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ARTICLE

Use of granulocyte colony-stimulating factor in ART treatment does not increase the risk of adverse perinatal outcomes

BIOGRAPHY

Dr Antonio Requena achieved his MD at Complutense University of Madrid, and his obstetric and gynaecology specialism at La Paz Hospital, Autonoma University of Madrid. He undertook postdoctoral training in in-vitro oocyte maturation at John Eppig's laboratory in Bar Harbour, USA.

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KEY MESSAGE

Granulocyte colony-stimulating factor (G-CSF) is a promising immunological treatment option for recurrent miscarriage in patients with KIR-HLA-C mismatch. This retrospective study indicates that administration of G-CSF at embryo transfer and during early pregnancy does not carry a higher risk of perinatal complications. Further larger studies are needed to confirm this.

ABSTRACT

Research question: Granulocyte colony-stimulating factor (G-CSF) acts on reproductive function at different stages, but its effects on the early stages of embryo development are unknown. The aim of this study was to assess the effect of G-CSF administration during treatment with assisted reproductive technologies (ART) and early pregnancy on newborns.

Design: Retrospective study in women undergoing egg donation, with a study group including 33 live-born children from a pregnancy in which G-CSF was administered, and a control group of 3798 children in which this medication was not ordered during pregnancy. The analysis was of perinatal outcomes resulting from G-CSF treatment administered off-label compared with a control group.

Results: No significant differences were found in maternal age (40.9 ± 0.1 versus 38.9 ± 1.8, $P = 0.055$), body mass index (23.2 ± 0.2 versus 22.6 ± 1.5, $P = 0.503$), infant birthweight (2952 ± 200 versus 3145 ± 270 g, $P = 0.184$), gestational age (38 ± 1 versus 37 ± 1 weeks, $P = 0.926$) or length (50.2 ± 1.5 versus 48.7 ± 2.7 cm, $P = 0.678$) (between the control group and women treated with G-CSF, respectively). The prematurity rates of births before week 36 (10.0% versus 9.5%, $P = 0.783$) or week 32 (2.2% versus 0.0%, $P = 0.585$) were similar in the control and study groups, respectively. The incidence of low birthweight (<2500 g; 19.6% versus 11.8%, $P = 0.570$) or very low birthweight (1500 g; 2.5% versus 0.0%, $P = 0.454$) was not significantly different between non-treated and G-CSF-treated women, respectively.

Conclusions: Administration of G-CSF at embryo transfer and during early pregnancy in recurrent miscarriage patients with KIR-HLA-C mismatch undergoing egg donation ART treatment does not convey a higher risk of perinatal complications.

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KEYWORDS
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INTRODUCTION

Proper implantation is a restrictive factor in human reproduction. Although major improvements in treatment with assisted reproductive technologies (ART) have been achieved, many embryos are still lost after implantation or as clinical miscarriage. Although knowledge of the molecular mechanisms in the receptive endometrium and the implantation process itself has increased in recent last years, conversion of this basic research into daily clinical practice is rather limited.

The role played by the immune system in recurrent miscarriage and recurrent implantation failure is a controversial topic in reproductive medicine. Several studies (*Hiby et al., 2010*; *Jin et al., 2011*; *Zhu et al., 2019*) have shown a variation in immune cells and cytokine expression in women with these pathologies compared with those with successful pregnancies, suggesting that a disturbance in immune tolerance from the mother to the allogeneic fetus contributes to these conditions. The original idea implied the presence of some mechanism of immunosuppression that allowed the presence of the allogeneic fetus, but evidence proving this hypothesis has been elusive and controversial for both blood and uterine maternal natural killer (NK) cells (*Moffett and Shreeve, 2015*; *Sacks, 2015*).

During early pregnancy in humans, embryo extravillous trophoblast cells (EVT) occupy the uterine lining to reshape the uterine spiral arteries to ensure an adequately nourished fetus during the entire pregnancy. The prevailing immune cells present in the decidua are uterine NK (uNK) cells, CD56bright+CD16–, which have receptors that bind ligands on invasive EVT (*Moffett and Colucci, 2014*). Killer immunoglobulin-like receptors (KIR) expressed by uNK cells bind to the trophoblast human leukocyte antigen (HLA-C) molecules, promoting the activating or inhibitory signals responsible for the EVT invasion. A sufficient degree of uNK activation is needed to facilitate invasion by the EVT and to transform the uterine arteries (*Cristiani et al., 2016*). Diminished trophoblast invasion and vascular conversion leads to poor placentation, and this procedure is thought to be the primary defect of pregnancy disorders, such as recurrent miscarriage, pre-eclampsia and fetal growth restriction (*Alecsandru and García-Velasco, 2017*; *Hiby et al., 2014*; *Moffett et al., 2016*). Genetic conditions have been suggested to be linked to combinations of polymorphic KIR genes expressed by maternal decidual NK cells and HLA-C genes expressed by the fetal trophoblast (*Alecsandru et al., 2014*; *Hiby et al., 2008*). It has also been reported that a KIR-HLA-C mismatch could be a risk factor for pregnancy conditions (*Hiby et al., 2014*; *Moffett et al., 2016*). Women who have a KIR AA genotype are at risk of recurrent miscarriage or pre-eclampsia, especially when there is a paternally or egg donor derived fetal C2-HLA-C and double embryo transfer (*Alecsandru et al., 2014*). Activation of KIR2DS1+dNK by HLA-C2 stimulated the synthesis of soluble products like G-CSF, which enhanced the migration of primary trophoblast cells *in vitro*, providing a molecular mechanism that explains how recognition of HLA class I molecules on fetal trophoblast by an activating KIR on maternal dNK may be valuable for placentation (*Xiong et al., 2013*).

Finally, the role of cytokines, such as G-CSF, has been described to enhance the migration of primary trophoblast cells because of the HLA-C binding to the appropriate KIR receptor (*Hiby et al., 2008*), suggesting how NK allo-recognition may determine successful placentation. These cytokines play a role in reproductive function through various effects on local inflammation and immunomodulation. G-CSF is a glycoprotein that mainly stimulates granulocyte colony development. It has an important function in endometrial decidualization, trophoblastic development and placental metabolism (*Rahmati et al., 2014*). It supports the mobilization, migration and differentiation of stem cells, enables endometrial regeneration by promoting angiogenesis, and reduces apoptotic activity. Finally, it also has a central role in embryo implantation and the maintenance of pregnancy (*Wurfel et al., 2010*).

In recent years, G-CSF has been part of pioneering therapies in reproductive medicine. As this cytokine is an important factor in EVT invasion and placentation, a therapeutic role for couples with recurrent miscarriage and KIR-HLA-C mismatch has been evaluated (*Santjohanser et al., 2013*; *Scarpellini and Sbracia, 2009*; *Wurfel et al., 2010*). Despite unequivocal interest in this study area, the actions of these CSF are still not clarified, especially the long-term effects on the early stages of embryo development. The objective of this study was to assess the effect of G-CSF administration in newborns resulting from ART.

MATERIALS AND METHODS

This was a non-interventional, large-sample, retrospective multicentre cohort study. The final sample size included all patients (control and study group) who underwent an egg donation treatment and had a newborn between January 2014 and December 2016. As study group, there were 33 live-born children from a pregnancy in which G-CSF was administered off-label in couples with KIR-HLA-C mismatch and recurrent miscarriage (2 or more) after previous egg donation cycles; on the other hand, the control group comprised 3798 children from couples also undergoing ART with egg donation in the same clinics in which this medication was not ordered during pregnancy. All of the women received treatment at one of the 11 private clinics belonging to the IVI Group. All patients provided written informed consent. All procedures and protocols to perform the retrospective analysis were approved by an Institutional Review Board (1603-MAD-021-AR). The study complied with the Spanish law governing ART (14/2006).

The hormone replacement protocol for oocyte recipients was described previously (*Requena et al., 2014*). Briefly, a baseline transvaginal scan was performed before down-regulation to ensure that the uterus was normal. For all recipients who were still cycling, down-regulation was performed using an intramuscular dose of 3.75 mg triptorelin (Decapeptyl®; Ipsen Pharma, Spain) during the mid-luteal phase of the previous cycle. Hormone therapy was initiated on Days 1–3 of the following cycle with increased doses of oestradiol valerate. On Day 15, an ultrasound was performed to evaluate endometrial growth. On the day after oocyte retrieval, after fertilization was confirmed, 800 mg/day of micronized intravaginal progesterone was added to the regimen.

Patients in the study group did not show any evidence of an autoimmune

TABLE 1 DEMOGRAPHIC CHARACTERISTICS AND PERINATAL OUTCOMES OF PATIENTS DIAGNOSED WITH RECURRENT MISCARRIAGE AND TREATED WITH G-CSF COMPARED WITH A CONTROL GROUP

	Control (*n* = 3798)	G-CSF treated (*n* = 33)	*P*-value
Age (years)	40.9 ± 0.1	38.9 ± 1.8	0.055
BMI (kg/m^2)	23.2 ± 0.2	22.6 ± 1.5	0.503
Gestational age (weeks)	38 ± 1	37 ± 1	0.926
Premature (<week 37)	10.0%	9.5%	0.783
Very premature (<week 32)	2.2%	0.0%	0.585
Length (cm)	50.2 ± 1.5	48.7 ± 2.7	0.678
Weight (g)	2952 ± 200	3145 ± 270	0.184
LBW (<2500 g)	19.6%	11.8%	0.570
VLBW (<1500 g)	2.5%	0.0%	0.454
Congenital anomalies	2.1%	0.0%	0.325

Data are presented as mean ± SD or proportions.

BMI = body mass index; G-CSF = granulocyte colony-stimulating factor; LBW = low birthweight; VLBW = very low birthweight.

condition, including the presence of antiphospholipid antibodies. Furthermore, other possible causes of recurrent miscarriage, such as anatomical, endocrinological or genetic causes, were excluded as far as possible. Finally, KIR and HLA-C typing was performed on the patients, egg donor and the male partner. They had normal results after a complete screening for thrombophilia and immune factors and they were diagnosed as patients with recurrent miscarriage of unknown aetiology. At this stage, they were tested for KIR and HLA-C. These women had an inhibitory KIR AA and their partners carried HLA-C2. Due to this medical condition, egg donors were tested for HLA-C in order to confirm that they were carriers of HLA-C2.

The KIR haplotypes were analysed as part of a routine investigation in women with recurrent implantation failure or recurrent miscarriage referred for immunology consultation. Genomic DNA was obtained from maternal blood. All samples were obtained from Spanish individuals and all were of Caucasian origin. KIR and HLA-C typing was performed by polymerase chain reaction using sequence-specific oligonucleotides (PCR-SSO) on Luminex devices (LIFECODES; Immucor, Norcross, GA, USA). 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS4N, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1 and 3DP1 were analysed using two amplification multiple mixes. KIR haplotype regions were defined by the presence of the following KIR genes: Cen-A/2DL3; Tel-A/3DL1 and 2DS4; Cen-B/2DL2 and 2DS2; as well as Tel-B/2DS1 and 3DS1. The HLA-C genotypes were analysed in all women, their partners and the corresponding egg donors. HLA-C genes for all the subjects were assigned to C1+HLA-C or C2+HLA-C groups. The HLA-C typing kit is able to define the C1+HLA-C and C2+HLA-C ligands for KIR receptors.

Patients with KIR-HLA-C mismatch (KIR AA mother and HLA-C2 embryo) and a diagnosis of recurrent miscarriage were treated off-label with a single subcutaneous administration of 13 mIU filgrastim (NEUPOGEN®; Amgen, Thousand Oaks, CA, USA) starting the day of the embryo transfer and then every 3 days until the end of the 9th week of pregnancy. This dose is 5 to 10 times lower than the dose used for patients with congenital neutropenia or neutropenia as a side-effect of chemotherapy, or than the dose used in bone marrow donation.

Pregnancies were followed up every 3 weeks through ultrasound scans until the 12th gestational week to observe the heartbeat and embryo growth or to register a miscarriage. The live birth of a healthy baby without major or minor congenital anomalies was considered the primary outcome. Other perinatal data, including gestational age, length and newborn weight, as well as congenital anomalies, were considered as secondary outcomes.

Data from clinical outcomes are presented as descriptive statistics. Clinical results were analysed using the Student's *t*-test for comparison of means and the chi-squared test for proportions. Differences in congenital anomalies between the study groups was assessed using Fisher's exact test. A *P*-value <0.05 was considered significant. Statistical analyses were performed using SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

TABLE 1 presents the demographics and perinatal outcomes of patients diagnosed with recurrent miscarriage and treated with G-CSF compared with the control group, which included couples undergoing conventional ART. Patients undergoing an egg donation treatment included in the study group had an average of five previous failed cycles. Couples were contacted a month after delivery to obtain perinatal information; these data are based on voluntary disclosures, questionnaires and telephone interviews.

No significant differences were found in age or body mass index between the control group and women treated with G-CSF; for age, results were 40.9 ± 0.1 years versus 38.9 ± 1.8 years, $P = 0.055$) and for body mass index were 23.2 ± 0.2 kg/m^2 versus 22.6 ± 1.5 kg/m^2, $P = 0.503$). According to the perinatal information, no significant differences were observed in the child's weight (2952 ± 200 versus 3145 ± 270 g, $P = 0.184$), length (50.2 ± 1.5 versus 48.7 ± 2.7 cm, $P = 0.678$) or gestational age (38 ± 1 versus 37 ± 1 weeks, $P = 0.926$). The data also showed no increased rate of preterm births, regardless of whether <37 weeks (10.0 versus 9.5%, $P = 0.783$) or week 32 (2.2 versus 0.0%, $P = 0.585$) was used as the cut-off, or in the percentage of newborns with low weight <2500 g (19.6 versus 11.8%, $P = 0.570$) or very low weight <1500 g (2.5 versus 0.0%, $P = 0.454$).

Finally, none of the newborns derived from pregnancies in which G-CSF was administered had any major or minor abnormalities or malformations, compared with 2.1% of children in the control group being affected by some congenital anomaly.

DISCUSSION

Embryo implantation and early placental development are strongly influenced by the endometrial environment. In normal pregnancy, maternal immune tolerance allows the maintenance of the semi-allogeneic fetus, KIR and HLA-C being important factors in the establishment of this interaction (*Hiby et al., 2010*; *Moffett and Shreeve, 2015*; *Wurfel, 2015*). Decline of trophoblast invasion and vascular conversion in decidua are thought to be the primary defects of common pregnancy disorders including pre-eclampsia and fetal growth restriction. KIR-HLA-C mismatch, which means a KIR expressed by the maternal uterine NK cells and the embryo's HLA-C inherited as a non-self-antigen from the father, is a known risk factor for obstetrical complications (*Hiby et al., 2014*; *Moffett et al., 2016*). Patients carrying a KIR AA haplotype have a lower live birth rate after an egg donation treatment compared with patients carrying a KIR AB/BB haplotype (*Alecsandru et al., 2014*). Moreover, recent studies have described better reproductive outcomes in patients with inhibitory KIR genotype after the administration of G-CSF (*Eapen et al., 2019*; *Wurfel et al., 2010*).

The origin of this immune deregulation remains poorly understood. No standardized procedures are available for detecting immunological disorders in recurrent miscarriage patients or to rule out and treat recurrent miscarriage patients with immunological disorders. Classical immunological factors such as cytokines are peptides or glycoproteins that have pleiotropic regulatory effects on many cell types (*Romagnani et al., 1997*). A reduced concentration of these molecules could be responsible for poor obstetric outcomes, including recurrent miscarriage; therefore, administration of G-CSF in these situations could be considered an option for immunomodulatory therapy. As has been pointed out previously, G-CSF is a cytokine that stimulates neutrophilic granulocyte proliferation and differentiation. Notably, this molecule has been used safely in the treatment of neutropenia during cancer chemotherapy, and no toxic effects on human embryos have been observed (*Dale et al., 2006*; *Gomez Raposo et al., 2006*). The results were confirmed in later studies (*Santjohanser et al., 2013*; *Scarpellini and Sbracia, 2009*), which provided increasing evidence that G-CSF could not be toxic during pregnancy, although they recommend being careful in its use, as not enough women have been treated with it to exclude any possible teratogenic effect.

Within this retrospective cohort study, the perinatal data of newborns from couples treated with G-CSF were compared to a control population that received no adjuvant treatment during pregnancy. The data did not show any relevant changes between the two groups in the analysed variables, suggesting that this therapy could be safe for a select group of patients. These results concur with other studies (*Santjohanser et al., 2013*; *Scarpellini and Sbracia, 2009*; *Wurfel et al., 2010*) that have shown no significant differences in obstetrical or neonatal complications and confirmed that off-label administration of G-CSF would be safe for future children. Finally, regardless of its use in reproductive medicine, the innocuous use of G-CSF during a pregnancy had already been demonstrated, as G-CSF is common practice in the management of neutropenia during cancer chemotherapy, with no damage to the embryo reported (*Cottle et al., 2002*; *Dale et al., 2006*).

Finally, a recent randomized clinical trial (*Eapen et al., 2019*) showed no benefit of G-CSF for women with unexplained recurrent pregnancy loss, but this study included women with recurrent miscarriage of unknown aetiology and did not select the patients by the mother's KIR and paternal or egg donor HLA-C match. The Severe Chronic Neutropenia International Registry (SCNIR) provides relatively extensive data on the administration of G-CSF during pregnancy. The duration of treatment in patients with chronic neutropenia is roughly two trimesters and no indications of higher mortality or morbidity have been observed (*Dale et al., 2006*).

Importantly, the dose and timing of G-CSF administration in cases of infertility are not standardized. Effect of CSF on inflammatory and immunological processes seems to depend on the dose (*Dale et al., 2006*); thus, incorrect timing in the pre-implantation stage may interfere with a local uterine immune environment and be detrimental to embryo implantation and development.

G-CSF is probably associated with female physiology. Among the possible explanations related to the beneficial effect of G-CSF on the outcomes of ART is that G-CSF exerts a direct effect on trophoblast invasion, improving placentation (*Hiby et al., 2014*). G-CSF administration would increase the expression of G-CSF receptors on the trophoblast surface. This cytokine is also involved in modulating genes necessary for embryo implantation and may induce appropriate immune tolerance in pregnancy (*Zhao et al., 2016*).

The dataset in this study has some limitations. With regard to the retrospective setting, this study cannot control for data generation. Another limitation is the small sample size, which makes it difficult to draw definitive conclusions.

In summary, G-CSF appears to be a promising immunological treatment option for recurrent miscarriage patients with KIR-HLA-C mismatch. Administration of G-CSF during early pregnancy does not confer a higher risk of adverse perinatal outcomes in offspring. Maintenance of pregnancy in patients treated with a low dose of G-CSF seems to be uneventful, with no reports of increased malformation rates thus far, although further studies are needed to show the harmlessness of this drug in newborns.

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