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Article

Soluble HLA-G in IVF/ICSI embryo culture supernatants does not always predict implantation success: a multicentre study

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Abstract

Several reports have described an association between the presence of soluble human leukocyte antigen G (sHLA-G) in human embryo culture supernatants (ES) and implantation success. However, not all studies agree with these findings. To further document this debate, a multicentre blinded study was performed to investigate, on a large number of IVF ES and ICSI ES, whether sHLA-G is a useful criterion for embryo selection before transfer. A total of 1405 ES from 355 patients were collected from three assisted reproductive technique (ART) centres and evaluated for their sHLA-G content in a single laboratory, using a chemiluminescence enzyme-linked immunosorbent assay. In only one centre was a significant association between sHLA-G-positive ES and successful implantation established ($P = 0.0379$), whereas no such association was observed in the other centres. It was found that the percentages and concentrations of sHLA-G-positive ES varied between centres, depending on culture media and ART conditions. The percentage of sHLA-G-positive ES was significantly higher in IVF ES than ICSI ES ($P < 0.001$ and $P < 0.01$ for two centres). These data demonstrate that substantial variations of sHLA-G content in ES occur between different ART centres, highlighting the influence of several technical parameters that differ from one centre to another.

Keywords: *embryo transfer, ICSI, IVF, soluble HLA-G*

Introduction

Since the first report indicating that soluble human leukocyte antigen G (sHLA-G) produced by some IVF/intracytoplasmic sperm injection (ICSI)-derived embryos could be a predictive marker of embryo implantation potential (Fuzzi *et al.*, 2002), a considerable interest has been aroused and several other assisted reproductive technology (ART) groups have performed similar kinds of studies (Sher *et al.*, 2004, 2005a,b; Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Yie *et al.*, 2005; Desai *et al.*, 2006; Fisch *et al.*, 2007; Rebmann *et al.*, 2007; Sageshima *et al.*, 2007; Shaikly *et al.*, 2008). However, not all investigators agree with their conclusions, which raised some concerns; these conclusions have been listed and thoroughly discussed in

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recent reviews (Sargent *et al*., 2007; Vercammen *et al*., 2008). Technical differences including IVF/ICSI culture conditions, duration of embryo culture, number of embryos transferred, enzyme-linked immunosorbent assay (ELISA) methods used and their sensitivity to detect sHLA-G in embryo culture supernatants (ES) are the most likely explanations for such discrepancies. As pointed out by Sargent *et al*. (2007), 'to understand and unravel these differences will not be achieved by researchers working in isolation, but requires collaborations involving exchange of samples, standards and technique for validation'. The issues dealing with sHLA-G ELISA protocols have already been addressed by specific workshops (Rebmann *et al*., 2005). The aim of this report was to set up an ART multicentre blinded study on a large sample size to further document whether sHLA-G detection in ES can be considered as a reliable predictive marker of potential clinical pregnancy.

Materials and methods

Patients

A total of 1405 ES were collected from 355 cycles, including 87 IVF and 268 ICSI, performed in CHU Poissy-St Germain-en-Laye (France), Toulouse (France) and CHR La Citadelle, Liège (Belgium), ART centres from September 2005 to March 2007 (**Table 1**). The mean age of the patients and numbers of previous IVF/ICSI attempts are indicated in **Table 1**. Ovarian stimulation was carried out using different standard protocols (Jennings *et al*., 1996; Lesourd *et al*., 2006; Tarlatzis *et al*., 2006) (**Table 1**). Response to ovarian stimulation was monitored by serial blood tests and ultrasound assessment of follicular and endometrial growth. Oocytes were retrieved by aspiration under vaginal ultrasound guidance 35–36 h after ovulation. Mean implantation rate, mean clinical pregnancy rate and multiple pregnancy rates in each respective ART centre are also indicated in **Table 1**.

Oocyte fertilization and embryo culture conditions

Media used in each ART centre for oocyte collection, removal of cumulus oophorus, culture of cumulus–oocyte complexes, sperm washing, and IVF or ICSI, are indicated in **Table 2**. In the Liège Centre, media from two different companies (Irvine Scientific, Santa Anna, CA, USA; Cook, Brisbane, Australia) were used on alternate days to minimize the effect of any potential batch quality problem. The fertilization culture media used for IVF or the manipulation of gametes for ICSI were Early Cleavage Medium (Irvine Scientific) and Cleavage Medium (Cook) in 70% and 30% of the attempts, respectively.

Embryo and oocyte culture supernatants

A total of 1405 ES (including frozen and transferred embryos and those leading to embryos which were not suitable for either transfer or freezing and subsequently destroyed) and 40 unfertilized oocyte culture supernatants were collected (**Table 3**). Culture of single embryos was performed in 50-µl droplets under mineral oil in the three centres. Poissy and Toulouse centres transferred their embryos on day 2, and the Liège centre on day 3. Culture supernatants from 550 day-2 embryos and 176 day-3 embryos, which were fully documented and traceable up to the time of implantation, were studied. The mean number of oocytes collected, embryos obtained, and embryos transferred are detailed in **Table 1**. ES (40 µl) were collected in the three centres less than 15 min after the transfer, freezing or destruction of the corresponding embryos, immediately frozen in liquid nitrogen and subsequently stored at –80°C until assay. Before being used for ELISA measurements, all ES were centrifuged at 21,000 *g* for 1 min. In the Poissy centre, oocytes were retrieved individually. All samples were anonymous at the time of the collection and analysed blind.

Embryo quality

Embryo morphology was evaluated and scored in two grades: 'high quality' was defined by 4–5 cells on day 2, 8–9 cells on day 3, less than 10% fragmentation and regular cells. All other embryos were classified as 'medium–low' quality.

Evaluation of clinical pregnancies

Clinical pregnancy was defined at 7 weeks after embryo transfer when a yolk sac was viewed by ultrasound. The implantation rate (IR) of the embryos corresponding to each sample tested was defined as the number of yolk sacs/number of embryos transferred. Clinical and biological data were stored in central Embryo Implantation Control Network anonymous database (ART application website: www.medifirst.fr; MédiFirst, Poissy, France). All patients gave fully informed consent, and each institutional review board approved this investigation.

sHLA-G chemiluminescent ELISA

Concentrations of sHLA-G in ES collected from the different ART centres were evaluated in Toulouse by a specific sandwich ELISA previously described (Fournel *et al*., 2000) and with improved sensitivity. Maxisorp-treated plates (384-microwell; Nalge Nunc International, Rochester, NY, USA) were coated with 50 µl anti-HLA-G monoclonal antibody MEM-G9 (immunoglobulin G1 (IgG1), Exbio, Prague, Czech Republic) or IgG1 isotype control at 2 µg/ml in 0.1 mol/l carbonate buffer pH 9.5 for 1 h at 37°C, then overnight at 4°C, as previously described (Fournel *et al*., 2000). After three washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS–Tween), plates were saturated with 100 µl PBS/4% bovine serum albumin overnight at 4°C. After 10 washes with PBS–Tween and three washes with PBS, embryo culture supernatants (40 µl) were added to each well. After incubation for 2 h at 37°C, plates were washed six times with PBS–Tween. Biotinylated W6/32 (Fournel *et al*., 1999), at 5 µg/ml in PBS–Tween containing 1% mouse serum (50 µl), was added to each well and incubated for 1 h at 37°C. Plates were washed six times with PBS-Tween and further incubated with a peroxidase-conjugated avidin (Dako, Denmark) diluted in PBS–Tween containing 1% mouse serum for 15 min at 37°C. After 10 washes with PBS–Tween and three washes with PBS, 50 µl SuperSignal ELISA Femto Maximum Sensitivity substrate (Pierce, Rockford, IL, USA) was added. The relative concentrations of sHLA-G were estimated from relative light units released over a 20 s period of time, 1–5 min after adding the substrate, using a Victor light luminescence counter for microplate application (Perkin Elmer, MA, USA). An affinity-purified

Table 1. Patient characteristics and assisted reproductive technique data (total of 355 infertile women).

Parameter		*Centre Poissy*	*Toulouse*	*Liège*
Number of women/cycles (*n*)		78	196	82
Age (years)		32.6	32.3	35.8
Range (years)		24–41	21–39	21–45
No. of attempts		1.8 ± 0.12	2.0 ± 0.15	1.7 ± 0.11
ART type (*n*)	IVF	0	74	13
	ICSI	78	122	69
Stimulation protocol (*n*)	Agonist/long protocol	60	141	35
	Agonist/short protocol	8	0	47
	Antagonist	9	55	0
	Recombinant FSH	73	196	23
	Urinary FSH/LH	4	0	59
Mean no. of oocytes retrieved		9.2	8.8	10.6
Mean no. of embryos obtained		4.9	3.8	6.5
Mean no. of embryos transferred	Fresh transfer	2.1	2.1	1.7
	Frozen–thawed transfer	–	–	1.5
Clinical pregnancy rate (%)[a]		30/77 (40.0)	60/196 (30.6)	27/82 (32.9)[a]
Multiple pregnancies (%)		9/30 (30.0)	14/60 (23.3)	4/27 (14.8)
Implantation rate (%)[b]	Fresh transfer	23	18	27.8
	Frozen–thawed transfer	–	–	22.7

[a]Cumulative pregnancy rate: fresh transfer and subsequent frozen–thawed transfer if relevant. [b]Implantation rate: number of yolk sacs at 8 weeks per the total number of embryos transferred. Absolute numbers are not reported since each embryo is defined by its own percentage of implantation (0, 100%, 50%, 66.66% [two implantations for three transfers], or 33.33% [one implantation for three transfers]); thus only the mean percentage of implantation of the group observed (fresh or frozen–thaw transfer) is reported.
ART = assisted reproductive technique.

Table 2. Characteristics of the media used in the different assisted reproductive technique centres.

Medium	*Centre Poissy*	*Toulouse*	*Liège Medium 1 (Irvine Scientific, USA)*	*Liège Medium 2 (Cook, Australia)*
Medium for oocytes[a]	Ferticult HEPES (JCD, France)	G fert (VitroLife[b])	Modified human tubal fluid	Oocyte wash buffer
Medium for sperm washing	Fertipro (JCD, France)	Spermafix (Eurobio[b])	Sperm washing medium	Sperm buffer
Fertilization medium	ISM1 (MediCult, France)	G (VitroLife[b])	Human tubal fluid	Fertilization medium
Cleavage medium	ISM1 (MediCult, France)	G (VitroLife[b])	Early cleavage medium	Cleavage medium

ICSI = intracytoplasmic sperm injection.
[a]Medium used for oocyte washing, removal of cumulus and ICSI.
[b]VitroLife, Göteborg, Sweden; Eurobio, Montpellier, France.

Table 3. Characteristics of the collected samples.

Parameter		*Centre* *Poissy*	*Toulouse*	*Liège*
No. of women/cycles		77	196	82
ART procedure		ICSI	IVF or ICSI	IVF or ICSI
No. of samples analysed		360[a]	450[a]	595[a] + 40[b]
No. of embryos transferred	Fresh	146	404	132
	Frozen–thawed	–	–	44
Day of transfer or freezing		Day 2	Day 2	Day 3

[a]Embryo supernatants
[b]Unfertilized oocyte supernatants
ART = assisted reproductive technique; ICSI = intracytoplasmic sperm injection.

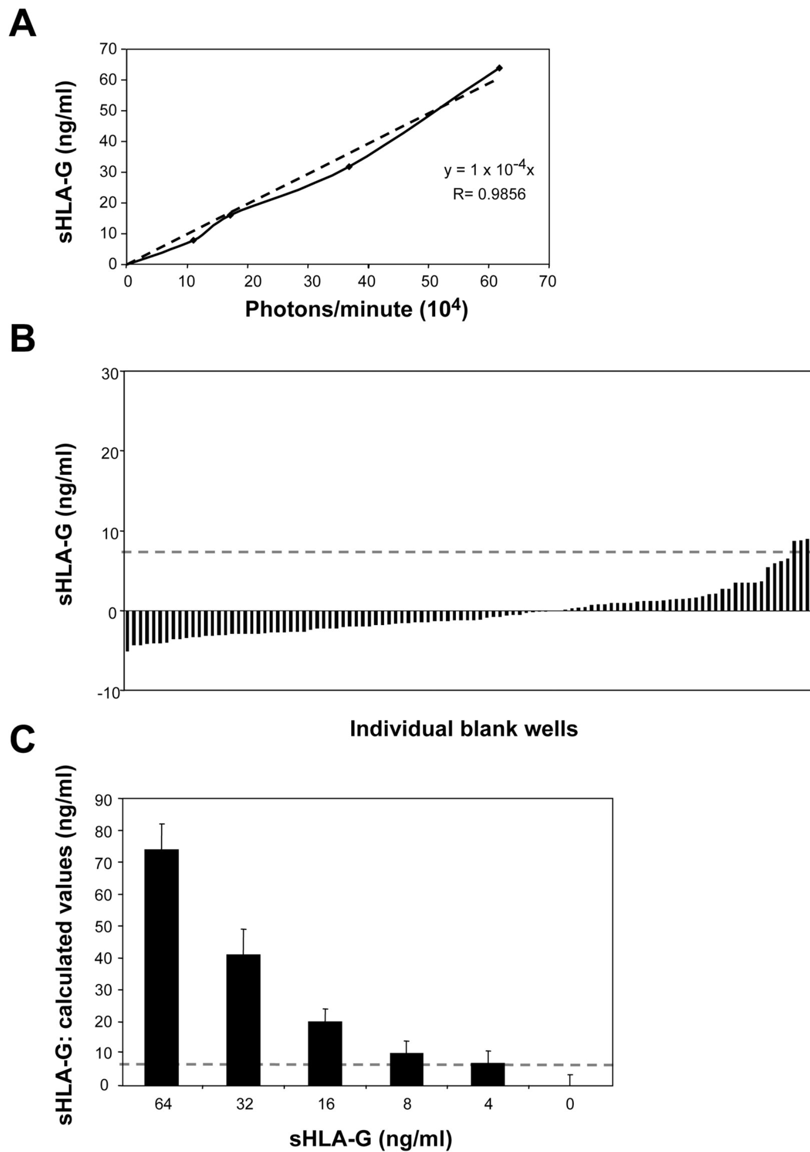

Figure 1. Representative soluble human leukocyte antigen G (sHLA-G) standard curve obtained with a chemiluminescence enzyme-linked immunosorbent assay (ELISA) procedure described in Materials and methods. (**A**) Typical standard curve obtained by making serial dilutions of a standard recombinant sHLA-G (duplicates) of known concentrations (vertical axis) and photons/minute on the horizontal axis, using a sandwich ELISA with MEM-G9 anti-HLA-G capture monoclonal antibody (mAb) and biotinylated W6/32 detection mAb. The dotted line indicates the linear regression. This representative standard curve revealed linearity (R = 0.9856) for sHLA-G between 2 and 65 ng/ml. No binding activity was detected when wells were coated with a control mouse immunoglobulin G1 (not shown). (**B**) Sensitivity test: sHLA-G detection limit was calculated on 106 blank wells filled with medium and scattered throughout the plate. In this case, three blanks are at the limit of positivity and four blanks are false positives. Calculation of sHLA-G concentrations using the standard equation curve (**A**) indicates that the negative wells have a mean of 0.2 ng/ml ± 3.5 SD. The lower detection limit of the assay is 7 ng/ml (dotted line). (**C**) Positive control wells containing serially diluted standard recombinant sHLA-G (quadruplicates). sHLA-G concentrations were calculated using the standard equation curve. In this representative plate, all samples which contained more than 7± 2 ng/ml sHLA-G were taken into account. Signals below the detection limit were considered as negative.

standard recombinant sHLA-G (rsHLA-G) isolated from 221-sHLA-G transfectant (Fournel *et al.*, 1999) was used to establish standard curves for each plate. Each standard curve revealed linearity for the range of rsHLA-G between 2 and 65 ng/ml (**Figure 1A**). To rule out possible artefacts from the position of the samples in the plate, positive controls (rsHLA-G) of known concentrations were evenly distributed within each plate. At least 60 blank wells (culture medium without embryo) were also scattered throughout each plate and simultaneously evaluated (**Figure 1B**). These wells were used to evaluate the limit of detection. The detection limit was overestimated as the values of the 'highest' blanks (generally one to a maximum of four out of 60) were always taken into account. A mean of one false positive for 60 blanks was obtained. The detection limit of the assay, evaluated by dilution of the standard reagent, was <10 ng/ml (**Figure 1C**). The mean intra-plate coefficient of variation was <1%. According to the sHLA-G concentrations measured and the limit of detection in each plate, samples were divided into 'detectable sHLA-G' or 'undetectable sHLA-G'. In the Liège centre, supernatants of immature, unfertilized or atretic oocytes were also used as negative controls.

Statistics

All measurements were analysed blindly and anonymously. Means and percentages were calculated for each group and the statistical differences between pairs of groups were assessed using analysis of variance (ANOVA) followed by Fisher test or chi-squared. Values of $P < 0.05$ were considered significant.

Results

Variability of sHLA-G-positive embryo culture supernatants in the different ART centres

Using the chemiluminescence ELISA, 1405 ES from three different ART centres were evaluated for their content of sHLA-G (**Table 4**). In the Toulouse centre, sHLA-G (>7 ng/ml) was identified in 153 of 450 ES (34%). In the Liège centre, the proportion of sHLA-G positive ES was 262 of 595 ES (44%). The mean sHLA-G concentration in all ES tested was similar in these two centres (18 ng/ml and 16.2 ng/ml, respectively). However, the sHLA-G concentrations in sHLA-G-positive ES were much higher (53.7 and 34.57 ng/ml, respectively). In contrast, in the Poissy centre the percentage of sHLA-G-positive ES (19%, 68 of 360 ES) was lower than those of the two other centres. The mean sHLA-G concentrations in all ES tested and in sHLA-G-positive ES were much lower (3.3 and 17.7 ng/ml, respectively). These data demonstrate that the proportions of sHLA-G-positive ES and concentrations of sHLA-G may substantially vary among different ART centres. Although the ovarian stimulation protocols differed in the three centres (**Table 1**), no difference in the percentages of sHLA-G detection was observed in the different centres according to either the protocols used or the types (recombinant or urinary) and concentrations of hormones administered (data not shown).

sHLA-G in embryo cultures is influenced by ART procedures

Since the Poissy ES were obtained solely from ICSI, it was postulated that the low percentage of sHLA-G-positive ES and their lower concentration of sHLA-G observed in this centre might be related to the ART procedure. In order to test this hypothesis, the percentages of sHLA-G-positive ES obtained after IVF (281 ES) and ICSI (718 ES) performed in the Liège and Toulouse centres were compared (**Figure 2**). In both centres, the percentages of sHLA-G-positive ES were significantly higher after IVF than after ICSI (61% versus 40%, $P < 0.001$, for Liège; and 42% versus 29%, $P < 0.01$, for Toulouse). These results demonstrate the influence of ART procedures on sHLA-G secretion in ES.

Relationship between sHLA-G in ES and implantation rate: differences between ART centres

It was investigated whether sHLA-G detection in ES was associated with embryo implantation potential in the three ART centres (**Table 5**). It was found that, despite producing the lowest concentrations, the detection of sHLA-G in ES from the Poissy centre was significantly associated with higher implantation rates after ART: 34% of HLA-G-positive but only 19% of HLA-G-negative embryos resulted in implantation after embryo transfer ($P = 0.0379$). In contrast, the presence of sHLA-G in ES was not associated with higher implantation after embryo transfer in either the Toulouse or the Liège centre (**Table 5**). These results indicate that the presence of sHLA-G in ES is not always associated with higher implantation potential, depending on the ART centres' procedures.

Embryo quality is independent of sHLA-G in embryo cultures

It was then evaluated whether sHLA-G secretion could be related to the embryo quality. As shown in **Table 6**, no statistically significant relationship (Fisher's exact test) was established between embryo morphology, classified as 'high' or 'medium–low' quality, and sHLA-G detection in ES.

sHLA-G in ES is influenced by culture medium

A comparison was made between two different culture media and the secretion of sHLA-G in ES. Media from two different companies were used on alternate days to minimize the effects of variations in quality in either of them. A statistically significant difference in the percentages of sHLA-G-positive ES was observed between the two culture media ($P < 0.0001$; **Table 7**), independently of IVF or ICSI ART procedure (data not shown). Furthermore, a tendency to higher implantation rate was observed with culture medium 2. These results demonstrate that culture medium influences the concentration of sHLA-G secretion in ES, and likely the implantation rate after embryo transfer.

Table 4. sHLA-G in embryo culture supernatants.

Parameter	Centre Poissy	Toulouse	Liège
Day	2	2	3
n	360	450	595
No. of sHLA-G-positive ES (%)	68 (19)	153 (34)	262 (44)
sHLA-G concentration (ng/ml) in sHLA-G-positive ES[a]	17.74 ± 11.2	53.70 ± 3.0	34.57 ± 28.0
sHLA-G concentration (ng/ml) in all sHLA-G-positive and negative ES tested[a]	3.3 ± 0.45	18.0 ± 1.5	16.2 ± 1

[a]Values are means ± SD.
ES = embryo culture supernatant; sHLA-G = soluble human leukocyte antigen G.

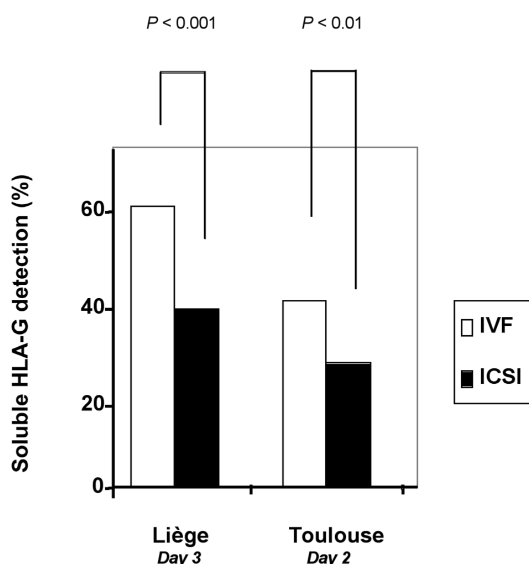

Figure 2. Soluble human leukocyte antigen G (sHLA-G)-positive embryo culture supernatants differ between IVF and intracytoplasmic sperm injection (ICSI) procedures. sHLA-G detection in embryo culture supernatants was evaluated by the enzyme-linked immunosorbent assay procedure described in text. Liège centre: IVF n = 130, ICSI n = 465; Toulouse: IVF n = 151, ICSI, n = 253. P was defined by chi-squared test.

Table 5. sHLA-G in embryo culture supernatants and implantation rates.

Centre	Day (n)	Implantation rate[b] (%) sHLA-G-positive ES	sHLA-G-negative ES	P-value
Poissy	2 (146)	34	19	0.0379[a]
Toulouse	2 (404)	17	18	NS
Liège	3 (176)	17	18	NS

[a]Analysis of variance.
[b]Only mean implantation rate of the group observed (HLA-G positive or negative, implantation rate of each reproductive unit) is reported; for explanation, see footnote b in Table 1.
ES = embryo culture supernatant; sHLA-G = soluble human leukocyte antigen G;
NS = not statistically significant.

Table 6. sHLA-G in embryo culture supernatant, and embryo quality.

Parameter	Centre Poissy (n = 349)		Liège (n = 595)	
Embryo quality	High	Medium–low	High	Medium–low
No. of embryos observed	166	183	147	448
No. of sHLA-G-positive ES (%)	30 (18)	36 (20)	57 (39)	206 (46)

ES = embryo culture supernatant; sHLA-G = soluble human leukocyte antigen G.

Table 7. Influence of the culture medium on sHLA-G detection in embryo culture supernatants.

Parameter	*Liège (595 embryo supernatants)* Culture medium 1 (Irvine Scientific)	Culture medium 2 (Cook)	P-*value*[a]
sHLA-G-positive ES (%)	77/328 (23.5)	187/267 (70.0)	<0.0001
sHLA-G-positive ES among embryos which were transferred (%)	34/122 (27.9)	33/54 (61.1)	<0.0001
Implantation rate[b] (%)	15.6	23.1	NS

[a]Fisher's exact test.
[b]Only mean implantation rate of the group observed (HLA-G positive or negative, implantation rate of each reproductive unit) is reported; for explanation, see footnote b in Table 1.
ES = embryo culture supernatant; sHLA-G = soluble human leukocyte antigen G; NS = not statistically significant.

Discussion

To the best of the authors' knowledge, this study is the first multicentre investigation performed on a large sample size of singly cultured embryos to evaluate a possible association between sHLA-G in ES and improvement of implantation rate of assisted reproduction. This study is unique as all sHLA-G measurements in ES collected from three centres in two different countries were performed blind in the same laboratory, using the same ELISA method. This differs from other reports which collected more limited numbers of ES from a single or two ART centres from the same country (Fuzzi *et al.*, 2002; Van Lierop *et al.*, 2002; Sher *et al.*, 2004, 2005b; Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Yie *et al.*, 2005; Desai *et al.*, 2006; Rebmann *et al.*, 2007; Sageshima *et al.*, 2007; Shaikly *et al.*, 2008). Another difference between other previous reports and the present study is the chemiluminescence ELISA method used to detect sHLA-G in 40 µl sample volume, using an automated light luminescence counter for microplate application.

This study shows a higher pregnancy rate in sHLA-G positive embryos in one centre, confirming some previous data, but it also indicates variability of percentages and concentrations of HLA-G-positive ES among the different centres. It further established that such variability of sHLA-G concentrations was largely influenced by ART conditions, including culture media and ICSI/IVF procedures. Only in the Poissy centre was a statistically significant association between sHLA-G-positive ES and implantation rate detected, although the percentage of sHLA-G-positive ES was rather low (19%). This association could be related to ICSI, as the Poissy centre used only this ART procedure and not IVF, confirming previous observations showing that the presence of sHLA-G in ES was highly associated to clinical pregnancy after ICSI (Rebmann *et al.*, 2007). The proportion of sHLA-G-positive ES and association with pregnancy varied between IVF and ICSI procedures. Fewer sHLA-G-positive ES were found after ICSI than after IVF, but there was a significant association between the presence of sHLA-G in ES and clinical pregnancy after ICSI. The observation that the ICSI centre was the only one to find an association further demonstrates the inherent limitations of measuring sHLA-G in ES as, in contrast to the hypothesis, the concentrations were lower rather than higher in this group. Of note, in the three centres, a similar implantation rate (18–19%) was observed with sHLA-G-negative ES, confirming a previous study showing a similar percentage (19%) of women conceiving after sHLA-G-negative embryo transfer (Rebmann *et al.*, 2007).

The concentration of sHLA-G in ES is a matter of debate. A variety of sHLA-G concentrations were found in ES from the different centres, ranging from 3.3 to 18 ng/ml. Knowing that embryos were cultured in ~50 µl medium, these amounts correspond to ~55–82 pg (lower concentrations) or ~300–450 pg (higher concentrations) of sHLA-G secreted per day per embryo (depending whether embryos were cultured for 2 or 3 days). These concentrations of sHLA-G secretion are much lower than those mentioned in a recent controversial report based on estimated total protein content of the preimplantation embryos and suggesting that sHLA-G detection in ES was artifactual (Ménézo *et al.*, 2006). Possible explanations that could lead to overestimated concentrations of sHLA-G have been previously discussed (Sargent *et al.*, 2007). Such variability of sHLA-G concentration detected in ES and also described in another recent report (Shaikly *et al.*, 2008) is unlikely to be due to the ELISA method, as only one type of assay was used throughout the study, although a limit cannot be excluded in the detection accuracy due to the analysis of ES samples in only one well, as there was only 40 µl of each ES sample. Moreover, this assay was always performed in the same centre, thus limiting possible bias. Other conditions reduced the effect of uncontrolled parameters, including the use of standard curves in each plate tested to estimate protein concentrations, and the addition of many blanks scattered throughout the plates to produce a mean intra-plate coefficient of variation that was always <1%. The use of MEM-G9 capture monoclonal antibody (mAb) and W6/32 detection mAb, first described as the best mAb combination to detect sHLA-G (Fournel *et al.*, 2000) and used in other studies (Vercammen *et al.*, 2008), was further shown to exclude non-specific reactions due to possible secretion of sHLA class I molecules different from HLA-G (Sageshima *et al.*, 2007). The chemiluminescence intensity always increased linearly, even at the lower (0–10 ng/ml) concentrations (**Figure 1A**).

Variability was also observed in the percentages of HLA-G-positive ES in the three centres, the highest percentage (44%) being detected in the Liège centre where only day-3 ES were collected. This extended culture of embryos to day 3 may explain why day-2 ES collected in Toulouse and Poissy centres exhibited lower percentages of sHLA-G positivity

(34% and 19%, respectively). Another report indeed indicated a significantly higher detection of sHLA-G in day-3 ES (Desai *et al.*, 2006). However, in the present study, it was observed that day-2 and day-3 ES were not significantly different in terms of implantation rate. Further investigation is in progress using embryos at the day-5 blastocyst stage, with only one embryo transferred.

The results also indicate that concentrations of sHLA-G depended on the culture medium. These data suggest that sHLA-G detection is very likely to be related to the ART conditions. In the same way as stimulation protocols will affect egg quality, different culture media may also affect embryo quality and hence the amount of sHLA-G expressed. However, it was found that the presence of sHLA-G in embryo cultures was not related to embryo quality determined morphologically, confirming previous studies (Noci *et al.*, 2005; Sher *et al.*, 2005a; Yie *et al.*, 2005; Desai *et al.*, 2006), but contrasting with another report (Rebmann *et al.*, 2007). It is unlikely that the different culture media produced artefacts as it was observed that crude values (photons/minute) of media without embryos were always similar to those of culture media with embryos that were considered as sHLA-G negative (data not shown).

In summary, this report shows that sHLA-G secretion in ES could be a useful tool to set optimal in-vitro embryo culture conditions and that it has some use in identifying embryos with enhanced developmental potential. However, the presence of sHLA-G alone cannot predict implantation potential after ICSI with certainty. This study clearly indicates that measurement of sHLA-G in ES is dependent on multiple parameters, including ART procedures and the culture media. Very similar conclusions were drawn in a recent report based on a statistical meta-analysis of 11 cohort studies previously published (Vercammen *et al.*, 2008). Further multicentre research is needed to reach a consensus on the best conditions to be used to definitely determine if sHLA-G detection could be one of the predictors of pregnancy outcome.

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