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

F. Scarpellini and M. Sbracia

Hungaria Center for Endocrinology and Reproductive Medicine (CERM), 00198 Rome, Italy

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 μg/kg/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, TNFα) 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 (Stem and Coulam, 1993) and i.v. immunoglobulin immunotherapy (IVIG, Mueller-Eckhardt et al., 1989; Christian 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, its effects on patients with unexplained RM (Scott, 2003; Porter meta-analysis has shown that none of these therapies showed significant

Materials and Methods

The patients with unexplained primary (no previous successful pregnancy) RM referred to the Hungary 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 fulfill 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 several examinations and only the couples in whom no abnormalities were found. The karyotype of both parents was normal, semen analysis of the male partners was also normal, hysteroscopy and/or pelvic

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 $\chi^2$ 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

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

Data are mean ± SD.

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

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

*Pre-eclampsia.

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
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., 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-abortive 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.

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.

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/.

**Funding**

Sponsored by University of Florence.
colony stimulation factor (G-CSF) suppresses interleukin (IL)-12 and/or IL-2 induced interferon (IFN)-gamma production and cytotoxicity of decidual mononuclear cells. Am J Reprod Immunol 2003;50:83–89.

Submitted on October 20, 2008; resubmitted on May 24, 2009; accepted on June 10, 2009.