Late Fertilization of Unfertilized Human Oocytes in In Vitro Fertilization and Intracytoplasmic Sperm Injection Cycles: Conventional Insemination versus ICSI¹

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Purpose: The aim of this study was to evaluate the efficacy of intracytoplasmic sperm injection (ICSI) in comparison with conventional reinsemination using fertilization failed oocytes by conventional in vitro fertilization (IVF).

Methods: Oocytes were collected from patients of IVF or ICSI cycles. Patients were grouped by fertilization techniques: group 1: conventional IVF; group 2: reinsemination after conventional IVF failure; group 3: regular ICSI; group 4: 1-day-old ICSI after conventional IVF failure; group 5: 2-day-old ICSI after conventional IVF failure; group 6: re-ICSI after regular ICSI failure.

Results: In different insemination groups, normal fertilization rate was higher ($P < 0.001$) in 1-day-old ICSI (47.1%) and 2-day-old ICSI groups (40.0%) than in reinsemination (14.7%). Abnormal fertilization rate was higher ($P < 0.05$) in re-ICSI group (21.7%) than any other groups (range: 0–8%). Cleavage rate was higher in 1-day-old (36.7%) and 2-day-old ICSI groups (36.0%) than in reinsemination (5.3%, $P < 0.001$) or re-ICSI groups (17.4%, $P < 0.05$). Pregnancy rate was 27.6% and 20.0% in conventional IVF and regular ICSI groups, respectively. However, 1-day-old ICSI (group 4) and 2-day-old ICSI (group 5) were attempted once embryo transfer (ET) but failed pregnancy occurred in each group.

Conclusions: In fertilization failure cycles, late ICSI increases the rate of fertilization and embryonic development and may rescue the completely failed attempt of pregnancy.

KEY WORDS: Conventional IVF; reinsemination; 1-day-old ICSI; 2-day-old ICSI; re-ICSI.

INTRODUCTION

On average, up to 40% of human oocytes remain unfertilized after in vitro insemination. These oocytes are not suitable for further clinical use and are usually discarded (1). In non-male factor infertility in vitro fertilization (IVF) cases, complete fertilization failure still may occur (2). Consequently, reinsemination of unfertilized oocytes can be carried out to achieve some late fertilization, that is, to “rescue” what otherwise would be a completely failed attempt at pregnancy and to get some embryos for embryo transfer (ET) or for later ET by cryopreserved embryo(s) (2). Most often the reason for failure of fertilization is poor cytoplasmic and zona pellucida quality of oocyte and poor sperm quality (1,2).

Palermo et al. (3) described the use of intracytoplasmic sperm injection (ICSI) for the treatment of severe male factor infertility. During the last few years, much research has focused on oocyte factors influencing the fertilization process; these factors may provide to be even more in the area of ICSI. This technique has become fully established as the insemination technique of choice for all forms of male factor infertility and unexplained fertilization failure treated by conventional IVF (2,4–6).

The aim of this work was to evaluate the fertilization and the subsequent cleavage characteristics of oocytes treated by conventional reinsemination or ICSI after failure to fertilize through the standard IVF and ICSI procedures.

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MATERIALS AND METHODS

Design of Experiments

Patients were grouped by fertilization techniques: group 1, conventional IVF, $n = 29$; group 2, reinsemination after conventional IVF failure, $n = 29$; group 3, regular ICSI, $n = 24$; group 4, 1-day-old ICSI after conventional IVF failure, $n = 17$; group 5, 2-day-old ICSI after conventional IVF failure, $n = 6$; and group 6, re-ICSI after regular ICSI failure, $n = 14$.

Unfertilized oocytes after conventional IVF or regular ICSI cycles in which fertilization failure were complete or partial were subjected to attempts to fertilize by reinsemination, 1-day-old ICSI, 2-day-old ICSI, or re-ICSI to attempt late fertilization and to rescue otherwise failed IVF-ET cycle.

Informed consent was obtained from each patient before this study start.

Patient Selection

A total of 109 cycles at the Kyungpook National University Hospital were used in this study. The mean ages of the female patients in six study groups were 32.1 ± 1.10 (25–44); 32.4 ± 1.0 (25–40), 32.2 ± 0.71 (26–40), 31.7 ± 1.60 (30–35), 33.3 ± 2.10 (30–40), and 32.7 ± 0.82 (26–41) years, respectively.

Follicular Stimulation

Ovarian stimulation was performed using a concomitant gonadotropin-releasing hormone-agonist (GnRH-a)—human menopausal gonadotropin regimen. Busereilin (900 μ g) (Suprecur, Hoechst, Germany) was administered starting at the mid-luteal phase. Human menopausal gonadotropin (hMG) (Pergonal, Serono, Italy) and/or follicle-stimulating hormone (FSH) (Metrodin, Serono, Italy) were started after down-regulation of the pituitary was confirmed by serum estradiol. The subsequent dosage of hMG was adjusted according to the individual ovarian response in terms of daily estradiol concentration and follicle number and size. Ovulation was induced by administering 10,000 IU of human chorionic gonadotrophin (hCG) (LG, Korea) when one or more follicles >17 mm in diameter were present and estradiol concentration was >900 pg/ml. Oocyte retrieval was performed 34–36 hr after hCG injection under transvaginal ultrasound control.

Procedure of IVF and ICSI

Semen samples were obtained by masturbation and collected before oocyte retrieval. Semen parameters were evaluated according to World Health Organization (WHO) criteria (7). After complete liquefaction, the sperm were washed twice with F-10 nutrient mixture medium (Ham's F-10, 11550-043, Gibco, USA) supplemented with 10% heat-inactivated human follicular fluid (hFF) at 300g for 30 min and 0.5% antibiotics (Penicillin-G, P3032; Streptomycin sulfate, S9137, Sigma, USA). The pellet was then resuspended in 1 ml of Ham's F-10, centrifuged, and placed to swim up for 30 min to 1 hr at 37°C incubator (3158, Forma, USA). Sperms were collected in 5 ml tube (2003, Falcon, USA).

In cases of conventional IVF and reinsemination, up to five oocytes were inseminated with approximately 200,000 sperm/2 ml in each culture dish (3037, Falcon, USA).

In cases of regular ICSI, retrieved oocytes were exposed briefly to 80 IU/ml hyaluronidase in Dulbecco's phosphate-buffered saline (DPBS) (21300-025, Gibco, USA) and mechanically cleaned their surrounding cumulus cells (CCs) by repeated aspiration through a glass pipette (CO-7095B-5X, Corning, USA) with an 200 μ m inner diameter. In cases of 1-day-old ICSI and 2-day-old ICSI of fertilization-failed oocytes by conventional IVF, oocytes CCs were removed by glass pipette. Denuded oocytes were washed in the culture medium [Dulbecco's modified Eagle medium (DMEM) 11966-025, Gibco, USA] supplemented with 10% hFF and 0.5% antibiotics. These procedures were performed in a culture dish. All denuded oocytes were examined under an inverted microscope (Diaphot-300, Nikon, Japan) at a magnification of $\times 200$, and those showing a first polar body were selected for micromanipulation. Sperm were placed in a Petri dish (3002, Falcon, USA) under paraffin oil (294365H, BDH, England), approximately 100 sperm cells/ μ l. The microinjection procedure was performed using an inverted-phase microscope that was equipped with a differential interference contrast and a set of micromanipulators (TDU 500, Research Instrument, England). The injection micropipette (ICSI micropipette, Humagen, USA) was lowered in the DPBS with 3% polyvinylpyrrolidone (PVP) (PVP-360, Sigma, USA), and a morphologically normal motile spermatozoon was chosen and immobilized by touching its tail near the tail tip with the injection pipette. The immobilized spermatozoon was aspirated, tail first, into the injection pipette. After the oocyte was secured in posi-

tion with the holding pipette, the injection pipette was introduced at the 3-o'clock position (polar body at the 6- or 12-o'clock position) through the zona pellucida and the oolemma until it reached up to two thirds of the cytoplasm and then was withdrawn to the center. Thereafter, some of the cytoplasm was aspirated to verify that the oolemma had been broken, and then the spermatozoon was injected slowly.

The presence of pronuclei was assessed about 18 h after insemination or injection. Oocytes were considered normally fertilized if they exhibited clear two pronuclei or abnormally fertilized when three or more pronuclei presented.

Culture of Vero Cells

The technical aspects of Vero cells maintenance have been described by Ouhibi et al. (8). Briefly, from the frozen cells, flasks were seeded with $2-3 \times 10^6$ cells and reached confluency within 4 days ($6-8 \times 10^6$ cells/flask). After trypsinization by trypsin-EDTA (25300-054, Gibco, USA), the cell suspension was divided into two to three aliquots. One was used to seed a new flask, one was frozen using a freezing medium (cell culture freezing medium, 11101, Gibco, USA), and the remaining aliquot was used to seed culture dishes at a concentration of 100,000 cells/well. Thus confluency was reached in 3 days. The culture medium was medium 199 (11150-059, Gibco, USA) containing 10% fetal bovine serum (FBS) (26140-079, Gibco, USA) and antibiotics.

Embryo Coculture on Vero Cell Monolayer

The culture medium, DMEM supplemented with 20% hFF, was changed on day 1. In the conventional IVF and reinsemination groups, zygotes were grown in coculture dishes from day 1. In the ICSI groups, oocytes were grown in coculture dishes after microinjection. Zygotes were cocultured on a monolayer of Vero cells until embryo transfer or cryopreservation. Cleavage rate of zygotes was examined.

Monitoring Pregnancies

Embryos showing the best morphological development were chosen for transfer. In our study, pregnancy was defined as serum β -hCG level of > 10 mIU/ml, and a clinical pregnancy was defined by the presence of gestational sac on vaginal ultrasound examination.

Statistical Analysis

The Microsoft Excell 97 was used to compare normal fertilization, abnormal fertilization, embryonic development ($\geq$ two cell), and clinical pregnancy rates in the six different fertilization techniques. The results were considered statistically significant when P value was less than 0.05. Statistical analysis was performed using χ^2 test to compare ratios.

RESULTS

The results of the late fertilization by conventional reinsemination or ICSI are presented in the Table I.

The average normal fertilization (2PN) rate from all groups was 61.6% (14.7–78.9%). In conventional IVF (group 1) and regular ICSI groups (group 3), normal fertilization rate (77.6% and 78.9%, respectively) appeared to be higher ($P < 0.001$) than that in reinsemination (group 2, 14.7%), 1-day-old ICSI (group 4, 47.1%), 2-day-old ICSI (group 5, 40.0%), and re-ICSI (group 6, 30.4%).

Of three different treatments after fertilization failure by conventional IVF, normal fertilization rate of 1-day-old ICSI (group 4, 47.1%) and 2-day-old ICSI groups (group 5, 40.0%) appeared to be higher ($P < 0.001$) than that of reinsemination group (group 2, 14.7%), which demonstrated the superiority of late ICSI in comparison with reinsemination for the treatment of fertilization failure.

Normal fertilization rate of re-ICSI group (group 6, 30.4%, 7/14) not only was lower ($P < 0.001$) than that of conventional IVF and regular ICSI groups (group 1, 77.6%, and group 3, 78.9%, respectively) but also lower ($P < 0.005$) than that of 1-day-old ICSI and 2-day-old ICSI groups (group 4, 47.1%, and group 5, 40.0%, respectively), but was higher ($P = 0.059$, not significant) than that of reinsemination group (group 2, 14.7%).

Abnormal fertilization (multi-PN) rates was higher in group 6 (21.7%) than the other groups (range: 0–8%).

Mean percentage of embryo development per number of allocated oocytes was higher ($P < 0.001$) in conventional IVF (group 1, 76.0%) and regular ICSI (group 3, 75.6%) than in any other treatment groups (5.3–36.7%). In different reinsemination groups, cleavage rate of 1-day-(36.7%) and 2-day-old ICSI groups (36.0%) was higher ($P < 0.001$) than that of reinsemination (5.3%) or re-ICSI groups (17.4%). The cleavage rate of re-ICSI group was higher ($P < 0.05$) than that of reinsemination group.

Table I. Comparison of Results in IVF, ICSI, and Late Fertilization of Fertilization-Failed Oocytes

Variable (<i>n</i>)	Treatment ^a					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Cycles	29	29	24	17	6	14
Used oocytes	192	95	209	68	25	23
Fertilized oocytes (%)						
2PN ^b	149 (77.6)	14 (14.7)	165 (78.9)	32 (47.1)	10 (40.0)	7 (30.4)
Multi-PN ^c	13 (6.8)	0	9 (4.3)	4 (5.9)	2 (8.0)	5 (21.7)
Cleaved embryos (%) ^d	146 (76.0)	5 (5.3)	158 (75.6)	25 (36.7)	9 (36.0)	4 (17.4)
ET cycles	29	—	20	1	1	—
Transferred embryos	128	—	69	4	2	—
Range	3–7	—	3–7	4	2	—
Mean $\pm$ SEM ^e	4.4 $\pm$ 0.5	—	4.5 $\pm$ 0.4	4	2	—
Clinical pregnancies (%) ^f	8 (27.6)	—	4 (20.0)	0 (0)	0 (0)	—

^a Group 1, conventional IVF; group 2, reinsemination after conventional IVF failure; group 3, regular ICSI; group 4, 1-day-old ICSI after conventional IVF failure; group 5, 2-day-old ICSI after conventional IVF failure; group 6, re-ICSI after regular ICSI failure.

^b $P < 0.001$ (group 1, 3 vs. 2, 4–6; 2 vs. 4, 5), < 0.005 (6 vs. 4, 5).

^c $P < 0.01$ (group 1, 3 vs. 6), < 0.05 (4 vs. 6), < 0.001 (6 vs. 2, 5).

^d $P < 0.001$ (group 1, 3 vs. 2, 4–6; 2 vs. 4, 5), < 0.05 (2 vs. 6).

^e P , Not significant.

^f P , 0.0207 (group 1 vs. 4, 5).

Clinical pregnancy rate was 27.6% and 20.0% in conventional IVF (group 1) and regular ICSI group (group 3), respectively. However, 1-day-old ICSI (group 4) and 2-day-old ICSI groups (group 5) were attempted once embryo transfer but failed pregnancy occurred in each group. The number of clinical pregnancies was higher in group 1 ($P = 0.0207$) and group 3 ($P = 0.0798$, not significant) than group 4 and group 5.

DISCUSSION

Our study proves that oocytes that failed to fertilize through standard IVF or ICSI can be fertilized after ICSI (1-day-old ICSI and 2-day-old ICSI in IVF cycles and re-ICSI in regular ICSI cycles) with a high rate of success. This shows that ICSI, although it is an invasive method, is no more detrimental when performed on older oocytes than on fresh ones (9).

Fertilization failure was associated with an absence of both paternal and maternal chromatic transitions (chromatin decondensation and anaphase onset, respectively). Most unfertilized oocytes after ICSI contained either decondensed sperm heads or intact spermatozoa or the spermatozoon had been ejected from the oocyte. The majority contained a decondensed sperm head, indicating that failure of oocyte activation and not technical error was the principal cause of fertilization failure (10,11). It appears that the state of the nuclear chromatin is relatively more important than

count and motility of sperm in routine conventional IVF (12) and ICSI (11). There were several other interesting aspects of unfertilized oocytes. First, despite alignment of the first PB at 12 o'clock prior to injection, decondensed sperm heads were found among the metaphase chromosome in ~10% of the oocytes (10,11). Second, ~7% of the metaphase II oocytes that contained a decondensed sperm head, the sperm DNA had the appearance of premature chromosome condensation (11). This originally was described in unfertilized IVF oocytes and probably indicates oocyte immaturity (13,14). It has been described in other studies of unfertilized ICSI oocytes, although the frequency varied from 2.5% (15) to 8% (10), and as high as 28.6% (16).

Repeated conventional fertilization failure can be a good reason to apply ICSI (2,17), but such failure is difficult to anticipate. If ICSI replaced conventional IVF insemination completely, any concerns over when ICSI should be applied would be obviated, although the question has been raised whether ICSI is justifiable when it might generate higher numbers of abnormal offspring and also cost more. Although ICSI reinsemination of fertilization-failed oocytes in 1st-day ICSI has been shown to cause high rates of mosaicism, it appears that ICSI rescue of failed conventional fertilization oocytes is feasible and moderately successful. If ICSI reinsemination was an effective means to rescue failed conventional IVF cycles, then automatic application of ICSI would not be needed; ICSI would be used only in those questionable cases in which

conventional insemination initially has failed (2). Tsirigotis et al. (18) have shown that late (24 hr) ICSI can give good fertilization and cleavage rates, but the potential of the generated embryos to achieve pregnancy seems to be low. This may be expanded by the high percentage of abnormal karyotypes observed in embryos with delayed fertilization (2,18,19). In another case, half the embryos derived from 2-day-old reinsemination oocytes presented chromosomal abnormalities (20). Although the pregnancy and implantation rates for patients with a history of idiopathic fertilization failure were significantly lower than those for other patients undergoing ICSI (21), ICSI is a relatively successful means of rescuing conventional IVF cycles in which fertilization fails completely (2,9,18). When patients were grouped according to the fertilization outcome after initial insemination or according to indication for IVF treatment, no difference was seen in normal or multiple PN formation after ICSI. This may mean that ICSI makes it possible to more easily overcome certain obstacles connected with the zona or oolemma of the oocytes or with the quality of the spermatozoa than is the case with other types of reinsemination (9). Flaherty et al. (11) reported that most couples consistently achieve high rates of fertilization after ICSI, and failed fertilization occurs rarely and is usually associated with the injection of only one or two oocytes. Once the oocyte has been fertilized, the percentage of normal embryo cleavages did not differ between conventional IVF and ICSI (5).

Concerning the safety of using polyvinylpyrrolidone (PVP), some reports have suggested that PVP solutions are toxic to mouse embryo development (22–24). On the other hand, it has been suggested that toxicity of PVP is related to its impurities. Van Steirteghem et al. (25) and Fujii et al. (26) have used dialyzed PVP for human ICSI procedures, and high fertilization and pregnancy rates have been achieved. However, we could obtain high fertilization and normal live birth using nonpurified PVP in ICSI cycles (data not shown). The phenomenon of sperm “swimming across” usually is seen in the 8–10% PVP droplet during the regular ICSI procedure (27,28). Fujii et al. (26) reported that 8% PVP could clearly separate motile spermatozoa but was not useful for weakly progressive motile spermatozoa, and they tried to use a droplet intermediate between 3% PVP droplet and 8% PVP. However, Dozortsev et al. (29) reported that PVP at a concentration of 10% could indeed reduce the fertilization rate. Dozortsev et al. (30) suggested that the presence of PVP in the oocyte together with the injected spermatozoon explains the delay between sperm injection and

the beginning of calcium oscillations observed after ICSI (31). Schwartz et al. (32) reported that oocyte degeneration is not caused by the penetration of a glass needle into the ooplasm but by an injury to the meiotic spindle or by an excessive dose of fluid PVP or medium during sperm injection. Thus the use of an unnecessarily high concentration of PVP should be avoided. In our preliminary experiment, we tried sperm preparation using 10, 8, 5, and 3% PVP droplets. When sperms were placed in the 3% droplet, the sperms showed longer motility than in other concentration of PVP (data not shown). We have used a 3% PVP droplet at ICSI (33) and Testicular sperm extraction–ICSI cycles (34) effectively.

In ICSI procedures, oocytes were exposed to hyaluronidase, intense light, and fluctuations in temperature and were subjected to the creation of an artificial breach in the zona pellucida and oolemma. This opening may increase the risk of introducing toxins and debris into the perivitelline space and oolemma, all of which may affect oocyte quality (35). Van de Velde et al. (36) suggested that a concentration as low as 10 IU/ml of hyaluronidase in combination with a pipette of at least 1000 μ m inner diameter can be used successfully to denude oocytes for microinjection. A different timing of cumulus–corona cell removal also has no effect on the outcomes of ICSI (37). Our previous study (33) showed that the degree of oocyte denudation (complete or partial) did not affect the outcomes of ICSI, and the denuded oocytes with incubation period of more than 2 hr show better outcomes of ICSI than those with the incubation period of less than 2 hr.

It is concluded that late ICSI appears to be good treatment for overcoming fertilization failure of oocytes and may rescue the completely failed attempt of pregnancy in conventional IVF or regular ICSI cycles because late ICSI increases the fertilization and embryonic development rates.

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