AMNIOTIC MESENCHYMAL STROMAL CELLS

Human amniotic mesenchymal stromal cell transplantation improves endometrial regeneration in rodent models of intrauterine adhesions

LU GAN, HUA DUAN, QIAN XU, YI-QUN TANG, JIN-JIAO LI, FU-QING SUN & SHA WANG

Department of Minimally Invasive Gynecologic Center, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, China

Abstract

Background aims. Intrauterine adhesion (IUA) is a common uterine cavity disease characterized by the unsatisfactory regeneration of damaged endometria. Recently, stem cell transplantation has been proposed to promote the recovery process. Here we investigated whether human amniotic mesenchymal stromal cells (hAMSCs), a valuable resource for transplantation therapy, could improve endometrial regeneration in rodent IUA models.

Methods. Forty female Sprague-Dawley rats were randomly assigned to five groups: normal, sham-operated, mechanical injury, hAMSC transplantation, and negative control group. One week after intervention and transplantation, histological analyses were performed, and immunofluorescent and immunohistochemical expression of cell-specific markers and messenger RNA expression of cytokines were measured.

Results. Thicker endometria, increased gland numbers and fewer fibrotic areas were found in the hAMSC transplantation group compared with the mechanical injury group. Engraftment of hAMSCs was detected by the presence of anti-human nuclear antigen–positive cells in the endometrial glands of the transplantation uteri. Transplantation of hAMSCs significantly decreased messenger RNA levels of pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1β), and increased those of anti-inflammatory cytokines (basic fibroblast growth factor, and interleukin-6) compared with the injured uterine horns. Immunohistochemical expression of endometrial epithelial cells was revealed in specimens after hAMSC transplantation, whereas it was absent in the mechanically injured uteri.

Conclusions. hAMSC transplantation promotes endometrial regeneration after injury in IUA rat models, possibly due to immunomodulatory properties. These cells provide a more easily accessible source of stem cells for future research into the impact of cell transplantation on damaged endometria.

Key Words: amnion, endometrium, intrauterine synechiae, mesenchymal stromal cell transplantation, regeneration

Introduction

Intrauterine adhesion (IUA), also known as Asherman syndrome, is caused by damage to the basal layer of the endometrium, resulting in formation of adhesion tissues that partially or completely obliterate the uterine cavity [1,2]; it is associated with infertility, oligomenorrhea and recurrent pregnancy loss, among other concerns [3–6]. The dual aims of IUA treatment are to reestablish anatomy (removal of adhesions) and restore uterine function [7,8]. However, in severe Asherman syndrome when the basal layer has been damaged, even when the uterine cavity has been restored by surgery, the prognosis remains poor with a recurrence ratio of up to 62.5% [9] because of failure in regeneration of functioning endometrial tissue [10]. Stem cell therapy has been used to attempt to overcome this particular problem.

Several sources of stem cells have been proposed for the endometrial regeneration of Asherman syndrome, such as bone marrow mesenchymal stromal cells (BMSCs) [11–15], menstrual blood–derived mesenchymal stem cells (mbMSCs) [16] and adipose tissue-derived mesenchymal stem cells (ASCs) [17]. BMSC were first used in a severe refractory Asherman syndrome patient and resulted in a positive biomedical pregnancy [11]. Meanwhile, autologous mbMSCs also improved endometrial thickness and pregnancy rate in an experimental, uncontrolled, prospective 3-year clinical study involving seven severe cases [16],
whereas ASCs were used only in an animal model [17]. Although these autologous stem cells all demonstrate a strong propensity to recruit to the injured endometrium and increase the endometrial thickness, they are relatively unavailable. Furthermore, the use of BMSCs has been restricted by both cost and extreme invasiveness of bone marrow collection [18]; similar limitations apply to ASC (i.e., invasive adipose tissue liposuction procedures), and the application of mbMSCs requires greater attention to the sterility of the cell product [19].

Isolated from discarded tissue and ethically nonproblematic, human amniotic mesenchymal stromal cells (hAMSCs) are a readily available, abundant, immunoprivileged cell source [20]. Moreover, similar to other mesenchymal stromal cells, hAMSCs are capable of differentiating into cells of all three germ layers both in vivo and in vitro, in addition to differentiating into typical mesenchymal lineages and exhibiting immunomodulatory properties through paracrine effects [21]. Therefore, hAMSCs have become exciting candidates for transplantation therapy techniques and may present as an attractive choice over other classically established stem cells (e.g., BMSCs) for xenograft and allograft transplantation [20–23]. Currently, apart from the widespread use of hAMSCs for preclinical treatment of many diseases (i.e., neurological, pancreatic, vascular, muscle, heart, pulmonary, and liver diseases) [21,24,25], the role of hAMSCs in IUA has remained uncharacterized.

In the present study, we aimed to investigate whether the transplantation of hAMSCs improved endometrial regeneration in IUA rats and to explore the underlying mechanisms.

Methods

Animals

A total of 40 nine-week-old female Sprague-Dawley (SD) rats weighing 220–240 g were used in the experiments. They were purchased from Vital River Laboratory Animal Technology and housed four to five per cage in a controlled environment that was maintained at 22°C with 12-h light/dark cycles. Food and water were available ad libitum. After a 1-week adaptation period, interventions began when subjects were in diestrus, as determined by vaginal smear (Figure 1A).

Ethics

All animals were treated under an approved Institutional Animal Care and Use Committee Protocol of the Capital Medical University, Beijing, China. All experiments were complied with the ARRIVE guidelines and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1985). All researchers involved in animal husbandry and experimentation possessed animal experimentation licenses issued by the Beijing Association on Laboratory Animal Care (BALAC).

Isolation and expansion of hAMSCs

The hAMSCs were kindly provided by Professors Jing Wang and Zhong-Jie Sun (Newish Technology). hAMSCs were isolated from human amniotic membranes of healthy donor mothers during full-term cesarean deliveries. Briefly, the amniotic and chorionic layers were mechanically separated by peeling away from the amniotic membranes. The amnions were digested with 0.25% trypsin at 37°C for 30 min and then digested with 0.1% collagenase II for 1 h at 37°C in an air bath shaker. Isolated hAMSCs were then filtered, centrifuged and cultured in Dulbecco’s Modified Eagle Medium (DMEM) nutrient mixture F-12 supplemented with 10% fetal bovine serum (FBS) in an incubator at 37°C with 5% CO₂. hAMSCs were specifically identified by their capacity to differentiate into adipocytes and osteoblasts, and their mesenchymal phenotypes were assessed by fluorescence-activated cell sorting. hAMSCs of no more than three passages were used for experiments.

Before in vivo studies, cells were isolated and suspended in fresh medium at concentrations of approximately 3–5 × 10⁴ cells/μL. Two hundred microliters of the cell suspensions (6–10 × 10⁶ cells) were placed into individual sterile tubes before transplantation procedures.

Flow cytometry

Third-passage culture-expanded mesenchymal stromal cells were phenotypically characterized by flow cytometry as previously described [18]. Briefly, cells were harvested using TrypLE Express detachment solution (Invitrogen, Life Technologies), and 1.0 × 10⁵ cells were stained with the following monoclonal antibodies: CD29, CD31, CD34, CD44, CD73, CD90, CD105, HLA-DR and HLA-ABC, or isotype antibodies (Beckton Dickinson) for 30 min. Cells were washed twice by adding cold phosphate buffered saline (PBS) at pH 7.4 and centrifuged at 150g for 5 min at 8°C. Immunoreactivities to cell surface antibody markers were assayed by flow cytometry (FACSScan, Beckton Dickinson).

Differentiation of hAMSCs into osteocytes and adipocytes

Osteogenic differentiation and alkaline phosphatase staining: third-passage culture-expanded cells at 80%
confluence were induced to differentiate in the following osteogenic mediums for 8 days: high-glucose DMEM supplemented with 10% FBS, 10 mmol/L β-glycerophosphate, 10 mmol/L dexamethasone and 0.2 mmol/L ascorbic acid. Mineralizations were determined by post-induction alkaline phosphatase (ALP) staining. Eight days after osteogenic induction, >70% of hAMSCs showed osteoblast-like morphologies. For detection of calcification, the induced cells in 24-well plates were washed twice with PBS and fixed with 95% ethanol (500 μL/well) for 20 min. Cells were then washed with PBS and incubated for 15 min with substrate solutions (0.2 mg/mL α-naphthyl-1-phosphate, 0.1 mol/L Tris buffer, pH 8.9, 0.01% magnesium sulphate and 0.6 mg/mL fast blue RR salt) to visualize osteogenic differentiation through alkaline phosphatase staining.

Adipogenic differentiation and oil red O staining: third-passage culture-expanded cells at 100% confluence were induced in the following adipogenic mediums for 8 days: high-glucose DMEM supplemented with 10% FBS, 1.0 μmol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine and 1.0 mmol/L ascorbic acid. Adipogeneses were determined by post-induction oil red O staining. Cells were washed twice with PBS and fixed with 10% formalin for 10 min. After fixation, cells were stained with aliquots of filtered oil red O solution (stock solution: 3 mg/mL in isopropanol; working solution: 60% oil red O stock solution and 40% distilled water) for 1 h. After staining,
cells were washed with water to remove unbound dyes, visualized using light microscopy and photographed. All reagents used in osteogenic and adipogenic differentiation were from Sigma-Aldrich.

Rodent model of intrauterine adhesions

Rat IUA models were established following mechanical injury, which consisted of (i) incision, (ii) curettage and (iii) suture. All rats were anesthetized with 5% pentobarbital sodium (5 mg/kg intraperitoneally). Right uterine horns were selected as the experimental side and exposed through abdominal midline incisions, whereas the left uterine horns were untouched. First, vertical incisions (~25–30 mm) were made longitudinally down the length of the right horns from 5 mm below the ipsilateral ovaries or uterotubal junctions to 2 mm above their bifurcations. Second, No. 21 surgical scalpels (Shanghai Pudong Jinhuan Medical Products) were used to scrape the entire endometrial linings of the inner uterine surfaces until the uterine walls became rough and pale to the naked eye, leaving the mesometrium intact. The uteri were then washed with saline solution and exposed to air for 30 min. Third, the uterine wounds were closed by continuous stitching with 6-0 Vicryl sutures (ETHICON, W9981, coated polyglactin 910 suture, Johnson & Johnson). Subsequently, interrupted 3-0 silk non-absorbable sutures (ETHICON) were inserted through the muscle and skin layers, respectively. After warming periods on 37°C constant-temperature operating facilities, the subjects were returned to their housing facilities.

Transplantation of hAMSCs

Preliminary experiments were performed to assess the functionality of rat IUA models. Twenty-four female Sprague-Dawley rats were randomly assigned to the following three groups: (i) a normal group without any treatments (NG, n = 8); (ii) a sham-operated group, in which abdominal surgery exposed the bilateral uterine horns, incisions and suturing were performed on the right horns, but the procedures did not include curettings (SO, n = 8); (iii) a mechanical injury group receiving all three surgical procedures (i.e., incision, curettage, and suture) described earlier (MG, n = 8). To determine the effects of hAMSC transplantation in IUA, 16 female rats simultaneously underwent induction of mechanical injury in the right uterine horn and were subsequently randomized into the other two groups immediately after suturing: (iv) the hAMSC transplantation group received intramuscular injections of 200-μL hAMSC suspensions into the myometrium (EG, n = 8) and (v) the negative control group received 200-μL saline vehicle suspension injections following the same procedure as in the hAMSC transplantation group (NC, n = 8; Figure 1B). All animals received intramuscular injections of penicillin (80 000 U/100 mg) for 3 days after surgery.

In each group, eight rats were sacrificed 7 days after surgery or transplantation, and complete uterine horns were obtained (Figure 1A). Portions of the uteri were sectioned and preserved in formalin, paraformaldehyde and/or liquid nitrogen for further examination.

Histology examination

Hematoxylin and eosin (H&E) staining: excised uterine horns were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Four-micrometer serial paraffin sections were stained with H&E by conventional methods in a Tissue-Tek DRS 2000 (Sakura Finetek Japan) according to the manufacturer’s instructions. Using quantitative image-processing software (Image-Pro Plus version 6.0, Media Cybernetics), endometrial thicknesses, glandular counts and endometrial morphologic features were evaluated and compared among the groups. Endometrial thickness measurements were obtained from the lateral sides of each cross-section; measurements of interest were defined as the vertical distances between luminal surfaces and serous membranes. Gland numbers were counted and averaged in four randomly selected high-power fields (HPF) taken from each of the slides to determine uterine gland abundance.

Masson staining: four micrometer tissue sections were stained in modified Weigert’s iron hematoxylin for 5 min, placed in 1% hydrochloric acid alcohol for 5 s, and then stained in Ponceau Acid Fuchsin (Goodbio Technology) for 5–10 min. Subsequently, sections were placed in phosphomolybdic acid solution for 3–5 min without washing, directly stained in aniline blue solution for 5 min and then placed in 1% acetic acid solution for 1 min. Negative and positive controls were processed simultaneously in each staining run. To quantitatively evaluate potential histological evidence of fibrosis, four random fields of each Masson-stained slide were chosen to calculate the ratios of endometrial fibrotic areas/total endometrial areas (excluding the uterine cavity) using Image-Pro Plus software (version 6.0). The ratios averaged per group were obtained.

Immunohistochemistry and immunofluorescence assays

After fixation in 10% formalin for 48 h, uterine horns were dehydrated and embedded in paraffin (Tissue-Tek VIP 5Jr. 5903; Tissue-Tek TEC 5; Sakura Finetek Japan), and 4-μm sections were prepared (Tissue-Tek IVS-410) and placed on micro slides (FRC-04, Matsunami). Sections were de-paraffinized in xylene, rehydrated through a series of alcohol gradients (100–70%) and rinsed in water. After antigen retrieval and
endogenous peroxidase activity blocking, specific primary antibody mouse anti-pan-cytokeratin (PCK, 1:50; Boster Biological Technology) was applied at 4°C overnight in humidified chambers. Sections were then incubated with goat-anti-mouse immunoglobulin G for 1 h at room temperature, then briefly counterstained with hematoxylin for 40 s and observed using an Olympus microscope. For each protein studied, negative controls were generated at each time point with PBS replacing the primary antibody.

Immunofluorescence staining was used to investigate mouse anti-human nuclear antigen (HuNu, 1:100; Chemicon, Millipore) antibody staining on the sections. For visualization, fluorescence-tagged secondary antibody (Cy3, 1:300; GoodBio Technology) was used to amplify the signal of the primary antibody. Cell nuclei were stained with 5 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 min. Images were obtained using appropriate excitation and emission filter sets for fluorescence microscopy (Nikon ECLIPSE TI-SR). Negative controls were generated using PBS in lieu of a primary antibody.

**RNA isolation and polymerase chain reactions**

Total RNA samples were extracted from excised uterine horns with RNAiso Plus (Takara Bio) and dissolved in water treated with diethyl pyrocarbonate. RNA concentrations were quantified using NanoDrop 2000 spectrophotometery (NanoDrop Technologies, Thermo Scientific). One thousand nanograms of total RNA per sample were reverse transcribed to generate cDNA. First-strand cDNA was generated with PrimeScript RT reagent kits in accordance with the manufacturer’s guidelines (Takara Bio), and using Applied Biosystems Veriti96 systems (Applied Biosystems, Life Technologies). Thermocycler protocols used for reverse transcription were 15 min at 37°C, 5 s at 85°C and ∞ at 4°C.

Subsequently, relative quantitative real-time polymerase chain reactions (qRT-PCR) using 96-well optical plates were performed and analyzed with Applied Biosystems 7500 Fast Real-Time PCR Detection Systems (Applied Biosystems). Each gene and sample was assayed in triplicate. To make a final volume of 20 μL, each well contained 10 μL of 2× SuperReal PreMix Plus, 1 μL of cDNA, 1 μL of forward primer (10 μmol/L), 1 μL of reverse primer (10 μmol/L) and 6.6 μL of RNAase-free ddH2O. Primer sequences used for each target gene are summarized in **Table I**. The cycling parameters for the qRT-PCR were as follows: an initial denaturation at 95°C for 15 min followed by 40 cycles of 10 s at 95°C and 32 s at 60°C. Analyses of relative gene expressions were performed using 2-ΔΔCT methods. Ratios of messenger RNA (mRNA) expression were given as fold-changes relative to untreated controls after normalizing to glyceraldehyde phosphate dehydrogenase housekeeping genes.

**Statistical analysis**

Numerical data were presented as means and standard deviations. One-way analysis of variance tests were used to compare normally distributed data (e.g., endometrial thicknesses, glandular numbers, fibrotic areas of uterine horns, and mRNA expression of cytokines), among the several groups followed by Tukey’s honestly significant difference (HSD) post hoc test for intergroup multiple comparisons. Data analysis was performed using an SPSS package 21.0 for Mac (IBM). P values < 0.05 were considered to be statistically significant.

**Table I. Primers of specific genes used in qRT-PCR analyses.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>COL1A1</td>
<td>5′-CCTCCCAGAACATCCTAT-3′</td>
<td>5′-CATCCCAAGCGGTGCTTAG-3′</td>
</tr>
<tr>
<td>TGFβ</td>
<td>5′-GCTGGAGACAGAGGTGTC-3′</td>
<td>5′-CACACCCCAAGACCTTAC-3′</td>
</tr>
<tr>
<td>bFGF</td>
<td>5′-GATCCCCAGCGGCTCTGCT-3′</td>
<td>5′-TCGACACACTCCTTCAGGT-3′</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>5′-TGACCACTCCATCCGCTCCT-3′</td>
<td>5′-CCAGAATGTCGCGGCTCAT-3′</td>
</tr>
<tr>
<td>TIMP</td>
<td>5′-AGCCTGCAATCTCCCGAGCA-3′</td>
<td>5′-AGGATCGAATCTCCCTTGAGCA-3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-TCTGCTGTACCTCCACCAT-3′</td>
<td>5′-GGGTATCTTGAGAGATGTC-3′</td>
</tr>
<tr>
<td>TNFα</td>
<td>5′-GTGCCCTGCGCTTCTCTCTATT-3′</td>
<td>5′-CATTTGGAACCTCCCTCTCTACT-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5′-CGACAGTGAGGAAATGACC-3′</td>
<td>5′-CACAGCCAATGAGTGCAG-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-AAGAGACCTCCAGCCAGTTC-3′</td>
<td>5′-TCTTGTGGTGGGTGATACCTC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TACCCAGGCGAAGTTCAAG-3′</td>
<td>5′-CACAGCAGTACCCCCATTTTG-3′</td>
</tr>
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</table>

GAPDH, glyceraldehyde phosphate dehydrogenase.

hAMSCs improve endometrial regeneration of IUA
Results

Characterization of hAMSCs

Culture-expanded cells derived from the amnion were plastic-adherent and showed fibroblast-like cell morphology, being spindle-shaped (Figure 2A,B). The cultured cells expressed CD29, CD44, CD73, CD90, CD105 and HLA-ABC (>95%) but not CD31, CD34 or HLA-DR (<5%; Figure 2C), similar to the phenotypes of MSCs derived from adult bone marrow. To evaluate the multi-potency of hAMSCs, we induced differentiation of culture-expanded cells into osteoblasts and adipocytes. The cultured cells differentiated into osteoblasts and adipocytes, as demonstrated by ALP and oil red O staining, respectively (Figure 2D,E).

Establishment of IUA models

Seven days after endometrial mechanical injury, a few fibrous adhesions formed (Supplementary Figure S1), as indicated by increased extracellular matrix (ECM) collagen depositions and scattered endometrial glands in the uterine cavities, which were confirmed by H&E staining (Figure 3A) and Masson staining (Figure 3B).

Endometrial thicknesses in the NG, SO and MG were 361.125 ± 38.635 μm, 318.375 ± 28.490 μm and 164.375 ± 36.257 μm, respectively, with significant differences among the three groups (P < 0.001). Endometrial thickness was significantly lower in the MG compared with the NG (P < 0.001) and SO (P < 0.001). Similarly, endometrial glands numbers were 15.375 ± 1.553 per HPF and 5.125 ± 1.126 per HPF in the three groups, respectively (P < 0.001). Post hoc multiple comparisons revealed that the number of endometrial glands was significantly lower in the MG compared with the other two groups (P < 0.001 and P < 0.001). As to the fibrotic areas ratio, MG uterine horns had the highest ratios (72.250 ± 4.89%) and were significantly different to NG horns (31.625 ± 4.47%, P < 0.001), and SO horns (34.500 ± 4.50%, P < 0.001; Table II).

Briefly, thinner endometria, lower gland number and increased fibrotic areas were observed in MG compared with the NG and SO, suggesting that endometrial morphologies failed to fully regenerate after injury.

Effect of hAMSC transplantation on histopathological parameters

One week after transplantation, endometrial thicknesses and glandular numbers were significantly higher in the EG horns (323.250 ± 29.300 μm, 14.875 ± 0.835 per HPF) compared with MG horns (164.375 ± 36.257 μm, 5.125 ± 1.126 per HPF; P < 0.001), whereas the NC (186.625 ± 25.025 μm, 5.750 ± 1.035 per HPF) did not show any improvement when compared with the MG (P < 0.001). Moreover, the level of endometrial thickness and glandular number in the EG reached that of the NG (361.125 ± 38.635 μm, 15.375 ± 0.916 per HPF; P = 0.147, P = 0.898; Table II).

Ratios of endometrial fibrotic tissue areas to total endometrial tissue areas were evaluated and ranged from 25% to 80% in each rat. The increase in positive Masson staining was indicative of fibrosis. EG horns showed significantly decreased fibrotic areas (37.875 ± 4.357%) compared with the MG horns (72.250 ± 4.89%; P < 0.001) and NC horns (66.250 ± 4.496%; P < 0.001; Table II). This may have contributed to the observed changes in endometrial function that are described below.

Location of transplanted hAMSCs

HuNu expressing cells, which demonstrated the presence of transplanted hAMSCs, were identified in the endometria of rats receiving hAMSC transplantation by immunofluorescence staining. Furthermore, HuNu-positive cells were detected in the endometrial glands of transplanted horns (Figure 4A); however, the number of labeled cells was limited.

Effects of hAMSC transplantation on gene expressions of cytokines

mRNA levels of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), were significantly decreased in the EG compared with the MG (P = 0.029; P = 0.013, respectively). However, no significant decreases were found between the EG and NC (P = 0.301; P = 0.471, respectively; Figure 5A). Meanwhile, expressions of anti-inflammatory cytokine mRNAs (basic fibroblast growth factor [bFGF] and interleukin [IL]-6) were significantly upregulated in the EG compared with the MG horns (P < 0.001, P < 0.001) and NC horns (P = 0.009, P = 0.015, respectively; Figure 5A).

Increased mRNA levels of ECM deposition related cytokines, for example, transforming growth factor-β (TGFβ), platelet derived growth factor (PDGF)-BB, tissue inhibitor of metalloproteinase (TIMP) and collagen type I alpha 1 (COL1A1) were apparent in the MG horns compared with NG (P = 0.008, P = 0.022, P < 0.001, and P < 0.001, respectively). Furthermore, hAMSCs transplanted uteri significantly down-regulated the accumulation of these cytokines compared with MG horns (P = 0.025, P = 0.019, P = 0.002, and P = 0.001, respectively; Figure 5B).

Evidence of hAMSC transplantation on the regeneration of endometrial cells

Epithelial cells, which were cytokeratin (CK) positive, were found in the luminal cavity and in the
Figure 2. Identification of hAMSCs. The morphology of primary (A) and third (B) passage hAMSCs. The cell is plastic-adherent and spindle-shaped growth. (C) Flow cytometry of hAMSCs. The phenotype of the cultured cells was conforming to the criteria of MSCs (i.e., CD29, CD44, CD73, CD90, CD105 and HLA-ABC positive (>95%) and CD31, CD34 and HLA-DR negative (<5%). (D) Osteoblasts differentiations (alkaline phosphatase staining). The purple reaction products indicated the calcifications. (E) Adipocytes differentiations (oil red O staining). The red products represented the lipid droplets. Scale bars = 200 μm.
endometrial glands of NG and SO rats. On the other hand, in the MG, no CK-positive epithelial cells were found in the endometrial glands or on injured thin luminal surfaces. After hAMSC transplantation following initial injuries, CK-positive epithelial cells were detected both in cavities and glands, which indicated the regeneration of endometrial epithelial cells (Figure 6A).

Vascular endothelial cell regeneration was confirmed by qRT-PCR analyses of vascular endothelial

Table II. Endometrial thickness, glandular numbers and percentages of fibrotic areas in each group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>NG (n = 8)</th>
<th>MG (n = 8)</th>
<th>SO (n = 8)</th>
<th>EG (n = 8)</th>
<th>NC (n = 8)</th>
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<tr>
<td>Endometrial thickness (µm)</td>
<td>361.125 ± 38.635</td>
<td>164.375 ± 36.257^*#</td>
<td>318.375 ± 28.490</td>
<td>323.250 ± 29.300^*^</td>
<td>186.625 ± 25.025</td>
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<tr>
<td>Number of glands (per HPF)</td>
<td>15.375 ± 0.916</td>
<td>5.125 ± 1.126^*#</td>
<td>14.125 ± 1.553</td>
<td>14.875 ± 0.835^*^</td>
<td>5.750 ± 1.035</td>
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<tr>
<td>Fibrotic areas ratio (%)</td>
<td>31.625 ± 4.470</td>
<td>72.250 ± 4.890^*#</td>
<td>34.500 ± 4.500</td>
<td>37.875 ± 4.357^*^</td>
<td>66.250 ± 4.496</td>
</tr>
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</table>

Value given are mean ± SD, comparison using one-way analysis of variance test and Tukey’s honestly significant difference post hoc test.

^*P < 0.001 MG versus NG and SO groups.

^#P < 0.001 the EG versus MG and negative control groups.
Figure 5. The mRNA expression of cytokines. (A) Inflammatory cytokines expression. Increased mRNA levels of anti-inflammatory cytokines (i.e., bFGF, IL-6) and decreased mRNA levels of pro-inflammatory cytokines (i.e., TNFα, IL-1β) were apparent in the mechanically injured uterine horns compared with normal horns ($P = 0.039$, $P = 0.048$, $P = 0.038$ and $P = 0.004$, respectively). Additionally, down-regulated pro-inflammatory cytokines and upregulated anti-inflammatory cytokines were observed in the EG compared with the MG group ($P < 0.001$, $P < 0.001$, $P = 0.029$ and $P = 0.013$, respectively). (B) ECM deposition-related cytokines. hAMSC-transplanted uteri significantly down-regulated accumulation of ECM deposition-related cytokines, namely, TGFβ, PDGF-BB, TIMP and COL1A1, compared with injured horns (MG) ($P = 0.025$, $P = 0.019$, $P = 0.002$ and $P = 0.001$, respectively). Values are expressed as mean ± SD ($n = 8$ animals per group).

* $P < 0.05$ MG versus NG. * $P < 0.05$ EG versus MG. ^ $P < 0.05$ EG versus NC. ** $P < 0.05$ NG versus NC. NS in the bar indicated nonsignificant.
Discussion

The findings of the present study indicated that (i) the transplantation of hAMSCs regenerated endometrium resulted in endometrial thickening, increased gland numbers, and fewer fibrotic areas; (ii) transplanted hAMSCs engrafted to injured endometrium led to greater expression of CK and vascular endothelial growth factor; and (iii) hAMSCs upregulated the expression of anti-inflammatory cytokines, as well as decreased the expression of pro-inflammatory and ECM deposition cytokines, compared with the mechanical injury and negative control group.

Previously, three methods had been proposed for the regeneration of endometrium. First, tissue engineering provided an alternative choice to endometrial repair. Because of the unique physical characteristics and complicated hormonal environment of the uterus, reports of uterine reconstruction are rare [26,27]. Second, endometrial epithelial cell infusions were rapid epithelial repair techniques used to prevent scar formation. However, collection of endometrial epithelial cells was not a simple feat because of the extremely limited in vitro proliferation capacity of these cells and highly invasive collection procedures [28]. Third, stem cell therapy showed great promise for repair and/or regeneration of damaged tissue [29].

Recently, rare populations of endometrial epithelial and stromal MSCs were identified in the basal layer of human endometria that appeared to resemble adult MSC [19,30–32]. The origin of endometrial stem cells remains unclear. Several sources have been postulated, including BMSCs [11–15], mbMSCs [16] and ASCs [17]. They all show a strong propensity to ameliorate tissue damage occurring after endometrial injuries [11–17,33,34] (Table III). Among these three types of stem cells, BMSCs are the most commonly used source of stem cells in human studies. In the first clinical case in 2011 [11], autologous BMSC transplantation was administered into the uterine cavity of a patient with severe IUA and primary infertility. Subsequently, the patient’s endometrial thickness increased from 3.2 to 6.9 mm, and a positive biochemical pregnancy was achieved through in vitro fertilization–embryo transfer. However, the exact mechanism through which the BMSC transplantation improves endometrial regeneration remains unknown. In addition, the inherent problems of BMSCs still need attention; namely, their low abundance in bone marrow, low overall number, decreased differentiation potential with age and the invasive isolation procedures needed to obtain them [18]. Similarly, mbMSCs have been found to improve endometrial thickness (5 of 7 subjects) and pregnancy rate (2 of 7 subjects) in an experimental, uncontrolled, prospective 3-year clinical study involving seven cases [16]. One concern regarding the use of mbMSCs is the sterility of the menstrual cell product and the methods for purification [19]. On the other hand, ASCs have not been used
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<th>Authors</th>
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<th>Administration</th>
<th>Design</th>
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<td>Nagori et al.</td>
<td>Human</td>
<td>Severe refractory AS</td>
<td>Autologous bone marrow stem cells</td>
<td>4 × 10^7 cells to uterine cavity</td>
<td>Case report (n = 1)</td>
<td>Endometrium thickness; pregnancy outcomes</td>
<td>SCs regenerate injured endometrium up to 8 mm and achieved positive biochemical pregnancy</td>
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<td>Singh et al.</td>
<td>Human</td>
<td>Refractory AS (grade III–IV)</td>
<td>Autologous BM mononuclear stem cells</td>
<td>(8–13) × 10^7 cells to uterine cavity</td>
<td>Prospective case series (n = 6)</td>
<td>Endometrium thickness; menstruation</td>
<td>SCs leads to endometrial regeneration reflected by restoration of menstruation (5/6)</td>
</tr>
<tr>
<td>Alawadhi et al.</td>
<td>Mouse</td>
<td>AS</td>
<td>BM-derived stem cell from male mice</td>
<td>1 × 10^7 cells to tail vein</td>
<td>Group 1: control (n = 10)</td>
<td>SC recruitment; pregnancy outcome</td>
<td>SCs represented 0.1% of total endometrial cells; in BM transplant group, 9/10 conceived</td>
</tr>
<tr>
<td>Kilic et al.</td>
<td>Rat (Wistar-Albino)</td>
<td>AS</td>
<td>ASCs from male rats</td>
<td>2 × 10^6 cells to uterine cavity by 3 intraperitoneal injections with 5-day intervals</td>
<td>Group 1: model group (n = 10)</td>
<td>Fibrosis, vascularization, inflammation; expression of VEGF, PCNA and Ki-67</td>
<td>In SC+ estrogen group compared with the SC group: less fibrosis but more Ki-67, PCNA and VEGF</td>
</tr>
<tr>
<td>Jing et al.</td>
<td>Rat (SD)</td>
<td>Thin endometrium</td>
<td>BMSCs from male rats</td>
<td>1 × 10^7 cells to tail vein</td>
<td>Group 1: experimental group (n = 24)</td>
<td>Endometrial thickness; endometrial receptivity; engraftment of SC; proinflammatory cytokines</td>
<td>SCs has thicker endometrium and improved expression of endometrial markers, possibly through migration and immunomodulation</td>
</tr>
<tr>
<td>Zhao et al.</td>
<td>Rat (SD)</td>
<td>Thin endometrium</td>
<td>BMSCs from male rats</td>
<td>1 × 10^7 cells to uterine cavity</td>
<td>Group 1: control group (NS)</td>
<td>Endometrial thickness; engraftment of SC; proinflammatory cytokines</td>
<td>SCs improved endometrium thickness, probably through their migration and immunomodulatory properties</td>
</tr>
<tr>
<td>Cervello et al.</td>
<td>Mouse</td>
<td>AS</td>
<td>Human CD133+ BM-derived stem cells</td>
<td>1 × 10^6 cells to intrauterine and tail vain</td>
<td>Group 1: intrauterine injection (n = 5)</td>
<td>Percentage and localization of engrafted SC; cell proliferation assay; specific paracrine factors and expression of ER and PR</td>
<td>SCs engraft around endometrial vessels, inducing proliferation of surrounding cells through paracrine molecules</td>
</tr>
<tr>
<td>Wang et al.</td>
<td>Rat (SD)</td>
<td>AS</td>
<td>BMSCs from rats</td>
<td>Intrauterine and tail vain</td>
<td>Group 1: control (n = 12)</td>
<td>ER and PR expression; numbers of endometrial glands and fibrosis area rate</td>
<td>SCs were effective to repair the damaged endometrium likely through promoting the ER and PR expressions</td>
</tr>
<tr>
<td>Tan et al.</td>
<td>Human</td>
<td>Severe AS (grade III–V)</td>
<td>Autologous mbMSCs</td>
<td>1 × 10^6 cells to uterine cavity</td>
<td>Experimental, uncontrolled, prospective 3-year clinical study (n = 7)</td>
<td>Endometrium thickness; pregnancy outcome</td>
<td>SCs increased endometrium thickness in five women to 7 mm; 2 conceived successfully</td>
</tr>
</tbody>
</table>

AS, Asherman syndrome; BM, bone marrow; IGF, Insulin-like growth factor; NOD, nonobese diabetic mice; PCNA, proliferating cell nuclear antigen; SC, stem cell; SD, Sprague-Dawley.
in human study, presumably because of the invasive nature of adipose tissue liposuction, low purification cells and decreased differentiation potential with age [19].

This is the first study to explore the effect of hAMSC transplantation on endometrial regeneration in an Asherman syndrome rat model. There are several advantages of these stem cells, which may allow them to replace other classically established stem cell lines (e.g., BMSCs). First, hAMSCs are an abundant and easily accessible source, isolated from discarded biological tissues without ethical concerns [20]. Second, they possess an immunoprivileged status that has been successfully used after xenotransplantation into immunocompetent animals that had not been previously treated with immunosuppressants in the absence of overt host responses [24] in a variety of clinical approaches including diabetes, muscular dystrophy, Alzheimer disease, cardiomyopathy, neuromyelitis, hepatic cirrhosis, bone injury, among others [21,25]. Third, hAMSCs have high proliferative capacity and expansion potential due to their youthful age [20]; thus, the in vitro expansion process can be reduced with regard to time and passage number without any morphological alterations. Fourth, they have immunomodulatory properties mediated by soluble factors [35,36]. Regarding the establishment of animal models, instead of previous reported methods in rabbits or other rodents such as physical injuries using Freon [37], Nd:YAG laser [38], and curettage [39]; chemical injuries with 10% formalin [40] and polyethylene sponges [41]; mechanical and infectious dual injuries using pairing curettage with lipopolysaccharide surgical suture [42], or copper wire [43]; we choose to induce pathologically relevant IUA using mechanical injury methods to mimic clinically observed human IUA, in which high-precision curettage procedures were performed. After the uterine horns were incised to expose endometrial surfaces, curettage was conducted under direct vision to obtain complete endometrial injuries that best resembled human conditions.

In the present study, the isolated hAMSCs conformed to the minimum criteria of the International Society for Cellular Therapy for the identification of mesenchymal stromal cells, that is, to be plastic-adherent; more than 95% expression of CD105, CD73 and CD90; lacking expression of CD34 and HLA-DR; and able to differentiate to osteocytes/adipocytes in vitro [18,44]. Moreover, the faithful capitulation of the models were confirmed by H&E and Masson staining after 1 week, in which injured uteri presented with thinner endometria, fewer glands, increased fibrotic areas and absence of epithelial cell markers, indicating the successful ablation of endometrial epithelial cells. The rat IUA model described here may adequately serve in future exploration of the efficacy and safety of new approaches to endometrial injury issues.

The effects of hAMSCs on injured endometrium were examined in this study. Compared with mechanical injury and injury accompanied by saline infusions (negative control), hAMSC transplantation produced thicker endometria, increased endometrial glandular tissues, produced lower ratios of fibrotic to normal tissue areas and increased expression of CK and VEGF. Alterations in the damaged endometria that we observed suggested that the processes of endometrial repair and regeneration were supported by hAMSC transplantation. In agreement with other studies using BMSCs [13,14,33,34], engraftment of hAMSCs into endometrial tissues was confirmed by detecting HuNu positive cells in the endometrial glands after hAMSC transplantation. Engraftment of hAMSCs may account for the endometrial histologic changes described here. Moreover, although the HuNu-positive cells were few, the transplanted cells may have secreted paracrine factors that aided in endometrial regeneration, rather than having undergone clonal expansion [45,46]. The secreted cytokines and growth factors were the basis of hAMSCs with immunomodulatory properties.

A relevant phenomenon is that hAMSCs reduce the expression of pro-inflammatory cytokines, such as IL-1β, TNFα and IL-8, and increase the expression of anti-inflammatory cytokines, such as IL-6 and IL-10, as well as other soluble factors, such as VEGF, bFGF, hepatocyte growth factor, insulin-like growth factor 1 and prostaglandin E2, both in vitro [25,36] and in animal disease models [21,22]. Considering the pathogeneses of IUA have been attributed to excess deposition of ECM resulting from inflammatory activity and uncontrolled fibroblast proliferation processes [9,10], the regulation of cytokines by hAMSCs may stimulate endometrial cell proliferation, inhibit excessive collagen production and exert anti-inflammatory effects. In the present study, the upregulation of anti-inflammatory cytokines (IL-6, bFGF) and down-regulation of pro-inflammatory cytokines (IL-1 β, TNFα) as well as ECM deposition-related cytokines (i.e., TGFβ, PDGF-BB, TIMP and COL1A1) after hAMSC transplantation were observed, when compared with the mechanical injury and saline infusion recipients. Thus, the suppression of inflammation and ECM deposition events may reveal elements of the mechanisms by which hAMSCs promoted endometrial regeneration in the present IUA models.

The particular strength of this study was the high-precision curettage procedure, which we believe was critical for successful modeling of endometrial injuries. However, the potential long-term implications of hAMSC transplantation on fertility cannot be drawn from this study. Moreover, the beneficial findings in
this study for the rat model cannot be extrapolated to humans. The doses of cells per infusion, cell transplantation techniques and routes of transplantation for use in humans would need to be optimized.

Conclusions

In conclusion, this study demonstrated that hAMSC transplantation may repair uterine horns and improve regeneration of endometria in a rat model of IUA by acting in an immunomodulatory manner. Given that hAMSCs are more readily available than other stem cells and can be used as both xenografts and allografts, it opens up an additional, more easily accessible source of stem cells for future research into the impact of stem cell transplantation on damaged endometrium. In future preclinical studies, minimal doses of cells per infusion need to be determined, and cell transplantation techniques need to be optimized. Finally, the long-term efficacy of hAMSC transplantation on fertility rates needs to be evaluated.

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References


Supplementary data to this article can be found online through distinct migratory and paracrine mechanisms. Respir Res 2008;3:doi:10.1371/journal.pone.0001886.


Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2017.02.003.