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Embryo sHLA-G secretion is related to pregnancy rate

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Research Article

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Summary

HLA-G expression has been detected in early preimplantation embryos and it has been postulated that a relationship between embryonic expression of this factor and successful pregnancy may exist. Forty-six patients were prospectively selected from our centre 'Unidad de Reproducción Humana, Hospital Universitario de Canarias' for conducting this study. In all cases, metaphase II (MII) oocytes were fertilized using intracytoplasmic sperm injection 2–4 h after retrieval. Embryos were cultured individually in 20 µl droplets of G-1 medium (VitroLife) under oil at 37°C and a 6% CO_2 environment. Fertilization was assessed at 18 h postinsemination and all oocytes fertilized were passed into a new culture plaque individually in 300 µl culture medium until day 3 of culture. The culture medium was examined for the expression and secretion of sHLA-G with a sandwich enzyme-linked immunosorbent assay kit (BioVendor, Heidelberg, Germany) according to the manufacturer's instructions. We found statistical significance between higher levels of sHLA-G secretion and pregnancy rate. When both groups were compared there was no difference in embryo quality of transferred embryos, but a significant difference in the number of oocytes and the embryo quality of the cohort existed that was greater in the pregnant group. A standardized sHLA-G assay with a specifically defined range and standard units provides a non-invasive method to identify the most competent embryos for transfer.

Introduction

The maternal immune system is a critical component of the implantation process. Human leukocyte antigens (HLA) are part of the major histocompatibility complex (MHC) of proteins, which is encoded by a series of grouped genes located on chromosome 6 and play a role in the control of adaptive immunity, particularly T-cell-mediated immunity towards pathogens. During normal pregnancy, the maternal immune system undergoes changes that lead to fetal tolerance.

Some evidence suggests that HLA-G may play a role in protecting the fetus from the maternal immune response (Bjorkman *et al.*, 1987).

Soluble HLA-G (sHLA-G) is present in many body fluids, and may confer immune tolerance to the embryo. HLA-G expression has been detected in early preimplantation embryos and it is postulated that a relationship between embryonic expression of this factor and successful pregnancy may exist (Sipak-Szmigiel *et al.*, 2007).

Classically, embryo morphology has been used as the major selection parameter for embryo transfer. But this not always relates to implantation potential.

Having a non-invasive marker of implantation potential could be an important tool to implement morphological evaluation to increase implantation rate and reduce the overall number of embryos transferred to control multiple pregnancies.

HLA-G expression has been detected in early preimplantation embryos suggesting the establishment of a successful connection with pregnancy (Fuzzi *et al.*, 2002). More recent studies indicate a relationship between sHLA-G secretion, embryo quality and pregnancy rate (Noci *et al.*, 2005; Desai *et al.*, 2006).

In the present study we analyzed sHLA-G secretion to culture medium of a group of embryos transferred on day 3 post retrieval and correlated levels with embryo score, and pregnancy outcome using enzyme-linked immunosorbent assay (ELISA) (sHLA-G ELISA BioVendor; Wiendl *et al.*, 2005).

Materials and methods

Patients and design

In total, 46 patients were prospectively selected from our centre (Unidad de Reproducción Humana, Hospital Universitario de Canarias) to conduct this study. The inclusion criteria

were: age under 38 years (medium age was 33 ± 3 years); during first or second IVF treatment; no female factor of infertility and with only male factor with light oligoasthenozoospermia; and subjected to ovarian stimulation with recombinant follicle stimulating hormone (rFSH) and/or human menopausal gonadotropin (hMG). Consecutive patients fulfilling all the inclusion criteria and that accepted and signed the consent form were included in the study. Ovulation was triggered with rhCG when at least two follicles were 18 mm and 50% of the remainder were ≥15 mm. Oocytes were recovered transvaginally under ultrasound guidance 36 h later. All embryo transfers were performed at day 3 post insemination.

Embryo culture

In all cases, metaphase II (MII) oocytes were fertilized using intracytoplasmic sperm injection 2–4 h after retrieval. Embryos were cultured individually in 20-µl droplets of G-1 medium (VitroLife) under oil at 37°C and in a 6% CO_2 in air environment. Fertilization was assessed at 18 h post insemination and all oocytes fertilized were passed into a new culture plaque individually in 300 µl culture medium until day 3 of culture.

On day 3, embryos were selected for transfer only under the morphology score criterion following the Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) criteria scored as A, B, C, D, (ASEBIR, 2015). Culture medium from the embryos selected for transfer was frozen at −80°C in Eppendorf tubes for later testing for sHLA-G using a specific ELISA.

In total, 83 embryos were analyzed for sHLA-G concentration in the supernatant on day 3 of culture.

Soluble sHLA-G assay

sHLA-G concentration in the culture medium was measured using a sandwich ELISA kit (BioVendor, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, the standards ($n = 6$) and samples were added to an anti-sHLA-G monoclonal antibody-coated 96-well plate. The plate was then incubated at 2–8°C for 16–20 h. After washing five times, monoclonal anti-human β2-microglobulin antibody labelled with horseradish peroxidase (HRP) was added and the plate was incubated at room temperature for 1 h. Following another washing step, the substrate solution (TMP) was added to react with the remaining HRP conjugate and incubated in the dark for 30 min. To stop the reaction, an acidic solution was added. Absorbance of the yellowish product was immediately measured using an ELISA reader at 450 nm. The absorbance is proportional to the concentration of the sHLA-G. Sample concentrations were quantified according to the standard curve (unit/ml).

The sandwich ELISA is commonly used to detect sHLA-G in embryo culture supernatants. The assay uses a capture antibody coated to a plate that binds the sHLA-G and then a detection antibody that binds a different epitope on sHLA-G is added. Various antibody combinations were tested and the reported sensitivity is usually around 1 ng/ml. Several studies have standardized the sHLA-G ELISA. In 2004, a wet laboratory workshop was conducted to establish optimum conditions for detecting sHLA-G (Rebmann *et al.*, 2010). Desai *et al.* (2006) have described a newly available ELISA kit (EXBIO; Czech Republic) used for their study.

Rebmann *et al.* (2010) recently used the Luminex assay in conjunction with a standard reference ELISA and obtained a 10-fold increase in sensitivity compared with the standard ELISA. The Luminex assay used microspheres coated with antibody in place of a plate-bound antibody to capture sHLA-G in solution. The assay also used a fluorescently tagged secondary antibody in place of the enzyme-catalysed reaction. Rizzo *et al.* (2011) have performed western blotting together with a newly developed ELISA to specifically distinguish shed HLA-G1 from secreted HLA-G5 molecules. There, various conditions have been used to test for the presence of sHLA-G in supernatants from IVF/ICSI embryos.

Statistical analysis

Results are expressed as means and standard deviations. Comparisons between pregnant and non-pregnant women in the quantitative and/or ordinal variables were performed using the Mann–Whitney test. We carried out two partial exact regression analyses. Only two independent variables were included in each partial model. LogXact 4.0 analysis was used for this study (Cytel Co., MA, USA).

A *P*-value less than 0.05 was considered to be statistically significant. Statistical analysis was carried out using SPSS for Windows (Version 17.0, SPSS Inc., Chicago, IL, USA).

Results

We registered 11 gestations (24% pregnancy rate), of which one was a twin pregnancy, one an ectopic pregnancy and one patient had a miscarriage during the first trimester.

We found statistical significance between higher levels of sHLA-G secretion and pregnancy rate (28.0 ± 18.8 vs. 19.0 ± 17.6 unit/ml for pregnant and non-pregnant patients respectively; $P < 0.005$); this result confirmed those in previous studies (Noci *et al.*, 2005; Desai *et al.*, 2006). We applied a partial exact regression logistics model using gestation as the dependent variable, sHLA-G secretion as the main dependent variable, and controlled for the number of oocytes and the number of mature oocytes MII, resulting in an odds ratio (OR) of 1.074 (95% CI 1.017–1.11; $P = 0.015$) for number of oocytes, and an OR of 1.65 (95% CI 1.015–1.106; $P = 0.014$) for number of mature oocytes MII.

When comparing both groups there was no difference between the embryo quality of the transferred embryos (1.7 ± 0.47 vs. 1.5 ± 0.48 for good quality embryos transferred for pregnant or non-pregnant patients respectively; $P = 0.70$). A significant difference in the number of oocytes retrieved was seen (11 ± 5 vs. 7 ± 3 for pregnant or non-pregnant patients respectively; $P = 0.001$), in the number of MII oocytes (10 ± 4 vs. 7 ± 3 for pregnant or non-pregnant patients respectively; $P < 0.001$). The embryo quality of the cohort was better in the pregnant group (3.8 ± 5 vs. 2.4 ± 1.3 of good quality embryos available from the cohort for pregnant or non-pregnant patients respectively; $P = 0.016$) (Table 1).

Discussion

Immune tolerance of pregnancy is a paradox as the mother's immune system does not reject a fetus even though it is a partially foreign tissue, or even if this tissue is from an oocyte donor. The non-classical MHC molecule HLA-G is essential for immune tolerance induction in pregnancy.

HLA-G is expressed as a membrane-bound protein, exhibiting very limited tissue distribution on extravillous cytotrophoblast

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Table 1. Comparison between pregnant and non-pregnant groups

	Pregnant group, $N = 26$ embryos	Non-pregnant group, $N = 57$ embryos	*P*-value
Age (years)	34 ± 4	34 ± 3	0.47
Number of oocytes	11 ± 5	7 ± 3	0.001[a]
Number MII	10 ± 4	7 ± 3	<0.001[a]
sHLA-G	28 ± 18.8	19 ± 17.6	0.038[a]
Embryos transferred	2 ± 0	2 ± 0.5	0.99
Good quality embryos cohort	3.8 ± 5	2.4 ± 1.3	0.016[a]
Embryo fragmentation rate at day 3	5.4 ± 6.3	4.9 ± 6.6	0.52
Good quality embryos transferred	1.7 ± 0.47	1.5 ± 0.48	0.70

[a]Statistically significant.

cells in the placenta, maternal spiral arteries, endothelial cells of fetal vessels in the chorionic villi, amnion cells, thymus, and interferon-γ-stimulated blood monocytes (Roussev and Coulam, 2007).

There are four membrane-bound HLA isoforms with a transmembrane region and an intracytoplasmic tail, and three secreted isoforms HLA-G5, HLA-G6 and HLA-G7. HLA is important in the immune response and also in modulation of the maternal–fetal immune relationship during pregnancy. Immunomodulation of cytokine secretion is believed to create a chemical dialogue between embryo and maternal immune tolerance.

The data presented by Rizzo *et al.* (2011) confirm the role of sHLA-G molecule, quantified in embryo culture supernatants as a marker for embryo selection. In pregnancy, several tolerance mechanisms have been demonstrated to counteract the maternal immune response.

Soluble HLA-G suppresses the functional activity of natural killer (NK) cells and inhibits NK-cell-mediated cytotoxicity (Marchal-Bras-Goncalves *et al.*, 2001) suggesting the importance of inducing immunotolerance, controlling trophoblast invasion and contributing to vascular remodelling of spiral arteries to allow implantation and pregnancy maintenance. All these activities point to the fundamental role of sHLA-G expression for invasive cytotrophoblasts and in creating tolerogenic conditions at the feto-maternal interface.

In a recent study (Giacomini *et al.*, 2017), the authors demonstrated for the first time that conditioned medium from non-manipulated human embryos cultured *in vitro* for at least 3 days contained extracellular vesicles with a diameter of 50–200 nm. The embryonic origin of these extracellular vesicles was confirmed by the presence of stemness gene transcripts and their enrichment in non-classical HLA-G protein. Extracellular vesicles can be easily taken up by the maternal side, giving interesting possibilities for future therapeutic use and for the establishment of a successful pregnancy.

A relationship has been shown between couples with recurrent miscarriage and a mutation in the HLA-G gene (Hackmon *et al.*, 2004), and attention has been focused on clinical application. A null mutation that results in no functional HLA-G1 or G5 protein isoforms has been associated with increased risk of recurrent miscarriage (Aldrich *et al.*, 2001; Pfeiffer *et al.*, 2001); HLA-G 725 promoter polymorphism is a risk factor for recurrent miscarriages. In the same line, Eskicioğlu *et al.* (2016) encountered a significant increase in TNF expression in patients with recurrent miscarriages, (Eskicioğlu *et al.*, 2016), and consequently increased cytotoxic activity in the maternal immune system that explained the miscarriages. In these patients, even with no change in HLA-G expression, the maternal immune system is unresponsive to HLA-G immunosuppressive signals from the embryo and fetus.

Other study conducted by Yao *et al.* (2014) provided also novel evidence that there is HLA-G expression in testicular tissues with different spermatogenic ability as well as in different stages of human embryos. This finding points to the importance HLA-G expression has in spermatogenesis, embryo development and implantation.

Focussing on clinical applications of measuring HLA-G, when the culture medium used was from embryo cultures from patients that became pregnant with no fetal loss, significantly higher amounts of HLA-G-positive sibling blastocysts were achieved compared with medium from patients who did not conceive (Jurisicova *et al.*, 1996). Absence of sHLA-G from human embryo culture medium is associated with reduced embryo development and pregnancy rate.

In our study we found a relationship between sHLA-G secretion and pregnancy rate as did other authors (Martí *et al.*, 2007). In this group of patients, our objective was to transfer the best quality embryos of the cohort, looking at morphological parameters only. Consequently, we found no differences in embryo quality for the transferred embryos between the two groups. But when comparing the total oocytes extracted, the number of mature MII oocytes and quality of all embryo cohorts, we found a significantly better oocyte number and embryo cohort in the group from pregnant patients.

Other authors (Desai *et al.*, 2006) have studied sHLA-G secretion using an ELISA kit and concluded that HLA-G-positive embryos have a greater ability to evade the cytotoxic activity of maternal T cells compared with embryos not secreting HLA-G.

This finding is according with our results of a positive correlation between higher rate of sHLA-G secretion in at least one of the embryos transferred and a positive pregnancy. sHLA-G secretion was measured for all embryos transferred, including both implanted and non-implanted embryos. This result should be confirmed with single embryo transfer, but despite this caveat, there was significant correlation between sHLA-G secretion and pregnancy.

In conclusion, a standardised sHLA-G assay with a specifically defined range and standard units should be made available as a non-invasive assay to identify the most competent embryo for transfer (Kotze *et al.*, 2013).

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Although important, these finding were not sufficient to be extrapolated to pregnancy development (Fuzzi *et al.*, 2002). ELISA can be a useful biochemical option coupled with embryo morphology for embryo selection and transfer for IVF when there are other embryos with the same morphology (Noci *et al.*, 2005; Rebmann *et al.*, 2010). In the absence of this assay or in cases of no determinant results for HLA-G concentration, major relevance is still given to traditional morphological grading for embryo selection and for which HLA-G concentration can be a complementary indicator of good embryo potential, especially for patients with implantation failure or recurrent miscarriage.

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Conflicts of interest. None.

Ethical standards. All patients were conveniently informed about the study and signed consent forms. The study was approved by the Ethics Committee of the Hospital Universitario de Canarias.

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