Journal of Assisted Reproduction and Genetics, Vol. 18, No. 12, 2001

CLINICAL ASSISTED REPRODUCTION

A Prospective Study on Oocyte Survival Rate After ICSI: Influence of Injection Technique and Morphological Features

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Submitted: May 2, 2001
Accepted: July 16, 2001

Purpose: *To determine the influence of technical pitfalls and oocyte morphology on survival rate and cleavage behavior after ICSI.*
Methods: *A total of 2210 injection procedures was examined for morphological and technical deviations. Survival rate and cleavage behavior were evaluated.*
Results: *In 77.8% of all cases ICSI was unsuspicous. Out of 491 deviations from optimal injection deep penetration of the oocyte and abundant presence of cumulus cells showed significant correlation with degeneration rate (*$p < 0.001$*). Morphological anomalies associated with the periphery of the oocyte were rather related to degeneration than cytoplasmic anomalies (*$p < 0.001$*). Early embryonic development was not impaired by technical or morphological parameters.*
Conclusions: *To conclude, these prospective data may be of prognostic value in regard of the number of embryos available for transfer and may help to improve treatment outcome.*

KEY WORDS: degeneration of oocytes; fertilization rate; ICSI technique; morphology of oocyte.

INTRODUCTION

While conventional IVF is successful in achieving pregnancies in couples without male factor infertility, it may fail in patients with severely compromised semen parameters. To overcome this drawback, certain methods of gamete micromanipulation have been introduced.

One approach was to pierce the oocyte zona pellucida, facilitating the access of spermatozoa to the perivitelline space (1). Although pregnancies could be achieved (2), results were not encouraging. Consequently, the next step was to inject motile spermatozoa under the zona pellucida (3). In fact, fertilization rates slightly increased (4) but the presence of a number of functional spermatozoa close to the oolemma often led to multiple sperm penetration (5). By depositing a single sperm directly into the cytoplasm, the last anatomical structure was bypassed. ICSI gave substantial fertilization and pregnancy rates (6,7).

Since intracytoplasmic sperm injection is more invasive than other micromanipulation techniques, there is a higher risk of irreversible damage to the injected oocyte such as lysis, shrinkage, and/or tanning of the egg. Although most embryologists have had the experience that a suboptimal injection technique may influence ICSI outcome as they progressed on their learning curve with micromanipulation, only few studies deal with degeneration of oocytes (8,9), none of them offering a solution of the problems.

This loss of potential embroys may not be of importance in patients with a high number of oocytes collected after ovarian hyperstimulation but it may reach clinical relevance if the number of harvested oocyte is low. In order to ensure embryo transfer even in women with low response every effort should be made, including practical experience and

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improvement in the technique of ICSI. Therefore, this prospective study was set up to evaluate whether problems in ICSI technique may cause degeneration after injection or impair early embryonic development.

MATERIALS AND METHODS

Two hundred and sixty patients, undergoing 320 ICSI cycles, were included in this prospective study. Immature oocytes showing a germinal vesicle were not included in the present evaluation. All couples suffered for tubal sterility and/or male subfertility. Women with polycystic ovaries and severe forms of endometriosis were excluded from investigation in order to minimize a possible influence of oocyte quality on the rate of degeneration in these patients. The mean age of the female patients was 32.5 ± 4.7 years.

All women were stimulated with a standard down-regulation protocol using either recombinant (Gonal F®, Serono, Vienna, Austria) or urinary medicamentation (Menogon®, Ferring, Vienna, Austria). Oocytes were aspirated 36 h after hCG administration by a transvaginal ultrasonographic procedure. Following follicular puncture the oocytes were cultured for at least 3 h in BM1 media (NMS Bio-Medical, Praroman, Switzerland) before they were exposed to 80 IU/mL hyaluronidase (2 min, MediCult, Copenhagen, Denmark) so as to facilitate mechanical removal of the cumulus cells. All morphological features of the oocytes were investigated immediately before ICSI. Anomalies related to cytoplasm (dark cytoplasm, refractile bodies, dark incorporations, vacuoles) and anomalies of the outer layer (fragile oolemma, dark zona pellucida, large perivitelline space, irregularity in shape) were pooled.

Semen was collected by masturbation and strictly analyzed. Ejaculates were washed and centrifuged in BM1 medium. A Mini swim-up technique was used to obtain a sufficient number of progressively motile spermatozoa for injection. The separated sperm of the supernatant was incubated in 10-μL droplets of fresh BM1 media on the injection dish (Falcon type 1006). Two small droplets of a PVP solution (MediCult, Copenhagen, Denmark) were also prepared. With this constellation of droplets under mineral oil contamination of the PVP, caused by debris carried by the sperm suspension, should be avoided.

The technique of ICSI has been described in detail (6). In brief, micromanipulation was performed on an inverted microscope ($\times$200 magnification, Olympus, Vienna, Austria) with Hoffman Modulation Contrast (Modulation Optics, Inc., Greenvale, NY), an electronically controlled heat stage and hydraulic micromanipulators (Luigs and Neumann, Ratingen, Germany).

At a magnification of $\times$400 a single spermatozoon, transferred from a BM1 droplet to one of the PVP droplets, was immobilized by mechanically damaging its tail with the injection pipette (outer diameter: 7 μm; Eppendorf, Hamburg, Germany). To perform ICSI, the oocyte was held in place with a holding pipette (outer diameter: 100 μm; Eppendorf, Hamburg, Germany) at 9 o'clock. The first polar body was located on the 6 o'clock position. As soon as the equatorial plane of the oocyte was focused the ICSI pipette was pressed against the zona pellucida creating a characteristic funnel at 3 o'clock. After penetrating both the zona and the oolemma a small volume of cytoplasm was aspirated into the glass tool to activate the egg. The single immotile spermatozoon was then gently placed near the horizontal axis. Withdrawal was done carefully to prevent the oocyte from leakage. Difficulties in penetrating the zona pellucida led us to replace a presumed low-quality glass tool.

All ICSI performances were done by one embryologist in order to minimize interindividual differences in technique (9,10). Recording on video (AG 7355, Panasonic, Vienna, Austria) guaranteed exact evaluation of the procedure. All deviations from a presumed optimal injection technique were analyzed by an independent embryologist. The main problems found were (i) difficult breakage of oolemma, (ii) spermatozoon remained attached to the ICSI pipette while being released, (iii) insufficient immobilization of the spermatozoon (as assessed by subsequent movement of the sperm's tail after injection), (iv) rejection of spermatozoon into perivitelline space after ICSI (as assessed by the sperm's tail protruding out of the zona pellucida), and (v) insufficient denudation of the oocyte.

At 16–20 h after injection, fertilization and survival were controlled. The presence of two pronuclei as well as two polar bodies characterized normal fertilization (2Pn). Early embryo development as assessed by the number of blastomeres and the percentage of fragmentation was evaluated 42 h after injection.

Fisher's exact test, chi-square test, and a stepwise logistic regression model were used to analyze variables in the form of frequency tables. All tests were two-tailed with a significance of 95% ($p < 0.05$).

Table I. Outcome of Unsuspicuous ICSI in Relation to Maturity and Morphology of Oocyte

Type of oocyte	*n*	2PN	0PN	Deg.	Blast.	Fragm.
No anomaly	810	607 (74.9)*	138	25 (3.1)*	3.7	12.4
Cytoplasmic anomaly	632	440 (69.6)	118	32 (5.1)	3.7	12.7
Anomaly in outer layer	241	161 (66.8)	50	30 (12.5)*	3.6	13.6
Metaphase I	65	20 (30.8)*	34	5 (7.7)	3.5	14.0

Note. blast.: mean number of blastomeres on day 2; deg.: degeneration; fragm.: mean number of fragments on day 2. Zygotes with abnormal fertilization (one or three pronuclei) are not listed.
* $p < 0.001$.

RESULTS

All in all, 2210 oocytes could be collected in 320 cycles, 65 of them being at metaphase I. The next day, 1493 (67.6%) zygotes (2Pn) were seen, whereas 414 (18.7%) eggs were not fertilized. One hundred and fifteen oocytes (5.2%) showed an abnormal number of pronuclei. The damage rate was 8.5% (188/2210).

In the group with an ICSI procedure considered as normal ($n = 1719$), 810 oocytes showed no morphological anomaly (47.1%). Eight-hundred and forty-four eggs showed either a cytoplasmic anomaly or an anomaly of the shell, 29 had both. All metaphase I oocytes were considered as normal, because no cytoplasmic anomalies were detected. A significant correlation was found between oocyte survival rate and anomalies in the outer layer of the egg ($p < 0.001$). Table I presents the results of all inconspicuous ICSI procedures in more detail.

With regard to the ICSI procedure, 77.8% of the injections were inconspicuous, while every fifth ICSI showed a deviation from the optimal condition described previously. Certain problems during ICSI could not be evaluated statistically because of a relatively small number (<10) of observations (attachment of ooplasm on spike after withdrawal of pipette; oocyte removed from holding pipette during injection). Insufficient denudation of the oocyte as well as difficult breakage of the membrane negatively influenced oocyte survival (Table II, $p < 0.001$).

Morphological features were not related to any of the observed deviations in ICSI procedure ($p > 0.05$).

DISCUSSION

Mechanical bypassing of both the zona pellucida and the oolemma may dramatically alter the dynamics of human gamete interaction but may, on the other hand, increase the risk of unprogrammed cell death. A lot of work has been done on fertilization rates in ICSI (11–15), whereas only few data are available, dealing with degenerated oocytes (9,10).

This is the first prospective study on a larger number of degenerated oocytes, taking into account both ICSI technique and morphological features.

Since the establishment of intracytoplasmic sperm injection in laboratory work (6,7) the number of couples who could benefit from ICSI has constantly been growing. Consequently, an increasing number of oocytes is available for injection, giving embryologists opportunity to practise. Thus, experience of the operator, a factor thought to influence ICSI outcome (9,14), may be limited as shown by the constantly high rates of oocyte survival (12,16,17).

Table II. Influence of Deviation in ICSI Procedure on Outcome

Type of deviation	*n*	2PN	0PN	Deg.	Blast.	Frag.
Difficult membrane breakage	136	78 (57.4)	16	40 (29.4)*	3.9	13.2
Attachment of sperm on pipette	120	87 (72.5)	15	11 (9.2)*,**	3.7	12.9
Insufficient sperm immobilization	63	46 (73.0)	12	2 (3.2)	4.0	13.8
Rejection of sperm in PVS	62	34 (54.8)	20	5 (8.1)	3.6	12.2
Insufficient oocyte denudation	110	75 (68.2)	12	20 (18.2)**	3.8	13.0
Total	491	320 (65.2)	75 (15.3)	78 (15.9)		

Note. blast.: mean number of blastomeres on day 2; deg.: degeneration; fragm.: mean number of fragments on day 2; PVS: perivitelline space. Zygotes with abnormal fertilization (one or three pronuclei) are not listed.
* $p < 0.001$; ** $p < 0.005$.

Prior to ICSI, oocytes have to be denuded, combining both an enzymatic and a mechanical method. Both steps hold a possible source of harm for the oocyte. To avoid a toxic effect of hyaluronidase on survival rate (13), concentration and incubation time were constantly kept low (2 min, 80 IU/mL) in the present study.

Another negative influence on survival during denudation may be caused by the usage of a hand-drawn glass pipette with a small inside diameter. A diameter of less than 250 μm (approximately twice the diameter of an oocyte) may irreversibly alter the morphology of the MII oocyte. Once the gamete is constricted, first polar body may move within the perivitelline space and would no longer be an accurate marker of spindle location (17), if it is at all (18). Thus, the ICSI pipette could pass the highly sensitive region of the presumed meiotic spindle and irreversibly harm further development. Another aspect to be taken into account is that compressing the oocyte during injection may somehow harm the cytoskeleton and therefore facilitate leakage of cytoplasm, with resultant degeneration (11). This may be favored by a bad quality injection pipette. Usage of uniform sterile glass capillaries with an outer diameter of approximately 7 μm causes a minimum of trauma and reduces the volume of PVP entering the oocyte.

Although good fertilization rates can be observed with the first polar body at almost any position during ICSI (15) we decided to constantly hold the first polar body at 6 o'clock. However, no difference in survival rate was found as compared to the 12 o'clock position (9,11).

A lot of work has been done to optimize outcome of ICSI procedure (12,14,16,19,20). In summary, these studies describe two factors being of importance for the establishment of a sucessful ICSI program: (i) immobilization of the spermatozoon before injection and (ii) aspiration of a small amount of cytoplasm into the injection pipette.

In approximately 3% of the cases, total immobilization of the spermatozoon could not be reached. By evaluating the oocyte at a higher magnification a local movement of the sperm's tail could be detected following ICSI. Probably, this problem resulted from touching the tail at an inappropriate angle (not parallel to the bottom of the injection dish). This three-dimensional problem may be solved by sucking the sperm in and out the injection pipette and thus breaking the 9+2 system of microtubules. However, in the case of motile spermatozoa, after ICSI the sperm's membrane must have been damaged because a constantly high rate of fertilization could be observed. Therefore, sperm decondensation and oocyte activation were not impaired (21,22).

In our study, four additional unexpected events leading to deviation from the recommended ICSI performance could be recorded.

Difficult breakage of the membrane, characterized by the impossibility to enter the egg without deforming it, was observed most frequently. Compared to the reports in Ref. (8), this deviation from standard ICSI procedure was less frequent in our study, although its influence was much more detrimental. This inconsistency may be the result of excessively deep penetration into the oocyte in the present study, possibly harming the opposite region of the oolemma. In order to overcome this form of penetration we improved survival rate by introducing a slightly adapted injection process combining a pressing phase (to the center of the oocyte) and a sucking phase (until rupture of the oolemma).

Moreover, 5% of all oocytes were insufficiently denuded either because of an excessively careful digestion process of the cumulus complex or because of a nonoptimal maturation of the oocyte (23). The problem caused by the abundant presence of cumulus cells was not blockage of the opening of the holding pipette or difficulties in observation of the oocyte (8), but much rather a hydraulic fixation problem on the holding pipette, resulting in a highly invasive injection process. Surrounding cells known to be without relevance for survival (13) may hinder optimal injection and, consequently, increase damage rate. Since spermatozoa may be injected almost at any position of the oocyte (15), this problem may be compensated by hydraulic manipulation of the oocyte until an optimal position for injection is reached.

Another phenomenon regularly observed was attachment of the sperm on the spike of the injection pipette during release of the gamete. Therefore, depositing the spermatozoon in the ooplasm required rotating movements of the injection pipete. This more or less invasive procedure did not influence ICSI outcome; it could rather be seen as additional activation of the oocyte.

If sperm release was not achieved after several attempts, the spermatozoon was frequently carried into the perivitelline space by the injection pipette. This situation somehow resembled the one seen with the SUZI technique (3), although the spermatozoon

was immotile. A decline in fertilization rate (not significant) may be due to different positions of the sperm within the oocyte. Some of the sperms must have been carried to the perivitelline space, whereas others, at least in part, remained within the oolemma allowing them to fertilize the egg.

None of these technical pitfalls did impair early embryonic development in vitro ($p > 0.05$). This finding is in line with previously published data (9). However, these authors found a decline in blastocyst formation rate in a subgroup with excessive cytoplasm aspiration. Since these results were based on a relatively small number of surplus embryos, its actual value still remains unclear.

In the absence of data indicating any correlation between single or multiple morphological anomalies and oocyte survival after ICSI (15,24,25) we decided to subdivide dysmorphic oocytes into two groups, depending on whether the anomaly was detected in the center or the periphery of the gamete. It could be demonstrated that anomalies associated with the ooplasm did not affect survival. On the other hand, anomalies of the outer layers correlated with degeneration. In most cases, brownish discoloration of the zona pellucida or sudden breakage of the oolemma indicated changes within the glycoprotein complex of the zona pellucida or the lipoproteic structure of the oolemma. These changes may be caused by the hormonal environment during controlled ovarian hyperstimulation (8). Therefore, a presumed protective sealing effect of the funnel cannot occur (26). What we called "anomalies of the outer shell" somehow resembles the "sudden breakage" pattern described by Palermo *et al.* (8). Consequently, the results in terms of survival are identical.

It is known that oocytes at metaphase I show no increased degeneration rate and may be fertilized with ICSI at a lower rate (27), probably because of the absence of first polar body extrusion. While our data confirm these findings, we have no information if these oocytes were collected at the germinal vesicle stage and matured in vitro or not.

To conclude, both morphological anomalies related to the zona pellucida and the oolemma and certain technical problems were found to negatively affect oocyte survival after ICSI. This study is the first to report a correlation between inadequate denudation of the oocytes and degeneration rate. These prospective data may be of prognostic value concerning a possible number of embryos available for transfer in patients with presumed poor prognosis ($\leq$3 oocytes collected).

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