CLINICAL ASSISTED REPRODUCTION

Assessment of Maximal Fertilization Rates with Intracytoplasmic Sperm Injection

SAI MA^{1,2} and BASIL HO YUEN¹

Submitted: April 12, 1999

Accepted: July 12, 1999

Purpose: Maximal fertilization rates following ICSI were assessed using two essential steps: immobilization of sperm and aspiration of oocyte cytoplasm.

Methods: ICSI procedure was performed for couples ($N = 42$) in whom the male suffered severe infertility or failure of fertilization in previous IVF cycle using different oocyte cytoplasmic aspiration and sperm immobilization methods. Outcome in four patient groups was measured by oocyte damage, fertilization rate, and pregnancy rate.

Results: Maximal fertilization (90%) were achieved from the group which used immobilization of sperm by hard-touching the tail with a pipette and optimal aspiration of oocyte cytoplasm.

Conclusions: The results suggest that if the immobilization of sperm and aspiration of oocyte cytoplasm are handled right during ICSI, this procedure can be expected to yield a 90% fertilization rate.

KEY WORDS: aspiration of oocyte cytoplasm; fertilization rates; immobilization of sperm; intracytoplasmic sperm injection.

INTRODUCTION

Intracytoplasmic sperm injection has become an established technique for treatment of severe male factor infertility. Although ICSI obtains high fertilization and pregnancy rates, the failure of fertilization still remains as high as 30%–40% in a successful ICSI program. Operator experience is undoubtedly important for the success, but other factors also exist.

During natural fertilization the naked sperm nucleus is incorporated into the oocyte cytoplasm following spermatozoon-egg membrane fusion, whereas during ICSI a whole sperm cell is deposited into the oocyte. There was enough evidence to support the hypothesis, that for ICSI to be successful, damage of the sperm plasma membrane should occur before intracytoplasmic injection. Dozortsev *et al.* (1) demonstrated that activation of the human oocyte after ICSI is due to a spermatozoon-associated oocyte-activating factor, and it has been suggested that the release of this factor into the ooplasm can only take place after the sperm plasma membrane has been removed or damaged (2). Oocytes, either unfertilized or activated, do not contain a factor which is able to remove the sperm plasma membrane (3). Furthermore, decondensation of the sperm nucleus is effected by the sperm nucleus decondensing-factor within the oocyte cytoplasm which is not able to penetrate the intact sperm plasma membrane (4). Thus, damage of the sperm membrane seems to be a prerequisite for both oocyte activation and male pronucleus formation following ICSI.

Another factor taken into account is the degree of aspiration of oocyte cytoplasm. Gearon *et al.* (5) and Tesarik and Sousa (6) have reported that the mechanical disruption of the ooplasm alone, and the degree of cytoplasmic aspiration, may enhance oocyte activation and subsequently increase the fertilization rate. The cytoplasmic aspiration itself may provide a mechanical stimulus to induce an important release of intracytoplasmic calcium stores (7).

Nowadays, two major steps in the injection procedure, i.e., immobilization of the spermatozoon and aspiration of cytoplasm, have been used in most IVF centers. By using these procedures, ICSI fertilization

¹ Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, British Columbia V5Z 1M9, Canada.

² To whom correspondence should be addressed at Room 313, Willow Pavilion, Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, British Columbia V5Z 1M9, Canada.

rates were similar to conventional IVF, but have not reached maximum. The aim of this paper is to report the importance of some technical parameters which could influence the outcome of ICSI in 42 attempts with couples having severe male factor infertility and dysfunction of fertilization.

MATERIALS AND METHODS

Patients

Between December 1995 and December 1996, 42 ICSI cycles were performed for couples in whom the male suffered severe male infertility or failure of fertilization in previous IVF cycles. The patient selection criteria for ICSI cycle with freshly ejaculated semen included either failed or very low fertilization rates in the previous standard IVF cycle(s) or poor sperm parameters as defined previously (8). Ovarian stimulation was carried out by a desensitizing protocol using the gonadotrophin-releasing hormone agonist synarel (Syntex, Inc., Mississauga, Ontario, Canada) in association with a human menopausal gonadotrophin (HMG, Fertinorm, Serono, Oakville, Canada or Humegon, Organo, Toronto, Canada) and a human chorionic gonadotrophin (HCG, Profasi, Serono, Oakville, Canada). Intravaginally administered progesterone was used for luteal phase supplementation.

Sperm Preparation

The evaluation of sperm concentration and motility was carried out according to World Health Organization criteria (9). Sperm morphology was determined using the strict Tygerberg criteria (10). In cases of severe oligoasthenoteratozoospermia, a modification of mini-swim-up method was used (11) to prepare the spermatozoa. The whole ejaculate was centrifuged at 400g in a 10-ml falcon tube for 2 min and resuspended in 0.5 ml Human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA) with 1% human albumin serum (Bayer, Etobicoke, Ontario, Canada). After transfer to an Eppendorf tube, the preparation was centrifuged, a second and third centrifugation for 2 min at 400g. Following these steps the supernatant was removed and the pellet was coated with 10–20 μ l medium and incubated at 37°C for 1–3 h. When fewer than 50,000 spermatozoa were present in the ejaculate, sperm suspension was concentrated in 5 μ l and transferred directly to the dish. Sperm was aspirated from the suspension and transferred into polyvi-

nylpyrrolidone (PVP; Irvine Scientific, Santa, Ana, CA) solution.

Oocyte Preparation

After oocyte retrieval, the oocytes were further incubated for 4 h. Immediately before micromanipulation, the cumulus-corona cells together with oocytes were replaced in an organ culture dish and digested in an HTF-HEPES-buffered medium (Irvine Scientific, Santa Ana, CA) containing 60 IU/ml of hyaluronidase (type VIII, Sigma, St Louis, MO) for no longer than 30 s. The free cells were removed by aspiration of the complex cumulus-corona into and out of a glass pipette with an inner diameter ranging between 180 and 220 μ m. Each oocyte was washed three times in HTF medium supplemented with 1% human serum albumin and then examined under the inverted microscope at 200 $\times$ magnification to assess integrity and maturation stage. The latter was determined by the presence of the first polar body (metaphase II stage). Intracytoplasmic sperm injection was only performed on oocytes that had reached metaphase II.

ICSI

Microinjection and holding pipettes with 20° angle were made by Cook IVF (Cook, Stouffville, Ontario, Canada). The outer and inner diameter of each injection pipette was 9.0 μ m and 7.0 μ m, respectively (Cook, K-MPIP-1020). The outer diameter of holding pipettes was 110 μ m and the corresponding inner diameter was 20 μ m (Cook, K-HPIP-1020). Prior to ICSI, 1 μ l of sperm suspension was diluted with 5 μ l of a 10% PVP solution in HTF-HEPES medium placed in the middle of the petri dish (Falcon type 3004). Each oocyte then was placed in a droplet of 5 μ l medium surrounding the central drop containing the sperm suspension-PVP. HTF-HEPES medium supplemented with 1% human serum albumin was used in the injection dish. The droplets were covered with mineral oil (Sigma, Oakville, Ontario, Canada). Up to four oocytes were placed in the injection dish to avoid exposure to the HEPES-buffered medium longer than 10 min.

The procedure was carried out on the heated stage of Zeiss inverted microscope with Hoffman Modulation Contrast Optics (Modulation Optics, Greenvale, NY). The microscope was equipped with two motor-driven coarse control manipulators and two hydraulic micromanipulators (MM-188 and MO-109; Narishige, Tokyo, Japan). The micropipettes were fitted to a tool holder controlled by two IM-6 microinjectors (Narishige). The holding and injection systems were

filled with light mineral oil. According to the states of sperm used and treatment for oocyte activation, 42 consecutive ICSI cycles were retrospectively analyzed in four groups.

In group A (14 ICSI attempts), sperm was immobilized by touching the tail either at its "tip" or in the "midpiece" (standard immobilization method). After holding the oocyte with the holding pipette and placing the polar body at the 12 or 6 o'clock position, the injection pipette was brought into contact with the zona pellucida of the oocyte at 3 o'clock, and the spermatozoon was moved to the tip of the injection pipette until the spermatozoon touched the zona pellucida. Then the injection pipette was pushed through the zona pellucida at the equatorial level until the opposite side of the oocyte was reached. The oocyte cytoplasm was slightly aspirated into the pipette at about 20–30 μm distance (slight aspiration). The cytoplasmic organelles and the spermatozoon then were slowly pushed back into the cytoplasm.

In group B (8 ICSI attempts), the spermatozoon was forcefully immobilized. The spermatozoon was positioned perpendicular to the tip of the pipette and was subsequently immobilized by rubbing its tail between the injection pipette and the bottom of the Petri dish until a bend, looped, or convoluted tail was observed (hard-touching). The oocyte cytoplasm was mildly aspirated not further than the border of the zona pellucida (mild aspiration).

In group C (10 ICSI attempts), method of sperm immobilization was the same as in group B, oocyte cytoplasm was aspirated further than the border of the zona pellucida (vigorous aspiration) (Fig. 1).

In group D (10 ICSI attempts), sperm immobilization was the same as groups B and C. Before expelling the immobilized spermatozoon, the oocyte cytoplasm was aspirated until an outflow of cytoplasmic organelles and the spermatozoon appeared in the injection pipette (optimal aspiration) (Fig. 2). The outflow of cytoplasm could occur either inside the border of the zona pellucida or beyond the border of the zona pellucida. After re-injection of the cytoplasm and immobilized spermatozoon, the injection pipette was withdrawn and the injected oocyte was released from the holding pipette.

Independent of the injection procedure used, the selected spermatozoon was aspirated tail first into the tip of the injection pipette. The injected oocytes were then washed four times in fresh HFT culture medium, placed in organ culture dishes, and incubated at an atmosphere of 5% CO_2 .

Assessment of Fertilization and Embryo Development

About 16–18 h after ICSI, the oocytes were observed under the inverted microscope for any sign of damage that may have resulted from microinjection and for the presence of pronuclei and polar bodies. Fertilization was considered normal when two distinct pronuclei with two polar bodies were present. On certain occasions one or three pronuclei were detected. Twenty-four h after ICSI, cleavage was assessed and the number and size of the blastomeres and the percentage of anucleated fragments were recorded. A further evaluation of cleavage was performed after an additional 24 h. Morphologically good quality embryos were transferred into the uterine cavity ~72 h after the microinjection procedure. A maximum of three embryos were transferred to each woman within the groups, with a mean age of 34 years (range 26–42). The mean age of women in group A was 34 years, 35 years in group B, 36 years in group C, and 34 years in group D.

Patients were subject to a serum β -HCG test 14 days after embryo transfer. Cycles in which the β -HCG concentration was elevated for a minimum of two consecutive tests at 7-day intervals were considered as positive β -HCG pregnancy. Pregnancies which were confirmed by the ultrasonic detection of a sac or a fetal heart beat 28 days after embryo transfer were recorded as clinical pregnancy.

Statistical Analysis

Outcome of ICSI in terms of fertilization and pregnancy rates in each group were analyzed with Pearson's χ^2 test and Yates' correction when necessary.

RESULTS

Of 444 oocyte-cumulus cell complexes retrieved in 42 ICSI cycles (mean 10.5 oocytes per cycle), 82.9% had extruded the first polar body, 13.5% were metaphase I, and 1.4% had the germinal vesicle. Among the 329 mature oocytes injected, 86% survived. Of 283 intact oocytes after ICSI, 68.2% showed two pronuclei, 3.5% one pronucleus, and 2.1% three pronuclei. One hundred and four embryos were transferred in 42 cycles (mean number per transfer 2.4). Remaining good quality embryos ($N = 42$) were cryopreserved in 14 cycles. A positive HCG titre was detected in 10 patients, in which a fetal heart beat was later detected. The overall pregnancy rate was 23.8%.

Fig. 1. Diagram presenting the procedure in group A, B, and C for intracytoplasmic sperm injection. (A) Slight cytoplasm aspiration and standard sperm immobilization methods were used. (B) Mild cytoplasm aspiration and hard-touching immobilization methods were used. (C) Vigorous cytoplasm aspiration and hard-touching immobilization methods were used.

A comparison of the ICSI procedure following injection of standard immobilization and hard-touching immobilization of sperm into cytoplasm with slight, mild, vigorous, and optimal aspiration of cytoplasm is summarized in Table I. Different rates of fertilization were observed among the groups, each corresponding to a change in the ICSI procedure used. The first increase in the percentage of two pronuclear oocytes

was observed between group A (where standard immobilization of sperm into cytoplasm with slight cytoplasmic aspiration was performed; 50% 39/76) and group B (where hard-touching method for immobilization of sperm and mild oocyte cytoplasmic aspiration were used; 66%, 41/62), although no statistical differences were found, most probably because of the limited sample size. When the same sperm immobilization method and vigorous cytoplasm aspiration was used (group C), there was no increase of normal fertilization rates between group B and group C (66% versus 63%).

A dramatic increase in the fertilization rate to 90% (73/81) was noted (group D) when an immobilized sperm (hard-touching method) was injected into ooplasm after optimization of oocyte aspiration, i.e., the oocyte cytoplasm was aspirated until an outflow of cytoplasm was visualized (Table I).

Independent of the semen categories, the fertilization rates corresponded to the changes in the ICSI procedure during the different time periods. The most remarkable increase was obtained when optimal aspiration and hard-touching immobilization methods were combined (Table II).

Oocyte damage dropped from 20%, 13.9%, 12.3%,

Fig. 2. Diagram presenting the procedure in group D. Sperm was immobilized with hard-touching method prior to sperm injection. Oocyte cytoplasm was aspirated until an outflow of cytoplasm was visualized in the injection pipettes.

Table I. Effect of Sperm Immobilization and Aspiration of the Cytoplasm on the Outcome of 42 Intracytoplasmic Sperm Injection Attempts

	A ^a	B	C	D	Total
No. of ICSI cycles	14	8	10	10	42
No. of injected oocytes	109	72	73	90	344
No. of intact oocytes	85	62	63	81	291
Percentages of intact oocytes (%)	78.0	86.1	86.3	90.0	84.6
Pronuclear (PN) status of the intact oocytes					
1PN (%)	6 (7.9)	3 (4.8)	1 (1.6)	0	10 (3.5)
2PN (%)	43 (50.6)	41 (66.1)	40 (63.5) ^b	73 (90.1) ^b	197 (67.7)
3PN (%)	3 (3.5)	0	0	3 (3.9)	6 (2.1)
No. of cleaved 2PN oocytes	30 (75.0)	39 (95.1)	36 (90.0)	68 (93.2)	173 (89.2)
No. of clinical pregnancies per ICSI cycle (%)	1 (7.7)	2 (25)	2 (20.1)	5 (50)	10 (23.8)

^a A = Group A; B = group B; C = group C; D = group D.

^b $P < 0.05$ (χ^2 test).

and 8.9% in group A through group D, respectively, over the four groups. Normal fertilization for each group was 50%, 65%, 62.5%, and 90.1%, respectively. Pregnancy rate per transfer was 7.7%, 22.2%, 20.0%, and 50.0% respectively. There was a significant difference between group D and other groups in terms of fertilization and pregnancy ($P < 0.005$). In addition, a significant difference was not observed in the proportion of parthenogenetically activated oocytes and oocytes displaying three pronuclei among the four groups. The mean proportion of single-pronuclear and three-pronuclear oocytes was 3.5% and 2.1%, respectively. After 42 transfers (87.5%; 2.4 embryos per transfer, maximum of three embryos), a total 10 clinical pregnancies were established, corresponding to a pregnancy rate of 23.8% per embryo transfer.

DISCUSSION

It has been a universal agreement that two essential steps determine the fate of fertilization following ICSI procedure, i.e., immobilization of sperm and aspiration

of oocyte cytoplasm (12). This study focused on whether the two steps could maximize the fertilization after ICSI.

To ensure normal sperm decondensation, partial sperm membrane damage prior to injection is required. However, when spermatozoa are immobilized in the standard way and used for injection, they are less successful than those used with forceful immobilization (13). It has been established that ICSI with sperm immobilization yields higher fertilization rates (14,15) and that external mechanical pressure increases permeability (1).

This study demonstrated that a modified immobilization method enables spermatozoa to achieve higher fertilization rates than those obtained with standard immobilization methods (50% versus 65%, group A and group B). This result is consistent with recent studies that have illustrated improved fertilization rates when a more extensive sperm tail disruption was performed prior to ICSI (13). This may promote membrane permeability and allow cytosolic sperm factors to access the oocyte and produce its activation (16). Tesarik *et al.* (7) showed that human oocytes undergo

Table II. Effect of Immobilization of the Spermatozoa and Cytoplasmic Aspiration on Survival, Fertilization, and Embryo Development Rates According to the Sperm Parameters or After Fertilization Failure

Total number of motile spermatozoa per ejaculate		A	B	C	D	Total
Immotile	N ICSI ^a	2	2	3	3	10
	2 PN (%)	7/13 (53.8)	4/9 (44.4)	18/27 (67.7)	27/30 (90.0)	56/79 (70.9)
<0.5 × 10 ⁶	N ICSI	5	2	2	3	12
	2 PN (%)	15/31 (48.4)	10/19 (52.6)	10/15 (66.7)	26/30 (86.7)	61/95 (64.2)
>0.5 × 10 ⁶	N ICSI	3	2			5
	2 PN (%)	11/23 (47.8)	8/12 (66.7)			19/35 (64.3)
Fertilization failure	N ICSI	4	2	5	4	15
	2 PN (%)	10/18 (55.6)	19/22 (86.4)	12/21 (57.1)	20/21 (95.2)	61/82 (74.4)

^a Number of ICSI cycles.

sperm-dependent multiple calcium oscillations after ICSI. Dozortsev *et al.* (1) demonstrated that sperm extracts can activate human oocytes. When a membrane intact spermatozoon was injected into an oocyte, only a monotonic increase of calcium was detected. In such a situation, the oocyte may fail to initiate the biochemical process necessary for oocyte activation (7). Furthermore, during oocyte activation, ooplasmic factors, such as thiol-reducing agents (17,18,19), are involved directly in the process of sperm chromatin decondensation. Immobilization of the spermatozoon by hard touching of its tail may initiate oocyte activation, while factors released from oocyte cytoplasm may lead to sperm chromatin decondensation.

In this study, after using the modified immobilization method and mild oocyte cytoplasm aspiration, a significant proportion of the oocytes still remained at metaphase II stage (35%). It seems that damage of the sperm membrane alone could not maximize fertilization rates after ICSI. Flaherty *et al.* (20), Tesarik and Sousa (21), and Ma and Ho Yuen (22) proposed that the failure of oocyte activation is the main cause of fertilization failure after ICSI. Flaherty *et al.* (20) showed that the oocyte contained a swollen sperm head which was arrested at various stages of decondensation, indicating that the oocyte had failed to activate and complete the second meiotic division. The data from the literature demonstrated that 11% (from 3% to 23%) of unfertilized oocytes contained prematurely condensed human sperm chromosomes after ICSI (22), which is a sign of immaturity of oocyte cytoplasm (23). It seems that the absence of oocyte activation in ICSI may be due to an inability of the oocyte to undergo cytoplasmic maturation or an inability of the oocytes to exhibit multiple Ca^{2+} oscillation after microinjection (7,23).

In natural fertilization, oocyte activation is triggered by the penetration of a spermatozoon. However, a variety of other triggers of mammalian oocyte activation have been described (24) and as yet there is still uncertainty as to which is the main initiating factor of human oocyte activation following ICSI. The data from the literature has provided evidence that the sperm cell itself contributes to the activation of oocytes by ICSI. However, the fact that some oocytes can also be activated in response to sham ICSI, indicates that pathenogenetic activation following ICSI may also occur (1). The hypothesis is that human oocytes can be activated at a high rate by needle puncture with the presence of a spermatozoon within the oocyte cytoplasm. In this study, after applying the improved immobilization method, further improvement of fertilization

was focused on oocyte cytoplasmic aspiration. Different oocyte aspiration methods were examined alone or together with either the standard immobilization method or the modified one. Although the vigorous oocyte aspiration combined with forceful immobilization of sperm was applied in group C, the fertilization rates remained similar to the former group (group B). It appears that the degree of oocyte cytoplasmic aspiration was not related to the enhancement of fertilization. Maximum fertilization (90%) was only achieved via the optimal aspiration of oocyte cytoplasm (the oocyte cytoplasm was aspirated until an outflow of cytoplasm was visualized), combined with the hard-touching sperm method. This data are in agreement with those obtained by Gearon *et al.* (5) and Tesarik and Sousa (21), who reported that the mechanical disruption of the ooplasm alone may increase the activation of the oocytes. Consequently, there was an increase in the fertilization rate.

It appears that improvement of the ICSI technique was the result of this success. Different hypothetical reasons may explain this success, for example an ability to undergo cytoplasmic maturation with optimal oocyte aspiration via alteration of maturation promoting factor (MPF) level, a release of cytosolic factors from the damage of sperm membrane with forceful immobilization of the sperm tail, or an ability of the oocytes to exhibit multiple Ca^{2+} oscillations resulting from the two crucial steps used in ICSI. The molecular mechanisms involved in the effect of damage of sperm membrane and optimal oocyte aspiration for this enhanced fertilization rates need further investigation.

In conclusion, although immobilization of sperm by "hard"-touching of the tail with the pipette is mandatory for enhancing fertilization, cytoplasm aspiration for oocyte activation is another crucial step. If these steps are handled correctly, ICSI can be expected to yield a 90% fertilization rate.

ACKNOWLEDGMENTS

We thank the clinical and laboratory staff of the UBC IVF Program in the Division of Reproductive Endocrinology and Infertility.

REFERENCES

1. Dozortsev D, Rybouchkin A, Sutter PDe, Qian C, Dhont M: Human oocyte activation following intracytoplasmic injection: The role of the sperm cell. *Hum Reprod* 1995;10:403-407

2. Swann K, Homa S, Carroll J: An inside job: The results of injecting whole sperm into eggs support one view of signal transduction at fertilization. *Hum Reprod* 1994;9:978–980
3. Meleszewski M: Decondensation of mouse sperm chromatin in cell-free extracts: a micromethod. *Mol Reprod Dev* 1990; 27:244–248
4. Dozortsev D, De Sutter P, Dhont M: Behavior of spermatozoa in human oocytes displaying no or one pronucleus after intracytoplasmic sperm injection. *Hum Reprod* 1994;9:2139–2144
5. Gearon C, Taylor A, Forman R: Factors affecting activation and fertilization of human oocytes following intracytoplasmic injection. *Hum Reprod* 1995;10:896–902
6. Tesarik J, Sousa M: Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca²⁺ fluxes and oocyte cytoplasmic dislocation. *Fertil Steril* 1995;64:770–776
7. Tesarik J, Sousa M, Testart J: Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod* 1994;9:511–518
8. Van Steirteghem A, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, Derde MP, Van Assche E, Devroey P: Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum Reprod* 1993;8:1055–1060
9. WHO Specific Programme of Research Development and Research Training in Human Reproduction: WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. New York, Cambridge University Press, 1992
10. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K: Sperm morphologic features as prognostic factor in vitro fertilization. *Fertil Steril* 1986; 46:1118–1123
11. Al Hasani S, Kupker W, Baschat AA: Mini-swim-up: A new technique of sperm preparation for intracytoplasmic sperm injection. *J Assis Reprod Gene* 1995;12:428–433
12. Vanderzwalmen P, Bertin G, Lejeune, B, Nijs M, Vandamme B, Schoysman R: Two essential steps for a successful intracytoplasmic sperm Injection: injection of immobilized spermatozoa after rupture of the oolema. *Hum Reprod* 1996;11:540–547
13. Palermo GD, Schlegel PN, Colombero LT, Zaninovic N, Moy F, Rosenwaks Z: Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum Reprod* 1996;11:1023–1029
14. Palermo G, Joris H, Derde MP, Camus M, Devroey P, Van Steirteghem A: Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Hum Reprod* 1993;4:826–835
15. Fishel S, Dowell K, Timson J, Green S, Hall J, Klentzteris L: Micro-assisted fertilization with human gametes. *Hum Reprod* 1993;8:1780–1783
16. Dozortsev D, Rybouchkin A, Sutter P, Dhont M: Sperm plasma membrane damage prior to intracytoplasmic sperm injection: A necessary condition for sperm nucleus decondensation. *Hum Reprod* 1995b;11:2960–2964.
17. Perealts S, Wolf R, Zirkin B: The role of disulfid bond reduction during mammalian sperm nuclear decondensation in vitro. *Dev Biol* 1984;101:160–167
18. Homa S, Swann K: A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. *Hum Reprod* 1994;9:2356–2361
19. Van Blerkom J, Davis P, Merriam J: A retrospective analysis of unfertilized and resumed parthenogenetically activated human oocytes demonstrates a high frequency of sperm penetration. *Hum Reprod* 1994;9:2381–2388
20. Flaherty S, Payne D, Swann N, Matthews C: Etiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1995;10:2623–2629
21. Tesarik J, Sousa M: More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. *Fertil Steril* 1995;63:343–349.
22. Ma S, Ho Yuen B: Prematurely condensed sperm chromosomes and In Vitro Fertilization. *Assisted Reprod Reviews* 1998; 8:26–130
23. Calafell JM, Badenas J, Egozue J. et al: Prematurely chromosome condensation as a sign of oocyte immaturity. *Hum Reprod* 1991;6:017–1021
24. Kaufman, MH: Early Mammalian Development: Parthenogenetic Studies. UK, Cambridge University Press, 1983