Comparison of Blastulation and Pregnancy Rates of Fertilized Human Oocytes Obtained after Conventional In Vitro Fertilization and Intracytoplasmic Sperm Injection

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Abstract: *A comparison was made of good quality blastocysts obtained after either conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). After IVF or ICSI, fertilized oocytes were kept in culture for a further 5 to 7 days before embryo transfer (ET) or embryo freezing. No differences were found in the number of oocytes showing two-pronuclei between conventional IVF (76.9%) and ICSI groups (85.1%). A cohort of 60 and 74 oocytes in the pronuclear stage were cultured after IVF and ICSI, respectively. The number of fertilized oocytes reaching the blastocyst stage was significantly higher (P<0.05) in the IVF group (73.3%) than in the ICSI group (56.8%). A total of 86 blastocysts were categorized into three grades depending on their morphology. The number of blastocyst embryos achieving blastocyst grade 1 (BG1) was significantly higher (P<0.05) in the IVF group than in the ICSI group, 63.3% and 13.5%, respectively. In the IVF group, 10.0% and 0% of 2PN oocytes developed to BG2 and BG3, and 2.7% and 4.1% in the ICSI group, respectively, with no significant differences between the two groups. Pregnancy rate was higher in the IVF group than in the ICSI group, 50% and 20%, respectively. It was concluded that fertilized oocytes resulting from ICSI cannnot be successfully cultured for 5-7 days for blastulation and blastocyst-ET.*

Key words: *In vitro fertilization, Intracytoplasmic sperm injection, Blastulation, Blastocyst grade (BG)*

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Physiologically, the human endometrium prepares itself to its optimum approximately 5 to 7 days after ovulation so as to receive a cavitated blastocyst from the fallopian tube for successful implantation [1, 2]. However, conventionally, in most programs offering in vitro fertilization (IVF), 4~8 cell stage embryos are quickly placed in the uterus [1, 3]. This asynchrony of embryonic stage and preparation of uterine endometrium may be one major contributory cause of increased abortion and low take-home baby rates in infertility patients [2–4].

Conventional IVF cannot help certain couples with male factor infertility [5]. In recent years, assisted fertilization techniques have been described for the treatment of such couples, but the results obtained with these techniques were not enough as demonstrated in several randomized studies by Calderon *et al.* [5], Goto *et al.* [6], Palermo *et al.* [7] and Bar-Hava *et al.* [8]. Palermo *et al.* [7] described the use of ICSI as for the treatment of severe male factor infertility. This technique is now used extensively worldwide and recent innovations have been published in a seriese of short articles [5–8].

The aim of this study was to evaluate the efficiency of conventional IVF in comparison with ICSI by developing embryos to blastocyst stage and transferring blastocyst(s) to the uterine cavity.

Materials and Methods

Patient selection

From May 1998 to May 1999, 18 infertile couples participating in IVF (n=8) or ICSI (n=10) cycles at the

Kyungpook National University Hospital were recruited to this study. The mean age of the female patients of IVF or ICSI cycles was 30.3 (25–35) years and 31.2 (25–35) years, respectively.

Follicular stimulation

Ovarian stimulation was performed using a concomitant gonadotrophin-releasing hormone agonist (GnRHa)-monotrophin regimen. Buserelin (300 μg twice daily; Suprefact, Hoechst, Germany) was administered starting at the mid-luteal phase. Human menotrophins [human menopausal gonadotrophin (hMG, Pergonal, Serono, Italy)] and/or follicle stimulating hormone (FSH, Urofollotropin, Metrodin, Serono, Italy) were given concomitantly with GnRHa starting on day 3 of the subsequent follicular phase for 4 days. The subsequent dosage of menotrophins was adjusted according to the individual ovarian response in terms of daily estradiol concentration, follicle number(s) and follicle size. Ovarian stimulation was monitored daily by ovarian ultrasonography and estradiol assay starting on days 6~8 of the stimulation cycle. Ovulation was induced by a 10,000 IU injection of human chorionic gonadotrophin (hCG, Profasi, Serono, Italy) when one or more follicles >17 mm in diameter were present. Oocyte retrieval was performed 36–38 hrs after HCG injection. Oocytes were obtained by ultrasonically-guided transvaginal follicular aspiration.

In vitro fertilization and intracytoplasmic sperm injection

Semen samples were obtained by masturbation and collected before ovum collection. Sperm density and motility were evaluated by Word Health Organization criteria [9]. After complete liquefaction, the sperms 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) and 0.5% antibiotics (Penicilln-G, P3032; Streptomycin sulfate, S9137, Sigma, USA), and centrifuged at 300 g for 30 minutes. The pellet was then resuspended at 1 ml and subsequently centrifuged in Ham's F-10, and swimmed up for 1 hr at 37°C under 5% CO_2 in an incubator. Sperms were collected in 5 ml tubes (2003, Falcon, USA). Up to five oocytes were inseminated with approximately 200,000 sperm cells/2 ml in each well.

When ICSI was carried out after oocyte retrieval, the oocytes were exposed briefly to 80 IU/ml hyaluronidase in Dulbecco's Phosphate-Buffered Saline (DPBS, 21300-025, Gibco, USA) and mechanically cleaned of their surrounding cumulus cells by aspiration through a glass pipette (CO-7095B-5X, Corning, USA) with an inner diameter of 200 μm. The denuded oocytes were washed five times in a culture medium (Dulbecco's Modified Eagle Medium, DMEM, 11966-025, Gibco, USA) supplemented with 10% hFF and 0.5% antibiotics. These procedures were all performed in a Petri dish (3260, Costar, USA) in 2 ml. All oocytes then were examined under an inverted microscope (Diaphot 300, Nikon, Japan) at a magnification of × 200, and those in which a first polar body was present were selected for micromanipulation. Sperm were injected in a Petri dish (3002, Falcon, USA) in 20 μl droplet (DPBS) with 3% polyvinylpyrrolidone (PVP, PVP-360, Sigma, USA) under paraffin oil (294365H, BDH, UK), and approximately 100 sperm cells/μl were added. 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, UK). The injection micropipette (ICSI micropipette, Humagen, USA) was lowered in the PVP, and the best morphologically normal motile spermatozoon was chosen and immobilized by touching its tail near the tail tip with the injection micropipette. The immobilized spermatozoon was aspirated, tail first, into the injection pipette. After the oocyte was secured in position with the holding pipette, the injection pipette was introduced at the 3-o'clock position (polar body at the 6-o'clock position), through the zona pellucida and the oolemma, until it reached into 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 the spermatozoon was then injected slowly.

The presence of two pronuclei was assessed exactly 18 hrs after insemination or injection. Oocytes were only considered fertilized if they exhibited clear pronuclei.

Culture of Vero cells

The technical aspects of *Vero* cell maintenance have been described by Ouhibi *et al.* [10]. Briefly, from the frozen cells, flasks were seeded at 2~3 × 10^6 cells and reached confluency within 4 days (6~8 × 10^6/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 by freezing medium (Cell Culture Freezing Medium, 11101, Gibco, USA), and the remaining part was used to seed culture dishes at a concentration of 100,000 cells/well. The culture medium was Medium 199 (11150-059, Gibco, USA) containing 10% fetal bovine serum (FBS, 26140-079, Gibco, USA) and antibiotics.

Embryo co-culture on Vero cell monolayer

In the IVF group, zygotes were grown in co-culture on day 1. In the ICSI group, zygotes were grown in co-culture after injection. Zygotes were co-cultured until day 5 to 7. For this procedure, zygotes were placed on a monolayer of *Vero* cells in a dish well. The culture medium was DMEM supplemented with 20% hFF and was changed on day 3. Up to three of the best blastocysts were transferred to the uterus on day 5 and those remaining were cryopreserved. When embryo development was delayed, embryos were kept in co-culture up to day 7 for full expansion to occur.

Blastocyst grading

At the blastocyst stage, embryo grading was based on the system devised by Dokras *et al.* [3]. Briefly, grade 1 blastocyst (BG1) demonstrated typical development with early cavitation occurring on or before day 6 of development resulted in an expanded single cavity lined with clear trophectoderm cells and a distinct inner cell mass (ICM) region. Grade 2 blastocyst (BG2) had the same apperance as BG1 embryos but their formation was delayed by 24~48 h after initial cavitation had begun. Grade 3 blastocyst (BG3) had dark degenerative areas within the ICM and trophectoderm and the cavity collapsed within 24 h often without prior expansion. An additional category of cavitating embryos was also identified. These were not considered to be blastocysts at all since they lacked a clear morula stage where the cells had undergone compaction and neither a distinct trophectoderm layer nor an ICM region was identifiable. These were often embryos of 6 to 8 cells that subsequently developed single or multiple cavities of different sizes and which were also unusual since they had well defined, smooth edges.

Monitoring of pregnancies

Embryos showing the most advanced morphological development (BG1) were chosen for transfer. For the purpose of our study, pregnancy was defined as serum β-hCG level of >10 mIU/mL, and a clinical pregnancy was defined by the presence of the gestational sac on vaginal ultrasound examination.

Cryopreservation of blastocyst

Freezing of blastocysts was based on the system devised by Menezo *et al.* [11]. First, the embryos were immersed in a 5% glycerol solution in DPBS + 10% FBS for 10 minutes. Next, the embryos remained 10 minutes in a solution of 9% glycerol in DPBS + 10% FBS containing 0.2 M sucrose. The programmed freezing curve was –2°C/min from 22°C to –6°C. Then manual seeding was made after a 30-second delay. After the seeding, the embryo was cooled slowly from –6°C to –37°C at a speed of –0.3°C/min. Then it was dropped directly into liquid nitrogen.

Statistical analysis

The SAS package was used to compare fertilization, blastulation, BG and pregnancies in the two categories of fertilization techniques. For statistical analysis, student's *t*-test was used. Results were considered statistically significant when the p value was less than 0.05.

Results

A total of 18 couples (IVF: 8; ICSI: 10) were included in this study. A total of 165 metaphase II oocytes were used in either conventional IVF (n=78) or ICSI (n=87).

The *in vitro* deveolpment of 134 bipronucleate embryos (IVF: 60; ICSI: 74) was analyzed to determine the number of embryos developing to the blastocyst stage. The results of fertilization, development of fertilized oocytes to blastocyst and clinical pregnancy are summarized in Tables 1, 2 and 3, respectively.

No differences were found in the number of oocytes showing two-pronuclei between the IVF group (76.9%, 60/78) and the ICSI group (85.1%, 74/87) (Table 1).

Of these oocytes, 73.3% (44/60) and 56.8% (42/74) developed to the blastocyst stage in the IVF group, and the ICSI group, respectively. The number was significantly higher ($P<0.05$) in the IVF group than the ICSI group (Table 2). The number of blastocyst embryos achieving BG1 was significantly higher ($P<0.05$) in the IVF group than in the ICSI group, at 63.3% (38/60) and 13.5% (10/74), respectively. In the IVF group, 10.0% (6/60) and 0% (0/34) of 2PN oocytes developed to BG2 and BG3, and 2.7% (2/74) and 4.1% (3/74) in the ICSI group, respectively. However, this difference was not statistically significant.

In the IVF group, 16 best quality blastocysts (BG1)

Table 1. Effects of fertilization methods on *in vitro* fertilization of human oocytes

Treatment	No. of cycles examined	No. of oocytes cultured	No. of oocytes fertilized (%)[a]
IVF	8	78	60 (76.9)
ICSI	10	87	74 (85.1)

[a] No significant differences.

Table 2. Effects of fertilization methods on development of *in vitro* fertilized oocytes

Treatment	No. of embryos cultured	No. of blastocysts (%)				
		Total	BG1	BG2	BG3	Early
IVF	60	44 (73.3)[a]	38 (63.3)[a]	6 (10.0)	0 (0.0)	0 (0.0)[a]
ICSI	74	42 (56.8)[b]	10 (13.5)[b]	2 (2.7)	3 (4.1)	23 (31.9)[b]

[a-b] Within columns treatments with different superscripts are significantly different ($P<0.05$).

Table 3. Effects of fertilization methods on clinical pregnancies

Treatment	No. of ET cycles	No. of embryos transferred (/cycle)	Pregnancies (%)[a]
IVF	8	16 (2)	4 (50.0)
ICSI	10	20 (2)	2 (20.0)

[a] No significant differences.

were transferred into 8 patients (2/patient). In the ICSI group, 10 BG1 and 10 early blastocyst were transferred into 10 patients (2/patient). The mean number of embryos transferred was two and the other embryos were cryopreserved. The clinical pregnancy rate per cycle in the IVF group was 50% (4/8) compared with 20% (2/10) in the ICSI group. There was a trend for pregnancy rates to be higher in the IVF group than that in the ICSI group, however due to the small number of cycles involved in embryo transfer, the differences were not statistically significant (Table 3).

Discussion

Currently, embryos are transferred to the uterine cavity on day 2 or 3 before the blastocyst stage is reached in conventional IVF or ICSI cycles [5, 7]. There are two main reasons why embryos are transferred at this particular stage of development. Firstly, culture for 2 days allows fertilization and early cleavage to be confirmed and secondly, further culture decreases the number of embryos available for transfer due to degeneration or arrest in cleavage [2, 3]. This may be a reflection of sub-optimal culture conditions [2], although inherent abnormalities like chromosomal anomalies will also contribute to the loss [12, 13]. Therefore embryos are routinely transferred at the 4~6 cell stage to the uterus where they remain for 90~99 h prior to implantation [3]. Following in vivo fertilization, the human embryo undergoes cleavage in the fallopian tube and enters the uterine cavity only at the morula or early blastocyst stage [2]. As a result the 4~6 cell embryo remains for 70~75 hrs in the oviductal environment and then enters the uterine cavity where it spends only 20~24 hrs before the onset of implantation [3].

The rate of development of sperm injected oocytes to the blastocyst stage was low in this experiment, in common with others [6, 14, 15]. Although an earlier report demonstrated that sperm microinjection does not increase the incidence of chromosomally abnormal embryos [16], it is well known that the frequency of chromosomal abnormalities is high in preimplantation embryos and that most abnormal embryos are lost before pregnancy is recognized clinically [13]. Furthermore, these oocytes are exposed to hyaluronidase, intense light, and fluctuations in temperature and are subjected to the creation of an artificial breach in the zona pellucida and oolemma. This opening may increase the risk of introduction of toxins and debris into the perivitelline space and oolemma, all of which may affect oocyte quality [8]. It should be noted that an 85% fertilization rate per injected egg was achieved in our laboratory during the study period, and that the result is similar to those reported by the other groups [6–8].

Concerning the safety of using PVP, some reports have suggested that PVP solutions are toxic to mouse embryo development [17–19]. However, Motoishi *et al.* [20] suggested that the ICSI procedures currently used for animal and human ICSI are neither detrimental to embryonic development nor detrimental to embryo quality (blastulation and number of cells per blastocyst). On the other hand, it has been suggested that toxicity of PVP is related to its impurities. Van Steirtegham *et al.* [21] and Fujii *et al.* [22] have used dialysed PVP for human ICSI procedures and high fertilization and pregnancy rates have been achieved. However, we obtained

high fertilization and normal live birth using non-purified PVP in ICSI cycles (data not shown).

The phenomenon of sperm 'swimming across' is usually seen in the 8–10% PVP droplet during the regular ICSI procedure [23, 24] Fujii *et al.* [22] reported that 8% PVP could clearly separate motile spermatozoa but was not useful for weakly progressive motile spermatozoa, and tried to use a droplet intermediate between a 3% PVP and 8% PVP droplet. However, Dozortsev *et al.* [25] concluded that PVP at a concentration of 10% can indeed reduce the fertilization rate. Dozortsev *et al.* [26] 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 [27]. Schwartz *et al.* [28] reported that oocyte degeneration is not caused by the penetration of the glass needle into the ooplasm but by injury to the meiotic spindle, or by an excessive dose of fluid [polyvinylpyrrolidone (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 preparations using 10, 8, 5 and 3% PVP droplets. When sperms were placed in the 3% droplet, the sperms were motile longer than others (data not shown). We used a 3% PVP droplet for ICSI [29] and TESE-ICSI cycles [30], effectively.

Shoukir *et al.* [31] has reported the overall rate of blastocyst development from embryos following ICSI and IVF (26.8% versus 47.3%). The inherent deficiency of ICSI may be linked to a post-embryonic genome activation event where the influence of the paternal genome appears. Spermatozoa that have the ability to fertilize may not necessarily be able to contribute to further embryonic development [31]. We believe, therefore, that it is highly unlikely that faulty technique would explain the findings of the current study. There is no doubt that embryo quality may be affected by a traumatic injection that causes excessive disruption of the cytosol.

There was a trend that more embryos developed to the blastocyst stage in the IVF group than in the ICSI group (Table 2). The number of blastocyst embryos achieving BG1 was significantly higher ($P<0.05$) in the IVF group than in the ICSI group. Moreover, the pregnancy rate was higher in the IVF group than in the ICSI group (Table 3). It might be expected that ICSI embryos have a poor growth potential.

The ICSI procedure is currently performed for two main indicators: severe male factor infertility [5, 7] and low fertilization rates (<30%) in previous standard IVF treatment [5, 8]. Therefore, it may be postulated that the paternal or maternal factors that led to this type of infertility have a deleterious influence on the developing ICSI embryo, resulting in reduced embryo quality and poorer outcomes in comparison with conventional IVF with its "natural" selection mechanisms [8]. In all three blastocyst grades the features of in vitro development were described by Dokras *et al.* [3]. Although the further *in vitro* developmental potential of each blastocyst appeared to be related to its grade (BG), a more objective assessment is needed which can be related to these morphological findings [3, 32]. It has been previously shown that hCG can be detected in blastocyst culture media from day 7 onwards [3, 32]. In Dokras *et al.* [3], hCG is secreted by most blastocysts with concentrations rising to a maximum by day 10 or 11 and declining thereafter. There was a difference in the initial pattern of hCG secretion between BG1 and BG2, with BG1 secreting significantly higher amounts of hCG on days 8 and 9 [3]. There is a correlation between both the amount of blastocyst and quality, and their developmental potential [3, 32]. Although this study indicates the importance of blastocyst formation for increasing pregnancy rates, another prerequisite will be to increase the number of embryos reaching the blastocyst stage. In several species, it has been reported that co-culture embryos using feeder cells such as human ampullary cells [1], monkey *Vero* cells [2, 31, 32] and bovine uterine fibroblasts [33], have greater numbers of cells and a full same 'cohesive' inner cell mass when compared with embryos cultured in a simple cell-free medium at the same developmental stage [34]. In this study, *Vero* cells will be useful the effects of co-culture on the morphological development of human blastocysts and pregnancy rates. An increase in the number of BG1 would allow the appropriate selection of blastocysts for transfer to the uterus, and thereby the evaluation of the potential of blastocyst transfers in humans. Moreover, it would permit cryopreservation [11] and preimplantation diagnosis of genetic and chromosomal disorders [12, 35] at this stage of development.

This work has shown that there is an overall improvement in human embryo development when fertilization is performed in normal IVF. A higher number of embryos reached the blastocyst stage and blastocyst grade 1 (BG1) of those blastocysts capable of implantation. On the basis of these results, ICSI affects development of embryos to the blastocyst stage and their quality relates with developmental ability. A great deal more work still remains to be done on understanding the low developmental ability of ICSI and whether the presence of a functional gene relates to an embryo's ability to implant *in vitro*.

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