Granulocyte colony-stimulating factor in repeated IVF failure, a randomized trial

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Abstract

Recent studies have revealed key roles for granulocyte colony-stimulating factor (GCSF) in embryo implantation process and maintenance of pregnancy, and some studies showed promising results by using local intrauterine infusion of GCSF in patients undergoing *in vitro* fertilization (IVF). This multicenter, randomized, controlled trial included 112 infertile women with repeated IVF failure to evaluate the efficacy of systemic single-dose subcutaneous GCSF administration on IVF success in these women. In this study, the Long Protocol of ovarian stimulation was used for all participants. Sealed, numbered envelopes assigned 56 patients to receive subcutaneous 300 µg GCSF before implantation and 56 in the control group. The implantation (number of gestational sacs on the total number of transferred embryos), chemical pregnancy (positive serum β-HCG), and clinical pregnancy (gestational sac and fetal heart) rates were compared between the two groups. This trial is registered at www.irct.ir (IRCT201503119568N11). The successful implantation (18% vs 7.2%, $P=0.007$), chemical pregnancy (44.6% vs 19.6%, $P=0.005$), and clinical pregnancy (37.5% vs 14.3%, $P=0.005$) rates were significantly higher in the intervention group than in the control group. After adjustment for participants' age, endometrial thickness, good-quality oocyte counts, number of transferred embryos, and anti-Müllerian hormone levels, GCSF treatment remained significantly associated with successful implantation (OR=2.63, 95% CI=1.09–6.96), having chemical pregnancy (OR= 2.74, 95% CI=1.11–7.38) and clinical pregnancy (OR=2.94, 95% CI=1.23–8.33). In conclusion, administration of single-dose systemic subcutaneous GCSF before implantation significantly increases the IVF success, implantation, and pregnancy rates in infertile women with repeated IVF failure.

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Introduction

In vitro fertilization (IVF) is the most successful assisted reproductive technology (ART) used to treat infertility. However, IVF is expensive and time-consuming, and the success rate is still less than 40%. Many couples still remain unsuccessful after several IVF attempts causing deep effect on quality of life and a heavy financial burden (Zeyneloglu & Onalan 2014). With the recent advances in understanding the important role of granulocyte colony-stimulating factor (GCSF) in reproduction, and the detection of GCSF receptors in placental cytotrophoblasts, syncytiotrophoblasts, decidual stromal cells, endometrial gland cells, and follicular cells (Saito *et al.* 1994, Miyama *et al.* 1998, McCracken *et al.* 1999, Salmassi *et al.* 2005, Ledee *et al.* 2008, 2013, Rahmati *et al.* 2014), GCSF started to attract attentions in gynecology, especially in patients with recurrent abortions and/or infertility.

In 2009, GCSF was successfully used in patients with recurrent abortions (Scarpellini & Sbracia 2009), and later another study reported promising results

by using GCSF in four women undergoing IVF who had demonstrated highly inadequate endometrium (Gleicher *et al.* 2011). Since then, several studies with controversial results have evaluated the effect of GCSF on endometrial thickness and IVF success (Scarpellini & Sbracia 2012, Gleicher *et al.* 2013, Barad *et al.* 2014, Eftekhar *et al.* 2014, Kunicki *et al.* 2014, Li *et al.* 2014, Xu *et al.* 2015). In the studies of Scarpellini and coworkers and Xu and coworkers, the implantation and pregnancy rates were significantly higher in IVF patients treated with GCSF (Scarpellini & Sbracia 2012, Xu *et al.* 2015), whereas in the studies of Barad and coworkers and Li and coworkers, treatment with GCSF did not affect endometrial thickness, implantation, or pregnancy rates in IVF patients (Barad *et al.* 2014, Li *et al.* 2014).

These studies had some limitations that might preclude the researchers from making a precise conclusion, including small sample size (Gleicher *et al.* 2013, Eftekhar *et al.* 2014, Kunicki *et al.* 2014, Li *et al.* 2014, Xu *et al.* 2015), no control groups (Gleicher *et al.* 2013, Kunicki *et al.* 2014), no

randomization (Gleicher *et al.* 2013, Eftekhar *et al.* 2014, Kunicki *et al.* 2014, Li *et al.* 2014), and unintended selection bias (Scarpellini & Sbracia 2012, Barad *et al.* 2014, Li *et al.* 2014, Xu *et al.* 2015). All these studies have suggested that large, randomized, controlled trials are still needed to evaluate the efficacy of GCSF administration on IVF success (Scarpellini & Sbracia 2012, Gleicher *et al.* 2013, Barad *et al.* 2014, Eftekhar *et al.* 2014, Kunicki *et al.* 2014, Li *et al.* 2014, Xu *et al.* 2015). The efficacy of GCSF in patients with repeated IVF failure has not been established yet. In addition, most studies have infused GCSF locally into the uterine cavity. Some authors have suggested that systemic administration of GCSF might be more effective than local infusion (Barad *et al.* 2014, Zeyneloglu *et al.* 2013).

We conducted this study on infertile women with repeated IVF failure to evaluate the efficacy of systemic subcutaneous GCSF administration on IVF success, implantation, and pregnancy rates.

Materials and methods

Study population and design

This multicenter, prospective, randomized, open-label, controlled trial was conducted on 112 infertile women who had repeated IVF failure. The study was conducted at the infertility departments of Shariati Hospital (46 patients), Bahman Hospital (36 patients), and Omid Infertility Clinic (30 patients), Tehran, Iran, from March 2015 through January 2016.

Repeated IVF failure is determined when transferred embryos fail to implant after several IVF cycles. However, there are no formal criteria defining the number of failed cycles or

the total number of embryos transferred in these IVF attempts (Simon & Laufer 2012). In our study, repeated IVF failure was defined as failure of implantation in at least three consecutive IVF attempts, in which three embryos of high-grade quality are transferred in each cycle (Simon & Laufer 2012).

The patients were considered eligible if they had the following inclusion criteria: age <40 years, having repeated IVF failure, absence of contraindications for GCSF treatment (sickle cell disease, chronic neutropenia, known past or present malignancy, renal insufficiency, upper respiratory infection, pneumonia, and congenital fructose intolerance), and absence of any systemic disease (including hypertension, diabetes, antiphospholipid syndrome, systemic lupus erythematosus, thrombophilia, and thyroid disorders). The exclusion criteria were the presence of an inadequate endometrium (<7 mm) on the day of hCG injection; the detection of Asherman's syndrome, fibroids, and/or polyps via diagnostic hysteroscopy; and/or refusing to give an informed consent to participate in the study.

A computerized random number generator was used for sequence generation. Simple randomization with a 1:1 allocation ratio was used. Consecutive opaque envelopes for the allocation concealment were performed. The envelopes were opaque when held to the light, opened sequentially, and only after the participant's name and other details were written on the appropriate envelope and implementation of assignments was carried out.

Intervention, data, and specimen collection

In this study, the Long Protocol of ovarian stimulation was used for all the participants. Gonadotropin-releasing hormone (GnRH) agonist 0.1 mg (Decapeptyl; Ferring) was administered

Figure 1 CONSORT flow diagram of the study.

subcutaneously once/daily, which was started 7 days before the next expected period and continued until the third day of the cycle. Next, a daily subcutaneous injection of 300 IU follicle-stimulating hormone (FSH) (Gonal-F; Merck Serono) was applied for 4 days starting from the third day of the cycle. On the 6th day of the cycle, 150–300 IU intramuscular injection of human menopausal gonadotropin (hMG) (Menogon; Ferring) was administered. The hMG dose was administered based on the participants' weight, FSH level, and antral follicle counts (AFC). Transvaginal sonography was performed every 1–3 days to evaluate the follicle size. When at least two follicles reached a minimum of 18 mm diameter, 10,000 IU intramuscular injection of human chorionic gonadotropin (hCG) (Choragon; Ferring) was given to the participants. All the participants received 5 mg prednisolone daily starting from the day of hCG injection until 3 days following oocyte retrieval. Transvaginal oocyte pickup was performed 36 h after hCG administration under transvaginal sonography. Embryo quality was evaluated using standard day-3 embryo grading; day-3 embryos with eight cells and <15% fragmentation were considered good. About one to three good embryos were transferred into the uterine cavity. The number of transferred embryos was chosen based on the criteria of the American Society for Reproductive Medicine (ASRM) (Practice Committee of the American Society for Reproductive Medicine 2013). The participants were randomly allocated to two groups: the intervention (GCSF) group and the control group. In the intervention group, a single dose of 300 µg GCSF (Neupogen; Roche) was administered subcutaneously 1 h before the embryo transfer. The participants in the control group did not receive any additional treatment before the embryo transfer. Luteal phase support was carried out on all participants using Cyclogest suppositories two times per day vaginally and 80 mg aspirin daily. After 2 weeks of the embryo transfer, serum β -HCG level was measured. After 4 and 6 weeks of embryo transfer, transvaginal sonography was performed on the participants who had a positive pregnancy test to detect the gestational sac and fetal heart rate (FHR) respectively.

The outcomes were chemical pregnancy, clinical pregnancy, and implantation rates. Chemical pregnancy was defined as having a positive serum β -HCG test, whereas clinical pregnancy was defined as the presence of gestational sac and fetal heart on ultrasound examination, and implantation rates were determined by the number of gestational sacs at least 28 days after embryo transfer based on the total number of embryos transferred per group.

This study was approved by the Research Deputy and the Ethics Committee of the Tehran University of Medical Sciences. This study was registered prospectively at the Iranian Registry of Clinical Trials (www.irct.ir), which is a Primary Registry in the WHO Registry Network (Registration Number=IRCT201503119568N11).

Statistical analysis

Presuming a clinical pregnancy rate of 15% in the control group and anticipating a clinical pregnancy rate of 40% with GCSF treatment based on the available literature, at least 50

participants in each study arm would be needed for 80% power, $\beta=20\%$ and $\alpha=0.05$. All the statistical analyses were performed using SPSS statistical software (version 18.0.0; PASW, Chicago, IL, USA). Data were expressed as mean, s.d., and percentage. Mean comparisons between the two groups and variables were performed using the *t*-test for independent samples and *t*-test for paired samples depending on the tested variables. Furthermore, the Pearson correlation coefficient, χ^2 analysis, Fisher's exact test, and one-way ANOVA were also used. The level of statistical significance was set at *P* value <0.05.

Results

Descriptive statistics

In total, 123 infertile women with repeated IVF failure agreed to participate in the study, 12 of whom were excluded. Of those excluded, six had inadequate endometrium, three were >40 years of age, and two had <3 failed IVF attempts. Therefore, 112 infertile women with ≥ 3 failed IVF attempts remained in the study. Of the 112 women, 56 were randomly assigned to the intervention group to receive a single dose of subcutaneous GCSF on the embryo transfer day and 56 were allocated to the control group (Fig. 1). Upon enrollment, the mean $\pm$ s.d. for the participants' age was 33 ± 4.8 years; their partners' age was 38.5 ± 8.2 years; the participants' weight was 67.9 ± 10.1 kg; the infertility duration was 8.1 ± 5 years; anti-Müllerian hormone (AMH) level was 2.8 ± 2.5 (median: 2.45) ng/mL; FSH level was 7.3 ± 4.1 mIU/mL; luteinizing hormone (LH) level was 8 ± 5.1 mIU/mL; antral follicular counts (AFC) were 10.7 ± 4.9 (median: 10); endometrial thickness on the day of embryo transfer was 11.3 ± 2.1 mm; retrieved

Table 1 Comparison of the demographics between the intervention and the control groups. Data are presented as mean $\pm$ s.d.

Demographics	GCSF-treated group <i>n</i> =56	Control group <i>n</i> =56	<i>P</i> value
Participants' age (years)	33.5 ± 4.2	32.4 ± 5.2	0.22
The partners' age (years)	40.1 ± 9.5	37 ± 6.4	0.08
Weight (kg)	66.7 ± 9.2	69.1 ± 10.9	0.19
Infertility duration (years)	8.1 ± 4.2	8.2 ± 5.8	0.89
AMH (ng/mL; median)	2.05	2.55	0.06
FSH (mIU/mL)	7.5 ± 4.6	7.2 ± 3.6	0.65
LH (mIU/mL)	8.9 ± 5.9	7.2 ± 4.2	0.09
AFC (median)	10	10	0.11
Endometrial thickness (mm)	11.2 ± 2	11.5 ± 2.3	0.9
Retrieved oocyte counts	15.8 ± 7.2	14.3 ± 6.5	0.1
Good-quality MII oocyte counts	9.1 ± 5.1	8.2 ± 6.1	0.07
Transferred embryos (number)	2.3 ± 0.6	2.5 ± 0.6	0.19
Failed IVF attempts (number; median)	3	3	0.62
Primary infertility (number (%))	51 (91.1%)	47 (83.9%)	0.25

GCSF, granulocyte colony-stimulating factor; s.d., standard deviation; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AFC, antral follicular counts; MII, metaphase II; IVF, *in vitro* fertilization.

Table 2 Comparison of the IVF outcomes between the intervention and the control groups.

Outcomes	GCSF group n=56	Control group n=56	Unadjusted OR (95% CI)	Adjusted OR [†] (95% CI)
Implantation (number of GS/total transferred embryos (%))	24/133 (18%)	10/138 (7.2%)	2.81 (1.29–6.15)	2.63 (1.09–6.96)
Chemical pregnancy (number (%))	25 (44.6%)	11 (19.6%)	3.29 (1.41–7.67)	2.74 (1.11–7.38)
Clinical pregnancy (number (%))	21 (37.5%)	8 (14.3%)	3.6 (1.43–9.06)	2.94 (1.23–8.33)
Ectopic pregnancy (number (%))	2 (3.6%)	1 (1.8%)	2.03 (0.17–23.13)	–

GCSF, granulocyte colony-stimulating factor; OR, odds ratio; 95% CI, 95% confidence interval; GS, gestational sacs.

[†]Adjusted for the participants' age, endometrial thickness, good-quality metaphase II oocyte counts, number of transferred embryos, and anti-Müllerian hormone levels.

oocyte counts were 14.9 ± 7 ; retrieved good-quality (metaphase II (MII)) oocyte counts was 8.5 ± 5.6 ; and the number of transferred embryos was 2.4 ± 0.6 . Of the 112 participants, 98 had primary infertility (87.5%), while 14 had secondary infertility (12.5%). Of the 112 participants, 57 had three failed IVF attempts (50.8%), 27 had four failed IVF attempts (24.1%), and 28 had ≥ 5 failed IVF attempts (25%).

There were no statistically significant differences in the demographics of the patients allocated to the intervention group versus those allocated to the control group (Table 1).

The effect of GCSF on IVF outcomes in patients with repeated IVF failure

The implantation rate in the intervention group was significantly higher than that in the control group (18% vs 7.2%, $P=0.007$). The chemical pregnancy rate was significantly higher in the intervention group than in the control group (44.6% vs 19.6%, $P=0.005$). The clinical pregnancy rate was also significantly higher in the intervention group than in the control group (37.5% vs 14.3%, $P=0.005$). One participant in each group had ectopic pregnancy (3.6% vs 1.8%, $P=1$) (Table 2).

Dependency of the obtained results

Logistic regression model was used to determine the dependency of the effect of GCSF treatment on IVF outcomes. When adjusted for the participants' age, endometrial thickness, good-quality MII oocyte counts, number of transferred embryos, and AMH levels, GCSF treatment remained significantly associated with successful implantation (OR=2.63, 95% CI=1.09–6.96), having chemical pregnancy (OR=2.74, 95% CI=1.11–7.38), and having clinical pregnancy (OR=2.94, 95% CI=1.23–8.33) (Table 2).

Discussion

GCSF and its use in obstetrics

GCSF is a glycoprotein with growth factor and cytokine functions that is produced in different tissues. GCSF was first cloned and used in the late 1980s and since then is being used mainly in patients with bone marrow

suppression and severe neutropenia (Morstyn *et al.* 1988). In 1989, Uzumaki and coworkers identified the GCSF receptors in human placental membranes and trophoblastic cells and suggested that GCSF might play some role in the fetoplacental unit during human development (Uzumaki *et al.* 1989). After that, studies began to detect GCSF receptors in different parts of the female reproductive system and at the feto-placental interface; GCSF receptors were identified in endometrial gland cells, follicular cells, placental cytotrophoblasts and syncytiotrophoblasts, decidual stromal cells, and fetal membranes (Saito *et al.* 1994, Miyama *et al.* 1998, McCracken *et al.* 1999, Ledee *et al.* 2008). These advances, in addition to the reports of the safety of GCSF use in pregnancy (Dale *et al.* 2003), made the researchers think of using GCSF in obstetrics. In 2009, Scarpellini & Sbracia for the first time used GCSF in 86 women with unexplained recurrent abortion and showed that 82% of the GCSF-treated women delivered a healthy neonate compared with 48% of the controls; additionally, in their study, GCSF was completely safe and was not associated with any adverse maternal or neonatal outcomes (Scarpellini & Sbracia 2009).

The effect of GCSF on implantation and pregnancy in IVF

In our study, subcutaneous administration of GCSF independently improved the successful implantation and pregnancy rates in IVF patients with normal endometrium who had repeated implantation failure after several IVF attempts. This was in accordance with the study of Scarpellini & Sbracia in women with repeated IVF failure that documented higher implantation rate in IVF women who had received GCSF compared with the placebo group (Scarpellini & Sbracia 2012). In contrast, in three other studies, GCSF administration did not affect the implantation or pregnancy rates (Barad *et al.* 2014, Eftekhari *et al.* 2014, Li *et al.* 2014). The differences in administration route, participants' age, endometrial thickness, and sample sizes could have contributed to the observed differences in the results; in the study of Scarpellini & Sbracia and our study, the sample size was larger, GCSF was administered systemically through the subcutaneous route, the study populations were younger, and had normal proliferating

endometrium (Scarpellini & Sbracia 2012), whereas in other studies, GCSF was administered locally through intrauterine perfusion, the sample sizes were smaller, the endometrium was thin in two studies, and the participants were older in the third study (Barad *et al.* 2014, Eftekhar *et al.* 2014, Li *et al.* 2014).

The role of GCSF in implantation and pregnancy

Our results indicate that GCSF might play an important role in the implantation process and in the maintenance of pregnancy. Salmassi *et al.* evaluated 93 women who underwent IVF; in their study, patients who became pregnant revealed a continuous increase in serum GCSF level from the day of embryo transfer to the day of implantation and from the day of confirmation of pregnancy to gestation; in contrast, those patients who did not become pregnant initially showed a slight increase in GCSF level, then with the failure of implantation as the GCSF level decreased significantly (Salmassi *et al.* 2005). The authors concluded that this cytokine could have an important function in achieving or maintaining pregnancy (Salmassi *et al.* 2005). In another study, Rahmati and coworkers demonstrated significantly lower levels of GCSF receptors at the maternal–fetal interface in infertile women with implantation failure, and interestingly, stimulation with high-dose GCSF was able to increase the expression of GCSF receptors in these patients (Rahmati *et al.* 2014).

GCSF might affect reproduction, implantation, and pregnancy through several possible mechanisms; GCSF has been shown to induce the trophoblast proliferation, invasion, and maintenance during pregnancy (Miyama *et al.* 1998, McCracken *et al.* 1999). GCSF also plays a key role during the embryo implantation process; it modulates fundamental genes responsible for the local embryo adhesion, cell migration, tissue remodeling, and angiogenesis, which are unavoidable for a successful implantation and further placentation (Rahmati *et al.* 2014). Finally, GCSF might be involved in the induction of adaptive changes that favor the immune tolerance in pregnancy; due to the semi-allogenic nature of the fetus, pregnancy represents an immune challenge to the mother. GCSF switches the T-cell cytokine secretion profile to the Th2 responses and promotes the IL-10-producing regulatory T cells and tolerogenic dendritic cell differentiation (Rutella *et al.* 2005), which are important parts of the immunoregulatory events that occur before and after the implantation in the uterus (Ledee *et al.* 2008).

The ideal route and dose of GCSF administration

The ideal route and dose of GCSF administration in obstetrics have not been identified yet. It seems that systemic administration is more effective and associated

with a better success rate than the commonly used local intrauterine infusion (Scarpellini & Sbracia 2012, Zeyneloglu *et al.* 2013, Barad *et al.* 2014). In this study and the study of Scarpellini & Sbracia GCSF was administered systemically through subcutaneous injection (Scarpellini & Sbracia 2012). Scarpellini & Sbracia used daily subcutaneous injection of 60 µg GCSF from the day of embryo transfer to the day of β-HCG test, and if it was positive, the treatment was continued for another 40 days, while we used a single-dose subcutaneous injection of 300 µg GCSF only before the implantation. In both studies, the intervention group had significantly higher implantation and pregnancy rates than the control group, while it is obvious that the financial burden in our study is lower and that a single-dose administration is much easier, safer, and more tolerable than daily administration for a long time.

Strengths and limitations

The main strengths of our study were its randomized, controlled design and to evaluate for the first time the efficacy of systemic subcutaneous GCSF administration on IVF success in women with repeated IVF failure. Assessing the administration of systemic and single-dose GCSF, which is easier, more tolerable and more cost-effective than repeated administration or local infusion of GCSF were other strengths of this study. This study, however, had some limitations that should be considered when interpreting the results; due to the nature of the study, it was unblinded and no placebo was used that could potentially affect the results of the study. In addition, we did not evaluate the pregnancy outcomes in the two groups of the study; this could help us in better interpretation of the results and establishing the safety of GCSF administration.

In conclusion, systemic administration of single-dose 300 µg GCSF subcutaneously 1 hour before embryo transfer significantly increases the IVF success, implantation, and pregnancy rates in infertile women with repeated IVF failure, which is independent of the women's age, endometrial thickness, good-quality oocyte counts, number of transferred embryos, and AMH levels. Further randomized, controlled trials are required to confirm these results and to establish the best route and dose of GCSF administration in IVF patients. Also studies are required to compare the efficacy, side effects, and pregnancy outcomes of the intrauterine perfusion versus systemic subcutaneous administration of GCSF in IVF patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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