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ORIGINAL ARTICLE *Infertility*

Autologous menstrual blood-derived stromal cells transplantation for severe Asherman's syndrome

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STUDY QUESTION: Does autologous transplantation of menstrual blood-derived stromal cells (menSCs) regenerate endometrium to support pregnancy in patients with severe Asherman's syndrome (AS)?

SUMMARY ANSWER: Autologous menSCs transplantation significantly increases endometrial thickness (ET) for women with severe AS.

WHAT IS KNOWN ALREADY: AS is a major cause of secondary infertility in women. Cell transplantation has been tried in a few clinical cases with encouraging results.

STUDY DESIGN, SIZE, DURATION: In this experimental, non-controlled and prospective 3-year clinical study involving seven patients with AS, autologous menSCs were isolated and cultured from menstrual blood of each patient within ~2 weeks and then transplanted back into their uterus. Endometrial growth and pregnancy were assessed after cell therapy.

PARTICIPANTS/MATERIALS, SETTING, METHOD: Infertile women, aged 20–40 years, diagnosed with severe AS (Grade III–V) by hysteroscopy and with menstrual fluid were recruited at the Shengjing Hospital affiliated to China Medical University. Autologous menSCs transplantation was conducted followed by HRT. Endometrial thickness was monitored with frozen embryo transfer (FET) as needed.

MAIN RESULTS AND THE ROLE OF CHANCE: We successfully cultured menSCs from seven patients and transferred the autologous cells back to their uterus. Our results showed that the ET was significantly ($P = 0.0002$) increased to 7 mm in five women, which ensured embryo implantation. Four patients underwent FET and two of them conceived successfully. One patient had spontaneous pregnancy after second menSCs transplantation.

LIMITATIONS, REASONS FOR CAUTION: Limited sample size, lack of rigorous controls or knowledge of underlying mechanism.

WIDER IMPLICATIONS OF THE FINDINGS: Autologous menSCs transplantation is a potential option for treating women with severe AS.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by Liaoning Provincial Science and Technology Program. The sponsor and authors declare no conflicts of interest.

TRIAL REGISTRATION NUMBER: Registered in the Chinese Clinical Trial Registry (ChiCTR-ONB-15007464).

Key words: Asherman's syndrome / menstrual blood-derived stromal cells / autologous transplantation / infertility / endometrial thickness

Introduction

Asherman's syndrome (AS) is a typical endometrial damage disease. It was first defined by Joseph Asherman in 1948, as intrauterine adhesions (IUAs) manifesting clinically as infertility, recurrent abortion or menstrual disturbances (Asherman, 1948; Conforti *et al.*, 2013). According to the diagnostic criteria and population demographics, the prevalence of AS varies from 2% to 22% in infertile women (Schenker and Margalioth, 1982; Yu *et al.*, 2008). The degree and localization of IUAs, referred to as fibrotic tissues adhering to the endometrial surface, determine the clinical symptoms of AS (Alawadhi *et al.*, 2014). Furthermore, IUAs are a major cause of

secondary infertility (Warembourg et al., 2015). According to the European Society of Gynecological Endoscopy (ESGE), AS is classified into Grades I–V based on hysteroscopy (Wamsteker et al., 1995). The leading cause of IUAs is pregnancy-related curettages, especially after a miscarriage that had the highest association with AS (Schenker and Margalioth, 1982). In addition, other genital tract surgeries such as cesarean section, myomectomy, hysteroscopic procedures as well as genetic susceptibility and pathological infections also contribute to the development of AS (Cenksoy et al., 2013).

Treatment of AS aims to restore uterine fertility and parturition. The traditional remedy for AS is surgical resection of adhesions combined with insertion of intrauterine devices or adhesion barriers to prevent recurrent adhesions and hormonal therapy to regenerate the endometrium (Deans and Abbott, 2010; March, 2011). However, in severe cases, the trauma goes deeply into the basal layer of the endometrium, which is resistant to hormonal interventions, resulting in high recurrence rate of IUAs (Preutthipan and Linasmita, 2000; Gargett and Healy, 2011). Some women with severe scars that resulted in genital obstructions are contraindicated for the surgery.

Severe damage to the basal layer of endometrium may cause loss of the local endometrial progenitor cells and leads to regeneration failure and adhesion formation (Gargett and Ye, 2012; Alawadhi et al., 2014; Verdi et al., 2014). Therefore, stem cell-like supplementation may represent a potential cure for severe AS.

The menstrual blood-derived stromal cells (menSCs) are adult stem cell-like cells generally comprise a mixture of MSC (mesenchymal stem cell) and stromal fibroblasts. As the name implies, menSCs are directly isolated from menstrual blood of women, which characterize its ease of noninvasive acquisition. MenSCs exhibit classical stem/progenitor cell characteristics of clonogenicity, high proliferative potential and multipotency *in vitro* (Rossignoli et al., 2013). They resemble MSC in terms of high surface expression of CD29, CD44, CD49f, CD73, CD90, CD105, CD117 and the absence of hematopoietic and endothelial markers (Meng et al., 2007), which cultured endometrial stromal cells also express. MenSCs are also positive for embryonic stem cell markers OCT-4 (Meng et al., 2007) and SSEA-3/4 (Li et al., 2013). MenSCs can be induced to differentiate into neurons (Meng et al., 2007), osteoblasts (Meng et al., 2007), chondrocytes (Kazemnejad et al., 2013), adipocytes (Meng et al., 2007), endotheliocytes (Meng et al., 2007), hepatocytes (Meng et al., 2007; Khanjani et al., 2015), cardiac myocytes (Meng et al., 2007), pulmonary epithelial cells (Meng et al., 2007), insulin-producing cells (Meng et al., 2007) and ovarian-like cells (Liu et al., 2014). Our previous results showed that menSCs can be reprogrammed to induced pluripotent stem cells (Li et al., 2013). Therefore, menSCs are promising candidates for cell-based therapy.

In this study, we investigated whether autologous transplantation of menSCs could regenerate the endometrium in women with severe AS and facilitated healthy parturition.

Materials and methods

Ethical approval

All the human samples and procedures involved in this study were approved by the Ethics Committee of Shengjing Hospital affiliated to China Medical University (2012PS09K).

Patients

Menstruating women, aged 20–40 years old, diagnosed with severe IUAs (Grade III–V) by hysteroscopy, and refractory to traditional treatments, were employed in the study. Study design is summarized in Fig. 1. Physical examinations were conducted to exclude women with active genital tuberculosis (TB). All the participants signed informed consent after fully understanding the details, possibilities of failure and procedural risks.

Preparation and identification of menSCs

MenSCs were isolated and cultured according to our previous report (Li et al., 2013). Briefly, menstrual blood samples were collected by catheter rinsed by penicillin/streptomycin from patients on Day 2 of their menses. The samples were transferred to phosphate buffered saline (PBS) containing penicillin/streptomycin and heparin. Mononuclear cells were fractionated in Ficoll (Sigma, St. Louis, MO, USA) and cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (Ham's) (1:1; HyClone, Logan, UT, USA) supplemented with 10% autologous serum (isolated from her own peripheral blood), 2 mM L-glutamine (Hyclone), 100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone). The culture medium was changed every 3–5 days, and the cells were passaged by trypsin digestion after reaching 80–90% confluence. The culture supernatant of the menSCs was collected when the medium was changed twice and sent to the clinical laboratory for testing of bacteria, fungi, lipopolysaccharides and hepatitis virus. The cells were abandoned upon detection of microbial contamination. The cells were cultured for about 14 days. MenSCs were transplanted before the third passage. All the culture in this study were conducted in the umbilical cord blood bank of Liaoning Province in Shengjing Hospital, which was certified for cell production under standard operating instructions with Good Manufacturing Practice facilities.

Flow cytometry was performed to identify the phenotype of menSCs on the day immediately before transplantation. FITC-conjugated anti-human antibodies for CD34, CD44, CD45, CD90 and CD105 as well as phycoerythrin-conjugated anti-human antibodies for CD38, CD73 and SSEA-4 (Pharmingen, San Jose, CA, USA) were used to characterize menSCs. Stained cells were analyzed with an FACScalibur™ Flow

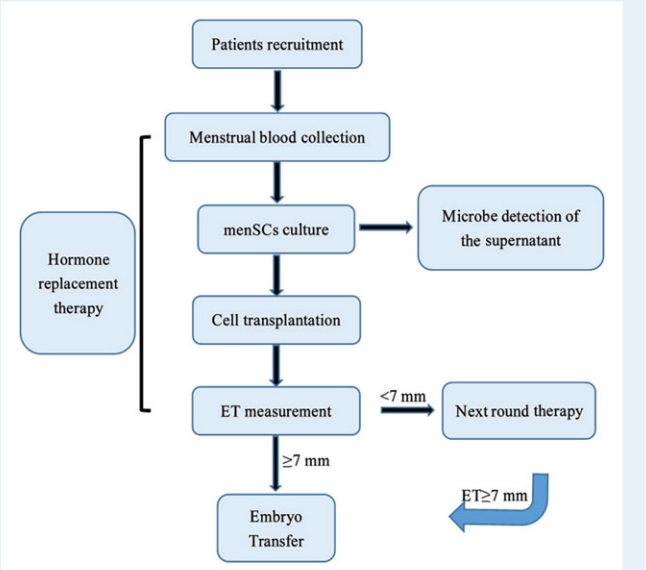

Figure 1 Study design of this research. ET; endometrial thickness.

Cytometer (Becton-Dickinson, Mountain View, CA, USA) using CellQuest software (Becton-Dickinson).

Autologous menSCs transplantation

On Day 16 of the same menstrual cycle, the patient was asked to stand-by in the operating room for menSCs transplantation. MenSCs were trypsinised, washed twice, counted and finally resuspended in sterile PBS at a concentration of 2×10^6 cells/ml. The patient was held in a lithotomy position. A mild scratch of the endometrium was done before transplantation. Then, a transvaginal probe was inserted into the cervix guided by transabdominal B ultrasound. An internal cannula (Frydman classic catheter 4.5; Laboratoire CCD, France), filled with 0.5 ml menSCs suspension, was delivered through the cervix to the fundus of the uterus to instill the menSCs. The internal tube followed by the external probe was withdrawn gently and slowly after 5 min. The patient was discharged after 2 h. The next round of cell therapy was conducted after three or more menstrual cycles if the endometrial growth was unsatisfactory.

HRT

HRT was adopted to stimulate endometrial growth. After Day 5 of menstrual blood collection, subjects were administered oral oestradiol valerate tablets of 4 mg daily for 14 days. After menSCs transplantation, the patients were treated with oral oestradiol valerate tablets of 6 mg daily for 21 days. If the endometrium thickness was <7 mm, an intra muscular injection of 40 mg progesterone was used.

Embryo transfer

When the endometrial thickness (ET) increased to 7 mm or more, revealing a triple-line appearance with sufficient subendometrial blood flow, frozen embryo transfer was performed following patient's consent. The embryos were preserved before this study in a stimulation cycle attempted for conception. Briefly two (<35 year olds) or three (≥35 years old) thawed 6- to 8-cell Grade I IVF embryos were transferred to the uterus. Luteal support was provided by i.m. administration of 40 mg progesterone twice daily after embryo transfer. Ethinyl oestradiol was continued at a dose of 6 mg daily.

Ultrasound measurement

Transvaginal ultrasound examination was performed before and intermittently on Day 16 of menstrual cycle after cell therapy to evaluate the ET. Transabdominal ultrasound was conducted to detect natural or assisted pregnancy outcome. ET was measured as the maximum distance between the myometrial interface of the anterior to the posterior wall in a median longitudinal plane of the uterus. All the data and photographs were preserved for data analysis.

Statistical analysis

Descriptive statistics and statistical analysis of study variables were carried out using GraphPad Prism 5 software (San Diego, CA, USA). Changes in ET values due to treatment were evaluated with two-tailed paired Student *t* test. Throughout the text, figures and figure legends, values of $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered as statistical significant.

Results

As shown in Tables I and II, seven infertile females with severe AS were recruited in this study. Patients were all of reproductive age (average 33.7 ± 1.5 years) with a mean infertile duration of 4.8 ± 1.2 years. History of pregnancy-related curettages including ectopic pregnancy surgery or artificial abortion was the main cause of AS. Adhesiolysis of IUAs resulted in recurrence or worsening of adhesions. Hormone therapy to stimulate the endometrial growth resulted in failure. The IUAs were assessed as Grade III–V by hysteroscopy (Fig. 2).

On Day 2 of menses, sterile menstrual blood was collected to culture menSCs. In women with normal menstrual flow, menstrual blood was collected and sufficient menSCs were cultured at once. In hypomenorrhea (bleeding lasting for <2 days), two or more cultures were performed to ensure an adequate amount of cells at one time. The cultured cells appeared typically adherent and showed a clonal cell phenotype. Flow cytometry analysis revealed that these cells were positive for CD44, CD73, CD90 and CD105 (Fig. 3). Autologous menSCs were transplanted into the uterus on Day 16.

Table I Patients information and clinical data.

Patient no.	Age (years)	Causes of AS (× times)	Infertile (years)	IUA grade	Previous treatment	Time from treatment to cell transplantation	Collected Menstrual volume (ml)
1	35	Spontaneous abortion × 2	3	III	Adhesiolysis + IUD + HRT	2 years	3
2	38	Artificial abortion × 1	12	Va	Adhesiolysis + IUD + HRT	2 years	2.5
3	39	Intestinal tuberculosis, lymphatic tuberculosis	3	Va	none	None	1.5 and 3
4	34	Hysteroscopic surgery × 2	3.5	Va	Adhesiolysis + IUD + HRT	2 years	2.5 and 3.5
5	32	Spontaneous abortion × 1, Artificial abortion × 1	4	Va	None	None	3
6	29	Artificial abortion × 1	5	Va	Adhesiolysis + IUD + HRT	2 years	3.5
7	29	Spontaneous abortion × 1, Artificial abortion × 1	3	Va	Adhesiolysis + IUD + HRT	1 years	3.5

AS, Asherman's syndrome; HRT, hormone replacement therapy; IUA, intrauterine adhesions; IUD, intrauterine device.

Table II Treatment information.

Patient no.	Transplanted menSCs number	HRT	Time of ET follow-up	Embryos transfer
1	1×10^6	oral oestradiol for 35 days	6 months	1 time
2	1×10^6	oral oestradiol for 35 days	3 months	1 time
3	1×10^6 and 1×10^6	oral oestradiol for 35 days + i.m. injection of progesterone × 2	4 months	3 times
4	1×10^6 and 1×10^6	oral oestradiol for 35 days + i.m. injection of progesterone × 2	4 months	none
5	1×10^6	oral oestradiol for 35 days	3 months	2 times
6	1×10^6	oral oestradiol for 35 days + i.m. injection of progesterone	3 months	1 time
7	1×10^6	oral oestradiol for 35 days + i.m. injection of progesterone	3 months	1 time

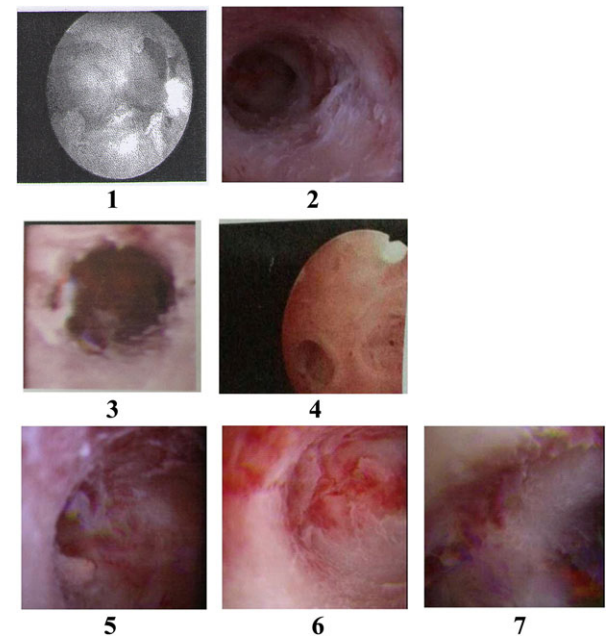

Figure 2 Diagnostic hysteroscopy of the uterus. The number under each image refers to the corresponding patient.

Before the program, patients' endometrium was extremely thin with rough morphology (Figs 4 and 5). Transplanting the menSCs into the wombs once or twice led to significant proliferation of the endometrium under estrogen stimulation. ET of five patients had attained 7–8 mm (Figs 4 and 5), which was adequate for embryo implantation (Reynolds *et al.*, 2010). Furthermore, the endometrium exhibited a smooth surface. Surprisingly, one woman (Patient 4) was naturally pregnant 3.5 months after transplantation. Embryo transfer led to successful conception in two of the other four patients; positive pregnancy twice in one (Patient 3) and developmental arrest of embryo in another (Patient 2) patient. No compilations or immune rejection was found in any of the patients. Therefore, three patients conceived after treatment either naturally or by IVF with one ongoing pregnancy.

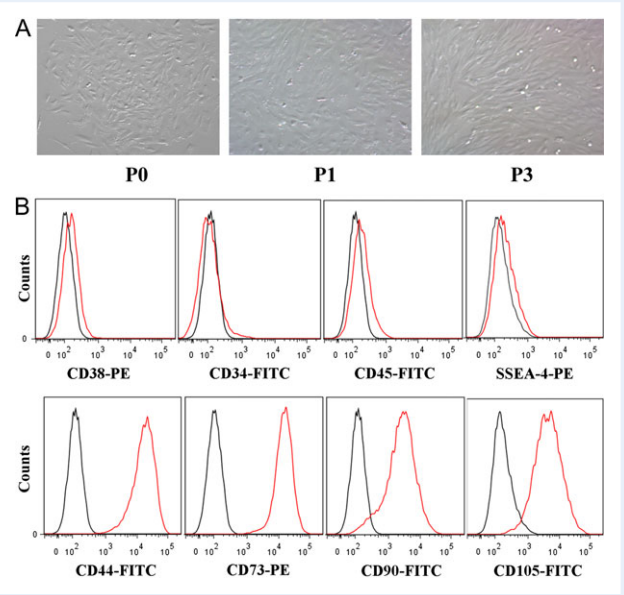

Figure 3 (**A**) Microscopic images of primary menSCs (P0), first passage (P1) and third passage (P3) (×100). (**B**) Flow cytometry of menSCs phenotype (isotype control: black histogram, specific antibody: red histogram).

Discussion

This clinical study included seven patients with severe AS; all of them showed positive results for menSCs culture. We transplanted those autologous cells back into the host uterus followed by hormonal stimulation. Our results demonstrated a significant increase in ET in all the patients. The ultrasound results suggested recovery of endometrial morphology to a normal status. In five women, the thickness of the endometrium reached 7 mm and showed a triple-line appearance. One patient had a spontaneous pregnancy. Embryo transfer was conducted in the remaining four patients and two of them became pregnant. A total of three patients (43%) conceived successfully. Our results suggest that autologous menSCs transplantation may be one of the best options for endometrial regeneration as long as sufficient menSCs can be cultured for treatment.

Stem cell therapy for endometrial restoration has been recently studied from bench to bedside. Bone marrow derived stem cells (BMSCs) are the most frequently used stem cell source (Cervelló *et al.*, 2015). They are directly isolated from the bone marrow through

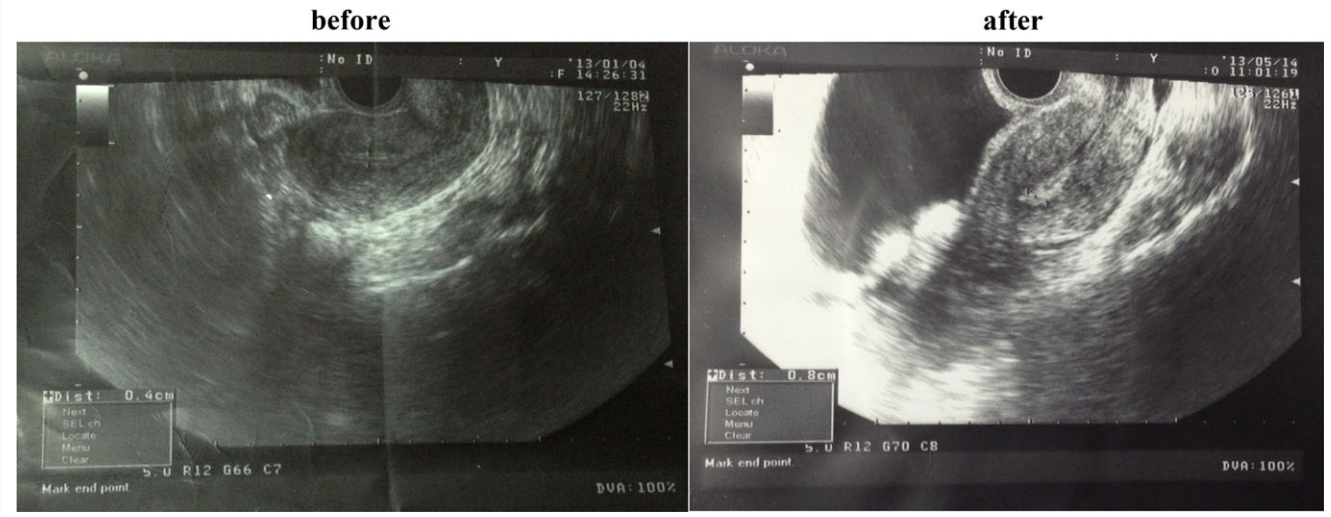

Figure 4 Transvaginal ultrasonography of the uterus before and after menSCs transplantation in Patient 3.

Patient No.	Thickness before (mm)	Thickness after (mm)
1	2	7
2	3	7
3	4	8
4	3	7
5	5	7
6	4	6
7	2	5

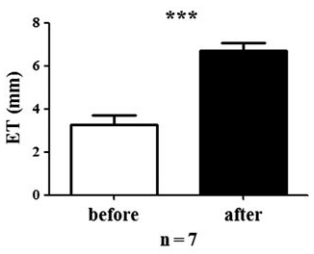

Figure 5 Endometrial thickness before and after menSCs transplantation. Data were shown as mean values ± SEM, ***$P < 0.001$.

aspiration. BMSCs can be obtained from both human and rodents, with potential for extensive migration and pluripotency. Researchers (Nagori *et al.*, 2011) first reported the use of autologous BMSCs to treat a patient with severe AS, refractory to conventional treatment for 6 months. The stem cells were purified from bone marrow by immuno-magnetic isolation and turned out to be effective for endometrial regeneration and angiogenesis. Finally, the endometrium was suitable for implantation. Another study (Singh *et al.*, 2014) investigated six patients with refractory AS and found that autologous BMSCs transplantation significantly increased the ET. They used a modified procedure involving transplantation of mononuclear cells in the bone marrow without a preceding curettage, which might be the reason of not an ideal endometrium (ET < 7 mm) for embryo implantation. Because the authentic stem cell proportion in the bone marrow was relatively low. Furthermore, it was difficult to immobilize the transplanted cells on the surface of endometrium. Therefore, we performed a mild scratch to induce menSCs implantation, although this procedure is still controversial (Shohayeb and El-Khayat, 2012; Dunne and Taylor, 2014). Endometrial scratch alone has been found to increase pregnancy outcomes in ART procedures (Nastri *et al.*, 2015). Therefore, well-designed control studies are needed to corroborate the effect of menSCs transplantation in this study.

Inspiringly, a recent study (Santamaria *et al.*, 2016) conducted autologous CD133+ BMSCs transplantation in 11 AS and five endometrial atrophies (ET < 5 mm) patients. Two months after cell therapy, all ET significantly increased with improved uterine cavity and menstrual flow, indicating endometrial functional restoration. Three spontaneous and seven artificial assisted pregnancies (63%) followed, resulting in two live births, two ongoing pregnancies, one ectopic pregnancy, two miscarriages and three biochemical pregnancies. These data demonstrated that cell therapy was effective in regenerating endometrium. In the study, a great amount of CD133+ BMSCs was collected through bone marrow mobilization and injected into the uterus by delivering into the spiral arterioles, which maximized the efficiency. Nevertheless, both of the procedures are traumatic, which is an inevitable limitation of BMSCs.

Transplantation of stem cells from bone marrow or adipose tissue to animal models of endometrial damage was also used to evaluate the effects. Transplanted cells migrated to the injured uterus compared with the sham group after intrauterine or tail vein injection (Cervelló *et al.*, 2015; Alawadhi *et al.*, 2014). The endometrial function in stem cell–transplanted mice was significantly restored, and the fertility rate improved eventually (Alawadhi *et al.*, 2014). In addition to promoting endometrial cell differentiation/proliferation and vascularization (Cervelló *et al.*, 2015), the stem cells also decreased fibrosis (Kilic *et al.*, 2014), which ensured tissue repair. These data demonstrated that cell therapy is a promising intervention for severe AS. However, the main drawback relates to aggressive approaches to obtain cells from the bone marrow or adipose tissue, which not only increases the risk of infection but also exacerbates pain. We selected menSCs due to the relative ease of availability noninvasively and repeatedly. The menSCs also show functional characterization compared with BMSCs (Alcayaga-Miranda *et al.*, 2015). MenSCs show a higher expression of adhesion molecule CD49, frequency of progenitors, paracrine response, migration and angiogenesis. They manifest higher potential

for support expansion of hematopoietic stem cells (HSCs) *ex vivo*. The menSCs are a promising source of therapies in regenerative medicine.

MenSCs have been used in cell therapy against several diseases in animal models, such as pelvic organ prolapse (Ulrich *et al.*, 2013), heart disorders (Ulrich *et al.*, 2013), ischemic conditions (Ulrich *et al.*, 2013), diabetics (Wu *et al.*, 2014), colitis (Lv *et al.*, 2014), ovarian dysfunction (Lai *et al.*, 2015) and sepsis (Alcayaga-Miranda *et al.*, 2015). These reports demonstrated that menSCs were similar to BMSCs in promoting tissue regeneration via cell proliferation and differentiation or immunomodulation. These findings support our speculation that menSCs offer unique advantages and might be a better alternative to BMSCs. As expected, menSCs transplantation was effective in increasing ET in patients with AS.

Artificial hormone therapy with estrogen is used to promote the endometrial proliferation and angiogenesis (Chen *et al.*, 2013). Low estrogen may be a risk factor for endometrial fibrosis by increasing the proliferation of fibroblasts. However, most of the patients with severe AS showed no response to estrogen alone. The efficacy of menSCs in inducing endometrial growth under hormone stimulation suggested that stem/progenitor cell loss was the main cause of adhesions in AS. Therefore, hormone therapy alone is inadequate in severe AS, as adult stem cells are the source of new endometrium during menstrual cycle. We postulated that the adult stem cells at the healthy site failed to migrate to the adhesion site due to unknown reasons, while transplanted menSCs supplemented stem cell deficiency at the adhesion. Further investigation of menSCs proliferation and differentiation under hormone stimulation *in vivo* and *in vitro* are essential to validate this hypothesis. Besides, the paracrine effects of menSCs including anti-inflammatory (Luz-Crawford *et al.*, 2016), anti-fibrotic (Zhang *et al.*, 2013) and immunomodulatory (Bozorgmehr *et al.*, 2014) in AS cannot be ignored, which needs to be clarified thoroughly in further study.

In conclusion, there are still three study limitations: (1) The study only investigated seven patients, which is not adequate to evaluate the application of cell therapy in a controlled study. Long-term randomized controlled trials with several patients are needed. (2) The pregnancy outcome was not satisfactory probably due to the low number of implanted menSCs in the endometrium after transplantation. We are finding ways to solve this problem. Also PGD should be done to distinguish pregnancy loss caused by aneuploidy. (3) Other techniques in addition to flow cytometry are required to ensure the transplanted cell function. Finally, studies should elucidate the mechanisms of menSCs in animal models and their long-term benefits or adverse effects.

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Authors' roles

J.T. did the literature search, designed the whole work plan, recruited AS patients and conducted cell transplantation. P.L. wrote the manuscript. Q.W., who was in charge of the umbilical cord blood bank of Liaoning Province, supplied place and guidance for cell culture. Y.L. and X.L. did all the cell culture, microbe detection and data collection. D.Z. prescribed medicine for HRT and supervised endometrial morphology. X.X. and L.K. performed frozen embryos thawing, transfer and pregnancy follow-up. The article was reviewed by all authors.

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Conflict of interest

None declared.

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