Influence of early ICSI-derived embryo sHLA-G expression on pregnancy and implantation rates: a prospective study

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BACKGROUND: We have previously reported the retrospective observation that when at least one embryo, transferred on day 3, expressed sHLA-G above the geometric mean (sHLA-G1) 46 h post-ICSI, there was a marked improvement in both pregnancy (PR) and implantation (IR) rates. METHODS: The media surrounding individual embryos derived from ICSI performed on oocytes from 482 women ≤43 years of age were tested for sHLA-G expression by specific ELISA. RESULTS: We report here prospective results showing improved IVF results following the transfer of ‘good quality’ embryos (7–9 cells with <20% fragmentation) by preferentially including at least one sHLA-G+ embryos. PR and IR for women ≤38 years were 63% and 32% when one transferred embryo was sHLA-G+, and 69% and 36% when at least two embryos were sHLA-G+. When none of the embryos transferred was sHLA-G+, PR and IR were 25% and 13%, respectively. Comparable PR and IR for women 39–43 years were 29% and 11% when none of the transferred embryos were sHLA-G+; 38% and 15% when at least one sHLA-G+ embryo was transferred; and 61% and 26% when at least two sHLA-G+ embryos were transferred. The data were stratified by patient age. CONCLUSIONS: PR and IR increased with the addition of each sHLA-G+ embryo, regardless of age. While there are significant barriers to routine embryo sHLA-G testing, we believe that if implemented, this would provide a mechanism for optimizing IVF PR while minimizing the risk of multiple pregnancies.

Key words: embryo/ICSI/implantation/pregnancy/sHLA-G

Introduction

Implantation rates (IR) following IVF have not improved significantly over the last decade (Ziebe et al., 1997). This is in spite of significant improvements in the methods used to assess embryos morphologically (Ng et al., 1999; Richter et al., 2001; Fisch et al., 2003), and advances in preimplantation chromosomal assessment of cleaved embryos for aneuploidy (Gianaroli et al., 2003; Munne, 2003; Pehlivan et al., 2003). Clearly, none of these methods is capable of accurately assessing embryo implantation potential (i.e. ‘embryo competency’). As a result, in spite of the risk of multiple pregnancies, many physicians elect to transfer multiple embryos in an effort to maximize their IVF success rates.

The dilemma that commonly confronts IVF practitioners is whether to sanction the transfer of multiple embryos in an effort to optimize success rates, or to limit the number of embryos transferred in order to minimize the risk of multiple gestations. This is no trivial decision, especially in countries such as the USA, where IVF is an expensive procedure that usually presents as an out of pocket expense (since health insurance rarely covers it), and in a highly competitive practice arena where the very survival of assisted reproduction technology (ART) programs often hinges on perceived success rates.

In an attempt to reduce the incidence of multiple pregnancies (especially high-order multiple gestations, i.e. triplets or greater), several countries have mandated that the number of embryos transferred be restricted to no more than two (Hviid, 2004). While this has indeed led to a slight reduction in the incidence of IVF multiple births, it has somewhat compromised IVF success rates, especially in women over 35 years of age and those with diminished ovarian reserve, where the advancing biological clock exacts a disproportionate toll on egg/embryo ‘competency’. In an attempt to bypass this mandate, many women (who have sufficient financial resources) currently choose to travel abroad to countries such as the USA where no such legislation currently exists.

sHLA-G, a non-classic type I human leukocyte antigen, was first identified in the media surrounding groups of embryos and blastocysts in culture (Jurisicova et al., 1996) and in clusters of 3-day-old embryos (Menicucci et al., 1999). Approximately 3 years later, it was shown that concentrations of sHLA-G in the culture media harbouring groups of 3-day-old cleaved embryos were positively correlated both with cleavage rate and with subsequent implantation potential (Fuzzi et al., 2002).

We recently reported on a positive correlation between sHLA-G expression in the media surrounding individual 46-h
embryos, and their subsequent pregnancy generating potential (Sher et al., 2004; 2005).

We now report on a cohort study to evaluate how IVF results [pregnancy rates (PR) and IR] were influenced when one versus two of the morphologically ‘good quality’ embryos transferred on day 3 post-ICSI are positive for sHLA-G expression.

Materials and methods

Patients

We conducted a prospective cohort study that evaluated 482 women into whom 7–9 cell, minimally fragmented (<20%), ICSI-derived embryos, tested for sHLA-G expression (Sher et al., 2004) 46-h post-ICSI were transferred on day 3. Oocyte providers ranged in age between 28 and 43 years. The study was conducted in five affiliated IVF programs. We define a clinical pregnancy as one where a viable intrauterine pregnancy is confirmed by ultrasound performed after the seventh week of gestation and an ongoing gestation as one where ultrasound performed after 12 weeks gestation confirms the presence of a viable intrauterine pregnancy. IR was determined by the number of embryos transferred divided by the number of viable clinical pregnancies (as defined above).

Ovarian stimulation

Patients underwent ovarian stimulation using similar protocols at all sites. All patients received Lupron (TAP Pharmaceuticals, St Louis, MO, USA) in a long protocol after pretreatment with oral contraceptive pills for 1–3 weeks. Ovarian follicular development was stimulated with recombinant human FSH at doses of 225–450 IU per day. Ovulations were triggered when at least two follicles reached 18 mm in mean diameter and half the remaining embryos were ≥15 mm. Oocytes were recovered transvaginally under ultrasound guidance, 34.5 h later. In each case, monitoring of controlled ovarian hyperstimulation, oocyte recoveries and embryo transfers were performed by one of the five physicians participating in the study.

Embryo culture

Metaphase II (MII) oocytes were fertilized by ICSI 4–6 h after retrieval in all cases (Fisch et al., 2001). Embryos were cultured individually in 50 μl droplets of P1 (Irvine Scientific, Santa Ana, CA, USA) under oil at 37°C in a 6% CO2, 5% O2, 89% N2 environment. All embryos were transferred to blastocyst medium 46-h post-ICSI (Keskiintepe et al., 2003). All embryos were microscopically evaluated for cell number and fragmentation 72 h following ICSI. The original droplets of P1 media were tested for sHLA-G expression using a specific ELISA.

sHLA-G assay

A monoclonal antibody (mAb) (MEM-G9 MCA2044; Serotec, Raleigh, NC, USA) against sHLA-G was used to coat a 96-well Nunc-ImmunoPlate (Fisher Scientific, Chino, CA, USA) using a concentration of 2 μg/ml in 0.1 mol/l carbonate buffer pH 9.5 for 1 h at 37°C. The plate was then refrigerated at 4°C overnight. On the following day, the plate was thoroughly washed twice using 100 μl phosphate-buffered saline (PBS) + 0.05% Tween-20. The wash was repeated twice using 100 μl of PBS + 5% bovine serum albumin (BSA) for 15 min each. A 50 μl aliquot of PBS + 5% BSA was added to each well prior to adding the sample of 50 μl embryo supernatant. JEG-3 cell line (which secretes HLA-G) supernatant was used as a positive control (Bamberger et al., 2000). Fifty micro-

litres of JEG-3 supernatant and 50 μl of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) were incubated for a period of 1 h at 37°C.

Following incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin-conjugated mAb (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS + 1% BSA for 45 min at 37°C and then washed five times with PBS. Streptavidin–alkaline phosphatase conjugate (BD Bioscience Pharmingen, San Diego, CA, USA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 min 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 min. 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 Inc., Winooski, VT, USA). A standard supernatant of sHLA-G/221 transfectant served as reference in standard calibration curves (Fournel et al., 2000).

Individual embryos whose surrounding media expressed sHLA-G with an OD ≥0.190 ± 0.6 (the geometric mean) were defined as sHLA-G+, while those with OD <0.190 ± 0.6 were designated as sHLA-G−.

Patients had up to three embryos transferred on day 3. The remaining embryos were allowed to continue in blastocyst culture media for up to three additional days. Those that developed into expanded blastocysts with a normocellular inner cell mass were cryopreserved for subsequent dispensation. All embryo transfers were performed under direct transabdominal ultrasound guidance using a Wallace catheter.

The study groups (n = 482)

We compared IVF results in women of two age categories. Group A: ≤38 years (n = 387); and group B: 39–43 years (n = 95). Groups A and B were further subdivided into subgroups 1, 2 and 3 as follows. Group A1 (n = 150): the total number of embryos transferred was 457; and group B1 (n = 38), the total number of embryos transferred was 124. In A1 and B1, all transferred embryos were sHLA-G+. Group A2 (n = 95): the total number of embryos transferred was 250; and group B2 (n = 21), the total number of embryos transferred was 71. In A2 and B2, one out of the embryos transferred per patient was sHLA-G+. Group A3 (n = 142): the total number of embryos transferred was 390; and group B3 (n = 36), the total number of embryos transferred was 112. In A3 and B3, two of the embryos transferred per patient were sHLA-G+. The clinical and demographic characteristics, as well as the number of embryos transferred, in subgroups A1, A2 and A3 were similar, as was the case for subgroups B1, B2 and B3 (see Tables I and II).

Statistical analysis

Differences between groups were evaluated using Student’s t-test. Differences in rates and proportions were evaluated with χ2-tests and Fisher’s exact test where appropriate. Significance was set at P < 0.05.

Results

Table I presents the demographic, clinical and embryologic characteristics, as well as comparing PR and IR in women of subgroups A1, A2 and A3 who underwent embryo transfer. Table II presents the same parameters for subgroups B1, B2 and B3.
Apart from the sHLA-G expression of the embryos transferred and the number of transferred embryos that were sHLA-G+, the clinical, embryologic and demographic characteristics of patients comprising the subgroups of group A were highly comparable. The same applied for group B.

Tables I and II, as well as Figure 1, demonstrate that, regardless of the age of the egg provider, the PR and IR per embryo increased progressively with the selection and transfer of each additional sHLA-G+ embryo.

### Discussion

Morphologic evaluations do furnish clues that can enhance proficiency at choosing the best pre-embryos for transfer. However, such analyses are severely limited in their ability to predict the implantation potential of individual embryos (Munne et al., 2003) using fluorescence in-situ hybridization (FISH) appears to be similarly flawed (Munne, 2003).

Presently, we know little about the genetic make-up of pre-embryos. However, evidence suggests that fragmented, multinucleated or poorly developing pre-embryos usually exhibit numerical chromosomal abnormalities or aneuploidy (Munne, 2003). Conversely, based on the observation that most early miscarriages are associated with karyotypically abnormal concepti (Pehlivan et al., 2003), it follows that many pre-embryos graded as morphologically normal are in all likelihood aneuploidic. The problem with using blastomere biopsy with FISH to identify aneuploidic embryos is that currently, commercially available FISH probes only target eight to nine chromosome pairs, and accordingly lack the ability to identify aneuploidy involving the remaining 14 pairs, which in reality may be at least equally likely to compromise embryo developmental ‘competency’.

HLA-G, a non-classic type I human leukocyte antigen, produced by early developing embryos (Vigano et al., 2003), and the interstitial trophoblast is believed to play a pivotal role in immunoprotection of the semiallogenic conceptus (Lea and Clark, 1989; Ludwig and Frauli, 1990). HLA-G’s...
probable role is to prevent allore cognition by maternal cytotoxic lymphocytes and to protect against natural killer cell-mediated lyses of target cells (LeMaoult et al., 2004). As such, its expression on embryonic cells probably plays a pivotal role in the development of pregnancy (Hackmon et al., 2004). We postulate that it does this by initiating a crucial ‘chemical dialogue’ between the embryo on the one hand and the maternal immune tolerance mechanisms on the other.

We demonstrated that the concentration of sHLA-G in the media surrounding individual embryos 46 h following insemination by ICSI correlates well with the embryo’s subsequent potential to implant successfully and produce a viable pregnancy (Sher et al., 2004). As such, sHLA-G expression in the media surrounding 46-h embryos could serve as a valuable ‘marker’ of embryo competency, or for selecting those embryos most likely to implant from a particular cohort of individually cultured embryos.

Noci et al. (2005) demonstrated the absence of a correlation between sHLA-G expression and embryo morphology. This finding is consistent with our own observations (unpublished); however, in their study, these workers concluded that only sHLA-G+ embryos are capable of establishing viable pregnancies. In contrast, our data demonstrate that viable pregnancies do indeed follow the transfer of sHLA-G− embryos (albeit at a significantly lower rate; see Figure 1). This discrepancy could perhaps be explained by the small sample size of women in the report by Noci et al. (i.e. 40 women received sHLA-G+ embryos and 26 women had only sHLA-G− embryos transferred). Moreover, both the overall PR as well as the PR for women into whom sHLA-G+ embryos were transferred were inordinately low (14% and 23%, respectively) in Noci et al.’s study, while our data were derived from a large sample of women (n = 482), 387 of whom were of a comparable age to those reported on by Noci et al. In this subset of women, the overall clinical pregnancy rate was 50% (195/387), the pregnancy rate in the women who received one or more HLA-G+ embryos was 67% (158/237) and the pregnancy rate in the women to whom no HLA-G+ embryos were transferred was 25% (37/150). Finally, Noci et al. (2005) measured sHLA-G expression on day 3 ICSI- and IVF-derived embryos, while we measured sHLA-G expression on day 2 embryos that were all ICSI-derived.

More recently we reported a prospective cohort study that demonstrated a three-fold improvement in embryo implantation and pregnancy potential when exclusively sHLA-G+ embryos were transferred, as compared with when all transferred embryos were sHLA-G− (Sher et al., 2005).

The results of this study strongly suggest that IR and PR following the transfer of ‘morphologically sound’, 7–9 cell embryos can be significantly enhanced through the preferential incorporation of one or two sHLA-G+ embryos (Figure 1; Tables I and II).

The IR for sHLA-G− embryos, as depicted in Tables I and II, and Figure 1, probably accurately reflects the competency of such embryos, since all the embryos transferred in subgroups A1 and A2 were sHLA-G−. However, the same cannot be said for the IR of sHLA-G+ embryos shown in the same tables (subgroups A2 and A3, and B2 and B3), because of a dilutional effect brought about by the transfer combination of both sHLA-G− and sHLA-G+ embryos. As such, the ‘actual’ implantation potential of sHLA-G+ embryos has been significantly understated here.

The present findings offer a compelling argument for incorporating sHLA-G testing in the selection process of embryos for transfer and provide a rational basis for limiting the number of embryos transferred to two or less. This could minimize the risk of high-order multiple pregnancies without compromising success rates.

However, there are indeed significant barriers to the implementation of routine, across the board early embryo sHLA-G testing, as this would require major adjustments and adaptations to currently entrenched ART laboratory protocols. For example, it would require that IVF laboratories (most of which currently culture embryos in clusters or groups) convert to an individual embryo culturing system in order to measure the concentration of sHLA-G surrounding each individual embryo.

Implementation of this methodology would require deviation from the current practice of only transferring embryos to blastocyst media after day 3. In order to sample media, 46-h post-insemination, it will be necessary to introduce the additional step of moving individual embryos to blastocyst media on day 2 in preparation for a day 3 transfer.

It would also require that ICSI be performed in all cases of IVF. Unless this is done, it would not be possible to determine with precision the 46-h interval between insemination and sHLA-G sampling, and we do not know whether or to what extent inability to precisely pinpoint fertilization would influence interpretation of embryo sHLA-G expression, since we perform ICSI in virtually all cases. Thus, unless a 100% ICSI policy is adapted, it will be necessary to first determine how and to what extent the use of conventional IVF methodologies might influence embryo sHLA-G expression.

Conclusions

These findings suggest that assessing early embryo sHLA-G expression in combination with morphologic embryo evaluation on day 3 post-ICSI significantly enhances the ability to select the most ‘competent’ embryos for transfer. This could have the effect of improving PR and IR, reducing the number of embryos transferred and minimizing the incidence of high-order multiple pregnancies.

References


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