The effect of the biochemical marker soluble human leukocyte antigen G on pregnancy outcome in assisted reproductive technology—a multicenter study

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Objective: To determine whether the presence of soluble human leukocyte antigen G (sHLA-G) affects implantation and pregnancy outcomes in vitro.
Design: A multicenter retrospective study.
Setting: Six certified in vitro fertilization (IVF) units.
Patient(s): Embryos obtained from 2,040 patients from six different IVF clinics.
Intervention(s): Soluble HLA-G determination on day-2 embryos after intracytoplasmic sperm injection, with embryos transferred on day 3 using the sHLA-G data.
Main Outcome Measure(s): Ongoing pregnancy rate (10- to 12-week ultrasound finding).
Result(s): All embryos were individually cultured, and a chemiluminescence enzyme-linked immunosorbent assay was used to detect the presence of sHLA-G in the culture medium surrounding the embryos. Embryos were selected based on a positive sHLA-G result and a graduated embryo scoring (GES) score >70, or on embryo morphology if the test was negative. In all centers, a positive sHLA-G result was associated with an increase in the odds of an ongoing pregnancy. The incidence of an ongoing pregnancy was 2.52 times greater in embryos transferred on day 3 with a positive sHLA-G test result than the incidence of an ongoing pregnancy in embryos with a negative sHLA-G test result.
Conclusion(s): Data from this multicenter study confirm that sHLA-G expression is a valuable noninvasive embryo marker to assist in improving pregnancy outcomes, with the theoretical potential to reduce multiple pregnancies. (Fertil Steril® 2013;100:1303–9. ©2013 by American Society for Reproductive Medicine.)
Key Words: ART, ICSI, embryo selection, noninvasive embryo testing, pregnancy outcome, sHLA-G testing

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Despite improvement of in vitro embryo culture conditions and in stimulation and transfer techniques over the last decade, no significant improvements in implantation rates have been achieved. Early studies of noninvasive criteria reported selecting embryos for transfer by use of embryo morphology (1–3), extended embryo culture to the blastocyst stage (4), and the detection of soluble human leukocyte antigen G (sHLA-G) in the culture medium surrounding embryos (5–7). Furthermore, invasive advances in preimplantation genetic screening (PGS) of embryos for aneuploidy have been reported (8–12). Up to now, in an effort to improve in vitro fertilization (IVF) success rates, multiple embryos have

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been transferred, resulting in the subsequent risk of high-order multiple pregnancies. To limit these risks, there has been an ongoing search for an accurate and precise tool to identify the specific embryos that have an improved potential to develop into a live baby.

The implantation mechanism, a very poorly understood aspect of reproduction, has been described as a highly complex dialog (cross-talk) between the embryo and the endometrium (13–16). Implantation requires the successful suppression of the maternal immune system. The human body identifies and discriminates against foreign tissue via major histocompatability complex (MHC) human leukocyte antigen (HLA) originating from a group of genes located on the short arm of chromosome six. The MHC evokes T-cell intervention to nonself antigens expressed by an individual of the same species. The human fetus is considered a nonself tissue by the maternal uterus because of the paternal MHC antigens. However, during pregnancy the immune system develops fetal tolerance (16).

Human leukocyte antigen G (HLA-G), produced by the extravillous cytotrophoblast (the only fetal contact with maternal uterine cells), confers immunotolerance through interaction with maternal uterine membrane lymphocytes. This scenario would suggest that HLA-G protects the fetus from maternal immune response attack. Human leukocyte antigen G is expressed by the placenta throughout gestation and is also present in amniotic fluid. Soluble HLA-G (sHLA-G), a spliced isoform of membrane-bound HLA-G, is in suspension and can be detected in culture medium. This nonclassic type I HLA was first identified in the media surrounding groups of embryos and blastocysts in culture by Jurisicova et al. in 1996 (5). Menicucci et al. (6) were the first to identify sHLA-G in the culture media surrounding a group of day-3 embryos. Fuzzi et al. (7) showed that the presence of sHLA-G in the culture media harboring groups of 3-day-old cleaved embryos correlated with both their cleavage rate and their overall subsequent implantation potential.

In 2004, Hviid et al. (17) postulated, as did Saito et al. (18) later, that the presence of sHLA-G protects the conceptus from destruction by the maternal immune response. Several studies on sHLA-G and its effect on pregnancy outcome have been reported since (19–28). Reviews have thoroughly evaluated and discussed the previous studies of sHLA-G and assisted reproductive technology (ART) outcomes (29, 30). Furthermore, Rebmann et al. (31) addressed specific issues dealing with sHLA-G enzyme-linked immunosorbent assay (ELISA) protocols. We analyzed the retrospective data from 2,040 patients from six different IVF clinics to determine whether the presence of sHLA-G had an effect on implantation and pregnancy outcomes in vitro.

MATERIALS AND METHODS

Patients

The patients included all consenting assisted reproduction technology (ART) patients (male and female infertility factors) who underwent intracytoplasmic sperm injection (ICSI) between July 2003 and December 2010. Patients treated at our institution receive routine ICSI to minimize the risk of nonfertilization. For the majority, a specific ELISA was used 46 hours after ICSI to determine the sHLA-G expression.

Study Design

All patients in the program who consented to the sHLAG-G ELISA were accepted into the sHLA-G study. Those who did not consent for their embryos to be tested were excluded. Data were retrospectively gathered from six fertility clinics that performed an sHLA-G assay/test on the majority of their consenting ART patients between July 2003 and December 2010. (The clinics were designated A–F in no specific order.) Because the protocols at all the clinics are standardized, the procedural variabilities were limited. We retrospectively compared IVF outcomes in all patients regardless of age or diagnosis, as our goal was to compare ART outcomes for sHLA-G-positive and sHLA-G-negative cohorts. The embryos were selected using the sHLA-G result. If the result was negative, the embryos were selected based on embryo morphology. All clinics transferred embryos on day 3. We also collected data for embryos transferred on day 5/6 (blastocyst) as well as single-embryo transfers but excluded the data from this analysis because our study's focus was on comparing day-3 transfers among all clinics.

For each group, the data consisted of the number of chemical pregnancies observed, the number of clinical implantations observed and whether these were single, twin, triplet, or quadruplet implantations, and the number of ongoing pregnancies together with whether these were single, twin, triplet, or quadruplet pregnancies. In addition, we recorded the mean age (± standard deviation [SD]) of the women in each group, and the number of embryos transferred per group, as well as the average number of embryos transferred per individual for each group. We were interested in any association between the outcome of the sHLA-G test and the pregnancy outcomes (chemical pregnancy, clinical implantation, and/or ongoing pregnancy).

Ovarian Stimulation

Patients were stimulated using similar protocols at all sites. All patients received Lupron (TAP Pharmaceuticals) in a long protocol after pretreatment with oral contraceptive pills for 1 to 3 weeks. Ovarian follicular development was stimulated with recombinant follicle-stimulating hormone (FSH) at doses of 225–450 IU/day. Ovulation was triggered when at least two follicles were 18 mm, and half the remainder were $\geq$15 mm. Oocytes were recovered transvaginally under ultrasound guidance 34.5 hours later. All monitoring of controlled ovarian hyperstimulation as well as egg retrievals and embryo transfers were performed by the same physician at each center.

Embryo Culture

All metaphase II (MII) oocytes were fertilized using ICSI 4 to 6 hours after retrieval. This is a policy followed by all clinics in the group to reduce the risk of fertilization failure. All embryos were cultured individually in 35-μL droplets of P1 (Irvine Scientific) supplemented with 10% serum substitute

supplement (SSS; Irvine Scientific) using Nunc 60 × 15 mm dishes. Since 2007, we have cultured embryos individually in 35-μL droplets of Global (LifeGlobal) supplemented with 10% SSS (Irvine Scientific) using Embryo Corral (SunIVF) dishes under oil at 37°C in a 6% CO_2, 5% O_2, 89% N_2 environment. All embryos were sequentially microscopically evaluated over a period of 72 hours after ICSI and were graded by graduated embryo scoring (GES) (Table 1).

The embryos were transferred into extended culture medium 44 to 46 hours after ICSI. Initially, individual embryos whose surrounding media expressed sHLA-G within an optic density (OD) range of 0.190 ± 0.006 (the geometric mean) were defined as having positive sHLA-G expression, and those outside this range were designated as sHLA-G negative. Each center applied different criteria for "negative" to "positive" sHLA-G ranges (Table 2).

The original droplets of culture medium (35 μL) were collected in 0.5 mL snap-cap microcentrifuge tubes (VWR-Scientific), frozen immediately at −20°C, and shipped on ice for sHLA-G expression testing to a central location where identical sHLA-G assays were performed (west of Mississippi to Las Vegas: LV; east of Mississippi to New York: NY) using the same specific ELISA. Furthermore, all embryos were graded by applying the GES score; the GES ≥70 embryos combined with positive sHLA-G expression were selected for transfer (28).

Soluble HLA-G Assay

A sHLA-G assay monoclonal antibody (mAb) (MEM-G9 MCA2044; Serotec) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific) using a concentration of 2 μg/mL in 0.1 mol/L carbonate buffer (pH 9.5) for 1 hour at 37°C. The plate was then refrigerated at 4°C overnight. On the next day, the plate was thoroughly washed twice using 100 μL phosphate-buffered-saline (PBS) plus 0.05% Tween-20. The next wash was repeated twice using 100 μL of PBS + 5% bovine serum albumin (BSA) for 15 minutes each. A 50-μL aliquot of PBS + 5% BSA was added to each well before adding the sample of 50-μL embryo supernatant. The JEG-3 cell line (which secretes HLA-G) supernatant was used as a positive control (32). We incubated 50 μL of JEG-3 supernatant and 50 μL of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) for a period of 1 hour at 37°C.

After incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin-conjugated mAb (w6/32 MCA81B; Serotec) at a 1:1,000 dilution in PBS + 1% BSA for 45 minutes at 37°C and then washed five times with PBS. Streptavidin-alkaline phosphatase conjugate (BD Bioscience Pharmingen) at a concentration of 1:1,000 in carbonate buffer was incubated for 30 minutes at 37°C and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/mL in 10% diethanolamine pH 9.8 for 30 minutes. The colorimetric reaction was then stopped by the addition of 50 μL of 3 mol/L NaOH.

The relative concentration of sHLA-G was estimated from absorbance measured at 405 nm on an EL800-ELISA microplate reader (Bio-Tek Instruments). A standard supernatant of sHLA-G transfectant served as the reference in standard calibration curves. The turnaround time of the test was 5 to 6 hours. It is important to note that the pH value is crucial and must be very accurate. The preparation of the substrate medium and the reading process must be performed in a dark room because this chemiluminescence step is extremely sensitive to light exposure.

TABLE 1

Mean age, mean number of embryos returned, number of chemical pregnancies, clinical pregnancies, and ongoing pregnancies by site and sHLA-G test result for individuals with day-3 transfers only.

Site	sHLA-G	Mean age (y)	Mean no. of embryos[a]	Chemical pregnancy[b]	Clinical implantation[b]	Multiple clinical[b]	Ongoing pregnancy[b]	Multiple ongoing[b]	Total (n)
A		35.1	1.8	248 (79.7)	222 (71.4)	103 (33.1)	220 (70.7)	101 (32.5)	311
	Negative	34.7	2.0	55 (70.5)	46 (59.0)	22 (28.2)	44 (56.4)	22 (28.2)	78
	Positive	35.3	1.7	193 (82.8)	176 (75.5)	81 (34.8)	176 (75.5)	79 (33.9)	233
B		34.6	3.7	87 (55.1)	80 (50.6)	47 (29.7)	72 (45.6)	38 (24.1)	158
	Negative	33.9	3.8	33 (50.8)	31 (47.7)	15 (23.1)	27 (41.5)	9 (13.8)	65
	Positive	35.1	3.6	54 (58.1)	49 (52.7)	32 (34.4)	45 (48.4)	29 (31.2)	93
C		33.7	3.0	238 (61.5)	225 (58.1)	57 (14.7)	202 (52.2)	44 (11.4)	387
	Negative	33.1	3.1	57 (38.0)	49 (32.7)	14 (9.3)	44 (29.3)	7 (4.7)	150
	Positive	34.0	2.9	181 (76.4)	176 (74.3)	43 (18.1)	158 (66.7)	37 (15.6)	237
D		36.6	1.7	53 (38.4)	48 (34.8)	21 (15.2)	47 (34.1)	19 (13.8)	138
	Negative	39.0	2.2	8 (23.5)	8 (23.5)	1 (2.9)	7 (20.6)	0 (0.0)	34
	Positive	35.8	1.6	45 (43.3)	40 (38.5)	20 (19.2)	40 (38.5)	19 (18.3)	104
E		37.5	3.3	282 (47.9)	248 (42.1)	53 (9.0)	206 (35.0)	26 (4.4)	589
	Negative	38.0	3.0	119 (39.3)	101 (33.3)	13 (4.3)	65 (21.5)	4 (1.3)	303
	Positive	37.0	3.6	163 (57.0)	147 (51.4)	40 (14.0)	141 (49.3)	22 (7.7)	286
F		35.2	3.6	102 (47.7)	88 (41.1)	31 (14.5)	72 (33.6)	26 (12.1)	214
	Negative	35.1	3.7	30 (31.9)	23 (24.5)	5 (5.3)	8 (8.5)	3 (3.2)	94
	Positive	35.2	3.5	72 (60.0)	65 (54.2)	26 (21.7)	64 (53.3)	23 (19.2)	120
Total		35.7	2.9	1,010 (56.2)	911 (50.7)	312 (17.4)	819 (45.6)	254 (14.1)	1,797

[a] Mean number of embryos transferred.
[b] Count (percentage of total).

Kotze. sHLA-G, noninvasive predictor of pregnancy. Fertil Steril 2013.

TABLE 2

Soluble HLA-G ranges used by each Sher Institute for Reproductive Medicine (SIRM) clinic.

Clinic	Soluble HLA-G range	SIRM testing laboratory
A	0.148–0.210	LV
B	NEG ≤ 0.99 ≤ POS	LV
C	0.150–0.210	LV
D	0.190–0.210	NY
E	0.190–0.210	NY
F	0.184–0.196	LV

Note: When the sHLA-G results fell within the range, it was considered positive; outside the range, it was considered negative. LV = results for clinics west of Mississippi to Las Vegas; NY = results for clinics east of Mississippi to New York.

Kotze. sHLA-G, noninvasive predictor of pregnancy. Fertil Steril 2013.

Embryo Transfer

Embryo transfers were performed on day 3 (70 to 72 hours after ICSI), or blastocyst transfers on day 5/6 (84 to 96 hours after ICSI), depending on each center's preference. All centers used a Wallace Trail, followed by a Wallace Sure View catheter, both under direct ultrasound guidance.

Pregnancy Testing

Serum β human chorionic gonadotropin (β-hCG) levels were measured 11 and 13 days after the date of the egg retrieval. An initial value of >5.0 IU followed by a doubling of this number was considered positive. The pregnancy rates were expressed as chemical (β-hCG positive concentration 10 days after transfer), clinical (6–7 week ultrasound that includes a sac + fetal heartbeat [FHB]), and ongoing (10–12 week ultrasound including developing fetus + FHB).

Institutional Review

Since January 2005, all embryos at Sher Institute for Reproductive Medicine (SIRM) clinics have undergone routine sHLA-G assay/testing to determine the expression of this potential biochemical marker in the culture medium surrounding embryos. All patients were counseled regarding the risks, benefits, and alternatives to sHLA-G testing. For the purpose of this Ph.D. study, ethics approval was obtained from the ethics committee of the University of Stellenbosch (N06/07/119). Furthermore, all clinical research conducted was in full compliance with the guidelines of the American Society of Reproductive Medicine and met the ethics principles involving human subjects as defined by the Declaration of Helsinki in 1964.

Statistical Analysis

The data was analyzed using STATA 12 (StataCorp). Descriptive tables of the sHLA-G grouping by site and day of transfer were compiled.

Mixed-effects logistic regression models. A series of mixed-effects logistic regression models were fitted, in which site was included as a random effect. A mixed-effects logistic regression model was fitted for each of the three outcomes: chemical pregnancy, clinical implantation, and ongoing pregnancy. Each outcome was regressed against the sHLA-G test result with mean age and mean number of embryos returned included as covariates in the analysis. The median odds ratio (OR) is reported to reflect the variability between sites.

Mixed-effects Poisson regression models. Mixed-effects Poisson regression models were fitted for the outcomes of number of clinically implanted embryo sacs and number of ongoing embryo sacs for day-3 transfers. The outcomes were regressed against the mean age of individuals with the sHLA-G test result and the number of embryos transferred per group as the exposure. The site was included as a random effect. Because all clinics did not perform a day-3 embryo transfer, only day-3 embryo transfers were statistically analyzed so that results from all the centers could be compared.

A paired *t*-test by site was conducted to test for an association between mean age and sHLA-G test result for day-3 transfers only to see whether age and the sHLA-G test result were confounded. $P<.05$ was considered statistically significant.

RESULTS

Out of the 3,036 women studied, 2,040 women had an sHLA-G test result, and 1,797 of these women had a day-3 embryo transfer. Table 1 provides the mean age, mean number of embryos transferred, number of chemical pregnancies, clinical implantations, and ongoing pregnancies by site and sHLA-G test result for day-3 transfers only. The women with sHLA-G test results and day-3 transfer have a mean age of 35.7 years (SD ± 1.7), and the mean number of embryos transferred for these women, across sites, is 2.9 embryos (SD ± 0.8). The sHLA-G ranges used by each clinic are shown in Table 2.

The mixed-effect logistic regression models indicate that the sHLA-G test result is associated with the outcome of a chemical pregnancy, clinical pregnancy, and ongoing pregnancy. A positive sHLA-G result is associated with an increase in the odds of a chemical pregnancy (OR 2.62; 95% confidence interval [CI], 2.14–3.22; $P<.001$), an increase in the odds of a clinical pregnancy (OR 2.72; 95% CI, 2.22–3.33; $P<.001$), and increased odds of an ongoing pregnancy (OR: 3.56; 95% CI, 2.88–4.40; $P<.001$) (Table 3).

The results of the mixed-effects Poisson regression models indicate that mean age and sHLA-G test results are associated with the number of clinical pregnancies and the number of ongoing pregnancies. The incidence of clinical pregnancies is 2.00 times greater (95% CI, 1.76–2.26) in embryos with a positive sHLA-G test result than the incidence of clinical pregnancies in embryos with a negative sHLA-G test result, while keeping age constant. The incidence of an ongoing pregnancy is 2.52 times greater (95% CI, 2.19–2.91) in embryos with a positive sHLA-G test result than the incidence of an ongoing pregnancy embryo with a negative sHLA-G test result, while keeping age constant (Table 4).

A paired *t*-test by site conducted to test for an association between mean age and the sHLA-G test result was not statistically significant, which implies that age and sHLA-G

TABLE 3

Mixed-effects logistic regression for the outcomes: chemical pregnancy, clinical implantation, ongoing pregnancy for day-3 transfers.

	Chemical pregnancy			Clinical implantation			Ongoing pregnancy		
Effects	**Odds ratio**	**Lower CI**	**Upper CI**	**Odds ratio**	**Lower CI**	**Upper CI**	**Odds ratio**	**Lower CI**	**Upper CI**
Fixed									
Mean embryo	0.77	0.52	1.15	0.82	0.56	1.21	0.88	0.60	1.29
Mean age	1.01	0.84	1.21	1.04	0.86	1.25	0.91	0.75	1.10
Soluble HLA-G (positive)	2.62	2.14	3.22	2.72	2.22	3.33	3.56	2.88	4.40
	Median odds ratio	**Lower CI**	**Upper CI**	**Median odds ratio**	**Lower CI**	**Upper CI**	**Median odds ratio**	**Lower CI**	**Upper CI**
Random									
Site	1.73	1.33	2.86	1.65	1.29	2.69	1.55	1.25	2.35

Kotze. sHLA-G, noninvasive predictor of pregnancy. Fertil Steril 2013.

are not confounders in the regression model. See Table 2 for the mean age by site.

DISCUSSION

The statistical analysis of the data from 1,794 patients in this retrospective multicenter study indicates that a positive sHLA-G test result is statistically significantly associated with an increase in chemical pregnancy, clinical pregnancy, and ongoing pregnancy outcomes after adjusting for mean age, mean number of embryos used, and day of transfer. A positive sHLA-G result increased the odds of each of these outcomes by as much as 2.52 in the ongoing pregnancy group. This study confirms the observations of a prospective, randomized, controlled study by Kotze et al. (28) that emphasized the benefits of transferring sHLA-G–positive embryos, predicting an increased pregnancy outcome in ART.

This study has a number of limitations. First, this was a retrospective study. Second, the majority of centers transferred embryos on day 3, but two of the six sites transferred embryos on day 3 and also on day 5/6, so the day of embryo transfer could not be differentiated in all sites. For this reason, only day-3 embryo transfer data were statistically analyzed. Third, all centers used the same sHLA-G assay, performed at two locations; it would have been ideal to have one testing laboratory, but for logistic reasons this was not possible. Furthermore, each clinic applied their own range and threshold values (see Table 2). It also was not possible to obtain data about the number of babies born from the different centers; for that reason, only the ongoing pregnancy rate was reported.

There has been some criticism of the use of the optical density (OD) value as our sHLA-G unit value. It is interesting that no previous group reporting on applying an sHLA-G assay has reached a consensus on the appropriate way to report such results (22–25, 27, 29–33). We would like to report our results as an internationally agreed upon unit, but no such consensus exists. Other obstacles include a lack of commercially available and universally accepted standards to generate standard curves. Initially we performed and extensive studies and generated our own value range of OD = 0.184−0.196. With additional data we expanded the range of positive expression used in this study to OD = 0.170−0.210. The lower limit had high sensitivity, but the increased range improved the specificity of the test. This adjustment allowed for the addition to the "positive" value group of some embryos that would have been considered "negative" but still resulted in an ongoing gestation. Further, we have conducted several unpublished in-house studies using commercially available kits and have found that our mean value corresponded to approximately 4.6 ng, or 1 "unit" using the standard curve generated by the standards supplied in the commercial kit. In future studies, agreement and cooperation between established groups need to be reached to standardize the use of the sHLA-G assay successfully. Last, to ensure accuracy, daily controls were run using amniotic fluid from a pregnant woman as the sHLA-G positive control and culture medium as a negative control.

It is important to note that in the case of a positive sHLA-G, the number of embryos selected for transfer must be carefully considered. Although we have not shown an increased multiple pregnancy rate in our study, there is a theoretical risk involved when transferring more than two sHLA-G–positive embryos. In theory, transferring fewer embryos that have been selected based on sHLA-G results

TABLE 4

Mixed-effects Poisson regression for the outcomes: clinical implantation and ongoing pregnancy for day-3 transfers.

	Clinical implantation			Ongoing pregnancy		
Fixed effects	**Incidence risk ratio**	**Lower CI**	**Upper CI**	**Incidence risk ratio**	**Lower CI**	**Upper CI**
Mean age (y)	0.77	0.70	0.85	0.71	0.63	0.80
Soluble HLA-G (positive)	2.00	1.76	2.26	2.52	2.19	2.91

Kotze. sHLA-G, noninvasive predictor of pregnancy. Fertil Steril 2013.

combined with morphologic evaluation can be used to reduce the risk of higher order pregnancies without compromising overall pregnancy rates.

Additionally, the results of this retrospective study indicate that two centers (A and D) reported on transferring embryos on day 3 or day 5. However, we have decided not to analyze their data because of the small numbers. It is interesting to look at previously reported studies reporting on day-3 versus day-5/6 embryo transfers. In 2000, Coskun et al. (34) reported on data from a randomized trial that compared day-3 versus day-5 embryo transfer outcomes; they concluded that day-3 and day-5 transfers had similar pregnancy, implantation, and twinning rates and thus day-5 transfers have no advantage over day-3 transfers. Bungum et al. (35) reported similar findings in another prospective randomized study. In a Cochrane review, Blake et al. (36) concluded that blastocyst transfers significantly improved pregnancy rates compared with those of cleavage stage embryos, and similar findings were also reported by Papanikolaou et al. (37). In our opinion, blastocyst selection combined with a positive sHLA-G expression can be beneficial in patients when sHLA-G results are available. In cases where multiple embryos are available on day 3, extended embryo culture should be considered.

This multicenter study affirms the benefit of sHLA-G screening as part of the embryo selection criteria, but the threshold values must be standardized. Clinics should consider this assay as a valuable noninvasive embryo marker to assist in improving pregnancy outcomes, with the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to select single embryos for transfer and reduce the risk of multiple pregnancies without compromising the pregnancy rates.

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