

Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: a randomised controlled trial

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BACKGROUND: Recurrent miscarriage (RM) is defined as the occurrence of three or more clinically detectable pregnancy losses in the first trimester. In most cases of RM, its aetiology remains unexplained. Granulocyte colony-stimulating factor (G-CSF), a cytokine, and its receptor are expressed in placental tissue. To investigate the effectiveness of G-CSF in preventing embryo demise, we administered G-CSF to women with RM.

METHODS: A randomised controlled trial in women with RM treated with G-CSF or placebo was conducted in one private reproductive medicine clinic. Sixty-eight women with unexplained primary RM, all with at least four consecutive miscarriages and negative for all clinical investigations, were selected. Patients were randomized for s.c. treatment with G-CSF ($n = 35$) (1 $\mu\text{g}/\text{kg}/\text{day}$) starting on the sixth day after ovulation, or with placebo ($n = 33$). Patients were randomized using a computer-generated randomization number sequence. Pregnancy outcome (delivery of a healthy baby without major or minor malformations) was the primary outcome measure.

RESULTS: In the group treated with G-CSF, 29 out of 35 (82.8%) women delivered a healthy baby, whereas in the placebo group, this figure was only 16 out of 33 (48.5%) ($P = 0.0061$, odds ratio = 5.1; 95% confidence interval 1.5–18.4). Significantly higher β -hCG levels were found in gestation weeks 5–9 in women treated with G-CSF versus placebo ($P < 0.001$).

CONCLUSIONS: Our data show that G-CSF may be effective in the treatment of unexplained RM. However, further studies are needed to confirm the effectiveness of this treatment in women with unexplained RM, refractory to conventional treatment.

The study was registered with a ICMJE recognized registry, the Clinical Trial.gov Protocol Registry System, with the number NCT00772122.

Key words: recurrent miscarriage / granulocyte colony-stimulating factor / pregnancy outcome / β -hCG / trophoblast

Introduction

It has been estimated that more than 70% of human conceptions do not achieve fetal viability, and ~50% of them are lost before the first missed menses (Edmonds *et al.*, 1982), whereas ~15% of clinical pregnancies miscarry before the 20th week of gestation (Alberman, 1988).

Recurrent miscarriage (RM) (Rai and Regan, 2006) is defined as 'the occurrence of three or more clinically detectable pregnancy losses prior to the 20th week of gestation'. It has been estimated that the frequency of RM is 1% in women of childbearing age (Alberman, 1988; Stirrat, 1990). Recognized causes for RM are generally considered to be the following: parental chromosomal defects, mostly reciprocal or Robertsonian translocations (Portnoi *et al.*, 1988), infections (Rae *et al.*, 1994; Summers, 1994; Odland *et al.*, 2001), endocrinological

causes, such as thyroid defects, diabetes and polycystic ovaries (Kutteh *et al.*, 1999; Craig *et al.*, 2002; Arredondo and Noble, 2006), uterine abnormalities (Guimaraes Filho *et al.*, 2006), antiphospholipid antibody syndrome or other autoimmune conditions (Rai *et al.*, 1995; Kutteh, 1996). However, more than 40% of RM cases remain unexplained (Carrington *et al.*, 2005) and for these cases, several possible causes have been proposed, including the so-called immune dysfunction or allo-immune response. RM could be due to an imbalance in Th1/Th2 systems, with a prevalence during pregnancy in the uterine tissues of Th1 cytokine production (interleukin 2, TNF α) which plays a cytotoxic role, instead of Th2 cytokine production (interleukin 4, 6 and 10) with an immuno-suppression role, and the consequent rejection of embryonic allograft (Michimata *et al.*, 2003). Several treatments have been suggested in these cases, including paternal leukocyte transfusion

(Taylor and Faulk, 1981), trophoblast membrane vesicle extracts (Johnson *et al.*, 1988), seminal plasma suppositories (Stern and Coulam, 1993) and i.v. immunoglobulin immunotherapy (IVIG, Mueller-Eckhardt *et al.*, 1989; Christiansen *et al.*, 2002; Yamada *et al.*, 2003). However, all these treatments have not received general acceptance because controversial results have been published. A recent meta-analysis has shown that none of these therapies showed significant effects on patients with unexplained RM (Scott, 2003; Porter *et al.*, 2006).

Granulocyte colony-stimulating factor (G-CSF) is a cytokine which stimulates neutrophilic granulocyte proliferation and differentiation. It is expressed and produced by the decidual cells, and its receptor, *c-fms*, is expressed by the trophoblastic cells (Uzumaki *et al.*, 1989; Shorter *et al.*, 1992; Saito *et al.*, 1994; McCracken *et al.*, 1996, 1999). In addition, in an animal model, G-CSF showed a marked anti-abortion activity (Saito *et al.*, 1994; Litwin *et al.*, 2005). G-CSF is safely used in the treatment of neutropenia during cancer chemotherapy, and no embryotoxic effects of this substance have been reported (Dale *et al.*, 2003; Gomez Raposo *et al.*, 2006). Since experimental findings showed a positive effect on the trophoblast, we discussed with patients the possible use of G-CSF after the failure of a previous attempt of treatment with immunoglobulin, obtaining their consent.

In this study, we have tested the use of G-CSF in a series of women with unexplained RM in whom previous treatment with other conventional therapies (IVIG) had failed.

Materials and Methods

The patients with unexplained primary (no previous successful pregnancy) RM referred to the Hungaria Center for Endocrinology and Reproductive Medicine between January 2000 and January 2007 were considered eligible for the study. Miscarriage and RM were defined according to the European Society for Human Reproduction and Embryology (ESHRE) Special Interest Group for Early Pregnancy, updated and revised nomenclature for description of early pregnancy events (Farquharson *et al.*, 2005).

The patients had to fulfil the following inclusion criteria: woman's age <39 years, more than four previous miscarriages, failure of a previous treatment for RM (immunoglobulin infusion) and they had to be negative for all of the known causes of RM (abnormal karyotype, uterine defects, infections, endocrine problems, coagulation defects or thrombophilia and autoimmune defects, including antiphospholipid antibodies). All the patients underwent several examinations and only the couples in whom no abnormalities were found were included. The karyotype of both parents was normal, semen analysis of the male partners was also normal, hysteroscopy and/or pelvic ultrasound examination did not show any uterine abnormalities, and hormonal blood tests (FSH, LH, prolactin, 17β -estradiol, estrone, progesterone, Δ_4 androstenedione, dehydroepiandrosterone, testosterone and 17α OH progesterone, thyroid tests (TSH, free T_3 and T_4 , antiperoxisome and anti-tireoglobulin autoantibodies), glycaemic tolerance test and insulin were in the range of normal values. Indirect Coombs test, antiphospholipid autoantibody (lupus anticoagulant, anticardiolipin autoantibodies) and other autoantibodies (antigastric wall cell, anti-mitochondrium, anti-smooth muscle cell, anti-nucleus) were negative (a transient low positivity for one of these autoantibodies was considered as negative). The blood test for the screening of toxoplasma, rubella, cytomegalovirus, herpes virus I and II and infections other than for chlamydia and mycoplasma were negative. No abnormalities were found for antithrombin III, coagulative protein C and S, and blood coagulation factors II, V, VII, VIII and XII and homocysteine; furthermore, all patients were negative for Factor V Leiden prothrombin gene mutation.

In addition, the patients to be included in the study had to show a normal karyotyping of embryonic tissues in their last miscarriage (during treatment with IVIG: this treatment in our centre is reserved for women with unexplained RM, in which no abnormalities were found in the clinical tests previously reported), and they were 46/XX in 37 miscarriages and 46/XY in 31 miscarriages. Both partners of all the couples included in the study had to be fertile, without any fertility impairment, and none of them had undergone fertility treatments, such as IVF. During the trial, the women in both groups did not take any other medications, except folic acid.

The study was reviewed and approved by the Institutional Review Board, and the clinical study was conducted according to Italian law and the Declaration of Helsinki for Medical Research involving Human Subjects. A sample size calculation showed that a total of 32 patients per group were needed in order to have a difference of 33% between the two groups at $P < 0.05$ and a $\beta > 0.80$.

A total of 72 patients were considered eligible for the study and 68 of them agreed to participate, signing an informed consent form (see Supplementary Fig. 1 for flow diagram of study). All the patients were informed about the possible risks of this treatment for the mother and the fetus, other than the lack of information about the developmental toxicity of G-CSF. The patients were randomized by means of a computer-generated randomization number sequence. They were randomly assigned to the two arms of the study, one to G-CSF treatment and the other to placebo administration; the patients were blind to which treatment they were assigned to. The patients underwent randomization only once and were not allowed to re-enter in the study. The G-CSF group of 35 women underwent a daily s.c. administration of Filgrastim (Neupogen, Dompè, Italy), the recombinant G-CSF, at a dosage of $1 \mu\text{g}$ ($100\,000 \text{ IU}/\text{kg}/\text{day}$) from the sixth day after ovulation until the occurrence of menstruation or to the end of the ninth week of gestation. The placebo group, consisting of 33 subjects, was treated daily with s.c. saline solution at the same dosage, also from the sixth day after ovulation until the recurrence of menstrual loss or to the end of the ninth week.

All the patients conceived within 3 months from randomization and inclusion in the study.

During the pregnancies, patients underwent a serial weekly estimation of serum β -hCG levels from the fifth through ninth week of gestation, and both groups were compared with the values of β -hCG assessed with the same system during the pregnancy of 15 normal fertile women. The kit used for the β -hCG assay was a commercial immunoradiometric assay kit (Kp14ct β -hCG IRMA Radim, Rome, Italy), which showed an intra-assay variation of $8.4 \pm 1.5\%$ and an inter-assay variation of $9.3 \pm 2.1\%$.

All the patients were followed up during the pregnancy in our clinic, and every 3 weeks, they underwent transvaginal ultrasound scans from the 5th through 14th gestational week to observe heart beat and embryo growth, or to diagnose miscarriage (according to ESHRE published criteria). Biochemical miscarriages were not included in the study.

All the babies born underwent a paediatric examination to exclude congenital anomalies. The gestational age of miscarriage was calculated by ultrasound scan using the crown-rump length and the gestational sac dimension. The live birth of a healthy baby without major or minor congenital anomalies was considered the primary outcome. Side effects of the treatment, possible pregnancy complications (pre-eclampsia, pre-term delivery, gestational diabetes, pregnancy hypertension, bleeding and thrombosis) and newborn weight were considered as secondary outcomes.

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows (SPSS Inc., Chicago, IL, USA). For continuous variables, statistical significance was assessed by the use of the two-tailed Student's *t*-test for unpaired data with the Bonferroni correction for multiple comparisons. Fisher's exact test and χ^2 were used when appropriate for discontinuous variables. $P < 0.05$ was defined as statistically significant.

Results

The demographic data of the patients are reported in Table I. No differences were found between the two groups of patients for the women's age, number of previous miscarriages and gestational week of miscarriage. None of the patients dropped out of the study. The patients were strictly followed up during pregnancy and no violation of the study was recorded, according to patients' reports.

The number of live births in women treated with G-CSF was 29 out of 35 (82.8%), whereas in the controls, there were 16 out of 33 (48.5%): this difference was significant [$P = 0.0061$, odds ratio = 5.1; 95% confidence interval (CI) 1.5–18.4]. The number of patients needed to treat (NNT) for one additional live birth was 2.9 (95% CI 2.1–10.3). No significant differences were found for gestational age of miscarriage, neonatal weight, side effects of treatment and pregnancy complications: only in one case treated with G-CSF, a skin rash was observed and in two cases the leukocyte count was higher than 25 000/ml, whereas in the group treated with a placebo, a case of mild hypertension in pregnancy was observed. None of the newborns showed any major or minor abnormalities or malformations and showed a normal perinatal development. Data are reported in Table II. In 14 out of 23 miscarriages, the embryonic tissue was available for karyotype; karyotype abnormalities were observed in three cases, one in the G-CSF group and two in the placebo group (in the 11 cases with normal karyotype, 6 were 46XX and 5 were 46XY).

Table I Demographic data for women with unexplained recurrent miscarriage who were treated with G-CSF or placebo in the RCT

	G-CSF	Placebo
Number of patients	35	33
Age (years): when pregnancy started	34.9 ± 2.7	33.8 ± 2.9
BMI: when pregnancy started	27.4 ± 1.9	27.8 ± 1.8
Smokers (more than 10 cigarettes per day)	1	2
Number of previous miscarriages	5.5 ± 0.4	5.6 ± 0.3
Gestational week of miscarriage	6.1 ± 1.2	6.4 ± 1.1

Data are mean ± SD.

Significantly higher levels of β -hCG were found in the women with ongoing pregnancies who were treated with G-CSF versus the placebo group and the panel of normal pregnancies also followed from the fifth to ninth gestational week ($P < 0.001$, Fig. 1).

Discussion

All studies evaluating the effectiveness of treatments for RM showed a bias for the spontaneous resolution of the problem, estimated to range from 40% to 60% of cases, depending on the number of previous miscarriages. Furthermore, the limited number of patients in each individual study is another problem in order to evaluate the effectiveness of treatments tested. Another concern in this matter is the wide variation of inclusion criteria in the several studies published on the treatment of RM, in particular the age and the number of previous miscarriages in these women, since it has been shown by several authors that the risk of miscarriage increases with maternal age and the number of previous losses (Brigham *et al.*, 1999; Andersen *et al.*, 2000; Christiansen *et al.*, 2005). A relatively recent meta-analysis on this question showed that for all treatments suggested for unexplained RM, including treatment with immunoglobulins or leukocyte infusion, there was insufficient evidence to be able to consider them really effective (Porter *et al.*, 2006). It has been suggested that an ideal trial to test the effectiveness of a treatment for RM should take into account the maternal age and number of previous miscarriages, with a stratification of the patients for these covariates, and randomization between the control and experimental substances within each stratum of patients, other than to include a substantial number of patients for each group (Christiansen *et al.*, 2005). Clearly, all studies in the literature do not show these characteristics, and our study also shows in part this bias, especially for the limited number of patients, even though we tried to include women with more strict criteria for the number of previous miscarriages and maternal age.

We included in this study only women <39 years old and with no fertility problems in either partner in order to avoid confounding factors, such as woman's age and fertility treatment (IVF, etc.), as well as having greater chance of becoming pregnant in a shorter time. Furthermore, our patients failed a previous cycle of treatment for RM, with a further miscarriage in which the embryo karyotype was normal; all these criteria make our trial closer to the ideal trial, as

Table II Results of the study in patients treated with G-CSF and controls (placebo)

	G-CSF	Placebo	P-value
Number of live births (%)	29 (82.8)	16 (48.5)	0.0061
Number of miscarriages (%)	6 (17.2)	17 (51.5)	0.0061
Gestational week of miscarriage (mean ± SD)	6.0 ± 1.1	6.2 ± 1.0	0.6989
Newborn weight (g, mean ± SD)	3050 ± 220	3125 ± 240	0.3098
Side effects			
Skin rash	1	0	0.5147
Leukocytosis	2	0	0.2617
Pregnancy complication*	0	1	0.3535

*Pre-eclampsia.

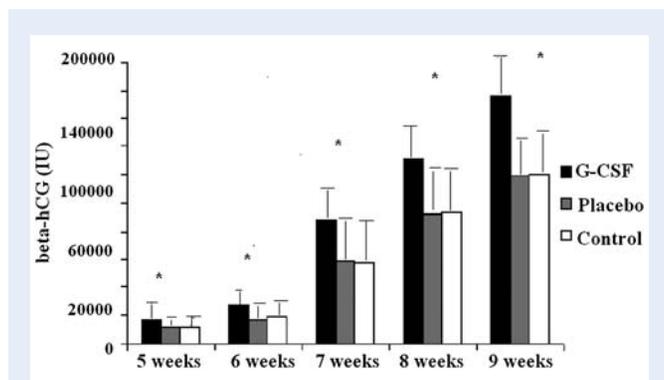


Figure 1 The data of β -hCG levels (mean + SD) were reported for each gestational week from the fifth to the ninth in the three groups: women with recurrent miscarriage (RM) treated with granulocyte colony-stimulating factor (G-CSF) ($n = 29$), women with RM treated with a placebo ($n = 16$) and normal pregnant women ($n = 15$). A statistical significant difference was observed ($P < 0.001$) in all weeks between the experimental group versus the placebo and normal pregnant women.

suggested by Christiansen *et al.* (2005). Consequently, our study has a limited patient number in order to meet the inclusion criteria: a larger number of patients, in a multicentre trial, may lead to less selective criteria for patient inclusion with an increase in confounding factors.

In our study, the G-CSF treatment showed an evident effect on the pregnancies of women with RM, with a remarkable increase in success rate and a consequent reduction of miscarriages. In our group of patients with RM, the NNT for one additional live birth was 2.9. This result is considered very interesting, since for other conventional treatments, namely paternal leukocyte transfusion and IVIG, an NNT of 10 and 6, respectively, has been reported (Scott, 2003; Porter *et al.*, 2006). These data may be a result of the small size of our group of women treated, or to the more strict inclusion criteria of our study with respect to other trials published, and they should not be generalized for all RM women. However, our results are important, and G-CSF should be tested in a larger multicentre trial to fully evaluate its therapeutic potential in these patients. This treatment in our hands was safe, since no major side effects were observed, except for a mild local skin rash which cleared in a few days, and two cases of a leukocyte count higher than 25 000/ml. However, it is important to remember that there is a lack of data on possible toxicity in pregnancy of G-CSF. Experimental data on animal models showed placental embolism only in rabbits (Kato *et al.*, 2001), with a dosage 1000 times higher than we used here, whereas in rats, mice and monkeys, no adverse effects were observed (Novales *et al.*, 1993; Okasaki *et al.*, 2002). Furthermore, in a review of cases who were treated for a long time with G-CSF for chronic neutropenia, no adverse effects on pregnancy or the fetus were reported in a series of 125 women (Dale *et al.*, 2003). These data, as well as our results, seem to show (at time of writing) the safety of G-CSF use in pregnancy, even though it should be used carefully, and reserved for refractory cases of RM only.

Human G-CSF is a 177 amino acid polypeptide with a molecular weight of c. 25 kDa, stimulating principally polymorphonuclear

granulocytes with a high affinity receptor, known as *c-fms*, of 183 amino acids with a molecular weight of ~ 14 kDa, present on the target cell surface (Nagata *et al.*, 1986). In early reports, the expression of G-CSF has been found on trophoblast and also in decidual cells of several mammals, including human placenta. The G-CSF receptor was instead localized only on the trophoblast cell surface (Uzumaki *et al.*, 1989; Shorter *et al.*, 1992; Saito *et al.*, 1994). An anti-abortion role was found for G-CSF in the animal, as well as its lack in expression on trophoblast of human early miscarriage (Novales *et al.*, 1993; Sugita *et al.*, 2003; Litwin *et al.*, 2005). It has also been shown from several studies that G-CSF has a positive effect on trophoblast growth and placenta metabolism (McCracken *et al.*, 1996, 1999).

In our study, we observed a low rate of pregnancy complications: these findings may be a result of the strict surveillance of these pregnancies performed by physicians, and the extremely safe life style of the patients during pregnancy, as well as their relative young age.

Furthermore, we observed a significant increase of β -hCG levels in the ongoing pregnancies during the fifth to the ninth gestational week in G-CSF treated women versus the placebo and the normal pregnancy groups. These data seem to reveal a direct trophic effect of this cytokine on the trophoblast cells, probably mediated by its natural receptor, *c-fms*, expressed on the trophoblast (Uzumaki *et al.*, 1989). In the literature, no data are reported about the effects of G-CSF on hCG production by trophoblasts, even though several papers reported the positive role of G-CSF on trophoblast metabolism and survival (Novales *et al.*, 1993; Sugita *et al.*, 2003; Marino and Roguin, 2008). However, a possible synergistic immunological effect cannot be excluded by our results, since no data were recorded on this issue in our clinical study.

However, our study is far from having demonstrated the effectiveness of G-CSF for the treatment of all patients with unexplained RM, and its safety. Even though there is increasing evidence that G-CSF is not toxic in pregnancy, this substance should be used very carefully as its safety is still under question and there are not enough women treated with G-CSF in pregnancy to exclude any possible teratogenic effects. However, it must be said that the present study may show a possible way to overcome the problem of RM, even though it needs to be confirmed in larger studies.

Further studies are needed to conclusively show the effectiveness of G-CSF treatment, especially in women with unexplained RM which is refractory to conventional treatments. Owing to a lack of data on the role of G-CSF in human reproduction, studies to elucidate the mechanism(s) of action of G-CSF on human trophoblast cells and the possible interaction with the immune system and embryo growth are warranted.

Supplementary Data

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

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