

A pilot cohort study of granulocyte colony-stimulating factor in the treatment of unresponsive thin endometrium resistant to standard therapies

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STUDY QUESTION: Is thin endometrium unresponsive to standard treatments expandable by intrauterine perfusion with granulocyte colony-stimulating factor (G-CSF)?

SUMMARY ANSWER: This cohort study is supportive of the effectiveness of G-CSF in expanding chronically unresponsive endometria.

WHAT IS KNOWN ALREADY: In a previous small case series, we reported the successful off-label use of G-CSF in four consecutive patients, who had previously failed to expand their endometria beyond 6.9 mm with the use of standard treatments.

STUDY DESIGN, SIZE AND DURATION: In a prospective observational cohort pilot study over 18 months, we described 21 consecutive infertile women with endometria <7 mm on the day of hCG administration in their first IVF cycles at our center. All previous cycles using traditional treatments with estradiol, sildenafil citrate (Viagra™) and/or beta-blockers had been unsuccessful. G-CSF (Nupogen™) was administered per intrauterine catheter by slow infusion before noon on the day of hCG administration. If the endometrium had not reached at least a 7-mm within 48 h, a second infusion was given following oocyte retrieval. Primary and secondary main outcomes were an increase in endometrial thickness and clinical pregnancy, respectively. Endometrial thickness was assessed by vaginal ultrasound at the most expanded area of the endometrial stripe.

PARTICIPANTS/MATERIALS, SETTINGS AND METHOD: This study was uncontrolled, each patient serving as her own control in a prospective evaluation of endometrial thickness. The mean \pm SD age of the cohort was 40.5 ± 6.6 years, gravidity was 1.8 ± 2.1 (range 0–7) and parity was 0.4 ± 1.1 (range 0–4); 76.2% of women had, based on age-specific FSH and anti-Müllerian hormone, an objective diagnosis of diminished ovarian reserve and had failed 2.0 ± 2.1 prior IVF cycles elsewhere.

MAIN RESULTS AND THE ROLE OF CHANCE: With 5.2 ± 1.9 days between G-CSF perfusions and embryo transfers, endometrial thickness increased from 6.4 ± 1.4 to 9.3 ± 2.1 mm ($P < 0.001$). The Δ in change was 2.9 ± 2.0 mm, and did not vary between conception and non-conception cycles. A 19.1% ongoing clinical pregnancy rate was observed, excluding one ectopic pregnancy.

LIMITATIONS AND REASONS FOR CAUTION: Small sample size (but a highly selected patient population) in an uncontrolled cohort study and in unselected first IVF cycles at our center.

WIDER IMPLICATIONS OF THE FINDINGS: This pilot study supports the utility of G-CSF in the treatment of chronically thin endometrium and suggests that such treatment will, in very adversely affected patients, result in low but very reasonable clinical pregnancy rates.

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materials and the manuscript presented here. N.G. and D.H.B. are listed as co-inventors on a number of awarded and still pending U.S. patents, none related to the materials presented here. N.G. is on the board of a medically related company, not in any way associated with the data presented here.

Key words: IVF / thin endometrium / granulocyte colony-stimulating factor / clinical pregnancy rate / diminished ovarian reserve

Introduction

Chronically thin endometrium resistant to standard treatments affects a small number of patients undergoing IVF. This problem, nevertheless, is of considerable importance because endometrium below 7 mm in thickness is widely considered sub-optimal for transfer and associated with reduced pregnancy chances (Casper, 2011; Singh *et al.*, 2011; Revel, 2012).

Various remedies have been proposed, including extended estrogen administration if time allows (Chen *et al.*, 2006), low-dose aspirin (Weckstein *et al.*, 1997) and treatment with pentoxifylline and tocopherol (Lédée-Bataille *et al.*, 2002) and with vaginal sildenafil citrate (Viagra™) (Sher and Fisch, 2002). However, even utilizing these remedies, a small number of women remain unresponsive.

The prevalence of patients who remain unresponsive to such standard treatment modalities is unknown but on the basis of our own (unpublished) experience, we have estimated it to be <1% of the IVF patients. However, for such patients and their treating physicians, such a chronically thin endometrium offers considerable treatment challenges, resulting in cycle cancellations, unplanned cryopreservation of embryos and, in the most extreme cases, in the utilization of gestational carriers.

We previously reported the successful use of granulocyte colony-stimulating factor (G-CSF) in four IVF cycles, in which standard treatments to reach a minimal endometrial thickness of 7 mm had failed (Gleicher *et al.*, 2011). This preliminary experience led to the initiation of two prospectively RCTs of G-CSF at our center.

In our first four reported cases of G-CSF treatment, all the women conceived (one an intramural ectopic pregnancy), and therefore, in a first trial (NCT01202656) we decided to investigate whether endometrial perfusion with G-CSF, independent of endometrial thickness, in routine IVF cycles affects pregnancy rates. This trial is still underway, with approximately three-quarters of targeted cycles completed.

In a second clinical trial (NCT01202643), we planned to investigate in a prospectively randomized fashion whether, and to what degree, endometrial perfusion with G-CSF, indeed, is able to expand chronically thin endometrium, which is resistant to standard therapies. This trial is also still underway but recruitment is exceedingly slow and completion of this study is, therefore, not in the near future.

Therefore, as an interim step, here we report a series of 21 IVF cycles in women with chronically thin endometrium, who were treated with G-CSF outside the aforementioned trials because patients either did not qualify for the studies or refused participation.

Materials and Methods

We here report on 21 women in their first IVF cycle at our center. All the women on the day of ovulation induction with hCG demonstrated via ultrasound an endometrial thickness of <7 mm, despite treatment with

oral and vaginal ethinyl estradiol (E₂; 2 mg, per os twice daily and 1 mg per vagina three times daily) and sildenafil citrate (Viagra™, 25 mg, per vagina four times daily, Pfizer Inc., New York, NY, USA).

At the time of this study, two registered prospective RCTs were being conducted at our center (ClinicalTrials.gov ID NCT01202643 and ClinicalTrials.gov ID NCT01202656), as already described, and the patients described here refused participation in the first and were unqualified for participation in the second trial. They, therefore, were given the following options: (i) participation in a clinical trial (for details see Materials and Methods); (ii) IVF cycle cancellation; (iii) embryo transfer into an inadequately thin endometrium and (iv) the off-label use of G-CSF in the form of an intrauterine infusion as already mentioned. All the patients selected option (iv).

Table I summarizes the patient characteristics: Commensurate with our center's patient population, the mean \pm SD age of patients was 40.5 ± 6.6 years; their BMI was 23.6 ± 4.0 . Their mean gravidity was 1.8 ± 2.1 (range 0–7) and their mean parity 0.4 ± 1.1 (range 0–4). The cycles reported here represent exclusively the first cycles at our center, but it is important to point out that these patients had previously undergone 2.0 ± 2.1 IVF cycles elsewhere. The most common primary infertility diagnosis was diminished ovarian reserve, present in 16 (76.2%) of the women.

Table I Characteristics of patients in a study of the effect of G-CSF on endometrial thickness in IVF cycles.

| | |
|--|---------------------|
| Number of patients | 21 |
| Age (years) | 40.5 ± 6.5 |
| Parity (range) | 0.4 ± 1.1 (0–4) |
| Gravidity (range) | 1.8 ± 2.1 (0–7) |
| Failed prior IVF cycles (range) ^a | 2.0 ± 2.1 (0–9) |
| BMI (kg/m ²) | 23.6 ± 4.0 |
| FSH (mIU/ml) | 15.2 ± 19.3 |
| Anti-Mullerian hormone (ng/ml) | 1.5 ± 2.6 |
| Gonadotrophin dosage (IU) | 4491.7 ± 2241.4 |
| Race (n/%) | |
| Caucasian | 12/57.1 |
| African | 4/19.0 |
| Asian | 5/23.8 |
| Primary infertility diagnosis (n/%) | |
| Diminished ovarian reserve | 16/76.2 |
| Male factor | 2/9.5 |
| Uterine factors | 4/19.0 |
| Polycystic ovary syndrome | 1/4.8 |

Data are mean \pm SD.

FSH and Anti-Mullerian hormone levels were tested before cycle start. ^a 0.1 ± 0.4 (range 0–1) prior IVF cycles were cancelled because of thin endometrium.

A diagnosis of unresponsive thin endometrium was made on the day of administration of 10 000 IU hCG. Considering the adverse endometrial thickness in these patients, ovulation induction was induced even if only one follicle reached 19 mm. Once the decision to administer hCG in an IVF cycle was made after morning monitoring by ultrasound, women with endometrial thickness <7 mm were asked to select one of the aforementioned four options. Once they decided to proceed with off-label use of G-CSF, they received an endometrial infusion (by tomcat catheter) of 30 mU (300 mcg/1 ml) of G-CSF (Neupogen™, Filgastrim, Amgen Inc., Thousand Oaks, CA, USA) approximately 6–12 h before hCG administration.

The infusion was administered as previously described (Gleicher et al., 2011). In short, the content of the ampule was aspirated into a 1-ml insulin syringe, the Tomcat catheter was introduced into the endometrial cavity as performed during an intrauterine insemination and the content of the syringe was slowly injected into the cavity, while the catheter was gently moved back and forth. Upon completion of the injection, the syringe was disconnected, a small amount of air was aspirated, the syringe was reconnected to the catheter and the air bubble was injected, to pass whatever small amount of G-CSG was left in the Tomcat catheter into the endometrial cavity.

Two days later, during oocyte retrieval, the endometrial thickness was reassessed by ultrasound. If at that point the maximal endometrial thickness was still below 7 mm, a second, identical infusion of G-CSF was performed following oocyte retrieval. This was required only in 3/21 (14.3%) of cases.

A final endometrial measurement was made on the day of embryo transfer (universally Day 3). Endometrial thickness was always measured at the thickest point.

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 18.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean and SD and assessed by *t*-test. A *P*-value of < 0.05 was considered significant.

As noted earlier, patients in this study did not participate in our clinical trials of G-CSF, approved by the Institute Review Board. They received G-CSF infusion off-label and provided informed consent for the procedure we described as 'experimental.' All the patients also signed an informed consent that allows review of their medical records for research purposes, as long as the patient's anonymity and confidentiality of her medical record are maintained. Both conditions were met here.

Results

Our center cancels embryo transfers and cryopreserves all embryos if endometrial thickness on the day of embryo transfer is not at least 7 mm. All the patients underwent transfer. The clinical pregnancy rate in treated cycles was 19.1%.

None of the patient characteristics listed in Table I varied between conception (*n* = 4) and non-conception (*n* = 17) cycles. While this makes a contribution of other unrecognized factors to pregnancy success unlikely, the small study size does not preclude type II errors.

Table II summarizes the endometrial findings before and after G-CSG perfusion: As the table demonstrates, for all the patients the average time between first G-CSF infusion and embryo transfer was 5.2 ± 1.9 days. There was no difference between women who conceived and those who did not.

At the time of the first infusion, the endometrium thickness was 6.4 ± 1.4 mm. By the time of embryo transfer, it had expanded to 9.3 ± 2.1 mm (*P* < 0.001). There was also a significant difference between measurements at the time of the first infusion versus

Table II Endometrial thickness in women before and after intrauterine treatment with G-CSF.

| | All patients (<i>n</i> = 21) | Patients who conceived (<i>n</i> = 4) | Patients who did not conceive (<i>n</i> = 17) |
|---|----------------------------------|--|---|
| First G-CSF infusion to embryos transfer (days) | 5.2 ± 1.9 | 5.5 ± 2.9^1 | 5.2 ± 1.7^1 |
| Endometrial lining (mm) at first G-CSF infusion | 6.4 ± 1.4^2 | 5.6 ± 1.5^3 | 6.6 ± 1.4^4 |
| Endometrial lining (mm) at embryo transfer (mm) | 9.3 ± 2.1^2 | 8.8 ± 1.7^3 | 9.5 ± 2.2^4 |
| Δ endometrial thickness (mm) | 2.9 ± 1.9 | 3.2 ± 1.7^5 | 2.9 ± 2.1^5 |

Values are mean ± SD; Based on *t*-tests, *P* < 0.05 denotes significance.

¹*P* = 0.767.

²*P* < 0.001.

³*P* = 0.034.

⁴*P* < 0.001.

⁵*P* = 0.793.

embryo transfer in cycles leading to pregnancy (*P* = 0.034) and those not resulting in pregnancy (*P* < 0.001). The change in endometrial thickness for the whole group was 2.9 ± 2.0 mm.

Discussion

Despite the small cohort size and lack of a control group, the present data are supportive of our initial report in which we suggested that endometrial perfusion with G-CSF may be effective in expanding chronically unresponsive thin endometrium, which was resistant to traditional remedies, such as increased E₂ support and Viagra™ (Gleicher et al., 2011).

By presenting 21 first cycles of IVF treatment at our center in 21 women, we (for the first time) are able to report statistically sound data, which demonstrate a significant improvement in endometrial thickness after G-CSF treatment. As the study did not involve a control group, one could argue that the endometrium may have expanded even without G-CSF perfusions. Considering, however, that all the patients still presented with endometrium <7 mm on the day of hCG and that prior attempts to thicken their endometrium with E₂ and Viagra™ had failed, such an explanation appears highly unlikely. Moreover, 0.1 ± 0.4 (range 0–1) of previously failed cycles had been cancelled because of 'inadequately' thin endometrium (Table I).

In considering our results, it also appears important to, once more, consider the patient population in which these results were obtained: These were women of advanced age (40.5 ± 6.6 years), with prior IVF failures at other centers (2.0 ± 2.1) and diminished functional ovarian reserve in 76.2% of cases. These women nevertheless reached the stage of ovulation induction with hCG but at that point were diagnosed with treatment-resistant thin endometrium. Options at that point were limited to: (i) the patient agreeing to participate in the

center's registered prospectively RCT (ClinicalTrials.gov ID, NCT01202643); (ii) cycle cancellation; (iii) embryo transfer into an inadequate endometrium (<7 mm) or (iv) off-label use of G-CSF, based on the center's prior experience with four patients (Gleicher *et al.*, 2011).

None of the patients described here consented to participation in the clinical trial, and all the 21 selected option (iv) and provided appropriate informed consent.

These results raise the question of how G-CSF expands endometrial thickness in such a short time? In our initial publication, we reported that a growth spurt in endometrial thickness can be observed within 48 h of G-CSF administration (Gleicher *et al.*, 2011). This was confirmed here, as all but three patients reached a minimal thickness of 7 mm within ~48 h, by the time of oocyte retrieval. The remaining three patients reached this minimal thickness, after a second infusion, by the day of embryo transfer (Day 3).

How G-CSF accomplishes this is, however, unknown. Surprisingly, little is known about how G-CSF impacts on the endometrium. G-CSF is a glycoprotein with growth factor and cytokine functions and is produced in different tissues/cells, including endothelium, macrophages and in other immunocytes. In the central nervous system, G-CSF not only acts as a proliferant by inducing neurogenesis but also has anti-apoptotic functions (Schneider *et al.*, 2005). It, therefore, has been proposed as a potential therapeutic agent in neurodegenerative diseases (Pitzer *et al.*, 2008).

Investigating the effect of G-CSF on proliferation and differentiation of normal human endometrial stromal cells, Tanaka *et al.* (2000) concluded that G-CSF enhances cAMP-mediated decidualization of human endometrial stromal cells in both an autocrine and a paracrine fashion. In a follow-up study, the same authors demonstrated that macrophage (M)-CSF enhances G-CSF secretion from unstimulated human endometrial stromal cells but not from 8-BR-cAMP-stimulated cells (Tanaka and Umesaki, 2003). Fahey *et al.* (2005) reported that, among other cytokines and chemokines, G-CSF and granulocyte-macrophage CSF (GM-CSF) are secreted apically in polarized epithelial cells.

In clinical reproduction, G-CSF has been proposed as a treatment for implantation failure and repeated miscarriages (Scarpellini and Sbracia, 2009; Würfel *et al.*, 2010; Toth *et al.*, 2011), two indications for which a US patent has been issued (United States Patent, 2008). How these widely divergent alleged benefits are achieved is, however, still unknown. The patent mostly assumes immunological processes, which cannot explain the proliferative effects on the endometrium observed here.

G-CSF and GM-CSF appear to be involved in a wide variety of reproductive functions: Yanagi *et al.* (2002) reported cyclic changes of G-CSF mRNA in follicular fluid during the menstrual cycle. Salmassi *et al.* (2004) described G-CSF and its receptor in human luteinized granulosa cells. G-CSF in follicular fluid has been proposed as useful biomarker of oocyte competence before fertilization (Lédée *et al.*, 2011).

From basic research, slightly more data on the effects of GM-CSF on endometrium have been obtained. Zhao and Chegini (1999) suggested that expression of GM-CSF and its receptor during the menstrual cycle implies an autocrine and paracrine function of GM-CSF in the endometrium. Chegini *et al.* (1999) reported that GM-CSF is not mitogenic for endometrial cells, whether epithelial or glandular,

but in an interactive fashion with transforming growth factor (TGF)- β 1 it regulates its own expression and the expression of TGF- β 1 in the endometrium. In mice GM-CSF has been suggested as an essential regulator of T cell activation competence in uterine dendritic cells during early pregnancy (Moldenhauer *et al.*, 2010).

Claims have been made that supplementation of embryo growth media with GM-CSF promotes human embryo development to blastocyst stage (Sjöblom *et al.*, 1999). An embryo growth medium supplemented with GM-CSF became available commercially in Europe in 2011. Its approval in the USA is expected in late 2012, with claims of a 44% improvement in implantation rate (EmbryoGen[®], Måløv, Denmark) [<http://www.origio.com> <http://www.origio.com/products/medicult%20edia/embryogen.aspx> (13 May 2012, date last accessed)]. Takasaki *et al.* (2008) reported that use of M-CSF in combination with human menopausal gonadotrophin in poor responders improves follicle development.

G-CSF, GM-CSF and M-CSF are, however, distinct: G-CSF, in principle, facilitates stem cell and progenitor proliferation in neutrophilic granulocytes, while GM-CSF facilitates proliferation and differentiation in granulocytes, macrophages and eosinophils. Because of proliferative effects on fibroblasts, one, therefore, can hypothesize that GM-CSF may expand endometrial thickness even more than G-CSF. Such studies remain, however, to be performed. Moreover, because it is unknown how G-CSF achieves the rapid proliferation of endometrial architecture, any potential effects of M-CSF or GM-CSF at this point are just conjecture.

The matter is further complicated by the fact that one can also not rule out possible synergistic effects between G-CSF and sildenafil citrate (Viagra[™]), which all the patients in the study received before and during G-CSF administration. A better understanding of the action of G-CSF on the endometrium would, obviously, greatly strengthen the credibility of its utility in this indication.

The cohort of patients presented here, thus, provides further evidence that G-CSF perfusion of the endometrium may offer an effective last resort for treatment of thin endometrium that is resistant to more traditional treatment modalities. This observation, however, has to be confirmed by larger studies, preferably in the form of a prospective RCT.

The study, however, raises a number of additional questions: How does G-CSF thicken the endometrium within such a short time interval? As we have performed no dosaging studies, the question arises as to whether the treatment protocol we are utilizing is maximal? One has to assume that improvements should be possible.

In earlier noted treatments for implantation failure and repeat miscarriages, G-CSF has been administered by the subcutaneous rather than the intrauterine route (Scarpellini and Sbracia, 2009; Würfel *et al.*, 2010; Toth *et al.*, 2011). Which delivery method for the drug is superior remains to be determined. Moreover, in these studies G-CSF was administered for much longer time periods, suggesting that more frequent administration (maybe daily) may have superior benefits. Finally, as already noted, it is tempting to hypothesize that M-CSF or GM-CSF may have even better effectiveness.

Considering the age of our patient population, with a mean of 40.5 years and with established diminished ovarian reserve in 76.2% of women, and taking their endometrial characteristics into account, the ongoing clinical pregnancy rate of 19.1% has to be considered quite remarkable. Our 2011 ongoing clinical pregnancy rate for all

women at age 41 years in a very similar patient population was 25% (www.centerforhumanreprod.com). While our data are obviously preliminary, we would suggest that administration of G-CSF in women with treatment-resistant endometrium restores potential female fertility to a significant degree. Considering that the natural conception rate in a general infertile population has been estimated at ~1% per month (Gleicher et al., 1996), it is reasonable to assume that the pregnancy chance in a patient population, such as that presented here, is unlikely to be more than one-tenth of that figure. In an 18-month period, the time that patients were recruited for this study, one therefore, optimistically, could expect only a spontaneous pregnancy rate of, at most, ca. 3.6%.

This observation once again raises the question of whether, beyond endometrial expansion, G-CSF can, in general, beneficially affect implantation and pregnancy rates in women undergoing IVF. We hope to be able to answer at least this last question quite soon, upon completion of our ongoing clinical trial.

Authors' roles

N.G. and D.H.B. contributed equally to the manuscript, being involved in all the stages of study design execution, data analysis and manuscript preparation. N.G. took primary responsibility for manuscript preparation and D.H.B. for data analysis. Data extraction and primary analysis were performed by A.K., while the remaining authors contributed to the laboratory and clinical aspects of the study. All the authors approved the final manuscript.

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Conflict of interest

N.G. and D.H.B. are members of the board of the Foundation for Reproductive Medicine. N.G. is owner of CHR-New York, where the study was conducted. N.G. and D.H.B. have been recipients of research awards, travel grants and speaker honoraria from various pharmaceutical and medical device companies. None of these companies was, however, in any way associated with the materials and manuscript presented here. N.G. and D.H.B. are listed as co-inventors on a number of awarded and still pending US patents, none related to the materials presented here. N.G. is on the board of a medically related company, not in any way associated with the data presented here.

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