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ARTICLE

Two protocols to treat thin endometrium with granulocyte colony-stimulating factor during frozen embryo transfer cycles

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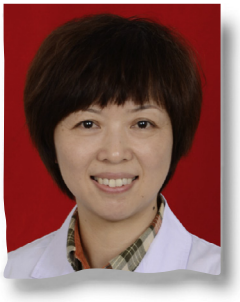

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Abstract The efficacy of two granulocyte colony-stimulating factor (G-CSF) protocols for thin endometrium were investigated. Eighty-two patients were diagnosed with thin endometrium (<7 mm). Thirty patients with previously cancelled embryo transfers received intrauterine G-CSF in subsequent frozen embryo transfer (FET) cycles. Patients were divided into the G-CSF only and G-CSF with endometrial scratch subgroups. Compared with previous cycles, endometrial thickness increased from 5.7 ± 0.7 mm to 8.1 ± 2.1 mm after G-CSF treatment ($P < 0.001$). Endometrial thickness increases were not significantly different between the two subgroups. The G-CSF with endometrial scratch subgroup established nominally higher though non-significant clinical pregnancy and live birth rates than the G-CSF only subgroup (53.8 % versus 42.9% and 38.5% versus 28.6%, respectively). Fifty-two patients underwent FET despite edometrial thickness less than 7 mm, and were included as controls. Significantly higher embryo implantation and clinical pregnancy rates were observed in the G-CSF group compared with the control group (31.5% versus 13.9%; $P < 0.01$; 48.1% versus 25.0%; $P = 0.038$, respectively). Endometrial scracth did not impair G-CSF treatment for thin endometrium and favoured pregnancy and live birth rates. For patients with thin endometrium, embryo transfer cancellation and G-CSF treatment in subsequent FET cycles is beneficial.

KEYWORDS: endometrial scratch, frozen embryo transfer, granulocyte colony-stimulating factor, pregnancy rate, thin endometrium

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Introduction

Endometrial thickness (TEM) is an important clinical marker of endometrial receptivity. A thin endometrium is often defined by a cut-off value of 7 mm (El-Toukhy et al., 2008; Kumbak et al., 2009; Miwa et al., 2009), and a previous report proposed that persistently thin endometrium may not respond normally to oestradiol (Casper, 2011). The latest systematic review and meta-analysis revealed that the probability of clinical pregnancy for a patient with TEM 7 mm or less was significantly lower than that for a patient with TEM over 7 mm (Kasius et al., 2014).

Various treatments to increase TEM have been proposed, including extended oestradiol administration; low-dose aspirin administration; intravaginal treatment with vitamin E, L-arginine and sildenafil citrate; and intrauterine administration of bone marrow stem and progenitor cells (Gargett and Healy, 2011; Senturk and Erel, 2008). A small number of patients, however, remain unresponsive, after these methods. Therefore, improved treatment methods are needed.

Granulocyte colony-stimulating factor (G-CSF), a member of the colony-stimulating factor family of cytokines, has been widely studied in assisted reproduction techniques. It contributes to oocyte and embryo development; therefore, it can be used as a biomarker to assess oocyte and embryo implantation potential (Ledee et al., 2008, 2011a, 2011b). Furthermore, G-CSF participates in ovulation mechanisms (Makinoda et al., 2008), enhances cyclic adenosine monophosphate-mediated decidualization of human endometrial stromal cells and trophoblast invasion into the maternal tissue (Litwin et al., 2005; Tanaka et al., 2000), and is predictive of IVF outcome. Outcomes of repetitive implantation failure can also be improved by G-CSF (Knieper et al., 2011; Wurfel et al., 2010) and decrease the incidence of recurrent spontaneous abortions (Santjohanser et al., 2013; Scarpellini and Sbracia, 2009).

In 2011, the first successful treatment of thin endometrium with G-CSF after standard treatments had failed was reported (Gleicher et al., 2011). Subsequent studies found that endometrial thickness increased significantly after G-CSF infusion compared with before infusion in the same IVF cycles; however, low ongoing clinical pregnancy rates were observed (19.1% and 18.9% in Gleicher et al., 2013 and Kunicki et al., 2014, respectively). In addition, the latest randomized controlled trial (RCT) showed that, although TEM increased significantly after G-CSF infusion, the increase in TEM in the G-CSF group was not significantly different from that in the saline group in IVF patients with normal endometrium. Embryo implantation rate and pregnancy rate were also similar between the two groups (Barad et al., 2014).

It is currently unclear whether G-CSF perfusion can increase TEM in women previously diagnosed with a thin endometrium who are unresponsive to regular programmes, and whether there is a better protocol using G-CSF to improve clinical pregnancy rate. Therefore, in the present study, preliminary results of intrauterine instillation of G-CSF to address these questions are presented. A literature review of cohort studies that have investigated the effect of G-CSF on endometrium and pregnancy outcome in IVF or frozen embryo transfer (FET) cycles is also presented.

Materials and methods

Participants

Patients referred to the Reproductive Medicine Center, Xiangya Hospital, Central South University, Hunan Province, China, for IVF treatment between July 2012 and July 2013 were considered eligible for the study. The trial received approval from the Xiangya Hospital Ethics Committee on 5 July 2012 (reference number 201207010). Endometrial thickness was defined as the maximal distance between the echogenic interfaces of the endometrium and the myometrium in the plane of the central longitudinal axis of the uterus. Thin endometrium was defined as TEM <7 mm, either during fresh embryo transfer cycles (on the day that HCG was administered), induced ovulation FET cycles (on the ovulation day despite normal serum oestradiol level) or hormone replacement therapy cycles (after a duration of over 10 days and the maximum dose of oral oestradiol valerate (Progynova; Bayer Schering Pharma, Roubaix, France), reaching 5–7 mg twice a day, was administered). The TEM referred to in the present study was defined as the measurement value on the day of ovulation or administration of progesterone or HCG, unless otherwise specified.

The patients had to meet the following inclusion criteria: age younger than 40 years and FSH less than 10 IU/L; failure of TEM to reach 7 mm by regular methods; no signs of submucosal uterine myoma, uterine malformations, endometrial polyps, or obvious intrauterine adhesion (IUA) by transvaginal ultrasound or diagnostic hysteroscopy, and no signs of other diseases, which could have affected endometrial growth; and no contraindications for G-CSF treatment (e.g. chronic neutropenia, sickle cell disease, renal disease and history of malignancy).

In our centre, patients with TEM less than 7 mm were generally given the following options: cancelling the cycle and proceeding to new FET cycles, or undergoing embryo transfer despite thin endometrium. In the present study, 30 patients who met our inclusion criteria and consented to receive G-CSF treatment in new FET cycles were prospectively included. They were randomized into the G-CSF only and G-CSF with endometrial scratch (EMS) groups by a randomized number table.

All patients were informed of their endometrial condition, present application status of G-CSF, possible risks (e.g. fever, nausea headache, weakness, rash, sore muscles, interstitial pneumonia or shock), uncertain efficacy, and non-indicated use of G-CSF and submitted informed consent forms. Fifty-two patients who underwent FET were retrospectively included despite thin endometrium as a control group.

Treatment protocols

In G-CSF cycles, a natural cycle was used in patients with normal ovulation; otherwise, an induced ovulation cycle was used. Oral letrozole (FuRui; HengRui Pharmaceutical Co., Ltd., Jiangsu, China) at a dose of 2.5–5.0 mg was administered each day from day 3 to day 8, and intramuscular human menopausal gonadotropin (Livzon Pharmaceutical, Zhuhai, China) at a dose of 75–150 IU was administered each day after day 12 if there follicle development was poor in induced ovulation

cycles. Follicle diameters were monitored by transvaginal ultrasound starting on day 8 of the patient's natural menstrual cycle or induced ovulation cycles.

On the day that one follicle became dominant (almost 12 × 12 mm in diameter), intrauterine instillation with G-CSF (Ruibai; QiLu Pharmaceutical Co., Ltd., Jinan, China) was carried out. All 30 patients were randomly divided into two subgroups: the G-CSF only subgroup and the G-CSF with EMS subgroup. An endometrial biopsy catheter was used to carry out EMS (Gynetics Medical Products N.V., Lommel, Belgium). For G-CSF with EMS, an endometrial biopsy catheter was inserted through the cervical orifice and advanced gently until it reached the uterine fundus. Then, the inner piston of the device was withdrawn to create suction, and the endometrium was lightly scratched once or twice up and down on every wall of the uterine cavity. The entire procedure was carried out gently and guided by abdominal ultrasound imaging (**Figure 1A** and **B**). Then, 300 μg of G-CSF (100 μg/0.6 ml) was aspirated into a 2-ml syringe, and an embryo transfer catheter (Laboratoire C.C.D., Paris, France) was introduced into the endometrial cavity. When the tip of the embryo transfer catheter made contact with the uterine fundus, the contents of the syringe were slowly injected into the cavity (**Figure 1C**). Afterwards, the catheter was gently moved back after most of the syringe contents had been injected into the uterine cavity. After completion, the syringe was disconnected, a small amount of air was aspirated, the syringe was reconnected to the catheter and air was injected to deliver the small amount of G-CSF remaining in the catheter into the endometrial cavity. For patients in the G-CSF only subgroup, the procedure was the same as that described above excluding EMS.

Endometrial thickness and endometrial pattern, as well as follicular diameters, were monitored daily or every other day after G-CSF perfusion by transvaginal ultrasound (DC-Expert; Mindray Medical International Co., Ltd. Shenzhen, China) (**Figure 1D–H**). The measurements were carefully and repeatedly performed by the same experienced sonographer (at least three to five times and 1–2 min). The mean value of every measurement was used for the analysis. Leucocytes and neutrophilic granulocytes levels in the blood were measured 24 or 48 h after G-CSF infusion. In addition, other possible side effects resulting from G-CSF were monitored during the whole course.

For both groups, 2 days after ovulation, 4 days after the luteinizing hormone concentration peaked, or 3 days after progesterone addition, two day-3 embryos (except for one patient with three embryos) were thawed and transferred. Day-3 embryos with eight cells and <15% fragmentation were considered good.

Luteal phase was supported by daily intramuscular injections of 60 mg progesterone (Zhejiang Xianju Pharmaceutical, China) for hormone replacement therapy cycles and 40 mg for induced or natural cycles. Starting on the embryo-transfer day, 200-mg progesterone soft capsules (Qining; Asen Pharmaceutical, Zhejiang, China) were taken orally every night. Fourteen days after embryo transfer, serum beta-HCG levels were evaluated. Four and 5 weeks after embryo transfer, transvaginal ultrasound was carried out for patients with positive beta-HCG results. Clinical pregnancy was considered with the presence of a gestational sac containing yolk sac at transvaginal ultrasonography, including ectopic pregnancy. Spontaneous abortion was defined as the loss of a clinical pregnancy of less than 20 weeks' gestation. Implantation rate was determined by the number of gestational sacs and the total number of embryos transferred at least 4 weeks after embryo transfer. Then, luteal support was administered to patients with intrauterine clinical

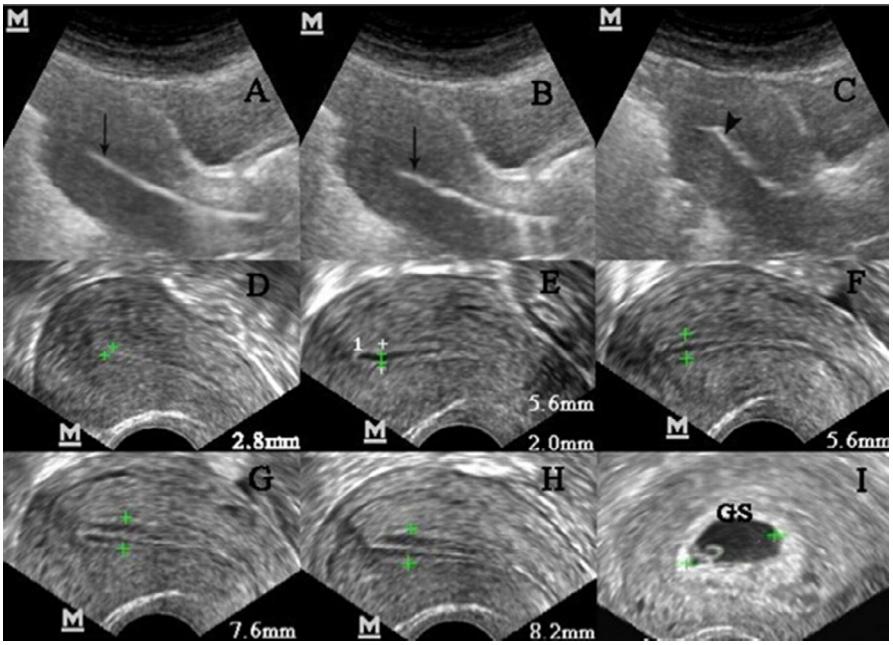

Figure 1 Treatment with granulocyte colony-stimulating factor (G-CSF) (with endometrial scratch) in a patient. (A, B) Endometrium was slightly scratched up and down with an endometrial biopsy catheter indicated by the black arrow; (C) Perfusion with G-CSF by an embryo transfer catheter indicated by the black triangular arrow; (D–H) the endometrial thickness on 0, 2, 4, 5, 6 days after G-CSF instillation; (I) a gestational sac in the uterine cavity on day 28 after embryo transfer.

pregnancy for an additional 4 weeks. The final pregnancy outcome was evaluated by telephone interview.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences version 19.0 (SPSS IBM Corp., USA). Continuous variables were assessed using the one-way analysis of variance test, multivariate analysis test and Kruskal–Wallis test, and data were presented as mean ± standard deviation. The numerical data were analysed using the chi-squared test and Fisher's exact test and presented as percentages. $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics and outcomes for the G-CSF and control groups are shown in **Table 1**. After G-CSF treatment, three out of 30 patients were advised to cancel embryo transfer because of poor TEM and pattern. Among these, one patient had a history of endometrial tuberculosis, and two had a history of severe IUA. No baseline characteristics were significantly different between the G-CSF group and the control group. A significantly higher embryo implantation rate and clinical pregnancy rate were observed in the G-CSF group compared with the control group (31.5% versus 13. 9% and 48.1% versus 25.0%; $P < 0.01$ and $P = 0.038$, respectively). The live birth rate, however, was not significantly different (33.3% versus 17.3%). In the two subgroups, the G-CSF with EMS group established nominally higher though non-significant clinical pregnancy and live birth rates than the G-CSF only group (53.8 % versus 42.9% and 38.5% versus 28.6%, respectively).

In **Table 2**, the changes in TEM in the G-CSF group, G-CSF only subgroup, G-CSF with EMS subgroup, and patients with and without a history of IUA are presented. In the G-CSF group, TEM increased after treatment from 5.7 ± 0.7 mm, measured during previous cycles with regular methods, to 8.1 ± 2.1 mm ($P < 0.001$), resulting in an overall change of 2.4 ± 2.2 mm. In the G-CSF only subgroup, TEM increased from 5.7 ± 0.7 mm to 8.1 ± 2.5 mm ($P = 0.003$), with an overall change of 2.4 ± 2.7 mm. In the G-CSF with EMS subgroup, TEM increased from 5.8 ± 0.7 mm to 8.1 ± 1.6 mm ($P < 0.001$), resulting in an overall change of 2.3 ± 1.6 mm. The change in TEM was similar between the two subgroups (**Figure 2**).

Furthermore, after G-CSF treatment, the TEM increased from 4.1 ± 0.9 mm at G-CSF infusion to 8.1 ± 2.1 mm in the G-CSF group ($P < 0.001$), with an overall change of 3.9 ± 2.0 mm. In the G-CSF only subgroup, TEM changed from 4.2 ± 0.9 mm to 8.1 ± 2.5 mm ($P < 0.001$), with an overall change of 3.9 ± 2.4 mm. In the G-CSF with EMS subgroup, TEM changed from 4.1 ± 0.9 mm to 8.1 ± 1.6 mm ($P < 0.001$), with an overall change of 4.0 ± 1.4 mm. The TEM change was also similar between the two subgroups.

The increase in TEM from previous cycles was significantly different between patients with and without a history of IUA (0.7 ± 1.2 versus 3.1 ± 2.1 mm; $P = 0.004$), but no difference was found in TEM increase in the same cycles (2.9 ± 1.3 versus 4.4 ± 2.1 mm).

In three patients, leucocytes and neutrophilic granulocytes in the blood exceeded the normal reference range 48 h after G-CSF perfusion; levels recovered to normal after re-examination the following day. One of the patients felt mildly sore muscles and dizziness, and one felt mildly weak with normal levels of leucocytes and neutrophilic granulocytes in the blood; these symptoms disappeared the next day (**Supplementary Table S1**).

Discussion

Since 2011, seven studies (Barad et al., 2014; Gleicher et al., 2011, 2013; Kunicki et al., 2014; Li et al., 2014; Lucena and Moreno-Ortiz, 2013; Zhao et al., 2013) have evaluated the effect of G-CSF on endometrium and IVF outcomes (**Table 3**). Consistent with previously reported data (Gleicher et al., 2013; Kunicki et al., 2014), TEM increased significantly after G-CSF treatment in the same cycles. An RCT (Barad et al., 2014) demonstrated the same result in normal IVF patients; however, the increase in TEM in the G-CSF group was not significantly different from that in the saline group. The reason would be that endometrium has a spontaneous increase during the interval between G-CSF administration on the HCG trigger day and embryo transfer day and the design of RCT avoided the bias. The results indicated that a comparison in the same cycle other than a RCT does not provide enough proof of the efficacy of G-CSF for thin endometrium. In our study, a self-controlled comparison was made between G-CSF treatment cycles and previous cycles to avoid the bias above mentioned. In patients who underwent FET with thin endometrium, G-CSF successfully increased TEM. Conclusive evidence from an RCT with a negative control to exclude other bias is required.

Colony-stimulating factor has been found to regulate endometrial growth, and macrophage colony-stimulating factor plays a role in the genesis of early endometriotic lesions (Jensen et al., 2010) and has been shown to exert a direct effect on endometrial epithelial cell proliferation and attachment to peritoneal mesothelial cells (Aligeti et al., 2011). In our animal study, a significantly thicker endometrium and stronger expression of cytokeratin and vimentin were found in the G-CSF-administration groups compared with the control groups (Zhao et al., 2013), indicating that G-CSF may promote proliferation of endometrial cells to increase TEM. However, no in vitro cell culture experiment has been conducted to confirm this effect.

Granulocyte colony stimulating factor is a mobilization inducer of haematopoietic stem and progenitor cells (Singh et al., 2012), and can increase mesenchymal precursor cell numbers in the bone marrow (Brouard et al., 2010). It also contributes to recruitment and homing of mesenchymal stem-like cells to the site of arterial lesions (Zhao et al., 2011). Human endometrium contains a small population of mesenchymal stem-like cells that may be responsible for endometrium cyclical growth and reconstruction (Chan et al., 2004; Schwab and Gargett, 2007). A thin endometrium may result from diminished numbers, function of endometrial stem cells, or both (Gargett and Healy, 2011; Gargett and Ye, 2012). Therefore, it was hypothesised that endometrial cells in a thin endometrium become conservative owing to various causes, such as injury. To avoid further impairment, a portion of endometrial cells may maintain a basal proliferation rate while another portion of endometrial cells or stem cells, responsible

Table 1 Baseline characteristics and outcomes of the granulocyte colony-stimulating factor and control groups.[a]

	G-CSF group (n = *27*)	*Control group* (n = *52*)	P	*G-CSF only* (n = *14*)	*G-CSF with EMS* (n = *13*)	P
Age (years)	31.4 ± 4.0	32.0 ± 3.9	NS	31.9 ± 4.1	30.8 ± 4.0	NS
BMI (kg/m^2)	21.8 ± 2.5	21.5 ± 3.0	NS	22.0 ± 1.9	21.5 ± 3.1	NS
Baseline FSH (IU/L)	5.9 ± 1.1	6.5 ± 1.3	NS	5.7 ± 0.9	6.0 ± 1.3	NS
Primary infertility (%)	33.3	32.7	NS	35.7	30.8	NS
Duration of infertility (years)	4.6 ± 2.9	5.5 ± 3.8	NS	5.2 ± 3.0	4.0 ± 2.7	NS
Gravidity (*n*)	1.9 ± 2.0	1.8 ± 1.9	NS	1.9 ± 2.2	1.9 ± 1.8	NS
Surgical abortion/evacuation (*n*)	1.4 ± 1.7	1.1 ± 1.6	NS	1.4 ± 1.9	1.3 ± 1.6	NS
Patients with history of IUA	25.9 (7/27)	NA	NA	14.3 (2/14)	38.5 (5/13)	NS
TEM (mm) before G-CSF	5.7 ± 0.7	6.5 ± 0.5[a]	<0.001	5.7 ± 0.7	5.7 ± 0.7	NS
TEM (mm) after G-CSF	8.4 ± 2.0[c]	NA	NA	8.5 ± 2.4	8.3 ± 1.6	NS
Embryos transferred (*n*)	2.00 ± 0.00	2.08 ± 0.33	NS	2.0 ± 0.0	2.0 ± 0.0	NS
Good embryos transferred (*n*)	1.96 ± 0.19	2.00 ± 0.40	NS	2.0 ± 0.0	1.9 ± 0.3	NS
Embryo implantation (%)	31.5 (17/54)[b]	13.9 (15/108)	<0.01	32.1(9/28)	30.8 (8/26)[b]	NS
Clinical pregnancy (%)	48.1 (13/27)[b]	25.0 (13/52)	0.038	42.9 (6/14)	53.8 (7/13)[b]	NS
Ectopic pregnancy (%)	7.4 (2/27)[b]	1.9 (1/52)	NS	7.1 (1/14)	7.7 (1/13)[b]	NS
Spontaneous abortion	11.1(3/27)	5.8(3/52)	NS	7.1(1/14)	15.4(2/13)	NS
Live birth (%)	33.3(9/27)	17.3(9/52)	NS	28.6(4/14)	38.5(5/13)	NS

BMI = body mass index; EMS = endometrial scratch; G-CSF = granulocyte colony-stimulating factor; IUA = intrauterine adhesion; NA = not available; NS = not statistically significant; TEM = endometrial thickness.

[a]After G-CSF treatment three out of 30 patients were advised to cancel embryo transfer, owing to very poor endometrium; data for these patients are excluded. Values are presented as means ± standard deviation or percentages. $P < 0.05$ denotes significance.

[b]Included a heterotopic pregnancy: the simultaneous existence of an intrauterine and an extrauterine pregnancy. After a laparoendoscopic salpingectomy, the intrauterine fetus was viable and the patient delivered a healthy baby.

[c]$P < 0.001$.

Table 2 Changes in endometrial thickness (mm) of patients after granulocyte colony-stimulating factor treatment.[a]

	Comparison of TEM between cycles				*Comparison of TEM in the same cycle*			
	Previous cycles	*G-CSF cycle*	P	*Change in TEM*	*Pre G-CSF*	*Post G-CSF*	P	*Change in TEM*
Total G-CSF group ($n = 30$)	5.7 ± 0.7	8.1 ± 2.1	<0.001	2.4 ± 2.2	4.1 ± 0.9	8.1 ± 2.1	<0.001	3.9 ± 2.0
G-CSF only subgroup ($n = 16$)	5.7 ± 0.7	8.1 ± 2.5	0.003	2.4 ± 2.7	4.2 ± 0.9	8.1 ± 2.5	<0.001	3.9 ± 2.4
G-CSF with EMS subgroup ($n = 14$)	5.8 ± 0.7	8.1 ± 1.6	<0.001	2.3 ± 1.6	4.1 ± 0.9	8.1 ± 1.6	<0.001	4.0 ± 1.4
Patients with history of IUA ($n = 9$)	5.9 ± 0.7	6.6 ± 1.3	NS	0.7 ± 1.2[b]	3.7 ± 0.7	6.6 ± 1.3	<0.001	2.9 ± 1.3
Patients without history of IUA ($n = 21$)	5.7 ± 0.7	8.7 ± 2.0	<0.001	3.1 ± 2.1[b]	4.3 ± 0.9	8.7 ± 2.0	<0.001	4.4 ± 2.1

EMS = endometrial scratch; G-CSF = granulocyte colony-stimulating factor; IUA = intrauterine adhesion; NS = not statistically significant; TEM = endometrial thickness.
[a]Values (in mm) are presented as means ± standard. $P < 0.05$ denotes significance.
[b]$P = 0.004$.

for endometrial development, may remain quiescent. Therefore, G-CSF may act to stimulate endometrial stem cells or to mobilize bone marrow stem cells, thus promoting thin, dysregulated endometrial development (Gargett and Healy, 2011; Gargett and Ye, 2012). This hypothesis provides a novel direction for future research: directly supplying some cytokine or mobilization factor, such as G-CSF, to endometrium to promote endometrial development. The in-vitro effect of G-CSF in endometrial cells warrants further research.

Human endometrium expresses G-CSF mRNA and its receptor throughout the menstrual cycle. Endometrial G-CSF protein production is stimulated by interleukin-1 beta (Vandermolen and Gu, 1996). Thus, G-CSF may play a physiological role in endometrial development throughout the menstrual cycle through interactions with other cytokines and ovarian steroid hormones. Oestrogen may be necessary to provide nutrition to the endometrium after stimulation by G-CSF. In the present study, G-CSF was administered once and probably acted as a stimulating factor. Therefore, we believe that G-CSF administration in the early endometrial proliferation phase should have significant results. In the present study, on the day one follicle became dominant, intrauterine G-CSF perfusion was carried out. The purpose was to provide much time for endometrial growth and subsequent endogenous oestrogen support. To avoid interference by other factors, we did not use exogenous oestrogen. Hormone replacement therapy cycles, however, can be controlled easily and may be a better protocol for the treatment of thin endometrium with G-CSF.

In using G-CSF to treat thin endometrium, clinical pregnancy rate is a major concern for clinicians. Previous studies have failed to demonstrate improved pregnancy rate in both patients with thin and normal endometrium with G-CSF treatment; however, in the present study, a significantly higher embryo implanatation rate (31.5%) and clinical pregnancy rate (48.1%), as well as a trend toward higher live birth rate (33.3%) was revealed. We believe that the significantly greater TEM in the G-CSF group attributed to the higher pregnancy rate. The clinical pregnancy rate per embryo transfer was higher in this study than that reported by Li et al. (2014) (30.30% in FET cycles), Gleicher et al. (2013) (ongoing clinical pregnancy rate: 19.1% in IVF cycles), and Kunicki et al. (2014) (18.9% in IVF cycles). This may be due to the low mean age of all patients in our study. A higher clinical pregnancy rate observed in our study does not necessarily mean that G-CSF can improve pregnancy rate directly after merely a single intrauterine perfusion; this finding of increased TEM is likely an indirect reason. When treating repititive implantation failure and recurrent spontaneous abortion, G-CSF was administered systemically by subcutaneous injection for a long period. In an RCT, women with unexplained primary recurrent spontaneous abortion underwent a daily subcutaneous administration of G-CSF, at a dosage of 1 μg/kg/day from the sixth day after ovulation to the end of the ninth week of gestation (Scarpellini and Sbracia, 2009). Another study also demonstrated that the results of G-CSF for the treatment of patients with a history of unexplained sterility or multiple unsuccessful IVF treatments were related to killer-cell immunoglobulin-like receptor defects (Wurfel et al., 2010). This may provide a reasonable explanation for the negative results of G-CSF on pregnancy rate in a study by Barad et al. (2014).

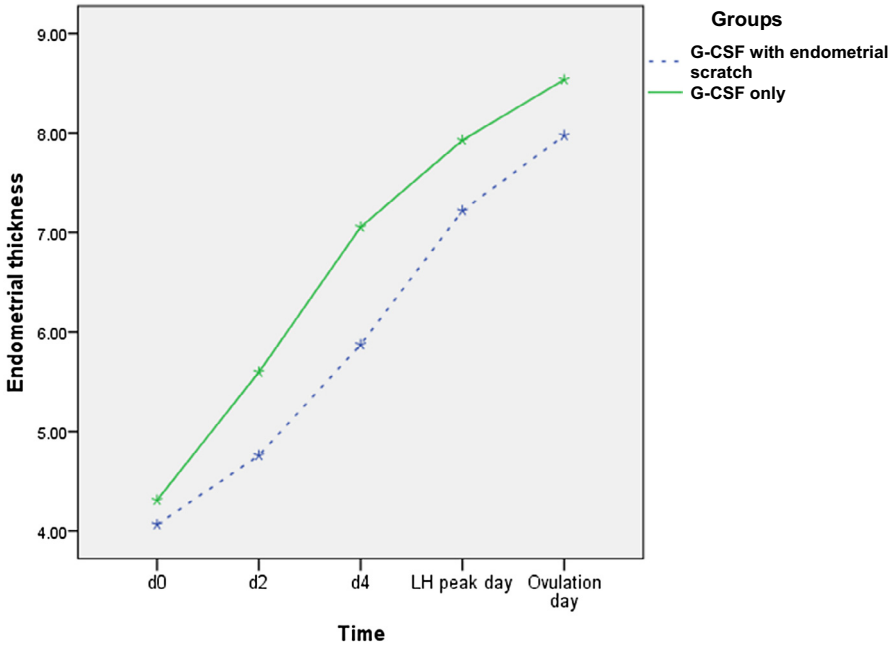

Figure 2 The trend of endometrial change with two protocols of granulocyte colony-stimulating factor (G-CSF) instillation. Day 0 is defined the day of G-CSF instillation. Multivariate analysis between two groups indicated no significant difference.

Our study also demonstrated that G-CSF has a limited effect in expanding thin endometrium in patients with a history of IUA. For these patients, the formation of severe endometrial injury may be prolonged, and the regenerative capability of endometrium was impaired seriously. It is hard to repair the injured endometrium with one G-CSF infusion. A dose of 300 μg G-CSF was used, based on a previous report (Gleicher et al., 2011). Perhaps repeated intrauterine G-CSF perfusion or administration in combination with other cytokines is feasible, but further studies are necessary to determine the perfect dose and treatment duration.

Another factor that may contribute to the clinical pregnancy rate observed in our studies is EMS. In a study of patients with severe Asherman syndrome who failed to achieve a functional receptive endometrium after six operative hysteroscopies combined with hormonal treatment, Barash et al. (2009) reported that TEM increased to 7 mm after four endometrial biopsies, and the subsequent IVF cycle resulted in the birth of a healthy baby. Local injury to the endometrium with irregular echo during an ovarian stimulation cycle improved the rates of embryo implantation, clinical pregnancy, and live birth in IVF and embryo transfer (Zhou et al., 2008). This also improved pregnancy outcomes of patients with repetitive implantation failure (Karimzadeh et al., 2009; Raziel et al., 2007; Tiboni et al., 2011).

In some studies (Gleicher et al., 2013; Kunicki et al., 2014), low clinical pregnancy rate was observed in the treatment of thin endometrium with G-CSF. Therefore, our team made additional efforts in an attempt to establish a better protocol. On the basis of the idea of 'scarification and fertilization', we first combined G-CSF and gentle EMS. Most patients and doctors who worry about causing further endometrium injury may find hard to understand the technique of EMS. In our study, however, EMS did not impair G-CSF treatment for thin endometrium, and it also improved clinical pregnancy rate and live birth rate. It can promote successful embryo implantation as mentioned above; after scarification by EMS, fibrous tissue, mucus on the endometrial face, and endometrium with a proliferation disorder may be removed so that G-CSF can be absorbed easily. Theoretically, intrauterine infusion of G-CSF with EMS is a better treatment in patients with thin endometrium.

We failed, however, to find a significant difference. This may be explained by the small sample size. In addition, our results, particularly in patients who were treated with G-CSF and EMS, suggested the percentage of patients with IUA history (38.5%) was relatively higher than that in the G-CSF only group (14.3%). Although EMS may also be a therapy for mild adhesions, a single gentle scratch cannot rescue a seriously injured endometrium. Therefore, the appropriate and maximum degree of EMS deserves further exploration.

Some may argue that a patient with a previously thin endometrium may have a normal TEM in a subsequent cycle, and letrozole may interfere with the results. Patients included in the current study, however, met strict criteria. Some failed to reach a TEM of 7 mm in previous fresh or human menopausal gonadotropin-induced cycles. Some suffered several embryo transfer cancellations because of thin endometrium. Among these, nine patients had a history of IUA, and embryo transfer was cancelled in three patients despite G-CSF treatment. Therefore, the possibility of this affecting our results is very small.

The imperfect control and the small sample size of patients constitute major limitations of our study. Moreover, patients who received G-CSF treatment had relatively low levels of FSH. Because of the FET treatment, basal FSH was not evaluated in the studied cycle, and these values were collected from the medical history of patients; therefore, these values may not be exact. In addition, pregnancy rate impact factors, including age, the number of embryos, and the number of good

Table 3 Cohort studies that have investigated the effect of intrauterine perfusion of granulocyte colony-stimulating factor on endometrium and pregnancy outcomes in IVF or frozen embryo transfer cycles.

Author(s)	*Year*	*Study design*	*Patient(s)*	*Endometrium*	*Programme*	*Dose (μg)*	*Time of intervention*	*Comparison*	*Main findings*
Gleicher et al.	2011	Case report	4	<7 mm at 5–10 days before embryo transfer	Egg donation ($n = 2$) FET ($n = 2$)	300	At 2–9 days before embryo transfer	Before versus after G-CSF infusion in the same cycle	TEM expansion to >7 mm; all four conceived
Gleicher et al.	2013	Prospective	21	<7 mm on day of HCG administration or day of ovulation trigger	IVF	300	On day of HCG administration or day of ovulation trigger and after oocyte retrieval	Before versus after G-CSF infusion in the same cycle	TEM increased significantly; ongoing clinical pergnancy rate: 19.1%
Li et al.	2014	Prospective controlled	34 (40 cycles)	≤7 mm on day of ovulation; on day of progesterone administration; on day of HCG administration; or day of ovulation trigger.	FET	100	On day of ovulation or on day of progesterone administration or day of HCG administration or day of ovulation trigger	Self-controlled group versus control group	No difference in implanatation rate and clinical pregnancy rate (30.3%) per embryo transfer
Lucena et al.	2013	Case report	1	<7 mm	In-vitro maturation	300	At the time of oocyte retrieval	Before and after G-CSF infusion in the same cycle	TEM quickly increased to >7 mm; a healthy baby was born.
Kunicki et al.	2014	Prospective	37	<7 mm on day of HCG administration or day of ovulation trigger.	IVF	300	On day of HCG administration or day of ovulation trigger	Before and after G-CSF infusion in the same cycle	TEM increased significantly; the clinical pregnancy rate: 18.9%.
Barad et al.	2014	Randomized controlled trial	73	Six patients <7 mm; others: normal on the morning of HCG administration or day of ovulation trigger.	normal IVF cycles	300	On the morning HCG administration or day of ovulation trigger	G-CSF versus saline group	TEM significantly increased in the G-CSF group; no difference in TEM increase, clinical pregnancy rate and implantation rate of the G-CSF group compared with the control group
Xu et al.	Present study	Prospective	30	<7 mm on day of ovulation; on day of progesterone administration; on day of HCG administration; or day of ovulation trigger in previous cycles	FET	300	On the day that one follicle became dominant (almost 12 × 12 mm in diameter)	G-CSF versus self-controlled and control group	TEM significantly increased for the G-CSF group; clinical pregnancy rate (48.1%) and implantation rate significantly greater than control group.

FET = frozen embryo transfer; G-CSF = granulocyte colony-stimulating factor; HCG = human chorionic gonadotropin; TEM = endometrial thickness.

embryos transferred, as well as other baseline characteristics, were similar between the G-CSF and control groups. These data are highly unlikely to affect pregnancy rate in FET cycles. The small sample size in the G-CSF group was another limitation of our study. This is mainly attributed to the low incidence of thin endometrium. Additionally, owing to the uncertain efficacy of G-CSF on thin endometrium, patients and doctors made their decisions carefully according to a relatively better TEM or appearance (e.g. endometrium <7 mm but >6.5 mm or has a triple-line pattern). According to our data (**Table 1**), patients in the control group had a thicker endometrium at baseline compared with patients in the G-CSF group, who had previously suffered embryo transfer cancellation ($P < 0.001$).

In conclusion, the present study sheds new light on the research and treatment of thin endometrium with G-CSF. We conclude that EMS did not impair the effect of G-CSF on increasing thin endometrium, and it produced relatively higher rates of clinical pregnancy and live birth than the G-CSF only protocol. For patients with thin endometrium, cancellation of embryo transfer and proceeding to undergo G-CSF treatment in a new FET cycle is beneficial, but the benefit is limited in patients with a history of IUA.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2014.12.006.

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