Embryo Developmental Stage at Transfer Influences Outcome of Treatment with Intracytoplasmic Sperm Injection

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Purpose: Our purpose was to determine if embryo cell stage at the time of intrauterine transfer correlates with pregnancy rate in patients treated with intracytoplasmic sperm injection (ICSI).

Methods: We conducted a retrospective analysis of 455 embryo transfer cycles following ICSI and 304 conventional in vitro fertilization (IVF) and embryo transfer cycles in women aged 40 years or less. Abstracted information included grading of the embryo cell stage and quality at the time of transfer.

Results: The overall ICSI pregnancy rate was 30.8%, while that of conventional IVF was 29.3%. However, the ICSI pregnancy rate fell to 9.3% for embryo transfers taking place at the two-cell stage but increased to 35.8% when at least one embryo had more than two cells, and this difference was statistically significant ($P \leq 0.0001$). The pregnancy rate following conventional IVF was 22.0% when only two-cell embryos were transferred and 32.0% when at least one of the embryos had more than two cells, but this difference in pregnancy rates was not significant ($P > 0.05$).

Conclusions: The stage of embryo development at transfer appears to exert a powerful influence on the successful establishment of pregnancy after ICSI.

KEY WORDS: embryo stage; embryo quality; intracytoplasmic sperm injection; in vitro fertilization; pregnancy rate.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has dramatically improved the outlook for infertile couples with male-factor infertility (1–3). The efficacy is such that traditional sperm parameters are less relevant to out-

come of ICSI provided sperm viability is documented (4,5). Indeed we reported recently that the pregnancy rate from ICSI with spermatozoa recovered through percutaneous epididymal sperm aspiration (PESA) is similar to that following the use of ejaculated sperm (3,6). However, ICSI bypasses all physiological sperm screening mechanisms and procures fertilization in a manner that is different from that of conventional in vitro fertilization (IVF). Furthermore, the biological characteristics of resulting embryos have not yet been fully assessed. We have observed that embryos derived from conventional IVF which have cleaved to the two-cell stage at the time of transfer have the same propensity to produce clinical pregnancy as do those that have cleaved to more than two cells. On the other hand, our preliminary impression seemed to be that the same did not hold for ICSI-derived embryos. We therefore examined our records to ascertain the influence of embryo stage and quality on subsequent pregnancy rates in patients who have had ICSI of their retrieved oocytes. This was to contribute to the body of knowledge on prognostic factors at embryo transfer following ICSI and stimulate further research into events that take place during embryo development after fertilization by ICSI.

MATERIALS AND METHODS

Patients

This study covers the period between January 1994 and October 1995 during which time 455 ICSI and 304 conventional IVF treatment cycles were carried out in women aged 40 years and below. We did not include data from older women to decrease the effect of the confounding factor of age on our results. All patients in our ICSI program either had at least one total failure of fertilization with conventional IVF in

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a previous treatment cycle at the center or had sufficiently abnormal sperm parameters such as severe oligo-, astheno-, and/or teratozoospermia.

Sperm Preparation

Spermatozoa used for ICSI in this study were recovered from the ejaculate or from PESA samples, for those with obstructive azoospermia. The percutaneous method for retrieval of spermatozoa from the epididymis has been described previously (3,6–8). Briefly, a 21-gauge butterfly needle (Venisystems; Abbot Ireland Ltd., Sligo, Republic of Ireland) was inserted directly into the epididymis, through the scrotal skin, under general anesthesia and an attached 20-ml syringe used to create a vacuum. The aspirate within the needle and tubing was subsequently flushed into sterile 1.5-ml Eppendorf tubes with equilibrated IVF culture medium (Medi-Cult, Copenhagen, Denmark). The ejaculate or PESA samples were prepared by washing twice with IVF culture medium and allowing the spermatozoa to swim up into the overlaid culture medium during 1 hr of incubation at 37°C. Alternatively sperm preparation was carried out by centrifugation of the sample through a discontinuous 40/80% Percoll gradient (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 10 min at 500g followed by two sperm washing cycles with IVF culture medium.

Ovarian Stimulation and Oocyte Retrieval

Multiple follicular development was stimulated with human menopausal gonadotropin alone or in combination with follicle stimulating hormone preparations using either the long or short protocol of pituitary desensitization. An injection of 5000 to 10,000 IU of human chorionic gonadotropin (hCG) was administered when the cohort of leading follicles measured between 18 and 22 mm or more in diameter. Transvaginal ultrasound-guided oocyte retrieval was performed 36 hr later with intravenous sedation. The luteal phase was supported with twice-daily administration of 400-mg progesterone pessaries (Cyclogest; Hoechst Ltd., Hounslow, UK).

Insemination Procedures

Following collection oocytes were incubated in IVF culture medium for 1 to 2 hr at 37°C. They were then denuded of their surrounding cumulus cells by a brief exposure to a 0.1% hyaluronidase solution (Sigma Chemical Co., St. Louis, MO) and drawing them in

and out of finely drawn sterile glass pipettes with an inner diameter of approximately 150 µm. Following assessment of maturational stage, those oocytes at the metaphase of the second meiotic division were cultured in fresh medium until injection. Oocyte holding and injection micropipettes were supplied by Cook-IVF (Queensland, Australia). Fine movements of the micropipettes were controlled by two Narashige MM-188 micromanipulators which were mounted on a Nikon Diaphot inverted phase-contrast microscope (Nikon Inc., London, UK).

Culture droplets and a droplet of sperm suspension in 10% polyvinylpyrrolidone (Medi-Cult, Copenhagen, Denmark) were placed in a petri dish and covered with liquid paraffin (Medi-Cult). The actual injection technique consisted of aspirating an immobilized spermatozoon tail first into the injection pipette, holding the oocyte with the holding pipette such that the polar body was at the 12 or 6 o'clock position, and inserting the injection pipette into the ooplasm. After aspirating a small amount of the ooplasm to confirm puncture of the oolemma, the spermatozoon was deposited in the ooplasm and the pipette withdrawn. Oocytes for conventional IVF were not denuded of their cumulus cells. Rather, they were inseminated between 38 and 40 hr from the time of administering the ovulation triggering hCG injection. The injected or inseminated oocytes were cultured for a further 16 to 18 hr and classified as being normally fertilized when two pronuclei and two polar bodies were clearly visible. Embryo development and quality were evaluated prior to embryo transfer (ET) 48 hr after insemination. A maximum of three cleaving embryos was transferred in each case and a serum β-hCG pregnancy test was carried out 12 days after ET. Women with positive results had ultrasound scanning 3 weeks later for the detection of fetal heartbeats and diagnosis or exclusion of multiple pregnancy.

Indices, Groups, and Statistics

Embryo quality in our study was defined as follows: Grade 1, >50% fragmentation of cells present in the embryo; Grade 2, <50 but >25%; Grade 3, <25%; and Grade 4, no fragments present. The embryo stage of development at transfer was defined as the number of cells in the embryo. No uncleaved fertilized egg was transferred. In this study we utilized a scoring system that gave an overall score for the transferred embryos. Thus embryo quality index (Q1) = $(Q_1 + Q_2 + Q_3)/n$, where Q_{1-3} indicate the quality grading of each transferred embryo and n is the number of

embryos transferred. Similarly, stage index (SI) = $(S_1 + S_2 + S_3)/n$, where S_{1-3} signify the stage of embryo development at transfer. Clinical pregnancy was defined as a definite fetal heartbeat evident on ultrasound scanning 3 weeks after the positive pregnancy test. Median and range were used because the data did not have a normal distribution. For the initial part of the analysis the data were arranged according to whether the SI was above two or equal to two. The chi-square test was used in checking for significant relationships between SI and the clinical pregnancy rate. Further analysis was carried out by subdividing each SI group into two, depending on the presence or absence of a clinical pregnancy. Group 1 consisted of ET cycles with a SI >2 that resulted in clinical pregnancies, while Group 2 had a similar SI but no pregnancies. In Group 3 the SI was 2 but no clinical pregnancy occurred, unlike Group 4, where there were clinical pregnancies despite having a similar SI. The Mann-Whitney *U*/Wilcoxon test was used in analyzing observed differences between the groups.

RESULTS

The overall clinical pregnancy rate was 30.8% after ICSI (140 of 455 embryo transfers) and 29.3% following conventional IVF (89 of 304 embryo transfers) (Table I). A higher rate of 35.8% was achieved in patients who had ICSI and transfer of at least one embryo with more than two cells (i.e., SI > 2), while it fell to 9.3% for those in whom all embryos that were transferred were at the two-cell stage (i.e., SI = 2) (Table I). This difference was highly significant ($P \leq 0.0001$). In contrast, a clinical pregnancy rate of 22.0% was achieved when, following conventional IVF, only two-cell embryos were transferred. Although this was lower than the rate of 29.3% obtained from the transfer of at least one embryo at more than the two-cell stage of development (Table I), the difference was not significant ($P > 0.05$). Transfer of ICSI-generated embryos

at the two-cell stage was associated with a significantly lower pregnancy rate, in contrast to transfer of two-cell embryos from conventional IVF (9.3 vs 22.0%; $P \leq 0.0001$).

Embryo quality was significantly better in patients who became pregnant (Group 1) than in those who did not (Group 2), when at least one embryo whose developmental stage was above two cells was transferred after ICSI ($P \leq 0.0005$) (Table II) or IVF ($P \leq 0.05$) (Table III). No difference in embryo quality was noted in the ICSI group when the embryo cell stage at transfer was equal to two. There was no significant difference in the number of embryos transferred in all groups after ICSI (Table II) but it was significantly lower in Group 2 (nonpregnant patients) than in Group 1 (pregnant patients) after conventional IVF ($P \leq 0.05$) (Table III).

DISCUSSION

Data presented in this study indicate that the stage of development of embryos at the time of ET does indeed have an effect on assisted conception treatment outcome following ICSI. When at least one of the embryos transferred was at greater than the two-cell stage (SI > 2), a pregnancy rate of 35.8% was achieved, in comparison to a rate of 9.3% when all the transferred embryos were at the two-cell stage. Such a significant difference was not found when pregnancy rates following transfer of embryos generated from conventional IVF were analyzed. A further finding related to the contribution of embryo quality to pregnancy rates in patients who had ICSI. For transfers at SI > 2 the embryo quality was significantly better in women who subsequently became pregnant (Group 1) than in those who did not achieve clinical pregnancies (Group 2) (Table II). This effect was not apparent when Groups 3 and 4 were compared, suggesting that the embryo cell stage at transfer was a more important

Table I. Embryo Cell Stage at Transfer and Achievement of Pregnancy

Stage index (SI)	ICSI			IVF		
	Pregnant	Nonpregnant	Total	Pregnant	Nonpregnant	Total
>2	132*	237	369	71	151	222
2	8	78	86	18	64	82
Total	140	315	455	89	215	304

* $P < 0.0001$ versus SI = 2 in the same treatment.

Table II. Embryo Stage at Transfer and Characteristics of Various Patient Groups Following ICSI^a

Variable	Stage index > 2		Stage index = 2	
	Group 1 (n = 132)	Group 2 (n = 237)	Group 3 (n = 78)	Group 4 (n = 8)
Age	32(19–40)	34(20–40)	34(21–40)	32(28–37)
Number of embryos transferred	3(1–3)	3(1–3)	3(1–3)	3(2–3)
Quality index	4.0(1.3–4.0)*	3.7(1.3–4.0)	4.0(1.0–4.0)	4.0(4.0–4.0)
Outcome	Pregnant	Nonpregnant	Nonpregnant	Pregnant

^a Values are medians, with ranges in parentheses.

* $P < 0.0005$ versus Group 2.

determinant of successful establishment of pregnancy than the quality of the embryos following ICSI.

We do not know for certain the reason for this observed differential performance of embryos transferred at different cell stages of development 48 hr after ICSI. The finding of a two-cell embryo at this time signifies either cessation or delay of embryo development. We have often observed that continued culture of ICSI-generated embryos for an additional 24 hr does not result in further cell division in a large number of cases (data not shown). However, the fact that clinical pregnancies still occurred in some women who had transfers of only two-cell embryos 48 hr after insemination (8 of 86 women) means that delay of embryonic development cannot be excluded completely. It is not possible for us to say at present whether we can improve pregnancy rates in such women by delaying ET for another 24 hr to select and transfer only those embryos that have cleaved to three or more cells. The suboptimal nature of most in vitro culture systems also has to be borne in mind.

Development of the human embryo after the two- to four-cell stage is controlled by the embryonic genome, in contrast to earlier stages, which are controlled by maternally encoded genomic products stored in the oocyte during the later stages of development

in the ovary. Although the mechanism that controls development of injected oocytes after ICSI is not clearly understood, it may be related in some way to the injected spermatozoon or the injection process. Oocyte activation following ICSI is widely believed to be by oscillation in intracellular calcium ions caused by the injected spermatozoon (9–11). Cessation of development of the two-cell embryo may be related to perturbation of the normal organization of ooplasmic structures caused by the injection process (12) and this could be by way of interference with activation of the embryonic genome. Oocytes activated pathenogenetically may demonstrate pronuclei and commence cleavage just as normally fertilized eggs do, but development usually ceases at later stages of early embryonic life. We hope that more work in this area will provide conclusive explanations.

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Table III. Embryo Stage at Transfer and Characteristics of Various Patient Groups Following IVF^a

Variable	Stage index > 2		Stage index = 2	
	Group 1 (n = 71)	Group 2 (n = 151)	Group 3 (n = 64)	Group 4 (n = 18)
Age	32(23–40)	34(24–40)	35(24–40)	33(28–37)
Number of embryos transferred	3(1–3)	3(1–3)	3(1–3)	3(2–3)
Quality index	4.0(2.0–4.0)*	3.7(1.0–4.0)	3.6(1.5–4.0)	4.0(4.0–4.0)
Outcome	Pregnant	Nonpregnant	Nonpregnant	Pregnant

^a Values are medians, with ranges in parentheses.

* $P \leq 0.05$ versus Group 2.

** $P \leq 0.0005$ versus Group 2.

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