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Research Article

The Effects of Subcutaneous Administration of Granulocyte Colony-Stimulating Factor on Pregnancy Outcome After Assisted Reproductive Technology: Clinical Trial

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Abstract

Background: Granulocyte-colony stimulating factor (G-CSF) is an innovative therapy in reproductive medicine. Although its mechanisms of action have remained unknown, G-CSF seems to be effective in the case of recurrent abortion or implantation failure and thin endometrium.

Objectives: This study was conducted to investigate whether subcutaneous administration of G-CSF has any effect on pregnancy outcome after assisted reproductive technology (ART).

Methods: Fifty women with male infertility factors undergoing ART treatment were enrolled and stimulated with the standard long protocol. The G-CSF group of women received one dose of subcutaneous G-CSF (Filgrastim, 300 μg/1 mL) on the day of embryo transfer and again two days later while the placebo group received normal saline.

Results: Seventeen patients had a positive β-human chorionic gonadotropin concentration after embryo transfer (8 and 9 in G-CSF and placebo groups, respectively) although the difference was not statistically significant. In addition, spontaneous abortion occurred in three patients (1 patient in the G-CSF group vs. 2 patients in the placebo group).

Conclusion: Overall, although G-CSF failed to affect the endometrial thickness, as well as implantation, or clinical pregnancy rates, a lower prevalence of abortion in G-CSF group may be due to the positive effect of G-CSF administration on the endometrium as compared to the placebo group.

Keywords: Assisted Reproductive Technology, Granulocyte-colony Stimulating Factor, Miscarriage, Implantation

Introduction

Despite all the developments in assisted reproductive technology (ART), many morphologically normal embryos fail to implant, which may be a consequence of embryo or uterine factors (1). Embryo implantation is an important step in the molecular events that require the development and trophoblast differentiation, adhesion and invasion and the formation of the placenta (2). In addition, endometrial receptivity for implantation necessitates a normal embryo, blastocyst stage, and the coordination between the mother and fetus. Human endometrium undergoes significant changes in order to prepare for implantation, and in this process, immune cells and their secreted substances such as Granulocyte-colony stimulating factor (G-CSF) play an important role in the luteal phase (3). G-CSF, as a glycoprotein with 177 amino acids which stimulate granulocytes, expressed by the trophoblast-decidual cells in mammals and its receptors also exits in trophoblast cells (4). Further, G-CSF is considered as an anti-apoptosis protein, which inhibits apoptosis in endometrial cells. Moreover, it increases the number of endometrial blood vessels in patients with thin endometrium, and therefore, prevents any damage. The other advantages of G-CSF include the improvement of the follicle in rats, increased fertility, ovarian response (in poor responders), and ovarian reconstruction (5-7).

Makinoda et al showed that serum G-CSF concentration significantly increased at the ovulatory phase and had a fundamental effect on ovulation mechanism (8). According to this hypothesis, further studies were conducted to investigate whether G-CSF can improve the outcome of in vitro fertilization (IVF). Recent studies have reported that G-CSF is involved in follicle development and ovulation (7) and can be a predictor of embryo implantation and

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IVF outcomes as well (9,10). On the other hand, the thin endometrium is one of the frequent finding and a cause of failed implantation in assisted reproduction. Unfortunately, its prevalence is unknown but some studies reported the prevalence of 1% or even lower in this regard (11,12). To increase the thickness of the endometrium, numerous treatment options are considered while the results are still questionable. Gleicher et al first used G-CSF in the successful treatment of unresponsive thin endometrium (13).

This was the beginning of evolution about reducing the abortion rate while increasing endometrial thickness, implantation, and pregnancy rate. Therefore, further animal and human studies were conducted to demonstrate the efficacy of G-CSF administration in infertile women with a thin endometrium (6,11,14-16).

Similarly, implantation is regarded as one of the important steps and a higher level of G-CSF can lead to a more successful rate of implantation (17). Furthermore, the endometrial tissue in human undergoes considerable changes to prepare for implantation and the immune cells and the secretions of this tissue (e.g., G-CSF) in the luteal phase play an important role in this process. Intrauterine G-CSF administration is a treatment option in reproductive medicine and the presumption of improving the survival of the transferred embryo and then decreasing miscarriage and promoting the regeneration of endometrial cells need to be confirmed as well (6,14,18,19). Thus, owing to the important role of G-CSF, the present study aimed to evaluate whether the subcutaneous administration of G-CSF affects the pregnancy outcome after ART.

Subjects and Methods

This is a prospective single-blinded randomized clinical trial which was conducted from March 2015 to December 2016. A number of 50 infertile women with male factor were selected based on the purpose of the study. The major inclusion criteria were infertile 20-40-year age group, the lack of a history of any underlying endocrine, systemic or gynecology disease, regular menstruation cycle, and male factor infertility. Accordingly, twelve women were excluded from the study (Figure 1). A written informed consent form was obtained from all patients and the study was approved by the Ethical Committee of Hamadan University of Medical Sciences.

All patients (38 women) were randomly divided into granulocyte colony-stimulating factor (G-CSF) and placebo groups who were all blind to the treatment. They were assigned to the standard "long" protocol with a high dose of gonadotropins and received oral contraceptive pills (OCP) for 21 days. Before discontinuing the OCP, gonadotropin-releasing hormone agonists were prescribed

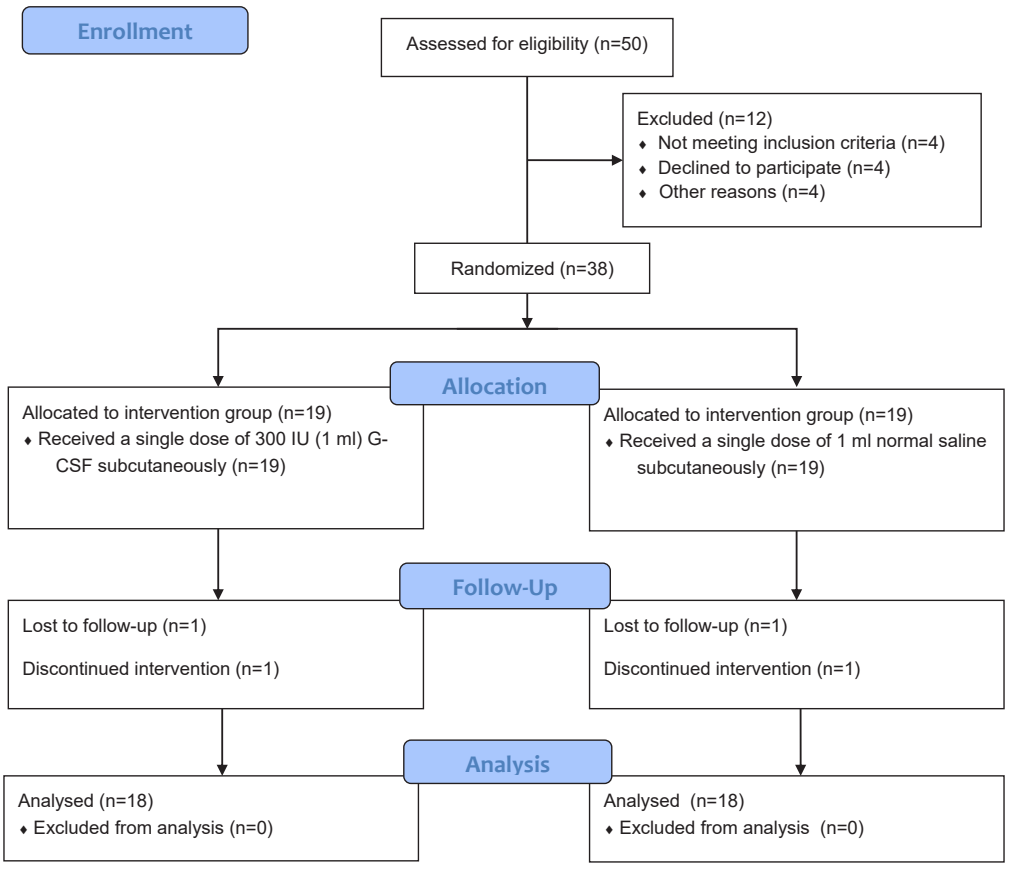

Figure 1. CONSORT Flow Diagram of the Study.

for 14 days, until menstruation bleeding, or until the day of gonadotropin stimulation, then it was reduced to half of the primary dose. Before gonadotropin stimulation, the regulation of the effective reduction of the pituitary was assessed under transvaginal sonography, when not observing follicular cyst with more than 10 mm in diameter. The pattern of gonadotropin response was monitored by serial serum estradiol levels and/or transvaginal ultrasound. When at least three follicles reached ≥18 mm, a 10,000 IU of human chorionic gonadotropin (hCG, 34-38 hours after hCG injection under ultrasound guidance and follicles larger than 15 mm in diameter were aspirated with a 17-gauge Cook needle, and oocytes were retrieved (20) and inseminated by intracytoplasmic sperm injection.

Likewise, the quality of cleavage stage embryos was graded as A, B, C, and D according to morphological criteria established by Hill et al (21). Three to 5 days after follicle puncture, embryo transfer was performed in the luteal phase while the embryos of grades C and D were not transferred.

In the treatment group, G-CSF was injected subcutaneously into the deltoid muscle on the day of embryo transfer, followed by another injection two days later. Each ampoule contained 300 IU (1 mL) G-CSF (Filgrastim, Pooyesh Daru). Moreover, G-CSF placebo group received the same dose of normal saline. It should be noted that the appearances of the injected ampoule were matched as well.

After embryo transfer, progesterone was similarly prescribed to both groups to continue the luteal phase. Clinical pregnancy was confirmed by blood β-human chorionic gonadotropin 14 days after embryo transfer as the detection of a gestation sac and positive fetal heart activity by a transvaginal ultrasound was performed 4 and 6 weeks after embryo transfer, respectively.

Statistical analysis was performed using SPSS. Quantitative data were presented as mean ± standard deviation and assessed by independent samples *t* test between the groups. Finally, the enumeration data were evaluated using the Chi-square and $P < 0.05$ was considered statistically significant.

Results

A total of 50 women with male factor infertility were included in this blinded randomized clinical trial. One patient among the G-CSF group and another one among the placebo group were excluded from the study because of interrupting the following-up after treatment. The baseline characteristics and outcomes are summarized in Table 1. There were no significant differences between the two groups concerning baseline characteristics.

Based on the results, 17 subjects had a positive β-human chorionic gonadotropin (βhCG) concentration after their embryo transfer, including 8 (44.4%) and 9 (50.0%) cases in G-CSF and placebo groups, respectively, and clinical pregnancy was established for all these subjects. Among these 17 established pregnancies, there were three spontaneous pregnancy losses (one in G-CSF and two in placebo groups) although no statistically significant differences were observed in the overall pregnancy rate between the placebo and G-CSF groups.

Additionally, the chemical pregnancy was not associated with a previous history of successful pregnancy and fresh embryos had higher chemical pregnancy rates compared to the frozen embryos group (Table 2).

Table 2 demonstrates the mean (SDs) of endometrial thickness, the level of follicle-stimulating hormone, luteinizing hormone (LH), and estradiol levels on the third day of the cycle although no differences existed among the positive and negative βhCG group in these clinical characteristics. Conversely, the LH level of the third day of

Table 1. Characteristics (Baseline and Outcome) of G-CSF and Placebo Groups

	G-CSF (n=18)	Placebo (n=18)	*P* Value
Age (y)	29.16±4.24	29.94±29.16	NS
BMI (kg/m^2)	25.88±2.40	26.89±4.37	NS
No. of transferred embryos	2.72±0.66	2.88±0.32	NS
No. of oocytes	10.72±5.27	11.38±4.70	NS
Endometrial thickness (mm)	8.13±1.82	8.81±1.85	NS
D_3 FSH (IU/mL)	6.31±1.87	6.73±1.77	NS
Previous IVF cycle (%)	38.9	44.4	NS
Number of embryos transferred	2.72±0.66	2.88±0.32	NS
Chemical pregnancy (positive βhCG) (%)	44.4	50.0	NS
Clinical pregnancy (%)	44.4	50.0	NS
Abortion (%)	5.6	11.1	NS

Note. * The $P<0.05$ was considered statistically significant. NS: Non-significant; BMI: body mass index; FSH: follicle-stimulating hormone; G-CSF: granulocyte colony-stimulating factor; HCG: Human chorionic gonadotropin; IVF: In vitro fertilization.
Values are presented as mean± standard deviation, and No. (%).

Table 2. Comparison of the Clinical Characteristics in the Negative and Positive βHCG Groups

	Positive βhCG (n=17)	Negative βhCG (n=19)	*P* Value
Endometrial thickness (mm)	8.44±1.57	8.5±2.10	NS
D_3 FSH (IU/mL)	7.0±6.21	6.04±5.14	NS
D_3 LH (IU/mL)	6.68±2.94	4.52±1.58	0.020*
Estradiol	55.85±23.14	59.96±25.47	NS
Infertility duration	7.58±4.82	6.23±4.32	NS
Positive history of previous pregnancies (%)	5.9	21.1	NS
Fresh embryo transfer (%)	70.6	57.9	NS
Freeze-thaw embryo transfer (%)	29.4	42.1	NS

Note: *The $P<0.05$ was considered statistically significant. FSH: Follicle-stimulating hormone LH: Luteinizing hormone; βhCG: β-human chorionic gonadotropin.
Values are mean± standard deviation, and No. (%).

the cycle was statistically higher in a βhCG positive group as compared to the negative group.

Discussion

More recently, G-CSF is found to have an essential role in innovative therapy in reproductive medicine, particularly CSF2/GMCSF and CSF3/G-CSF (22).

G-CSF receptors are out-spread in the human placental membrane, trophoblastic cells, fetal membranes, and female reproductive systems such as endometrial gland cells and follicular cells (4,23,24).

One of these aspects is using G-CSF as a therapeutic tool for the patient undergoing IVF treatment. A small number of IVF patients have thin endometrium resistant to conventional therapies (11) and the prevalence of thin endometrium is estimated to be about less than one percent and its etiology is still unknown (14,25). Gleicher et al were among the first to use G-CSF as a major innovation in treating thin endometrium (13). However, the best choices of route and optimal dose for G-CSF administration have not been identified yet. It seems that systemic administration is more effective and associated with a high success rate than the commonly used local intrauterine infusion (26,27). Due to the possibility of vaginal and subcutaneous administration of G-CSF, the researchers decided to utilize the subcutaneous injection of G-CSF.

Demographic variables in this study were not statistically different between the two groups. In addition, the results showed that the prevalence of previous IVF, the number of oocytes and embryo, fresh embryo transfer or frozen embryo, the duration of infertility, the history of a previous miscarriage, and the level of follicle-stimulating hormone and estradiol were equal in both groups and demonstrated no significant difference.

In accordance with the results of the study by Li et al, the prevalence of abortion in the G-CSF group (5.6%) was lower than that of the placebo group (11.1%) and this difference was not significant, which can be due to the small sample size of the present study. The findings of Barad et al also revealed that G-CSF prescription did not affect the endometrial thickness, implantation rates, or clinical pregnancy rates in patients who underwent IVF treatment (27). G-CSF plays a crucial role in implantation, establishment, and the maintenance of pregnancy (10, 28) and is regarded as a promising treatment in women with unexplained recurrent miscarriage (29). In a previous study conducted by Papanikolaou et al, the administration of intrauterine G-CSF did not only increase the endometrial thickness (20%) in two-thirds of patients and reduced the risk of miscarriage (19). However, the results of the present study represented that endometrial thickness in the placebo group was almost the same as that of the G-CSF group, and there were no statistically significant differences in this respect. The results of Rahmati et al also confirmed that simulating G-CSF receptor expression with a higher dose of G-CSF at the fetomaternal interface improved embryo adhesion, cell migration, tissue remodeling, and angiogenesis during embryo implantation process (30). In another study, Aleyasin et al (26) evaluated the efficacy of the single-dose administration of G-CSF on 112 infertile women with repeated IVF failure and found that a single-dose of G-CSF administration before implantation significantly contributed to the maintenance of pregnancy. Finally, they reported that G-CSF administration could cause a lower abortion rate in infertile women undergoing IVF.

As known, pregnancy rate increases when the G-CSF level is extremely greater in the follicular fluid because the increased serum level of G-CSF leads to ovulation stimulation (10). In the current study, the rate of clinical pregnancies represented no statistical differences in both the case and the control groups. Similarly, Aleyasin et al (26) demonstrated that the subcutaneous administration of G-CSF successful improved the pregnancy rates and implantation in repeated IVF failure patients with normal endometrium. The findings of Scarpellini et al (28) also indicated a higher implantation rate in IVF patients who received G-CSF when compared with the placebo group. Conversely, some studies explained that G-CSF administrations have no effect on implantation or pregnancy rate (11,27,31). This can be due to several parameters such as small sample size, patient's age, as well as endometrial thickness, a low dose of G-CSF, and one-time G-CSF administration (32).

Little information is available on the exact mechanism of G-CSF impact on endometrial thickness. It is proven that G-CSF receptors in the fetomaternal interface and endometrium are expressed and secreted by both fetal chorionic villous and maternal decidual tissues in the first trimester, indicating the important role of G-CSF in maintaining pregnancy (32,33).

Although the beneficial outcomes of prescribing subcutaneous G-CSF cannot be approved, a lower prevalence of abortion in the case group can be attributed to the positive effect of G-CSF administration on the endometrium when compared to the placebo group. Most of the preliminary studies which suggested the benefit of using G-CSF were nonrandomized trials while a high-quality randomized controlled trial showed no beneficial effect for G-CSF, though it included women with normal endometrium (34). It is noteworthy that the present study has a type of bias called volunteer bias. The selected subjects are not representative of a whole population and effective dose, timing, the frequency and route of G-CSF administration also remain unestablished (30, 32). Therefore, further studies with larger sample sizes contribute to a better understanding of the effectiveness

of G-CSF treatment. In conclusion, the subcutaneous administration of G-CSF may be beneficial in the ART treatment, especially for cases with recurrent implantation failure and abortion.

Authors' Contributions

Conception and design: MF; Data collection: MB and NM; Data analysis and interpretation: NM and AP; Manuscript drafting: MB, MF, and NM; Critical manuscript revision for important intellectual content: MF and MB; Statistical analysis: AP.

Conflict of Interest Disclosures

The authors declare no potential conflicts of interest relevant to this article.

Ethical Issues

This research was approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamedan, Iran. All the ethical standards of the Helsinki Declaration were observed and the study protocol was registered in the Iranian Registry of Clinical Trials under the code of IRCT201511269014N85. Finally, all data were analysed using SPSS software, version 16.

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