Low Blastocyst Formation Rates in Day-2 Fertilized Oocytes

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Purpose: To examine the blastocyst formation rates of day-2 fertilized oocytes.

Methods: A retrospective study of the outcomes/blastocyst formation of day-2 fertilized oocytes was undertaken.

Results: Fertilization rates of day-1 and -2 oocytes by intracytoplasmic sperm injection were similar. The development frequencies to four cells were similar. However, the blastulation rates were significantly lower from the day-2 fertilized eggs. The fertilization rates from day-2 conventional in vitro fertilization reinsemination were lower than the fertilization rates of day-1 oocytes. The blastulation rates from day-2 fertilized eggs were also lower than the rates from day-1 fertilized eggs in the in vitro fertilization group.

Conclusions: Fertilization is not a good indicator to predict the viability of fertilized oocytes. Day-2 fertilized oocytes had significantly lower blastocyst formation rates than the rates from day-1 fertilized oocytes.

KEY WORDS: blastocyst; development; fertilization; intracytoplasmic sperm injection; in vitro fertilization.

INTRODUCTION

Reinsemination of initially unfertilized oocytes has been reported to be unsuccessful (1–4). Chen *et al.* (3) reported a 7.5% fertilization rate with reinsemination by conventional methods followed by a 14.3% pregnancy rate per embryo transfer (ET). In the same study (3), reinsemination by subzona sperm insertion resulted in a 37.5% fertilization rate and a 0% pregnancy rate per ET. A recent study (4) showed a 46.5% fertilization rate by day-2 (the day after egg retrieval) of intracytoplasmic sperm injection (ICSI) and a 6.9% delivery rate per ET. Clearly, the success rate for

achieving pregnancy by second-day insemination has been consistently low. The mechanism that causes the low pregnancy rate is as yet unclear. This study is to examine whether the day-2 fertilized oocytes have the same developmental potential.

MATERIALS AND METHODS

This is a retrospective study including all assisted reproductive technology cases treated during June 1, 1996, to May 31, 1997.

The ovarian stimulation protocol has been reported previously (5). In brief, luteinizing hormone–releasing hormone (LHRH) agonist (leuprolide acetate) was given 1.0 mg/day subcutaneously for approximately 10 days, then 0.5 mg/day until the morning of human chorionic gonadotropin (hCG) injection. Purified follicle-stimulating hormone (FSH; Fertinex; Serono Laboratories Inc., Randolph, MA) was initiated when the serum estradiol level was less than 35 pg/ml. The dose of purified FSH was begun at 150–300 IU/day. On the sixth day of stimulation, the dosage was either reduced, maintained, or increased depending on the number, size, and synchrony of ovarian follicles. The criteria for hCG (Profasi; Serono) injection were that the leading follicle reach 1.7 cm in diameter or a progesterone concentration higher than 0.9 ng/ml. The oocyte retrieval was 35 hr after the hCG (10,000 U) injection. The conventional insemination is performed on the day of egg retrieval (day-1 in vitro fertilization [IVF]). Reinsemination by conventional IVF is performed approximately 24 hr after egg retrieval (day-2 IVF). The ET was on day 4 (morula stage) or day 5 (blastocyst stage).

ICSI is a manual procedure in which a single sperm is directly injected into an oocyte. A sperm is aspirated into a micropipet, which then penetrates the zona pellucida and oolema and injects the sperm into the cytoplasm. The ICSI procedure follows the report by Van

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Steirteghem *et al.* (6) using Narishige micromanipulators. The holding and injection micropipets were from Humagen Fertility Diagnostics (Charlottesville, VA). The definition of day-1 ICSI is the mature oocytes (metaphase II) being injected with spermatozoa on the day of egg retrieval. The denuded oocytes that did not display the first polar body were in either the germinal vesicle stage or the metaphase-I stage. Those oocytes were immature and were not exposed to any spermatozoa at all. They were cultured overnight. No hormonal supplementation was added to the culture medium, allowing the immature oocytes to undergo spontaneous maturation. If the oocytes displayed a first polar body on day 2, they were injected (day-2 ICSI).

Conventional insemination is adding 30,000 selected motile spermatozoa to the egg in a culture well with 0.7 ml of culture medium (5). The sperm selection procedure involves two steps, the first by density and the second by the motility. The density gradient media were 45 and 90% Enhance-S (Fertility Technology, Inc., Natick, MA). The low density spermatozoa, degenerated or dead, remain on top of the density gradient, while most of the viable spermatozoa form a pellet in the bottom—90% Enhance-S by centrifugation. The pellet was washed once and overlaid with culture medium. The active motile sperm swim up from the pellet into the supernatant fluid and form a sperm suspension. This suspension is then used for insemination. A fresh semen sample was requested and used for reinsemination or day-2 ICSI. The preparation time for a sperm sample was approximately 2 hr from reception to insemination. Reinsemination is simply the transfer of the unfertilized oocytes to a new well and their exposure to 30,000 motile spermatozoa/0.7 ml a second time (IVF reinsemination, day-2 IVF).

The culture medium used is minimal essential medium α (MEM; Irvine Scientific) with 10% synthetic serum substitute (Irvine Scientific). The data in this study refer to embryos that were not transferred. In our preliminary study, the highest frequency of blastocyst formation was approximately 120 hr after egg retrieval. Therefore, the embryos were cultured until either they reached the blastocyst stage or 7 days after egg retrieval. The definition of blastocyst is an embryo having a blastocoel with a diameter of at least one-quarter that of the embryo's diameter. Those that developed into blastocysts were cryopreserved. An embryo was considered unviable if it had not developed into a blastocyst after 7 days of culture.

The χ^2 test was used for statistical analysis. Fischer exact tests were used to analyze the blastocyst formation rates for both day-2 insemination groups.

RESULTS

Conventional IVF Insemination

As shown in Fig. 1, there were 724 oocytes from 66 IVF cases for day-1 insemination. The fertilization rate was 75% of 724 eggs. The blastocyst formation rate was 47% of the 543 fertilized oocytes. For reinsemination, there were 92 oocytes from 31 cases. The fertilization rate was 23% and the blastocyst formation rate was 1 of 20 fertilized eggs (5%). Both the fertilization rates and the blastocyst formation rates were significantly different ($P < 0.05$) between the day-1 and the day-2 inseminations.

ICSI: Pooled data

In Fig. 2, 542 oocytes were used from 52 patients for day-1 ICSI, resulting in a fertilization rate of 68% and a blastulation rate of 32% of 369 fertilized oocytes. Day-2 ICSI was performed on 75 oocytes from 33 cases, resulting in a fertilization rate of 52%. The blastulation rate of the day-2 ICSI group was 2.5% of 39 fertilized oocytes. Again, the blastulation rate was significantly different ($P < 0.05$) between day-1 and day-2 ICSI, yet there was no significant difference in the fertilization rate.

ICSI: Data of Both Day-1 and Day-2 ICSI from the Same Patients

As shown in Fig. 3, 33 patients had both day-1 and day-2 ICSI. For each patient, the results of paired ICSI

Fig. 1. The fertilization rates (hatched columns) of the day-1 and day-2 conventional inseminations are statistically significant ($P < 0.01$). The blastulation rate (filled columns) from the day-1 inseminations is significantly ($P < 0.05$) higher than the result from the day-2 inseminations. The number in each column is the number of oocytes or embryos used.

Fig. 2. The fertilization rates (hatched columns) are not significantly different between day-1 and day-2 ICSI. The blastulation rate (filled columns) from day-2 ICSI fertilized eggs is significantly lower ($P < 0.05$) than that of day 1. It should be noted that those oocytes in the day-2 ICSI were immature oocytes that had not been exposed to sperm before. Pooled data.

procedures were included. The day-2 ICSI group data are the same as in Fig. 2. However, the fertilization rate of the day-1 ICSI group was 67%, with a 33% blastulation rate. Again, the blastulation rates were significantly different ($P < 0.01$) between the day-1 and the day-2 ICSI, but there was no significant difference in the fertilization rates between day-1 and day-2 ICSI.

Spontaneous Maturation of Immature Oocytes

After overnight culture in MEM, 43% of 57 germinal vesicle-stage oocytes reached metaphase II. Of the

Fig. 3. Considering only the patients with both day-1 and day-2 ICSI procedures, the blastulation rate (filled columns) was significantly lower ($P < 0.01$) in the day-2 ICSI group than the day-1 ICSI group. There was no significant difference in fertilization rate (hatched columns) between the two groups.

60 metaphase-I oocytes, 80% spontaneously reached metaphase II after overnight culture (Fig. 4).

DISCUSSION

In this study, the day-1 fertilized egg has an approximately 40% (including all data pooled) chance to develop into a blastocyst, and the pooled data for day-2 fertilized eggs shows a blastulation rate of approximately 5%. In considering blastocyst formation, the day-2 group is only one-eighth (5%/40%) as efficient as the day-1 group. In this set of assisted reproductive technology cases, the clinical pregnancy rate was 44%, and one-eighth of 44% is 5.5%. This result is similar to the pregnancy rates of day-2 fertilized embryos (3,4). The decrease in the blastulation rate may well explain the low pregnancy rate with transfer of day-2 fertilized eggs. Therefore, it supports the concept that the primary factor for the low pregnancy by day-2 fertilized eggs is the low developmental potential of day-2 fertilized eggs.

There is a significant difference in the fertilization rates of the day-1 and -2 IVF oocytes, whereas there is no significant difference in the day-1 and -2 fertilization rates of the ICSI group. The oocytes chosen for the day-2 ICSI procedure were immature on the day of egg retrieval but had normal morphology and were cultured overnight for some reached metaphase II (Fig. 4), whereas the oocytes in the day-2 IVF group consisted of those that had reached maturity and those that remained immature. The oocytes for day-2 IVF had already been exposed to sperm from the day-1

Fig. 4. Immature oocytes were cultured overnight. Forty-two percent of the germinal vesicle (GV)-stage oocytes reached metaphase II (MII). Eighty percent of metaphase I (MI) oocytes reached metaphase II. The number in each column represents the number of oocytes in the group.

IVF. Those oocytes that did not fertilize may have had abnormal morphology, chromosomal abnormalities (7), zona pellucida defects (8), etc., in addition to being immature. In other words, the day-2 IVF pool of oocytes may have had many defects in addition to being immature that might have prevented fertilization. This is the reason that the day-2 IVF group yielded a lower fertilization rate compared to that of the day-2 ICSI group. The data showed that the subnormal quality of human oocytes resulted in subnormal preimplantation development by evidence.

The exact nutritional requirements for the development of the human egg, zygote, and preembryo are unclear. We have reported that MEM gives the best support among HTF, Ham's F-10, and NCTC-135 for mouse embryo development (9). MEM consistently supported more than 80% of two-cell mouse embryos that developed to blastocysts. As for humans, a 47% blastocyst formation rate was observed in the IVF group. Lopata and Hay (10) used MEM to culture surplus human embryos and obtained a 26.8% blastocyst formation rate, which is significantly better than the popular HTF, which produces a 14.5% blastulation rate. Muggleton-Harris *et al.* (11) reported that Earl's balanced salt solution with human serum achieved a 44.7% blastulation rate, while T6 obtained only a 22.3% blastulation rate. The commercial Medi-Cult M2/M3 medium achieved a 45 to 50% blastulation rate. In a newly developed medium, G1/G2 (12), zygotes were cultured for 48 hr in G1, then cultured in G2 for another 48 hr to achieve a 66% blastulation rate, and for comparison, Ham's F-10 and G2 achieved a blastocyst formation rate of 38%. Therefore, MEM is one of the best media to support the development of human embryos.

The mechanisms of fertilization and blastocyst formation must be considered separately. This is made clear by the fact that there is no significant difference in the day-1 and day-2 ICSI fertilization rates but a significant difference in the blastulation rates (Figs. 2 and 3), of which the day-2 rate was significantly lower. These results correlate with our previous observation that there was no significant difference in the rate of egg fertilization between the oocyte population from the old patient group (40 years and older) and that from the young patient group [younger than 40 (5)]. The old patients' embryos had a significantly lower blastocyst formation rate than the young group (13). Furthermore, the old patients had a lower pregnancy rate, which is indirect evidence supporting the separation of mechanisms for fertilization and development to blastocyst.

The reason for delaying insemination until day 2 was to allow the egg, immature upon retrieval, to mature spontaneously. This was determined morphologically by observing the extrusion of the first polar body, which indicates that the egg is in metaphase II and, thus, at least nuclear mature. Both nuclear and cytoplasmic processes are involved in oocyte maturation and completion of both is essential to produce an egg that is competent to undergo fertilization and embryogenesis (14). The definition of nuclear maturation is that the oocyte has undergone a process of disengaging meiotic arrest at prophase I, driving the progression from meiotic arrest, and continuing the progression of meiosis to metaphase II, where it normally becomes arrested again until fertilization. The dissolution of the germinal vesicle is the most obvious indication that nuclear maturation has been initiated. Cytoplasmic maturation refers to the processes that prepare the egg for activation, formation of pronuclei, and preimplantation development. This study clarifies the point that nuclear maturation and cytoplasmic maturation are not necessarily synchronized in the human egg. Once the egg reaches metaphase II, it may be fertilized, i.e., day-2 ICSI, but a normal fertilization does not guarantee the full potential of embryo development, i.e., day-2 embryo development. The data suggest that fertilization and blastulation have different control mechanisms. Complete cytoplasmic maturation is required to realize the full potential of embryo development. It is not clear, however, what kind of cytoplasmic factors respond to postfertilization embryo development, e.g., embryonic genome activation, morula compacting, blastulation, etc. We hypothesize that oocyte cytoplasmic immaturity is responsible for the low day-2 inseminated embryo blastocyst formation.

In conclusion, the primary reason for the low clinical pregnancy rate with day-2 fertilization is poor preimplantation embryo development to the blastocyst stage.

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