascular endothelial growth factor receptor, ER: estrogen receptor, PR: progesterone receptor, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of metalloproteinase-1, SMA: smooth muscle actin, VEGF: vascular endothelial growth factor, LIF: leukemia inhibitory factor, IGF: insulin-like growth factor, FGF: fibroblast growth factor.

SMAD1/5/8 and ERK1/2 pathways *in vivo*, thereby alleviating endometrial fibrosis via inhibiting TGFβ1/Smad3 signaling pathway (73). Additionally, in Xiaos' study, the injection of exosomes released from bone marrow mesenchymal stem cell (BMSC-EVs) into uterine cavity subjected to mechanical damage can repress endometrial fibrotic gene (collagen1α1 and α-SMA) expression and modulate the endometrium recovery process by increased the microRNA-340 level in endometrial stromal cells (74). Similarly, Yao et al. (75) found that BMSC-EVs decreased the fibrotic area and even reversed EMT process induced by TGF-β1 signaling pathway. MiR-29a in BMSCs-EVs also has an anti-fibrotic role during the repair process (76). In IUA model, treatment with exosomes isolated from adipose derived stem cell promoted endometrial regeneration, enhanced the expression of integrin-β3, LIF, VEGF and improved fertility (77). Moreover, lncRNA-MIAT in exosomes alleviated endometrial fibrosis by adsorbing miR-150-5p which up-regulated the expression of TGFβR1 and a-SMA (78). IUA treatment with EV from MSCs derived umbilical cord also has shown a promising alternative therapy. It was demonstrated that a construct of exosomes and collagen scaffold induced endometrium regeneration, collagen remodeling and restored fertility by facilitated CD163+M2 macrophage polarization, reduced inflammation, and increased anti-inflammatory responses *in vivo* and *in vitro* (79). Another study further compared the therapeutic effects of estrogen alone, UCMSCs-EVs alone and UCMSCs-EVs combined with estrogen on IUA model. They observed that the combined treatment group had the best therapeutic effect on promoting the regeneration of damaged endometrium and re-establishing endometrial function. Meanwhile, gene expression of inflammatory cytokines (IL-1, IL-6, and TNF-α) was significantly downregulated in combined treatment group (80). In addition, Saribas et al. (81) has investigated the effects of MSC- EVs on angiogenesis, which is essential for the physiological process of endometrium regeneration. They have shown that the expression of the angiogenic marker CD31 is markedly increased in the MSC-EVs group.

Taken together, at least three key functions are involved in the repair of damaged endometrium. Firstly, one of the therapeutic functionalities of MSC-derived EVs is anti-inflammatory efficacy. Some cytokines, chemokines and chemokine receptors contained in MSC-EV, limiting inflammation to prevent excessive tissue destruction. Secondly, on hypoxia stimulation, MSC-EVs activate the expression of genes related growth of blood vessels. Angiogenesis is a fundamental process for the delivery of oxygen, nutrients, and growth factors for the healing of damaged tissues (82). Thirdly, the release of MSC-EVs allows cells to communicate with other adjacent or distant cells by transferring RNAs into recipient cells via endocytosis and/or fusion. A variety of EV-encapsulated RNAs can regulate the expression of multiple target genes and participate in various cell signaling processes to modulate the fibroblast biology inhibiting excessive fibrosis (30, 83).

Conclusions

Although MSC-derived EVs application as a cell-free method can be a promising alternative to stem cell therapy, various challenges arise to be dealt with before clinical application. Given MSC-EVs have vast heterogeneity in size, composition, and origin, it is urgent to establish a standardized protocol for the isolation and purification of MSC-EVs. However, the technologies for the purification and isolation of EVs are still in their infancy due to insufficient specificity to distinguish EVs and the density or size of the lipoproteins contaminations can be very close to that of EVs, making it difficult to separate completely (84). The frequency of administration and dosage of MSC-EVs are still inconclusive, and continued in depth preclinical *in vivo* models is required. Furthermore, most of the traditional laboratory-scale methods used for EV isolation employ complex steps of isolation and are low throughput, which poses challenges for scaling up the processing to large volumes. In addition, currently, we know little about the complex molecular mechanisms of EVs in disease, the unknown negative effects of MSC-EVs have to be clarified. Therefore, further research is needed to clearly apply MSC-EVs. In conclusion, MSCs-EVs play a significant role in treatment of IUA. Although challenges and difficulties still remain, MSC-EVs are very promising for future clinical use and MSC-EVs-based may be an alternative to MSC-based treatments. However, more detailed investigation should be carried out before clinical application in the future.

Acknowledgments

None.

Potential Conflict of Interest

The authors have no conflicting financial interest.

Ethical Approval

This article does not contain any experiments involving human participants or animals.

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