# Unresponsive Thin Endometrium Caused by Asherman Syndrome Treated With Umbilical Cord Mesenchymal Stem Cells: A Pilot Study

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**Research Article**

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## Abstract

Background

Unresponsive thin endometrium caused by Asherman's syndrome (AS) is the major cause of uterine infertility. However, current therapies are ineffective. This study is to evaluate the effect of transplantation with collagen scaffold/umbilical cord mesenchymal stem cells (CS/UC-MSCs) on this refractory disease.

Methods

Eighteen infertile women with unresponsive thin endometrium, whose frozen–thawed embryo transfers (FET) were cancelled due to reduced endometrial thickness (ET ≤ 5.5 mm), were enrolled in this before and after self-control prospective study. Hysteroscopic examination was performed to confirm no intrauterine adhesions, then ten million UC-MSCs loaded onto a CS were transplanted into the uterine cavity in two consecutive menstrual cycles. Then uterine cavity was assessed through hysteroscopy after two transplants. FET were performed in the following cycle. Pregnancy outcomes were followed-up. Endometrial thickness, uterine receptivity and endometrial angiogenesis, proliferation and hormone response were compared before and after treatment.

Results

Seventeen patients completed the study. No treatment-related serious adverse events occurred. Three months after transplantation, the average ET increased from 4.11 ± 1.01 mm to 5.87 ± 0.77 mm ($P < 0.001$). Three of 15 patients after FET got pregnant, of whom 2 gave birth successfully and 1 had a miscarriage at 25 weeks' gestation. One of 2 patients without FET had a natural pregnancy at 36 weeks after transplantation. Immunohistochemical analysis showed increased micro-vessel density, upregulated expression of Ki67, estrogen receptor alfa and progesterone receptor, indicating an improvement in endometrial angiogenesis, proliferation, and response to hormones.

Conclusion

CS/UC-MSCs can promote the growth of unresponsive thin endometrium caused by AS, possibly through promoting endometrial proliferation and angiogenesis and enhancing the response of the endometrium to hormones.

Trial Registration

ClinicalTrials.gov (NCT03724617). Registered 26 October 2018-prospectively registered, https://register.clinicaltrials.gov/


## Introduction

Thin endometrium is often found in women with Asherman's syndrome (AS) because the basal layer is destroyed, and the functional layer fails to respond to hormonal stimulation, which is the major cause of uterine infertility (1, 2). Adequate endometrial thickness (ET ≥ 7mm) at the day of embryo transplantation represents the "fertile soil" for an implanting embryo, which is essential to accomplish a successful pregnancy (3). At present, there is no consensus on the exact definition of thin endometrium. The most widely acceptable measure is 7 mm, as an ET < 7 mm is negatively associated with the chance of implantation and pregnancy (4, 5).

Clinically, numerous strategies have been adopted to promote endometrial regeneration, including extended estrogen administration, low-dose aspirin, pentoxifylline, tocopherol, vaginal sildenafil citrate and intrauterine perfusions with granulocyte-colony stimulating factor (6-9). However, even with the use of these therapies, the endometrium of some patients still remained unresponsive, frozen–thawed embryo transfer (FET) cycles have to be cancelled repeatedly, or embryo implantations are failed. Effective treatment for thin endometrium is still a major challenge that has not been solved, and new therapeutic approaches for increasing endometrial thickness are urgently required.

In women at reproductive age, the endometrium undergoes repeated stripping and bleeding during the menses and can be built up without scarring in subsequent cycles (10). The regenerative capacity of the endometrium suggests that stem cells might play crucial roles in uterine homeostasis and regeneration (11). Previous research has identified the presence of epithelial- and stromal-derived stem cells in the human endometrium (12). Loss of endometrial stem cells might be responsible for the regeneration failure and adhesion formation in patients with AS (13). Basic and clinical studies on the application of stem cells to treat intrauterine adhesions are well under way (14-24). Our team demonstrated that stem cells could restore injured endometrium and improve fertility of the endometrial injury mice, which was partially attributed to angiogenesis and proliferation and macrophage immunomodulation induced by SCs (25-27). These led us to further explore the use of stem cells to treat refractory thin endometrium (≤ 5.5mm) caused by AS in clinic. Compared with other existing clinical studies (17-24), the characteristics of this study were that all patients included had no history of intrauterine adhesions at the time of enrollment, and endometrial thickness was still less than 5.5mm after traditional treatment and adjuvant treatment.

How to transplant stem cells is an important issue to be solved in clinical applications. Tissue engineering, which involves the use of living human cells on appropriate scaffolds for the repair and reconstruction of various tissue injuries and defects, has provided a new and reliable strategy for the transplant of SCs and has been widely recognized by the medical community (28). Collagen-based materials, with good cytocompatibility, can significantly improve the retention and survival of transplanted cells in damaged and necrotic sites and can induce dermal regeneration in situ (29, 30). Our previous studies have confirmed that collagen scaffold/umbilical cord mesenchymal stem cells (CS/UC-MSCs) could facilitate endometrial regeneration and restore fertility in rodents (25). Here, we investigated whether transplantation of CS/UC-MSCs could expand the endometrium of patients with AS who were unresponsive to conventional treatments and thereby enhance embryo implantation and gestation.

## Materials And Methods

# Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Stem Cell Clinical Research Institution of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (No. 20180801-1) and conducted at the Reproductive Medicine Center of Sir Run Run Shaw Hospital from February 2019 to April 2021. All the patients signed informed consents. This study was registered on ClinicalTrials.gov (NCT03724617).

# Patients

Patients were recruited from the Reproductive Medicine Center of Sir Run Run Shaw Hospital from February 2019 to April 2020. The inclusion criteria were: (1) age 20–40 years; (2) infertile patients who had received assisted reproduction treatment and had frozen embryos in store; (3) patients who underwent at least two rounds of hysteroscopic adhesiolysis (HSA) and the uterine cavity returned to normal; (4) women whose ET failed to expand beyond 5.5 mm with the use of 6–8 mg daily estradiol valerate, combined with at least one round of treatment with aspirin, granulocyte colony-stimulating factor (G-CSF), heparin, vaginal sildenafil or Chinese traditional medicine. Patients were excluded from recruitment if they had any of the following issues: (1) those who could not agree to the follow-up conditions required by the study; (2) contraindications for hysteroscopic surgery and estrogen therapy; (3) congenital uterine malformations, adenomyosis or uterine fibroids that could impair embryo implantation; (4) chromosomal abnormalities; (5) systemic diseases such as thrombosis, cardiopulmonary diseases, hematopoietic diseases and malignant tumors; and (6) no desire to be pregnant.

# Study Design and Power Calculation

This study was prospective with each patient serving as her own control. We assume that the mean endometrial thickness would increase from 5 mm to 7 mm with a standard deviation (SD) of ± 1.2 mm. Accepting type I errors (α) of 0.05, and type II errors (β) of 0.20 and assuming that the dropout rate would be 20%, the sample size should be at least 17.

### Isolation, Identification and differentiation of UC-MSCs

The UC-MSCs were of clinical grade, as recognized by the National Institutes for Food and Drug Control (Report number SH201702375, Supplemental Table 1) according to Chinese regulations and were provided by Zhejiang Gene Stem Cell Biotech Co. Ltd. Fresh umbilical cords (UC) of normal term fetuses (maternal hepatitis B, hepatitis C virus, human immunodeficiency virus, syphilis and other related infectious indicators are negative), were collected under sterile conditions, soaked in DMEM/F12 medium (corning cellgro, USA, NO. 10-092-CVR), and transported on ice to the cell preparation laboratory within 48 hours. UC tissue were washed with saline to remove blood stains. Residual blood, capsule and blood vessels were removed and the remaining tissue cut into about 4$mm^3$ pieces. Tissue fragments were inoculated in a petri dish, cultured in DMEM/F12 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA), and then placed in an incubator at 37°C with 5% $CO_2$ for 7 days. After 7 days of culture, we observed that the cells had crawled out under an inverted microscope (Olympus, Japan) and continued the culture to the 14th day. When the cell density reaches 80%-90%, the primary cells were passaged and resuspended by serum-free culture medium (ScienCell, Carlsbad, CA, USA, Cat. No. 7511) for use.

Flow cytometry was performed to identify the phenotype of UC-MSCs (Supplemental Figure 1A). Briefly, Cells were fixed with 4% PFA for 15 minutes at room temperature and blocked with 2% bovine serum albumin (BSA, Meilun Biological Technology, #MB4219, Dalian, P. R. China). The cells were stained with primary antibodies, followed by secondary antibodies diluted in PBS plus 2% BSA, Stained cells were analyzed with flow cytometer (BD) and analyzed using Cellquest pro software. The primary antibodies Anti-human CD105 (BD, 560839), anti-human CD73 (BD, 550257), anti-human CD34 (BD, 555822), anti-human CD45 (BD, 5554882), anti-human CD90(BD, 555595), anti-HLA-DR(BD,555560) and the secondary antibodies FITC mouse (BD, 555748), PE mouse (BD, 555749) were used.

For osteogenic and adipogenic differentiation, 1 × $10^5$ cells/well UC-MSCs were digested by trypsin, resuspended by fresh MSC culture medium and seeded into plates. MSC culture medium was replaced by adipogenic differentiation medium or osteogenic differentiation□BD□A10072-01 and

A1007001, respectively, prepared as instruction manual told at 100% confluence. Fat vesicles and calcium deposition could be observed 3 weeks later (Supplemental Figure 1B).

## Fabrication of CS/UC-MSCs

The CS/UC-MSCs were fabricated as follows: 4cm ´ 6cm collagen scaffolds with pores of 20–200 mm in diameter (Zhenghai Biotechnology Co., Shandong, P. R. China) were rinsed with serum-free MSC culture medium (ScienCell, Carlsbad, CA, USA, Cat. No. 7511); excess fluid was aspirated, and a suspension of $1 ´ 10^7$ UC-MSCs (1 mL) was dripped uniformly onto the scaffold. The seeded scaffolds were incubated under humid 5% $CO_2$ in air at 37 °C for 1 h before transplantation.

## Hysteroscopic Transplantation of CS/UC-MSCs

The CS/UC-MSC scaffolds were aspirated into a 10 F Foley catheter and placed into the uterine cavity. After being placed in the uterine cavity, a balloon filled with 3 mL sterile saline was inserted to assist the scaffold in attaching to the inner wall of uterine cavity. B-ultrasonography confirmed that the scaffold had adhered to the uterine wall. The patient was kept in the hospital for 2 hours after this procedure to observe vital signs. The balloon was left in place for 3 days before removal. Antibiotics were used to prevent infection in all patients 6 days after surgery.

## Study Procedure

The study procedure is outlined in the flow chart shown in Figure 1A. Specifically, hysteroscopic CS/UC-MSC transplantation was performed twice by the same gynecologist in two consecutive menstrual cycles. We observed the uterine cavity and whether the collagen scaffold had degraded using a third hysteroscopy 1 month after these two transplant procedures. The following month, patients were invited to undergo hormone replacement therapy at a dose of 6 mg/d estradiol valerate for 12 days and the 3-day progesterone use in patients whose embryos were frozen on the third day before transfer, the embryos were thawed and transferred on the following day. We collected endometrial biopsy specimens at the same location of the uterus at the first and third hysteroscopies. Endometrial receptivity (ER) assessed by transvaginal ultrasonography was compared before and after treatment at day 3 of progesterone administration for HRT.

## Follow-up and Data Collection

Patient follow-up was performed either in the clinic or by telephone consultation, ending in April 2021. Any surgical complications (e.g., uterine perforation or anesthesia accidents), and the patient's body temperature and hemograms before and after the transplantation were recorded. The hemograms, and liver and kidney function test results were recorded 1 week after the operation. All patients were followed up to determine whether there was any tumor formation.

The primary outcome was the ET measured on the day of starting progesterone before, and 3 months after surgery. Secondary outcomes included ER, pregnancy outcomes and endometrial histology. Ultrasonography was performed to evaluate uterine receptivity with a 5–9 MHz endovaginal probe using a GE Voluson E10 (GE Medical Systems, Milwaukee, WI, USA) by the same expert examiner at day 3 of progesterone administration during HRT cycles. The evaluation indicators for ER in this study mainly included: (1) endometrial thickness; (2) endometrial volume; (3) endometrial and sub-endometrial blood flow, which were observed and classified using the Applebaum classification (31); (4) uterine artery hemodynamic parameters, such as pulse index (PI), resistance index (RI) and systolic peak velocity/diastolic peak velocity ratios (S/D), which were measured as reported(32).

Pregnant women were followed up until the end of pregnancy, during which fetal conformation and aneuploidy screening and routine prenatal examinations were performed. Any placental complications were monitored by ultrasonography during pregnancy. Endometrial biopsies obtained before and at 2 months after treatment were stained for CD34, Ki67 antigen, estrogen receptor alfa (ERa) and the progesterone receptor (PR). Endometrial angiogenesis was measured as microvascular density (MVD, stained with CD34) as described (27). Endometrial proliferation and responsive sensitivity to hormones was reported by quantification of positive staining of Ki67, ERa and PR respectively.

## Histological Analysis

Samples used for this study were human endometrial formalin-fixed and paraffin wax-embedded biopsies obtained before and after treatment. All biopsies were taken during the proliferative phase. Hematoxylin and eosin (H&E) staining, and Immunohistochemistry were performed as described(27). The primary antibodies used in this study included CD34, Ki67, ERa and PR (Abcam, Cambridge, MA, USA). Images were captured and analyzed by microscopy (BX40, Olympus Optical Corporation, Tokyo, Japan). A semi-quantitative grading system (H-score) was used to evaluate the intensity and percentage of staining. This was calculated as: H-score = ΣPi (i + 1), where i indicates the intensity of staining with a

value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi stands for the percentage of stained cells in the whole image, with intensity ranging from 0% to 100%.

## Statistics

Statistical analysis was performed using GraphPad PRISM software (v. 7.04; La Jolla, CA, USA). Student's *t* test or the Mann–Whitney nonparametric *U* test were used for continuous variables. Fisher's exact test was performed for comparing categorical variables. A two-sided *P* value of <.05 was considered statistically significant.

# Results

## Participants and Baseline Characteristics

Between February 2019 and November 2019, after preliminary evaluation of 96 patients diagnosed with thin endometrium in our reproduction center, 77 of whom did not meet the enrollment criteria. Then 19 patients were enrolled in the study (Figure 1B) meeting the prescribed inclusion and exclusion criteria. Seventeen patients completed the study for analysis, because one declined to participate, and one patient withdrew from the study because of massive recurrent abnormal vaginal bleeding. All the 17 included patients had experienced an average of 3.2 attempted hysteroscopic surgeries. Specifically, four patients experienced two hysteroscopic attempts, eight had three, two had four, and three had five. The mean age of patients was 34.1 ± 3.6 years. The mean duration of infertility was 4.2 ± 2.5 years (range 1–12). Thirteen patients presented with hypomenorrhea, two had amenorrhea, and two patients had normal menstrual histories (Table 1).

## Adverse Events and Safety Assessment

To assess the safety of SC therapy, we determined surgical complications, local and systemic safety issues after treatment. None of the patients had surgical complications such as postoperative fever after surgery. So far, no patients have developed tumors during the follow-up period. All patients had normal hemograms with average leukocyte counts of 6.69 ± 1.22 $10^9$/L, lymphocyte counts of 2.37 ± 0.46 $10^9$/L, neutrophils 54.76 ± 6.74% and normal liver and kidney functions at 7 days after surgery (Supplemental Table 2). One patient withdrew from the study because of massive recurrent abnormal uterine bleeding after the first CS/UC-MSCs transplant. This patient had no postoperative infection and the symptoms improved with norethindrone tablets. Therefore, anovulatory abnormal uterine bleeding cannot be ruled out. After multidisciplinary discussions among our team, we concluded that there was no direct evidence that this patient's abnormal uterine bleeding was related to the SC therapy.

## Improvement in Endometrial Receptivity after Implantation with CS/UC-MSCs

Although our patient had no recurrence of adhesions at the time of enrollment, the endometrium was scarred due to repeated intrauterine adhesions and did not have a clear three-line structure under ultrasound. The endometrial thickness did not expand to 5.5 mm after many times of hormone replacement and adjuvant therapy. Endometrial thickness has been generally considered as an important component of endometrial receptivity (33, 34). In our study, the mean endometrial thickness increased from 4.11 ± 1.01 mm to 5.87 ± 0.77 mm after CS/UC-MSCs therapy. The difference was statistically significant ($P$<0.001, Figure 2). The endometrial thickness from 12 patients exceeds 5.5 mm after treatment and 8 patients exceeds 6 mm. Under the first hysteroscopy, the uterine cavity of all enrolled patients appeared normal, while most of the endometrium was thin and looked rough with some scar formation. After 2 months, the morphology of the endometrium looked better, with a hairy appearance (Figure 3).

The spiral artery of the uterus is the main blood vessel that nourishes the endometrium which characterized by low resistance blood flow spectrum. To a certain extent, the values of PI, RI, and S/D of the spiral artery of the uterus reflect the resistance of the nourishing arterial bed after implantation of the pregnant egg. The lower the value, the higher the viability of trophoblast cells (32). In our study, S/D measures of the uterine artery dropped from 8.03 ± 2.31 to 6.53 ± 1.21, indicating that the blood flow resistance of the uterine artery decreased after treatment, and the ability of endometrium to accept embryos increased. While PI and RI presented no significant improvement before and after the SC therapy (Table 2).

## Pregnancy Outcomes

By the end of December 2020, 15 of the patients had undergone 22 FETs. Three of these patients become pregnant, of whom two had delivered live babies with no obvious birth defects and without placental complications, and one had a spontaneous abortion at 25+ weeks. One of the two patients who did not undergo FET became pregnant naturally and was in the third trimester of pregnancy at the time of writing.

## Improvements in Endometrial Proliferation, Angiogenesis and Hormonal Responses

SCs can promote the proliferation of endometrial epithelial and stromal cells, thereby upregulating the expression of ERa and PR, which in turn further promote endometrial cell proliferation and vascular reconstruction(35). Therefore, we compared MVD (stained with CD34), and the expression of Ki67, ERa, and PR of endometrium in patients before and after treatment. These were all increased (Figure 4), indicating that SCs transplantation promoted proliferation and angiogenesis in the endometrium, and enhanced its biological response to hormones.

## Discussion

In our study, 17 infertile patients with unresponsive thin endometrium caused by AS underwent transplantation with CS/UC-MSCs and were followed up for two years. There was a significant increase in endometrial thickness after the therapy. In addition, transplantation with CS/UC-MSCs could increase MVD and the expression of Ki67, ERα and PR in endometrium, indicating that the possible mechanism of such therapy is to increase endometrial angiogenesis, proliferation and differentiation (Figure 5).

Our main outcome measure was an increase in ET. All 17 patients showed increased ET from a mean of 4.11 mm to 5.87 mm. The ET of patient #6 showed the biggest increase of 3.8 mm, from 2.3 mm to 6.1 mm. Although this patient was not pregnant, this increase of the endometrium was meaningful considering that our patients all had refractory thin endometrium and did not respond to conventional treatments. The secondary outcomes included endometrial receptivity, changes in endometrial biological indicators and pregnancy outcomes. There were improvements in endometrial volume, endometrial-sub-endometrial vascularization and uterine artery blood flow before and after therapy, with no statistical difference. The major reason is probably due to the insufficient sample size. In this study, only 12 patients received ultrasound examination on the 3rd day of progesterone-based HRT use. On the other hand, ultrasonography itself has limitations in evaluating endometrial function, and is only suitable for guiding embryo transfer in a very favorable uterus or for cancelling it in extremely poor cases(36). In addition, endometrial MVD and the expressions of Ki67, ERa and PR after treatment were up-regulated, indicating that SCs transplantation promoted proliferation and angiogenesis in the endometrium, and enhanced the biological response of the endometrium to hormones, consistent with our previous studies (25, 27). Functional repair of the endometrium is mainly reflected in pregnancy outcome. In this study, 4 of the 17 patients became pregnant, of which 2 babies was born, 1 was ongoing pregnancy and 1 had a miscarriage. Considering the different inclusion criteria from other studies, the pregnancy rate and the live birth rate are not comparable. In our study, the patient's uterine cavity does not have uterine adhesions, but the thickness has never exceeded 5.5mm after repeated hormone supplements combined with other adjuvant treatments before stem cell therapy. In view of this, the pregnancy rate (24%) and live birth rate (12%) are greatly improved.

In 2011, Nagori et al. reported the first case of AS treated with adult autologous SCs via intrauterine infusion for endometrial regeneration that resulted in conception after in vitro fertilization and embryo transfer (IVF–ET) (17). Eight clinical studies from six research centers have explored the effect of SCs in treating intrauterine adhesions and thin endometrium (17-24), of which one was a case report and seven were prospective studies (Table 3). What distinguishes our study from other studies is the inclusion of patients. In our study, all the patients we included have no intrauterine adhesions at the time of enrollment and no history of tuberculosis (TB) infection, with thin endometrium (≤ 5.5 mm) after conventional treatment adjuvant therapy. In detail, In four of seven prospective studies, transplantation of SCs followed surgery to separate adhesions, which might have enhanced growth of the endometrium, so it is impossible to distinguish the respective roles of surgery and SC therapy (19, 21-23). Therefore, our results better reflect the therapeutic effect of SCs, and avoided the false positives possibly caused by uterine surgery. Moreover, genital TB was the most common etiology of treated patients in two studies by Singh et al.; all three pregnancies were in the group that underwent D&C and none in the TB groups. Therefore, whether the inadequate growth of endometrium seen in those two studies was because of a history of endometrial TB cannot be confirmed (18, 23).

Another difference was in the way SCs were administered, although they were all transplanted locally into the uterus. In the above trials, three transplantation methods such as intrauterine perfusion, intravenous injection and basal layer injection were used as shown in Table 3. The shortcomings of intrauterine perfusion are that the SC suspension is easy to lose, with low retention and survival rates, so the long-term treatment effect is not ideal. Intravenous injection of SCs not only lowers the utilization rate, but also has greater side effects with safety risks. We also found that the number of SCs homing on the uterus is relatively small through the injection of SCs via the tail vein in a mouse model (27). The basal injection of SCs is difficult to verify, and the risk of secondary adenomyosis is higher. Compared with modes of administration such as intrauterine infusion and via uterine spiral arterioles. Collagen scaffold with a three-dimensional structure which can guide cells to grow into the scaffold has good histocompatibility, no inflammation and no immune rejection. What's more, collagen itself can promote vascularization, tissue regeneration and wound repair, and degrade simultaneously with the reconstruction of new tissue. Therefore, transplantation with CS/UC-MSCs is not only simple and easy to implement, but also reduces the loss of SCs and promote the tissue regeneration.

Finally, different types of SCs were used among these studies. As shown in Table 4, bone marrow mesenchymal SCs (BMSCs) were used in five studies, menstrual endometrial SCs (MenSCs) were applied in one study, UC-MSCs were used in one study, and adipose tissue stem cells (ASCs) were used in one. Although BMSCs have been used widely, their extraction is invasive and trauma is unavoidable, as is the use of ASCs. As for MenSCs, the number available is limited because of the thin endometrium and they are easily contaminated, which might lead to endometrial

inflammation, bleeding and abdominal pain. The use of SCs from umbilical cords, as a “waste product” of birth, can avoid such ethical limitations. UC-MSCs, with a wide range of sources, not only have a strong ability for self-renewal and multidirectional differentiation potential, but also have unique immune characteristics and capacity for repairing tissue damage. Therefore, they have been used widely to study the treatment of various diseases(37).

According to the above, although this study has some limitations such as lack of control and a small sample size, we can conclude that the collagen scaffold combined with umbilical cord mesenchymal stem cells can effectively promote the growth of unresponsive thin endometrium through this prospective self-control study. Randomized controlled studies to assess the application of CS/UC-MSCs in unresponsive thin endometrium will be further studied.

## Conclusions

In summary, our work describes that transplantation of CS/UC-MSCs could promote the growth of unresponsive thin endometrium caused by AS, possibly through promoting endometrial proliferation and angiogenesis and enhancing the response of endometrium to hormones.

## Abbreviations

CS/UC-MSCs: collagen scaffold/umbilical cord mesenchymal stem cells

ET: endometrial thickness

AS: Asherman syndrome

FET: frozen–thawed embryo transfer

SCs: stem cells

HRT: hormone replacement therapy

HSA: hysteroscopic adhesiolysis

G-CSF: granulocyte colony-stimulating factor

SD: standard deviation

ER: endometrial receptivity

PI: pulse index

RI: resistance index

S/D: systolic peak velocity/diastolic peak velocity ratios

ERa: estrogen receptor alfa

PR: progesterone receptor

MVD: microvascular density

PBS: phosphate-buffered saline

IVF–ET: In vitro fertilization and embryo transfer

D&C: dilatation and curettage

TB: tuberculosis

BMSCs: bone marrow mesenchymal stem cells

ASCs: adipose tissue stem cells

MenSCs: menstrual endometrial stem cells

## Declarations

### Study funding/Competing interests

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### Author Contributions

YZ and LS recruited and followed up the patients, obtained patient information and samples, analyzed the data, and wrote the article. XL and FZ performed embryo transfer. LX help to prepare CS/UC-MSCs scaffold. WX performed hysteroscopic surgery. HY and JL helped to collect patients' information. MP performed 3D color Doppler ultrasound. YP and YD supervised the study and revised the paper. YZ interpreted the data. JS and ML was responsible for the preparation and transportation of stem cells. LZ help to acquire data. SZ supervised and conceived the study, and critically revised the manuscript. All authors have read and approved the final manuscript.

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### Declarations

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of Stem Cell Clinical Research Institution of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (No. 20180801-1)

### Consent for publication

Consent for publication was also obtained from every patient.

### Availability of data and materials

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

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## Tables

Table 1. Clinical characteristics and outcome of patients.

| Patient | Age (years) | Symptoms | Etiology of AS (times) | Prior repair attempts | Score of AS at 1st HSA | Previous treatment received | ET (pre-/post therapy mm) | Pregnancy outcome |
|---|---|---|---|---|---|---|---|---|
| P1 | 39 | Infertility (3 years)<br>Hypomenorrhea | 5 D&C | 3 HSA | AS Stage II | Estrogen/Aspirin/Heparin/GH /Sildenafil/traditional Chinese medicine | 5.5/7.1 | 2 FET and implantation failure |
| P2 | 33 | Infertility (3 years)<br>Hypomenorrhea | 1 D&C | 3 HSA | AS Stage III | Estrogen/Aspirin/Heparin/ /Sildenafil | 3/6.1 | 1 FET and implantation failure |
| P3 | 30 | Infertility (4 years)<br>Hypomenorrhea | 1 HSP<br>1 D&C | 5 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/GH /Sildenafil/G-CSF/GM-CSF/traditional Chinese medicine | 5.1/6.6 | 3 FET and implantation failure |
| P4 | 37 | Infertility (3 years) | 6 D&C | 2 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/GH /G-CSF | 5.1/5.9 | 1 FET and implantation failure |
| P5 | 34 | Infertility (3 years)<br>Hypomenorrhea | 2 D&C | 2 HSA | AS Stage II | Estrogen/Aspirin/traditional Chinese medicine | 5.2/5.8 | 1 FET 4 months post treatment and cesarean section at 35+5 weeks girl,1950g |
| P6 | 34 | Infertility (6 years)<br>Hypomenorrhea | 2 D&C | 5 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/GH /Sildenafil | 2.3/6.1 | 1 FET and implantation failure |
| P7 | 35 | Infertility (4 years)<br>Hypomenorrhea | 4 D&C | 4 HSA | AS Stage II | Estrogen/Aspirin/Heparin/GH/G-CSF | 5.1/6.4 | 2 FET and implantation failure |
| P8 | 35 | Infertility (4 years)<br>Hypomenorrhea | 1 D&C | 3 HSA | AS Stage III | Estrogen/Aspirin/Heparin/ | 4.5/6.6 | 1 FET 2 months post treatment and abortion at 25+ weeks |
| P9 | 39 | Infertility (6 years)<br>Hypomenorrhea | 1 D&C | 3 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/GH /Sildenafil | 3.7/5 | 2 FET and implantation failure |
| P10 | 30 | Infertility (1 year)<br>Hypomenorrhea | 4 D&C | 2 HSA | AS Stage III | Estrogen/Aspirin/Heparin /Sildenafil | 3.5/4.5 | Spontaneous pregnancy 9 months<br>post treatment and 30 weeks pregnant |
| P11 | 37 | Infertility (3 years)<br>Hypomenorrhea | 3 D&C | 2 HSA | AS Stage IV | Estrogen/Aspirin/Heparin | 4.5/- | No FET |
| P12 | 36 | Infertility (4 years)<br>Hypomenorrhea | 3 D&C | 3 HSA | AS Stage III | Estrogen/Aspirin | 3.4/5.8 | 1 FET and implantation failure |
| P13 | 31 | Infertility (5 years)<br>Hypomenorrhea | 2 D&C | 3 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/Sildenafil/G-CSF | 4.9/6.4 | 2 FET and implantation failure |
| P14 | 33 | Infertility (5 years) | 8 D&C | 4 HSA | AS Stage III | Estrogen/Aspirin/Heparin /Sildenafil | 4.2/5.9 | 2 FET and implantation failure |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Hypomenorrhea | | | | | | |
| P15 | 26 | Infertility (5 years)<br>Amenorrhea | 2 D&C | 5 HSA | AS Stage VA | Estrogen/Aspirin/Heparin/Sildenafil | 2.5/4.7 | 1 FET and implantation failure |
| P16 | 37 | Infertility (2 years)<br>Hypomenorrhea | 1 D&C | 3 HSA | AS Stage IV | Estrogen/Aspirin/Heparin | 3.5/- | Withdrew |
| P17 | 32 | Infertility (1 years)<br>Amenorrhea | 1 D&C | 3 HSA | AS Stage Va | Estrogen/Aspirin/Heparin | 4.4/6.3 | 1 FET 3 months post treatment and 40+1 weeks, girl, 2900g |
| P18 | 39 | Infertility (12 years) | 1 D&C | 3 HSA | AS Stage IV | Estrogen/Aspirin/Heparin/G-CSF | 3/4.7 | 1 FET and implantation failure |

D&C, dilatation and curettage; HSP, hysteroscopic polypectomy; HSA, hysteroscopic adhesiolysis; AS, Asherman, syndrome; ET, endometrial thickness; GH, growth hormone; G-CSF, granulocyte colony stimulating factor; FET, frozen–thawed embryo transfer

**Table 2. Evaluation of endometrial receptivity parameters.**

| Parameters | Pre-treatment (*n* = 12) | Post-treatment (*n* = 12) | P value |
|---|---|---|---|
| Endometrial volume | 1.00±0.32 | 1.12±0.56 | P=0.52 |
| Subendometrial blood flow | | | P=0.21 |
| Sparse | 6 | 6 | |
| I | 6 | 3 | |
| II | 0 | 3 | |
| Endometrial blood flow | | | P=0.25 |
| Sparse | 9 | 7 | |
| I | 3 | 2 | |
| II | 0 | 3 | |
| PI | 2.66±0.50 | 2.57±0.58 | P=0.70 |
| RI | 0.87±0.05 | 0.86±0.06 | P=0.63 |
| S/D | 8.03±2.31 | 6.53±1.21 | P=0.06 |

PI, average value of left and right pulse index; RI, average value of left and right resistance index; S/D, average value of left and right systolic peak velocity/diastolic peak velocity ratios.

**Table 3. Summary of research on human stem cell therapy for thin endometrium.**

| Author | Year | Number of patients | Type of stem cell | Intervention | Method of stem cell administration | ET (pre-/post therapy) | Pregnancy outcome |
|---|---|---|---|---|---|---|---|
| Nagori et al. | 2011 | 1 AS | Auto-BMSCs | Stem cell therapy | intrauterine infusion | 3.2/7.1 | 8 weeks |
| N Singh et al. | 2014 | 6 AS (5/6 genital TB) | BM-MNCs | stem cell therapy | subendometrial zone injection | 1.38/4.05/5.46/5.48 | N/A |
| X Santamaria et al. | 2016 | 11 AS<br>5 EA | Auto-CD133+BMSCs | HSA + stem cell therapy | uterine spiral arterioles | IUA improve obviously.<br>EM for AS:4.3/6.7<br>EM for EA:4.2/5.7 | 2 babies born,<br>2 ongoing pregnancy<br>2 miscarriage<br>1 ectopic<br>3 biochemical pregnancy |
| Jichun Tan et al. | 2016 | 7 AS | Auto-MenSCs | stem cell therapy | Intrauterine infusion | EM :3/7 | 2 babies born,<br>1 ongoing pregnancy |
| Guangfeng Zhao et al. | 2017 | 5 AS | Auto-MNCs | HSA + stem cell therapy | loaded onto a collagen scaffold | IUA improve obviously.<br>EM:4.5/7.2 | 5 babies born |
| Yun Cao et al. | 2018 | 26 AS | UC-MSC | HSA + stem cell therapy | loaded onto a collagen scaffold | IUA score:9.12/5.52<br>EM:4.46/5.74 | 8 babies born,<br>1 ongoing pregnancy<br>1 miscarriage |
| Se Yun Lee et al. | 2019 | 6 AS | Auto-ADSCs | stem cell therapy | Intrauterine infusion | EM:3.0/6.9 | 1 miscarriage |
| N Singh et al. | 2020 | 12 AS (9/12 genital TB)<br>13 EA (6/13 genital TB) | BM-MNCs | HSA + stem cell therapy | Subendometrial zone injection | IUA improve.<br>EM for AS:2.6/4.2/4.6<br>EM for EA:3.6/5.9/6.5 | 3 babies born |

## Figures

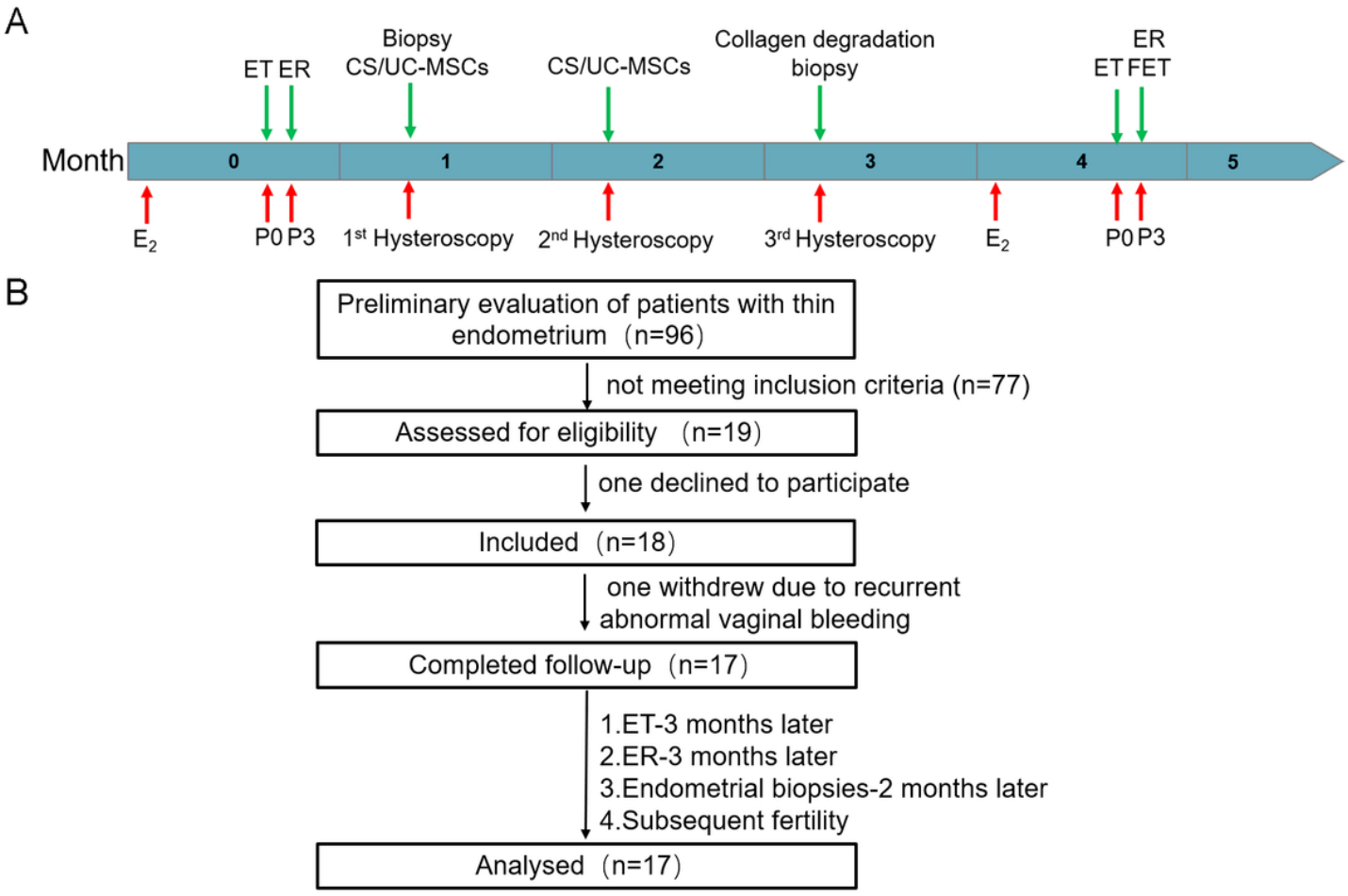


**Figure 1**

Study design and flow chart showing the patient enrollment. A. Flow chart showing the study procedure. B. flow chart showing the patient enrollment. CS/UC-MSCs, collagen scaffolds/umbilical cord mesenchymal stem cells; ET, endometrial thickness; ER, endometrial receptivity; FET, frozen–thawed embryo transfer; P0, on the day of starting progesterone-based HRT.

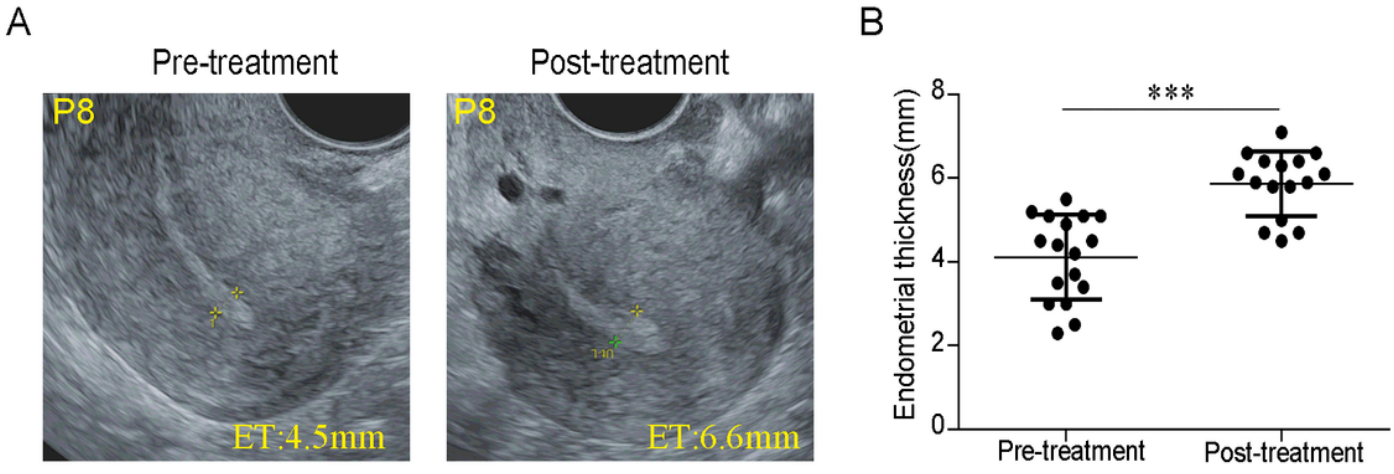


**Figure 2**

Improvement of endometrial receptivity after implantation with CS/UC-MSCs. A. Transvaginal ultrasonography of the uterus before and after transplantation of CS/UC-MSCs in Patient #8. B. Endometrial thickness before and after transplantation of CS/UC-MSCs. Results are shown as the mean ± S.D., ***P<.001. S.D., standard deviation.

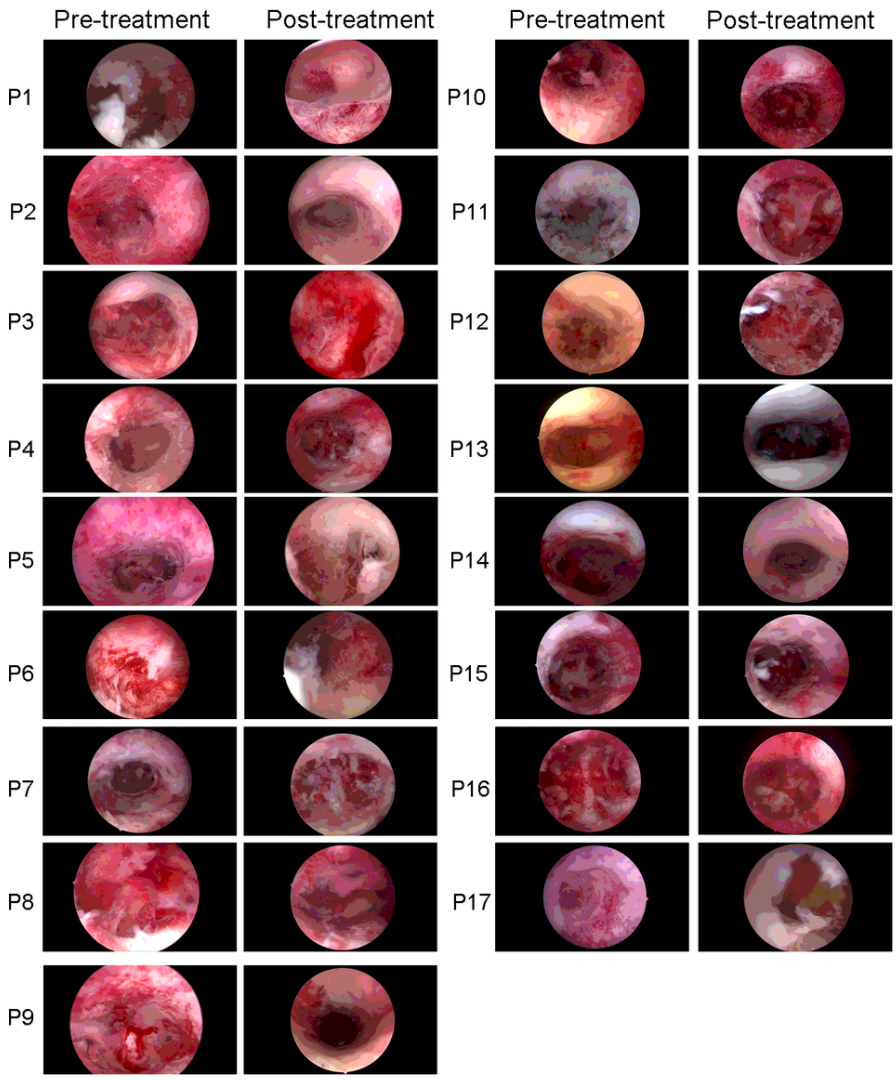


**Figure 3**

Hysteroscopy images from all 17 patients before and after CS/UC-MSCs treatment.

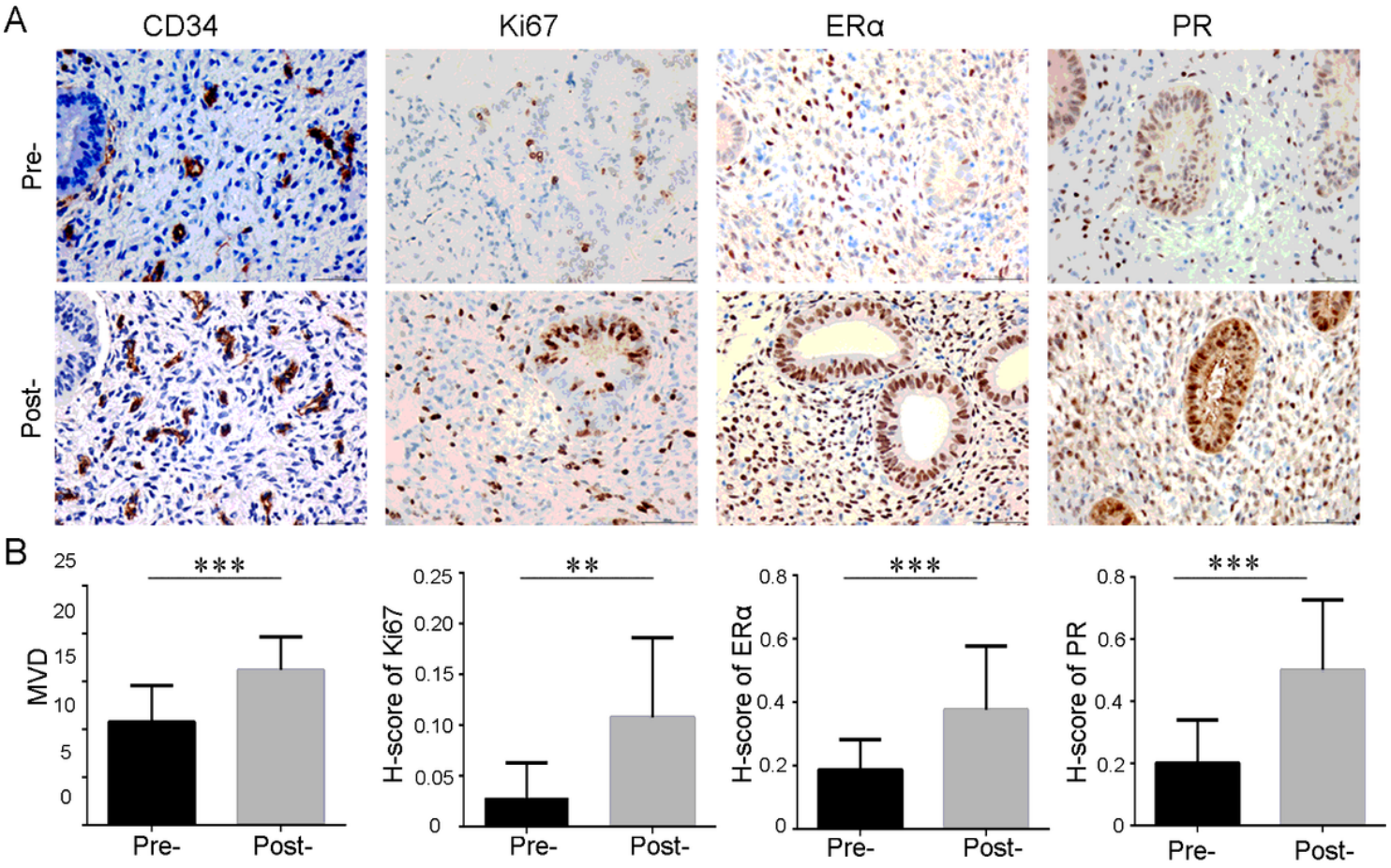


Figure 4

Immunohistochemical staining of CD34, Ki67, ERα and PR on endometrial biopsy samples obtained from patients before and after CS/UC-MSC treatment. MVD was determined by CD34 immunostaining. Scale bar = 50 μm. **P<.01; ***P<.001; ERα, estrogen receptor alpha; PR, progesterone receptor. Pre-, pre-treatment; Post-, Post-treatment.

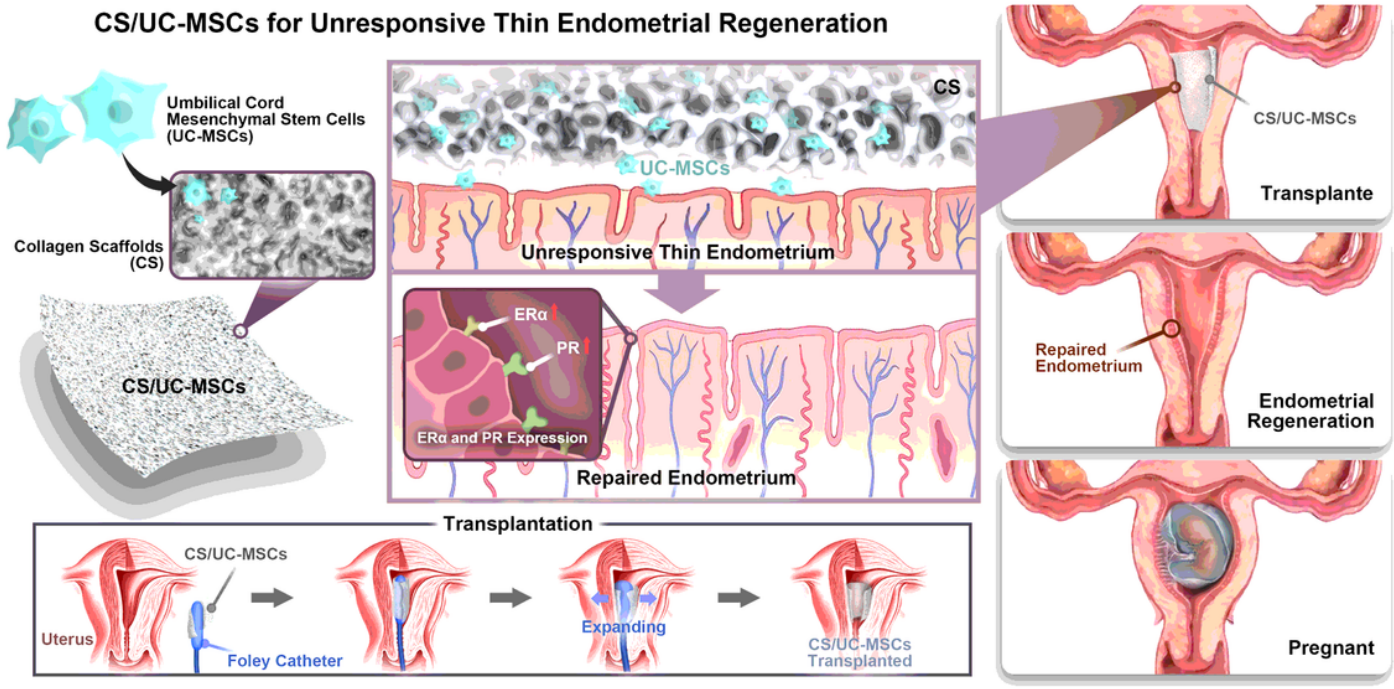


Figure 5

Schematic illustration of applying CS/UC-MSCs for endometrial regeneration and fertility restoration.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure1.tif
- Supplementaltable1.docx
- Supplementaltable2.docx