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ORIGINAL ARTICLE ***Reproductive biology***

Autologous cell therapy with CD133+ bone marrow-derived stem cells for refractory Asherman's syndrome and endometrial atrophy: a pilot cohort study

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STUDY QUESTION: Could cell therapy using autologous peripheral blood CD133+ bone marrow-derived stem cells (BMDSCs) offer a safe and efficient therapeutic approach for patients with refractory Asherman's syndrome (AS) and/or endometrial atrophy (EA) and a wish to conceive?

SUMMARY ANSWER: In the first 3 months, autologous cell therapy, using CD133+ BMDSCs in conjunction with hormonal replacement therapy, increased the volume and duration of menses as well as the thickness and angiogenesis processes of the endometrium while decreasing intrauterine adhesion scores.

WHAT IS KNOWN ALREADY: AS is characterized by the presence of intrauterine adhesions and EA prevents the endometrium from growing thicker than 5 mm, resulting in menstruation disorders and infertility. Many therapies have been attempted for these conditions, but none have proved effective.

STUDY DESIGN, SIZE, DURATION: This was a prospective, experimental, non-controlled study. There were 18 patients aged 30–45 years with refractory AS or EA were recruited, and 16 of these completed the study. Medical history, physical examination, endometrial thickness, intrauterine adhesion score and neoangiogenesis were assessed before and 3 and 6 months after cell therapy.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After the initial hysteroscopic diagnosis, BMDSC mobilization was performed by granulocyte-CSF injection, then CD133+ cells were isolated through peripheral blood aphaeresis to obtain a mean of 124.39 million cells (range 42–236), which were immediately delivered into the spiral arterioles by catheterization. Subsequently, endometrial treatment after stem cell therapy was assessed in terms of restoration of menses, endometrial thickness (by vaginal ultrasound), adhesion score (by hysteroscopy), neoangiogenesis and ongoing pregnancy rate. The study was conducted at Hospital Clínico Universitario of Valencia and IVI Valencia (Spain).

MAIN RESULTS AND THE ROLE OF CHANCE: All 11 AS patients exhibited an improved uterine cavity 2 months after stem cell therapy. Endometrial thickness increased from an average of 4.3 mm (range 2.7–5) to 6.7 mm (range 3.1–12) ($P = 0.004$). Similarly, four of the five EA patients experienced an improved endometrial cavity, and endometrial thickness increased from 4.2 mm (range 2.7–5) to 5.7 mm (range 5–12) ($P = 0.03$). The beneficial effects of the cell therapy increased the mature vessel density and the duration and intensity of menses in the first 3 months, with a return to the initial levels 6 months after the treatment. Three patients became pregnant spontaneously, resulting in one baby boy born, one ongoing pregnancy and a miscarriage. Furthermore, seven pregnancies were obtained after fourteen embryo transfers, resulting in

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three biochemical pregnancies, one miscarriage, one ectopic pregnancy, one baby born and one ongoing pregnancy.

LIMITATIONS, REASONS FOR CAUTION: Limitations of this pilot study include the small sample size and the lack of control group.

WIDER IMPLICATIONS OF THE FINDINGS: This novel autologous cell therapy is a promising therapeutic option for patients with these incurable pathologies and a wish to conceive.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the Spanish Ministry of Science and Innovation (SAF 2012-31017, Principal Investigator C.S.), Spanish Ministry of Health (EC11-299, Principal Investigator C.S.) and Regional Valencian Ministry of Education (PROMETEOII/2013/018, Principal Investigator C.S.). Four authors (X.S., I.C., A.P. and C.S.) are co-inventors of the patent resulting from this work (Application number: 62/013,121). S.C., C.A., F.R., J.F., J.P. and J.R. have no conflict of interest in relation to this work.

TRIAL REGISTRATION NUMBER: This study was registered with ClinicalTrials.gov (NCT02144987).

Key words: Asherman's syndrome / endometrial atrophy / cell therapy / bone marrow-derived stem cell / CD133+

Introduction

Asherman's syndrome (AS) is an uncommon gynecological disorder caused by the destruction of the endometrium due to repeated or aggressive curettages and/or endometritis (Yu *et al.*, 2008). As a result, there is a loss of functional endometrium in many areas and the uterine cavity is obliterated by intrauterine adhesions, leading to amenorrhea, hypomenorrhea, infertility, recurrent pregnancy loss and/or abnormal placentation, including placenta previa and accreta (Dmowski and Greenblatt, 1969). The incidence of AS varies between 2 and 22% of infertile women. Furthermore, 1.5% of women undergoing hysterosalpingography and 5% of women suffering recurrent miscarriages present with AS (Ventolini *et al.*, 2004). Several hysteroscopic techniques have been proposed to treat AS, but have yet to show consistent results.

Endometrial atrophy (EA) is another rare condition in which the endometrium is too thin and never grows more than 5 mm thick (Senturk and Erel, 2008). Factors that can cause EA include prolonged use of oral contraceptives and tamoxifen, but in many instances the cause of EA is unknown. The prevalence of this pathology is 0.5% of infertile women undergoing assisted reproductive treatments (ARTs) (Senturk and Erel, 2008). EA patients have a poor reproductive outcome, and although many therapies have been attempted (Sher and Fisch, 2002; Okusami *et al.*, 2007), none have proved effective.

Both pathological conditions share a common effect, the absence of functional endometrium. Two independent groups have demonstrated that endometrial cells with a side population phenotype, identified as having the capacity to extrude the DNA-binding dye Hoechst 33342 via the ATP-binding cassette transporter, contribute to human endometrial regeneration *in vitro* and *in vivo* (Cervelló *et al.*, 2010, 2011; Masuda *et al.*, 2010). Furthermore, bone marrow-derived stem cells (BMDSCs) have been shown to contribute to the repair and regeneration of tissues and organs (Pittenger *et al.*, 1999), including human (Taylor, 2004; Du and Taylor, 2007; Mints *et al.*, 2008; Ikoma *et al.*, 2009; Cervelló *et al.*, 2012) and murine (Brantincsak *et al.*, 2007; Du *et al.*, 2012; Morelli *et al.*, 2013) endometrium, primarily by forming endometrial stromal cells (Aghajanova *et al.*, 2010) and glandular and luminal epithelium. However, the BMDSC subpopulation(s) that promote(s) the repair of the endometrium remains unknown. Stem cell therapy targeting the endometrial niche with the ultimate aim of replenishing the cellular compartments of the functionalist layer offers a promising possibility for treating AS and EA.

Cells derived from the bone marrow expressing CD133/VEGFR2 represent a subpopulation of cells, with endothelial progenitor capacity and known as EPCs (Urbich and Dimmeler, 2004), that can be mobilized to the circulation and can improve neoangiogenesis afforded by pre-existing endothelium. CD133+ BMDSCs have been indicated in clinical trials for regenerative medicine in non-hematological applications (Rafii and Lyden, 2003), and have been shown to functionally contribute to neoangiogenesis during wound healing and limb ischemia, postmyocardial infarction, endothelialization of vascular grafts, atherosclerosis, retinal and lymphoid organ neo-vascularization and tumor growth (for review see Rafii and Lyden, 2003). In addition, the CD133 surface marker is currently used for the isolation of stem cells from brain (Uchida *et al.*, 2000), kidney (Sagrinati *et al.*, 2006), prostate (Richardson *et al.*, 2004) and liver (Kordes *et al.*, 2007).

In the interest of developing a cell-based therapy for these as-yet-incurable endometrial pathologies, we chose CD133+ BMDSCs for infusion into the spiral arterioles of the patient, based on several reasons. First, CD133 is a well-known endothelial progenitor cell marker with a proved safety profile in regenerative medicine for more than a decade (Rafii and Lyden, 2003). Recently, the efficiency and safety of EPCs in improving vascularization in humans has been documented in a randomized clinical trial to promote angiogenesis in patients with refractory angina (Jimenez-Quevedo *et al.*, 2014). Additionally, a pilot study described that intracoronary CD133+ instillation improves segmental myocardial perfusion with an excellent safety profile (Manginas *et al.*, 2007). Another important reason for this strategy is that, in humans, the endometrial stem cell niche is located at the endothelium of the spiral arterioles in the basal layer (Murakami *et al.*, 2014). Finally, the plasmaferesis kit to isolate CD133+ cells from peripheral blood has the CE (Conformité Européenne; European Conformity) mark of approval and thus is suitable for clinical purposes [CliniMACS® system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)].

Based on these premises, we conducted a pilot study designed to demonstrate the safety and efficiency, in terms of morphological and functional improvement of the treated endometrium, of using advanced cell therapy with autologous CD133+ BMDSCs in sixteen patients suffering from refractory AS or EA.

Materials and Methods

Study participants and study design

This first-in-human pilot study was approved by the Clinical Research Ethics Committee at the Hospital Clínico Universitario (HCU) Valencia, Spain (26/07/2012), and sponsored by the Spanish Ministry of Health (Ref EC

11-299). All participants provided written informed consent. This study was registered with ClinicalTrials.gov (NCT02144987).

We enrolled 18 patients, but one patient had no peripheral vein access, and in another the CD133+ BMDSCs were not properly mobilized from the bone marrow. Thus, 16 patients (ranging from 30 to 45 years of age) diagnosed with refractory AS ($N = 11$) or EA ($N = 5$) were included in our study. According to the study design, external diagnosis of AS or EA was confirmed by the same surgeon (J.F.) who also performed all hysteroscopies in the proliferative phase immediately before the BMDSC mobilization. For endometrial thickness, each patient acted as her own control since all of them underwent an HRT cycle before cell therapy. Endometrial thickness was measured and is presented in Tables I and II as maximal preoperative endometrial thickness. Patients diagnosed with AS were classified according to the American Fertility Society (AFS) classification of uterine adhesions (The American Fertility Society Classification, 1988), and endometrial biopsies were obtained. All patients experienced little or no menstrual bleeding during their natural cycles or after hormonal replacement therapy (HRT), and none of them had ever achieved a pregnancy after the diagnosis of AS or EA. Requirements for participation in the study included normal liver, heart and kidney function, absence of HIV, Hepatitis B or C, syphilis and psychiatric pathology, and willingness to complete the study. Patients were excluded in instances where there was no peripheral vein access or splenomegaly.

BMDSC mobilization was performed by granulocyte-CSF (G-CSF) injection, then CD133+ cells were isolated through peripheral blood apheresis and subsequently delivered into the spiral arterioles of the patient by a minimal invasive radiology intervention through each uterine artery using a microcatheter (Supplementary data, Fig. S1).

BMDSC mobilization and isolation

Mobilization of BMDSCs was induced by pharmacological administration of granulocyte colony stimulating factor (G-CSF) (10 μg/kg/day on Days-4, -3, -2 and -1). G-CSF is a cytokine extensively used for this purpose in both autologous and allogeneic donors (Gordon *et al.*, 2003). Five days after injection, isolation of mononuclear cells was performed by apheresis through peripheral venous access using the CobeSpectra separator (Terumo BCT, Lakewood, CO, USA) according to the manufacturer instructions. Once the volume was adjusted to 95 g, the mononuclear antibody was added to perform CD133+ cell selection and isolation using the cell sorter CliniMACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), no later than 24 h after extracting mononuclear cells. In all cases, a vial of CliniMACS CD133 was used since the number of CD133+ cells obtained was not $>0.6 \times 10^9$. In general, two blood volumes were processed unless the patient showed a suboptimal mobilization of CD133+ circulating cells ($<30/\mu l$), in which case, three blood volumes were processed. A unit of blood volume is equal to 10 l. We processed two to three times the blood volume of each patient according to the pH variation in peripheral blood. The mean amount of mobilized volume corresponds to 26 ± 2.09 l. All procedures were performed in a room with HEPA filters and controlled temperature at 22–24°C. The minimum of CD133+ cells to be obtained by selection was 50 million cells. Isolated CD133+ cells were diluted into 15–30 cm^3 of saline solution and transported in a sterile syringe to the radiology department for delivery into spiral arterioles. For this purpose, the Hematology Department of the Hospital Clinico has a CAT certification (Organismo de Certificación de la Calidad en Transfusión, Terapia Celular y Tisular; Quality Certification in Transfusion, Cell and Tissue Therapy Organism) which is an entity accredited by the ENAC (Entidad Nacional Acreditación; Spanish Accreditation Entity) for the Transfusion and Cell Therapy. Additionally, this Department has a JACiE (Joint Accreditation Committee of the International Society for Cellular Therapy and European Society for Blood and Marrow Transplantation) accreditation for the isolation and processing of progenitor cells.

Delivery of BMDSCs

After successful CD133+ isolation, patients were referred to the radiology department of HCU, where cell delivery to the endometrial stem cell niche via intra-arterial catheterization was performed using a technique routinely performed for embolization of fibroids (Goodwin *et al.*, 2008). The common femoral artery was approached using the Seldinger technique in which a 4 F introducer allowed catheterization of both hypogastric arteries with an angiographic catheter curve and a guide Terumo 0.035 in. Through the latter catheter, a 2.5 F microcatheter with a guide (0.014 in) was introduced to catheterize the uterine artery to the most distal spiral arterioles that the microcatheter could reach (Supplementary data, Fig. S2). Once the catheter position was stabilized and verified, 15 cm^3 of a saline suspension of the selected CD133+ cells (containing $42–200 \times 10^6$ cells, mean 123.56×10^6) was two injected through each uterine artery into the spiral arterioles.

Follow-up

All patients were given HRT (Progyluton™, Bayer, Berlin, Germany) before and after receiving cell therapy. Menstrual history was recorded monthly based on the following parameters: duration (days), volume (pads/day) and frequency (days). Endometrial cavity status was assessed by diagnostic hysteroscopy, vaginal ultrasound and histology before the intervention and 3 and 6 months after cell therapy. Hysteroscopies were performed 6–10 days after menstruation under HRT replacement and endometrial thickness was measured. Endometrial biopsies for histology and immunohistochemistry were obtained during hysteroscopy. All data are comparable for similar times of the HRT cycle before and after the intervention. Finally, patients were invited to undergo ART to attempt conception (Supplementary data, Fig. S1).

Endometrial immunofluorescence

We assessed blood vessel formation by CD31 and α-SMA immunofluorescence as previously described (Gee *et al.*, 2003) in formalin-fixed paraffin-embedded endometrial sections. Briefly, the sections were deparaffinized with xylenes, dehydrated with ethanol and heated in 10 mM Tris, 1 mM EDTA. The blocking solution used was composed by phosphate-buffered saline (Sigma-Aldrich, MO, EEUU)-bovine serum albumin 5% (Sigma-Aldrich, MO, EEUU)-NGS 5% (Sigma-Aldrich, MO, EEUU) and Tween-20 0.05% (Sigma-Aldrich, MO, EEUU). All samples were incubated for 1 h at room temperature with this blocking solution. Then, they were incubated with mouse anti-human CD31 (1:20 final concentration) (Dako, Glostrup, Denmark) for 30 min and a secondary Alexa-488 goat anti-mouse (1:500 final concentration) was then applied for 30 min at room temperature. Incubation with mouse anti-human α-SMA-Cy3 (1:300 final concentration) (Sigma-Aldrich, MO, EEUU) was performed for 30 min at room temperature. Slides were counterstained with DAPI (Invitrogen, CA, EEUU). Positive controls included in the assay were tonsil for CD31 and myometrium for α-SMA. Slides were examined for the staining pattern under a Nikon Eclipse 80i fluorescence microscope at 20× magnification. Three separate fields were used to analyze the total blood vessel formation per area by ImageJ software. Data are presented as specific values for every patient before and 3 and 6 months after cell therapy, as well as mean numbers with associated standard error (SEM).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (IBM, MD, USA). Student's t-test with paired data was used to analyzed endometrial thickness before versus after cell therapy in patients with Asherman Syndrome and EA. Paired sample *t*-test was used to analyze the differences observed in the counting of total mature blood vessels. A *P*-value obtained in a two-tailed

Table I Clinical characteristics and outcome of patients with AS.

Patient	Preoperative menstrual history	Etiology of Asherman	Prior repair attempts	Age	Maximum preoperative endometrial thickness (mm)	Hysteroscopy			Post-operative menstrual history	Maximum post-operative endometrial thickness (mm)	Pregnancy outcome
						First look before cell therapy	Second look after cell therapy	Third look after cell therapy			
1	Scant spotting	D&C	h/s × 6	39	4.5	AS Stage III	Stage II	Stage I	Regular with HRT	5.2	No
2	Scant spotting	D&C	None	30	4	AS Stage III	Stage II	Stage I	Regular with HRT	6.5	No
3	Scant spotting	D&C	h/s × 2	43	4.5	AS Stage II	Stage I	Stage I	Regular with HRT	7	Yes, BP
4	Amenorrhea	D&C	h/s × 5	37	4.5	EA + AS Stage II	Stage I	Stage I	Regular with HRT	6.1	No
6	Scant spotting	Unexplained	h/s × 1	45	5	EA + AS Stage I	Stage I	Uterine cavity normalized	Regular with HRT	5	No
7	Scant spotting	D&C	h/s × 9	34	3.5	EA + AS Stage II	Stage I	Stage I	Regular with HRT		Yes, SP premature rupture of membranes at 17 weeks
8	Amenorrhea	D&C; IUD (LNG 5 years)	h/s × 1	35	3.5	EA + AS Stage II	Stage I	Stage I	Regular with HRT	7.1	No transfer. All abnormal embryos
9	Scant spotting	D&C	none	40	4.7	AS Stage III	Stage I	Not performed	Regular with HRT	12	Yes, SP ongoing First trimester pregnancy
11	Scant spotting	lm	h/s × 2	40	5	AS Stage I	Stage I	Not performed	Regular with HRT	6	Yes, ongoing first trimester pregnancy
13	Scant spotting	D&C; lm	None	43	3	EA + AS Stage II	Stage I	Not performed	Regular with HRT	8 mm	Yes, EP
15	Scant spotting	D&C	h/s × 2	32	5	AS Stage II	Uterine cavity normalized	Not performed	Regular with HRT	6.8	Baby born, 39.4 weeks, 2860 g

D&C, dilatation/curettage; POF, premature ovarian failure; h/s, histeroscopy; hm, histeroscopic myomectomy; lm, laparotomic myomectomy; AS, Asherman's syndrome; EA, endometrial atrophy; BP, biochemical pregnancy; EP, ectopic pregnancy; SP, spontaneous pregnancy; ART, assisted reproductive treatment; LNG, levonorgestrel; HRT, hormone replacement therapy; The Asherman Syndrome Classification by 'The American Fertility Society classification of intrauterine adhesions, 1988'.

Table II Clinical characteristics and outcome of patients with EA.

Patient	Preoperative menstrual history	Etiology of Atrophy	Prior repair attempts	Age	Maximum preoperative endometrial thickness (mm)	Hysteroscopy			Post-operative menstrual history	Maximum post-operative endometrial thickness (mm)	Pregnancy outcome
						First look before cell therapy	Second look after cell therapy	Third look after cell therapy			
5	Scant spotting	hm	h/s × 3	42	5	EA	Normal endometrium	Normal endometrium	Regular with HRT	6.8	No
10	Amenorrhea	D&C	h/s × 2	38	4	EA	Normal endometrium	Not performed	Regular with HRT	7	Yes, clinical miscarriage at 9 weeks
12	Scant spotting	Unexplained	h/s × 2	35	4.3	EA	Normal endometrium	Not performed	Regular with HRT	5.7	Baby born, 39.6 weeks, 3560 g
14	Amenorrhea	POF; IUD (LNG 2 years)	h/s × 1	30	2.7	EA	EA	Not performed	Regular with HRT	3.1	No transfer for cell therapy failure
16	Amenorrhea	POF	h/s × 1	41	5	EA	Uterine cavity normalized	Not performed	Regular with HRT	5.7	No

D&C, dilatation/curettage; POF, premature ovarian failure; h/s, histeroscopy; hm, histeroscopic myomectomy; lm, laparoscopic myomectomy; AS, Asherman's syndrome; EA, endometrial atrophy; BP, biochemical pregnancy; EP, ectopic pregnancy; SP, spontaneous pregnancy; ART, assisted reproductive treatment; LNG, levonorgestrel; HRT, hormone replacement therapy; IUD, intra uterine device; The Asherman Syndrome Classification by 'The American Fertility Society classification of intrauterine adhesions, 1988'.

test ≤0.05 was considered statistically significant. In Supplementary data, Fig. S3, the KrusKal–Wallis rank sum test was used to compare all the data, and the Mann–Whitney test was used to test the difference between these data; *P*-values <0.05 were considered statistically significant.

Results

We enrolled 18 patients, but only 16 were included because one patient had no peripheral vein access, and in another CD133+ BMDSCs were not properly mobilized from the bone marrow (<40 million). No major complications were reported in any of the 16 participants.

There were 11 patients with the diagnosis of refractory AS (Table I). Menstrual history revealed amenorrhea in two patients and scant spotting in nine even with previous HRT treatment. Causes of AS were traumatic dilatation and curettage (D&C) in none of the cases, hysteroscopic myomectomy in one case and for one patient the cause was unexplained. The average number of previously attempted reparative operative hysteroscopies was two (with a range of one to nine). Despite previous surgical treatments, no patient reported significant improvement of their endometrial status and none had achieved pregnancy. Three patients were classified as AS Grade III, four patients were scored as Grade II plus EA, two patients were classified as Grade II and two patients were scored as Grade I with or without EA (Fig. 1A). The maximum endometrial thickness with high doses of HRT achieved before cell therapy was 4.3 + 0.74 mm (ranging from 2.7 to 5.0 mm; Table I).

The five patients with EA (Table II) enrolled in this study had a previous menstrual history of amenorrhea (in three patients) and scant spotting (in two patients). The etiology was previous D&C, unexplained, the use of a levonorgestrel IUD or a previous hysteroscopic myomectomy (for one patient each). The average number of previous reparative operative hysteroscopies attempted was two. Severe EA was observed in all cases (Fig. 1B). The maximum endometrial thickness with high doses of HRT reached before cell therapy was 4.2 + 0.8 mm (ranging from 2.7 to 5.0 mm; Table II).

Endometrial reconstruction after stem cell therapy

After autologous CD133+ BMDSC therapy, menstrual cycles resumed with HRT in all patients in the first month after cell therapy, except for one patient with EA. However, the duration and intensity of menstruation decreased progressively from a mean of 5.06 days (ranging from 3 to 7) in the first month to 3.25 days (ranging from 1 to 3) in the third month after cell therapy. Menstrual volume assessed by number of pads used also decreased from a mean of 2.69 (ranging from 1 to 5) to 1.75 (ranging from 1 to 4) pads per day in the third month (Supplementary data, Fig. S3).

Hysteroscopic observations performed before (first look) and 3 months (second look) and 6 months (third look) after cell therapy revealed improvements in the endometrium and the uterine cavity (Tables I and II; Fig. 1). Specifically, all patients diagnosed with stage III AS improved to stage I, while one of the two patients affected with stage II showed a completely normalized endometrial cavity and the other improved to stage I. The remaining patients initially with stage I improved in qualifying score as shown in Table I. The maximum post-operative endometrial thickness obtained was 6.7 mm (range 3.1–12 mm; $P = 0.004$; Table I, Fig. 1A). In the EA group, a normal endometrium was observed after cell therapy in four out of the five patients (Table II; Fig. 1B). The maximum endometrial thickness obtained after cell therapy was 5.7 mm (range 5–12 mm; $P = 0.03$; Table II).

The total number of mature blood vessels formed was assessed in eight patients by colocalization of CD31 and α-SMA performed before and 3 and 6 months after cell therapy (Fig. 2). An increase in blood vessel formation was observed after 3 months of treatment (patients #4, #5, #7, #12 and #13), while a similar number of mature blood vessels were found in some others (patients #6, #9 and #10). We compared results between the starting point of the experiment (referred to as control) and 3 months after the treatment with CD133+ cells using the corresponding averages and SEM. A two-tailed test revealed a significant increase in the total number of mature blood vessels (CD31+/α-SMA+) in patients 3 months after treatment ($P = 0.021$). These results suggest a characteristic neoangiogenesis after autologous injection of CD133 cells in patients with AS and EA, that progressively diminished after 6 months.

Functionality of the treated endometrium was assessed by the reproductive outcome of patients wishing to conceive after autologous CD133+ BMDSC therapy (Tables I and II). Three patients became pregnant spontaneously, 2, 4 and 19 months after cell therapy, resulting in a baby boy born by Caesarean section at 39.4 weeks (weight 2860 g, Apgar 9/10) (patient #15), a first trimester ongoing pregnancy (patient #9) and a miscarriage at the 17th week due to a premature rupture of membranes (patient #7). Seven positive pregnancies were obtained after 14 embryo transfers, resulting in three biochemical pregnancies, one miscarriage at the 9th week due to a chromosomally abnormal embryo identified after the analysis of the products of conception, one ectopic pregnancy, one baby born by vaginal delivery at 39.6 weeks (weight 3560 g, Apgar 9/10) (patient #12) and one first trimester ongoing pregnancy (patient #11). In one case, embryo transfer was cancelled due to chromosomal abnormalities in all of the embryos (patient #8), and in another case, transfer was not performed due to failure of cell therapy (patient #14).

Discussion

The first observations on intrauterine adhesions were made in Germany in 1894 by Dr Heinrich Fritsch (Fritsch, 1894), but Joseph G. Asherman later popularized this pathological condition (Asherman, 1948), now commonly referred to as AS. From a histological point of view, AS corresponds to a replacement of the endometrial stroma by fibrous tissue, whereas glands are usually replaced by an inactive cubo-columnar epithelium that it is generally non-responsive to hormonal stimulation. As a consequence, the complete disappearance of the endometrial structure (Donnez and Nisolle, 1994) alters the functionality of this organ in addition to obliterating the uterine cavity. In the initial 50–60 years, work on this disease focused on understanding its prevalence, etiology and pathology. The advent of endoscopy led to new methods for the diagnosis and treatment of this condition, but despite the technological advances, ~50% of AS cases have remained without a comprehensive cure (March, 2011).

Here, we describe the first instance of stem cell therapy specifically targeting the endometrial stem cell niche. Under steady-state conditions, circulating endothelial progenitor cells (cEPs) represent only 0.01% of cells in the circulation. Therefore, mobilization and isolation of cEPs

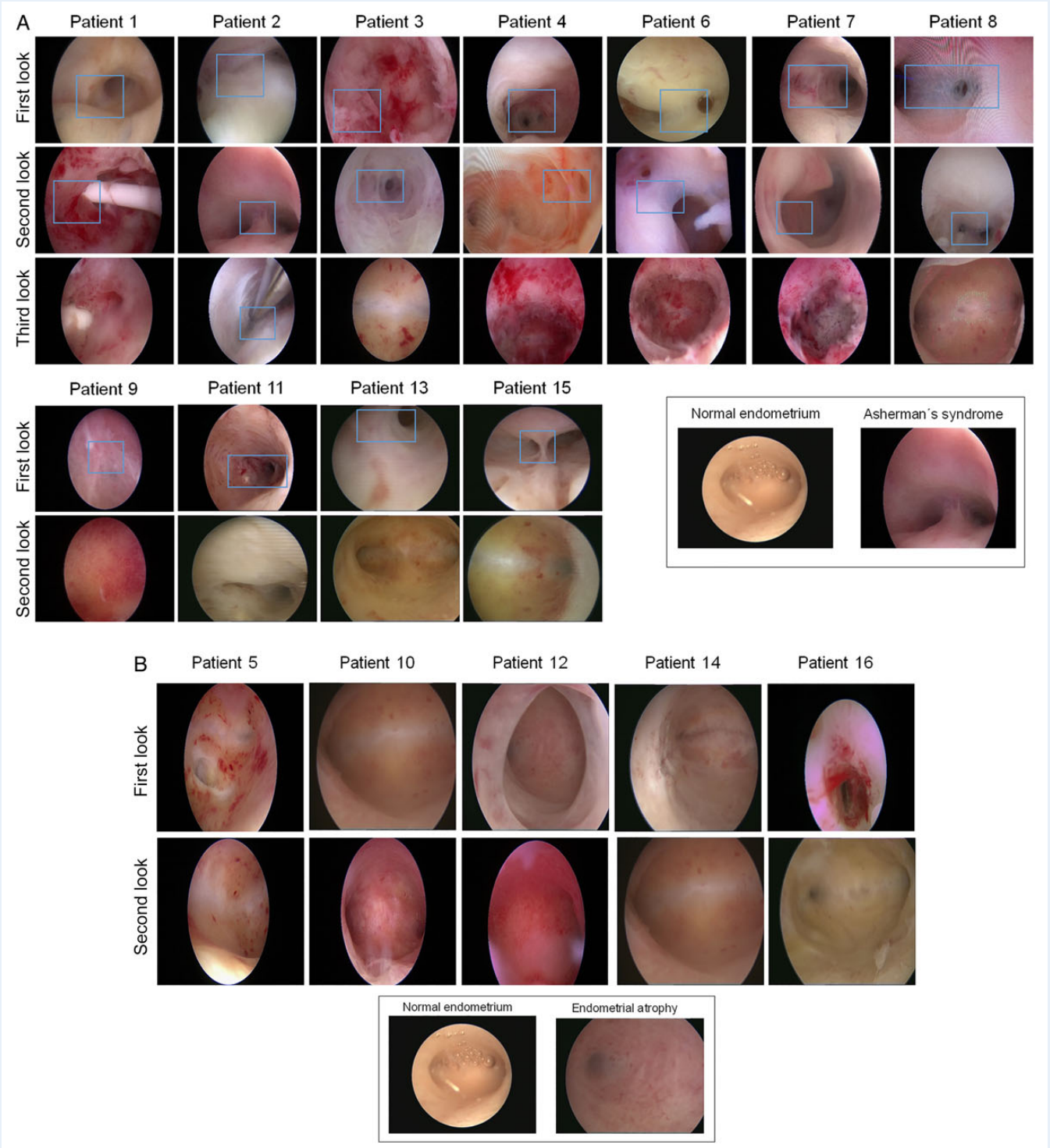

Figure 1. Preoperative and post-operative hysteroscopic images of patients with AS (**A**) and EA (**B**). Hysteroscopic findings are presented before stem cell therapy (first look) and 3 months (second look) and 6 months (third look) after stem cell therapy (boxes within the images shown adherences). Some patients lack the third look because either they became pregnant spontaneously or decide to advance the embryo transfer. Controls are also showed. The severity of the endometrial adhesions was graded according to the AFS classification (The American Fertility Society Classification, 1988).

coupled with direct infusion in the affected endometrial niche was planned. We isolated autologous CD133+ BMDSCs after mobilization with G-CSF and then reintroduced them into the spiral arterioles of the uterus in the same patient using non-invasive radiological procedures. CD133+ BMDSCs regenerate vascularization and induce endometrial proliferation leading to the creation of an autologous reconstruction of

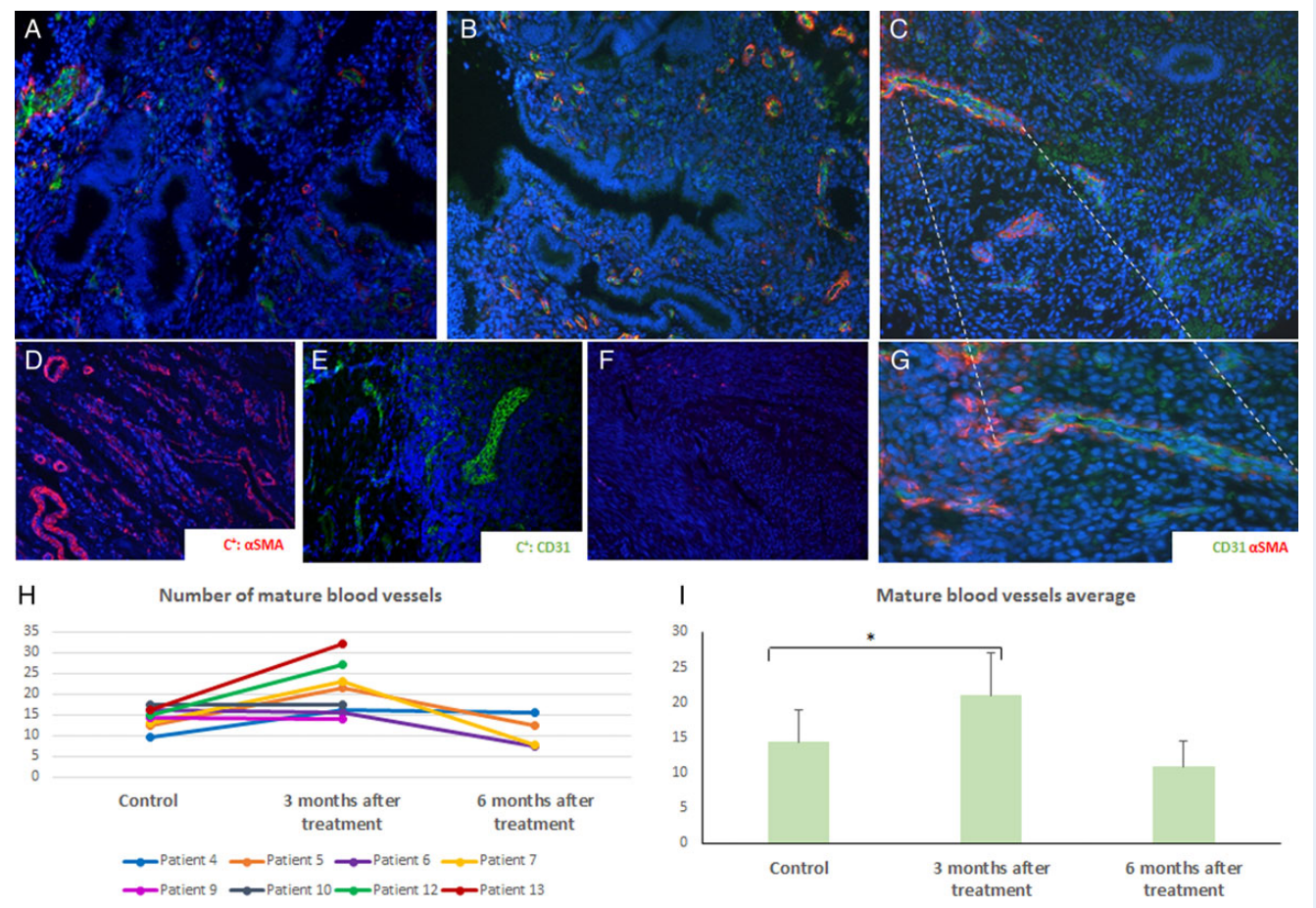

Figure 2. Tissue analyses. Immunohistochemical results for the detection of mature blood vessels in the endometrium from patient 7 before (**A**) and 3 months (**B**) and 6 months (**C**) after autologous cell therapy. α-SMA+, CD31+ positive cells identify mature blood vessels (20×). (**D**) Human myometrium was used as a positive control for α-SMA staining, and (**E**) human tonsil as a positive control for CD31. (**F**) Negative control was the absence of primary antibody. (**G**) Detail of the vessel identified in C (40×). (**H**) The dynamics of the total number of mature blood vessels from eight patients before and 3 and 6 months after cell therapy are presented, indicating a time-sensitive neoangiogenic effect. (**I**) Statistical analysis was performed using SPSS 17.0 software (IBM), * paired samples *t*-test indicated significant differences ($P = 0.021$).

the endometrium. CD133+ BMDSCs have been safely used for more than a decade in clinical trials for regenerative medicine in non-hematological applications (Rafii and Lyden, 2003).

Cell engraftment was our main concern because our IRB does not allow us to label CD133+ BMDSCs with superparamagnetic iron-oxide nanoparticles (SPIOs) to track the injected cells. Instead, we utilized a murine immunodeficient experimental model for AS (Cervelló *et al.*, 2015) for this purpose. An aliquot of 1 million CD133+ BMDSCs from patients involved in the study were incubated with 50 μg/ml Molday ION Rhodamine B for 18 h, resulting in a labeling efficiency >97% in all experiments. Then, SPIO-labeled cells were injected in an immunodeficient mouse model of AS through tail vein or intrauterine injection. Cell engraftment was detected by the identification of intracellular iron deposits using Prussian blue staining, revealing that CD133+ BMDSCs engrafted predominantly around endometrial blood vessels of the traumatized endometrium (Cervelló *et al.*, 2015). In this back-to-back animal study, we demonstrated a statistically significant increase of Ki67 in the epithelial glands of treated horns in comparison with non-treated horns, regardless of the administration route. After intrauterine injection, the proliferation rate increased from 14.00 to 23.15% ($P < 0.01$). After the tail vein injection Ki67+ cells increased from 6.92 to 20.55% ($P < 0.05$). These results demonstrate that human CD133+ BMDSCs induce proliferation of the neighboring endometrial cells in the damaged endometrium mainly at the epithelial compartment (Cervelló *et al.*, 2015). Our hypothesis for the mechanism involved is that engrafted CD133+ BMDSCs induce the secretion of the paracrine factors thrombospondin 1 and insulin-like growth factor, which activate mitosis of surrounding cells, thus inducing endometrial regeneration.

We searched PubMed and Ovid without language or date restrictions for articles with the following terms: AS, EA, autologous stem cell therapy, regenerative medicine, CD133+ cells and bone marrow-derived cells from January 1964 to January 2014. We identified two articles (Nagori *et al.*, 2011; Singh *et al.*, 2014) reporting autologous stem cell transplantation in AS. A case report suggested positive results in treating AS with autologous stem cells isolated from bone marrow by CD9,

CD40 and CD90 expression and placed into the endometrial cavity (Nagori *et al.*, 2011), while another case report described the direct placement of non-characterized mononuclear stem cells into the subendometrial zone with a needle (Singh *et al.*, 2014). However, there were no reports showing autologous cell therapy with CD133+ BMDSCs injected in the endometrial stem cell niche in refractory AS and EA in humans.

Our primary objective was the reconstruction of the endometrium, assessed first by the resumption of menstruation, which occurred in 15 out of 16 of our patients. Although the duration and intensity of menstruation decreased progressively 6 months after cell therapy, it was promising to observe that stem cell therapy made an immediate difference in endometrial morphology. Hysteroscopic visualization of the uterine cavity, endometrial thickness measured by vaginal ultrasound, and neoangiogenesis observed through immunofluorescence were consistent with an effective, although transitory, reconstruction of the endometrium. In terms of endometrial thickness, each patient acted as her own control since all of them had previously undergone an HRT cycle before cell therapy. Endometrial thickness was measured and is presented in Tables I and II as maximal preoperative endometrial thickness.

The secondary objective was to test the functionality of the newly formed endometrium by attempting to conceive. Several pregnancies were achieved spontaneously and through ART after cell therapy, and the two miscarriages observed in our study were not related to endometrial functionality. Two babies were born at term by Caesarean section and vaginal delivery with Apgar 9/10, and two ongoing first trimester pregnancy are progressing. Nevertheless, conception success remains to be improved.

On the whole, our study suggests that CD133+ BMDSC autologous cell therapy may be useful in treating patients with refractory AS and EA and a wish to conceive. We acknowledge that progression from first-in-human experiences in only 16 patients to a full integration into health systems involves many steps, including a large patient cohort with long-term follow-up, but this can be considered as the first focused proof-of-principle study.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

C.S., A.P., J.R., X.S. and S.C. did the literature search, planned the study, supervised all clinical and surgical tasks and clinical follow-up, and performed the reproductive treatment. I.C. performed the studies on the animal model and the immunohistochemistry. C.A. performed the apheresis and selection procedures. F.R. organized the required infrastructures and the gynecological follow-up. J.F. performed all hysteroscopies pre- and post-treatment. J.P. delivered the cells via intra-arterial catheterization and organized the required infrastructures. C.S. wrote the paper and it was reviewed by all authors.

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Conflict of interest

X.S., I.C., A.P. and C.S. are co-inventors of the patent resulting from this work (Application number: 62/013,121). S.C., C.A., F.R., J.F., J.P. and J.R. have no conflict of interest in relation to this work.

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