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Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures

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Objective: To examine whether sperm-borne and oocyte-borne oocyte activation failures can be overcome by mechanical means that entail modifying the ICSI technique.

Design: Case report series.

Setting: Private clinics.

Patient(s): Six infertile couples undergoing ICSI.

Intervention(s): Standard ICSI and modified ICSI based on mechanical manipulation that facilitated entry of calcium into the oocyte.

Main Outcome Measure(s): Fertilization rate and pregnancy outcome.

Result(s): In three cases of sperm-borne and three cases of oocyte-borne oocyte activation deficiencies, the modified ICSI technique enabled normal fertilization and development of embryos with good morphology. In terms of fertilization, the efficacy of modified ICSI was similar to use of a calcium ionophore, without producing extensive embryo fragmentation during postfertilization development. Term pregnancies resulting in the birth of normal children were achieved with the modified ICSI technique in five cases.

Conclusion(s): Sperm-borne and oocyte-borne oocyte activation failures can be overcome by modifying the ICSI technique. The modification obviates the need to use insufficiently tested and potentially harmful drugs. (Fertil Steril® 2002;78:619–24. ©2002 by American Society for Reproductive Medicine.)

Key Words: Oocyte activation failure, globozoospermia, intracytoplasmic sperm injection, calcium, ionophore

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Most fertilization failures after ICSI are caused by failure of oocyte activation (1, 2). Oocyte activation relies both on sperm-borne oocyte-activating factors and on oocyte-borne elements that transduce the sperm-derived signal to the oocyte's cell-cycle controlling systems via specific mechanisms (3).

Of the abnormalities that cause oocyte activation failure, sperm-borne failure associated with round-headed spermatozoa (globozoospermia) has been researched most extensively (4, 5). On the basis of the observation that fertilization failures after ICSI can be overcome by artificially increasing the free intracellular calcium concentration (2), ionophore-boosted oocyte activation was applied in assisted reproduction attempts using spermatozoa from globozoospermic patients (6, 7).

Little is known about oocyte-borne oocyte activation failures and how to treat them. Moreover, the clinical use of ionophores in assisted reproduction is limited by insufficient knowledge about their potential cytotoxic, teratogenic, and mutagenic effects on oocytes and embryos.

In a previous study, we showed that mechanical techniques of micromanipulation in ICSI can produce increases in intracellular calcium concentration leading to potentiation of oocyte activation similar to that produced by ionophores (8). In the current study, we sought to determine whether micromanipulation-

driven calcium entry into ICSI-treated oocytes can be used as an alternative to ionophores in cases of sperm-borne and oocyte-borne oocyte activation failure.

MATERIALS AND METHODS

We studied six cases of repeated oocyte activation failure. Three cases were caused by a sperm deficiency, and the other three originated from the oocyte.

Two modifications of the standard protocol, one involving the use of a modified ICSI procedure and the other using treatment of sperm-injected oocytes with calcium ionophore, were performed in an attempt to overcome oocyte activation failure. We obtained institutional review board approval for application of these modified ICSI procedures.

Basic Diagnostic Examinations

The diagnosis of male infertility was based on physical examination, basic sperm analysis, and measurement of serum FSH, LH, testosterone and estradiol. Female examinations included a basic gynecologic check-up; vaginal ultrasonography; hysterosalpingography; postcoital testing; and measurement of serum FSH, LH, prolactin, estradiol, progesterone, and testosterone.

Heterologous ICSI Testing

In cases in which the basic diagnostic examinations failed to distinguish between sperm-borne and oocyte-borne oocyte activation failure, heterologous ICSI testing was performed, as described elsewhere (4). In brief, metaphase II oocytes were obtained from mice superovulated by s.c. injection of 8 IU of pregnant mare serum gonadotropin, followed by intraperitoneal injection of hCG 48 hours later. The mice were killed by cervical dislocation 13 to 14 hours after hCG administration. After removal of cumulus and corona cells, oocytes were injected at laboratory temperature with the male partner's spermatozoa. The holding and injection pipettes used for mouse ICSI were prepared as described elsewhere (4).

For each case, oocytes from three to four animals were mixed together and separated at random into three groups: One group was to be injected with the male partner's spermatozoa, and the other two received living or dead donor spermatozoa. Oocytes injected with spermatozoa from a donor of proven fertility served as a positive control, and oocytes injected with spermatozoa from the same sample that were subjected to an additional cycle of freezing and thawing in phosphate-buffered saline (unprotected freezing and thawing) shortly before ICSI served as a negative control.

Each group consisted of at least 20 oocytes. Oocyte activation was assessed between 10 and 14 hours after ICSI by examining the oocytes for the presence of pronuclei and polar bodies.

Clinical Procedures

Controlled ovarian stimulation, oocyte recovery, surgical sperm recovery by fine-needle aspiration or open testicular biopsy, embryo transfer, and luteal phase support after embryo transfer were performed as described elsewhere (9).

Standard ICSI Procedure

The cumulus oophorus was removed from each oocyte–cumulus complex by brief incubation (20–30 seconds) in a solution containing 40 IU/L hyaluronidase (Hyase; Scandinavian IVF Science, Gothenburg, Sweden) that was prepared by diluting the original hyaluronidase solution (800 IU/L) in IVF-50 medium (Scandinavian IVF Science). The remaining corona radiata cells were then removed from oocytes by repeated aspiration into a finely drawn denuding pipette (SAGE BioPharma, Bedminster, NJ).

Oocytes were subjected to ICSI 4 to 8 hours after recovery by using techniques and instruments described elsewhere (10). Oolemma breakage was achieved by aspirating ooplasm into the microinjection needle (ICSI micropipette; Humagen Fertility Diagnostics, Charlottesville, VA) in a manner similar to "gentle aspiration" (8).

The ICSI oocytes were cultured at 37°C in IVF-50 medium equilibrated with 5% CO_2 in air. They were checked for signs of fertilization 12 to 16 hours after ICSI, and normally fertilized oocytes (those with two pronuclei and two polar bodies) were cultured for an additional 32 to 36 hours under the same conditions. Embryo transfer was performed on the second or on the third day after ICSI.

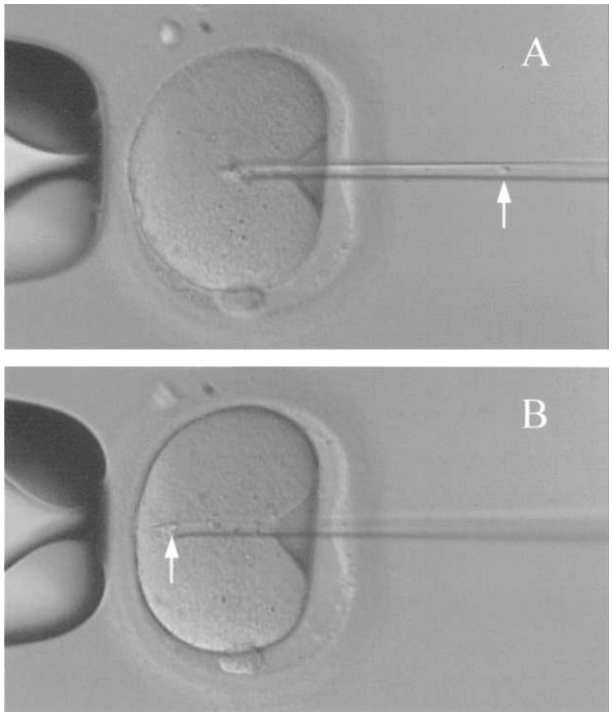
Modified ICSI Procedure

The only difference between the standard and modified ICSI procedures involved the micromanipulation technique between insertion of the microinjection needle into the oocyte and expulsion of the spermatozoon from the needle to the ooplasm. The insertion of the microinjection needle was performed with the spermatozoon located as close as possible to the tip of the needle. Oolemma breakage was achieved by vigorous aspiration of the ooplasm into the needle (8). This aspiration was performed with the needle tip located in the oocyte central area (Fig. 1A) to reduce the risk of disturbing the area occupied by oocyte chromosomes.

The ooplasm was aspirated until the distance between the spermatozoon in the needle and the outer surface of the zona pellucida was equal to the distance between the outer surface of the zona pellucida and the center of the oocyte (Fig. 1A). The needle tip was subsequently pushed as close as possible to the oocyte periphery opposite to the puncture site, and the aspirated ooplasm was reinjected to the oocyte until the spermatozoon reached the needle tip (Fig. 1B). The needle was then pulled back until the needle tip reached the central area of the oocyte, and the same aspiration and reinjection procedure was repeated once more. Care was taken to avoid lateral deviations of the needle from the axis of the original trajectory during these movements. The needle tip was

FIGURE 1

Modified ICSI procedure. (**A**), Culmination of the ooplasmic aspiration phase. The needle tip is located in the center of the oocyte, and the distance of the sperm head (*arrow*), marking the front of the aspirated ooplasm column, from the outer surface of the zona pellucida is equal to the distance between the outer surface of the zona pellucida and the oocyte center. (**B**), Culmination of the ooplasmic reinjection phase. The needle tip is located in the oocyte periphery opposite to the puncture site. The sperm head (*arrow*) is visible at the needle tip.



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pushed as close as possible to the oocyte periphery opposite to the puncture site again, and the spermatozoon was gently expulsed from the needle to the ooplasm.

Treatment of Sperm-Injected Oocytes With Calcium Ionophore

Within 10 to 30 minutes after ICSI, the injected oocytes were treated with ionophore A23187 (Sigma, St. Louis, MO) as described elsewhere (2). The ionophore was dissolved in cell-culture tested dimethylsulfoxide (Sigma) at a concentration of 1 mg/mL. The final solution containing 10 μM of ionophore A23187 was prepared by diluting the stock solution of ionophore in IVF-50 medium equilibrated with 5% CO_2 in air. The final solution was used immediately after preparation to avoid interaction of ionophore with proteins in the medium. The incubation time was 10 minutes, and the temperature was 37°C.

TABLE 1

Mouse oocyte activation after ICSI with spermatozoa from case-patients and from positive and negative control sperm samples.

Sperm source	No. of oocytes injected	No. of surviving oocytes	No. (%) of activated oocytes (one or two pronuclei)
Case 1	23	18	0 (0)
Case 2	21	17	0 (0)
Case 3	24	18	0 (0)
Case 4	24	17	15 (88)
Case 5	20	15	12 (80)
Case 6	21	17	16 (94)
Positive control[a]	131	107	99 (93)
Negative control[b]	130	105	2 (2)

[a] Spermatozoa from donors of proven fertility.

[b] Donor spermatozoa treated by unprotected freezing and thawing.

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RESULTS

Sperm-Borne Oocyte Activation Defects

Case 1

A 39-year-old man and a 37-year-old woman had primary infertility caused by male factor. Semen examination revealed 100% round-headed spermatozoa and moderate asthenozoospermia (22% motile spermatozoa). All other diagnostic tests had normal results. Heterologous ICSI testing with mouse oocytes revealed complete failure of oocyte activation by the patient's spermatozoa (Table 1). The couple had undergone one assisted reproduction attempt before presenting to our clinic. None of eight metaphase II oocytes subjected to ICSI was fertilized.

We then attempted ICSI using the standard protocol. The injected oocytes were exposed to ionophore A23187 to boost oocyte activation. Eight metaphase II oocytes were injected. On the day after ICSI, five oocytes showed signs of normal fertilization (two pronuclei and two polar bodies), one oocyte was fertilized abnormally (one pronucleus and two polar bodies), and two oocytes remained in metaphase II. Of the five normally fertilized oocytes, only two underwent cleavage and were transferred to the patient's uterus 3 days after ICSI. The remaining three zygotes became multifragmented and were discarded. No pregnancy occurred.

A third ICSI attempt was performed by using the modified ICSI technique, without ionophore treatment. Of 11 metaphase II oocytes injected, 7 showed signs of normal fertilization and 2 were fertilized abnormally (one pronucleus and two polar bodies). All 7 normally fertilized oocytes underwent cleavage. Three embryos with the best morphology and more than six blastomeres were transferred to the

patient's uterus 3 days after ICSI. A singleton pregnancy was established that resulted in the birth of a healthy boy.

Case 2

A 37-year-old man and a 35-year-old woman had primary infertility caused by male factor. Semen examination showed severe oligoasthenozoospermia with 100% immotile spermatozoa and 100% globozoospermia. Supravital eosin staining showed more than 99% dead cells among ejaculated spermatozoa. Heterologous ICSI testing with mouse oocytes revealed complete failure of oocyte activation by the patient's spermatozoa (Table 1).

An assisted reproduction attempt was performed using spermatozoa recovered from the testis by fine-needle aspiration. Fifteen percent of these spermatozoa showed slow, nonprogressive movement. Only motile spermatozoa were selected for ICSI.

Seven metaphase II oocytes were recovered. Four of them were microinseminated by using the standard ICSI technique followed by ionophore treatment, and the other three oocytes were microinseminated by using the modified ICSI protocol with no additional treatment. Three of the ionophore-treated oocytes were fertilized normally (two pronuclei and two polar bodies) and one was fertilized abnormally (one pronucleus and two polar bodies). Two of the three oocytes that were not treated with ionophore also fertilized normally, and the remaining oocyte remained in metaphase II.

A high degree of fragmentation was observed in all ionophore-treated fertilized oocytes during subsequent in vitro culture. On the third day after ICSI, two of the embryos created with standard ICSI were multifragmented and had no apparent blastomeres; these embryos were discarded. In contrast, both embryos that developed after fertilization by the modified ICSI technique without ionophore treatment showed excellent morphology. These two embryos were transferred to the patient's uterus along with the one remaining embryo from the ionophore group. No pregnancy occurred.

Case 3

A 35-year-old man and a 34-year-old woman had primary infertility caused by male factor. Semen examination showed severe oligoasthenoteratozoospermia but no round-headed spermatozoa. All other male and female diagnostic examinations were normal.

Heterologous ICSI testing with mouse oocytes revealed a complete failure of oocyte activation by the patient's spermatozoa (Table 1). The couple had undergone two assisted reproduction attempts before presenting at our clinic, both of which resulted in lack of fertilization.

At the third attempt, which we performed, 12 metaphase II oocytes were recovered. All of the oocytes were microinseminated by using the modified ICSI protocol with no additional treatment. On the day after ICSI, 10 oocytes showed signs of normal fertilization (two pronuclei and two polar bodies), one oocyte was fertilized abnormally (three pronuclei and one polar body), and one oocyte remained in metaphase II.

All 10 of the normally fertilized oocytes underwent cleavage. All of the cleaved embryos showed excellent (five embryos) or good (five embryos) morphology on days 2 and 3 after ICSI. Three of the embryos were transferred to the patient's uterus 3 days after ICSI. The remaining seven embryos were frozen. A singleton pregnancy was established and resulted in the birth of a healthy girl.

Oocyte-Borne Oocyte Activation Defects

Case 4

A 42-year-old man and a 38-year-old woman had primary infertility of unknown etiology. Semen examination revealed no anomalies. All other male and female diagnostic examinations had normal results.

Heterologous ICSI testing with mouse oocytes resulted in normal oocyte activation by the patient's spermatozoa (Table 1). The couple had undergone one assisted reproduction attempt before presenting at our clinic. None of seven metaphase II oocytes subjected to ICSI was fertilized.

During a second attempt, which we performed, eight metaphase II oocytes were recovered. All oocytes were microinseminated by using the modified ICSI protocol and no additional treatment. On the day after ICSI, six oocytes showed signs of normal fertilization (two pronuclei and two polar bodies) and two oocytes remained in metaphase II. Of the six normally fertilized oocytes, five underwent cleavage.

Three embryos with the best morphology were transferred to the patient's uterus 2 days after ICSI. The remaining two embryos were frozen. A singleton pregnancy was established and resulted in the birth of a healthy girl.

Case 5

A 40-year-old man and a 36-year-old woman had primary infertility of unknown etiology. Semen showed moderate oligoasthenozoospermia. The woman had mild endometriosis. All other diagnostic examinations were normal.

Heterologous ICSI testing with mouse oocytes resulted in normal oocyte activation by the patient's spermatozoa (Table 1). The couple had undergone two assisted reproduction attempts before presenting at our clinic. On both occasions, more than 10 metaphase II oocytes were obtained, but the fertilization and cleavage rates were poor. Only one embryo was transferred during the former attempt, and no embryo transfer was performed during the latter attempt, in which the only fertilized oocyte failed to cleave.

At a third attempt, which we performed, nine metaphase II oocytes were recovered. All of them were microinsemi-

nated by using the modified ICSI protocol with no additional treatment. On the day after ICSI, eight oocytes showed signs of normal fertilization (two pronuclei and two polar bodies) and one oocyte was fertilized abnormally (three pronuclei and one polar body). All of the eight normally fertilized oocytes underwent cleavage.

Three embryos with the best morphology were transferred to the patient's uterus 2 days after ICSI. The remaining six embryos were frozen. No pregnancy occurred after the fresh embryo transfer. The subsequent transfer of three of the six cryopreserved embryos resulted in a twin clinical pregnancy and the birth of two healthy girls.

Case 6

A 36-year-old man and a 32-year-old woman had primary infertility of unknown etiology. Semen analysis showed moderate oligoasthenozoospermia. All other diagnostic examinations had normal results.

Heterologous ICSI testing with mouse oocytes resulted in normal oocyte activation by the patient's spermatozoa (Table 1). The couple had undergone three assisted reproduction attempts before presenting at our clinic. On each occasion, more than 10 metaphase II oocytes were obtained (18, 16, and 14 oocytes at attempts 1, 2, and 3, respectively). The respective rates of normal fertilization were 2 of 18 oocytes (11%), 1 of 16 oocytes (6%), and 1 of 14 oocytes (7%). Only one embryo was transferred in the first two attempts, and no embryo transfer was performed during the last attempt, in which the only fertilized oocyte failed to cleave.

At a fourth attempt, which we performed, 14 metaphase II oocytes were recovered. All of the oocytes were microinseminated by using the modified ICSI protocol with no additional treatment. On the day after ICSI, 11 oocytes showed signs of normal fertilization (two pronuclei and two polar bodies), 2 were fertilized abnormally (one pronucleus and two polar bodies), and 1 remained in metaphase II. All 11 normally fertilized oocytes underwent cleavage.

Three embryos with the best morphology were transferred to the patient's uterus 2 days after ICSI. Seven of the remaining eight embryos were frozen, and one embryo became multifragmented and was discarded. A singleton clinical pregnancy was established and resulted in the birth of a healthy boy.

DISCUSSION

We found that a modified ICSI technique can be used to overcome oocyte activation defects of different etiologies. Two of the three cases of sperm-borne defects in our study were associated with globozoospermia. Spermatozoa from some (4–7) but not all (11–13) globozoospermic patients fail to activate oocytes after ICSI. Our two patients with globozoospermia belonged to the former category. In one case, sperm-borne oocyte activation failure was suspected in one case on the basis of fertilization failures in previous ICSI attempts, and it was definitely demonstrated in both cases by complete failure of oocyte activation on heterologous human/mouse ICSI testing.

The third case of sperm-borne oocyte activation failure in our study showed a similar deficiency in oocyte activation that was confirmed by heterologous ICSI testing. However, this functional anomaly was not associated with globozoospermia. In all three of these cases, the oocyte activation defect was overcome by a simple modification of the micromanipulation technique during ICSI, with no need to use chemical agents, such as ionophores.

The oocyte-borne origin of the three remaining cases of oocyte activation failure was revealed by the normal performance of spermatozoa on heterologous ICSI testing. In addition, the modified micromanipulation technique during ICSI overcame the oocyte activation defect, in contrast with the poor fertilization results obtained in previous attempts using standard ICSI.

This is the first study in which an oocyte-borne oocyte activation defect was unequivocally recognized and efficiently treated. Unlike for sperm-borne oocyte activation defects, it is not known whether alternative treatments for boosting oocyte activation, such as the use of calcium ionophores, would be effective for this indication.

It is interesting that oocyte activation defects of apparently different etiology can be overcome by using the same, relatively simple mechanical manipulation technique. Using confocal laser-scanning fluorescence microscopy in conjunction with fluorescent probes for calcium visualization in living cells, we have shown that vigorous aspiration of oocyte cytoplasm and repeated in-and-out movements of the microinjection needle within the human oocyte during ICSI produce a considerable influx of calcium ions from the surrounding culture medium into the oocyte (8). It is also possible that mechanical disruption of endoplasmic reticulum during the movements of the microinjection needle in the oocyte cytoplasm and repeated aspiration releases calcium stored in this organelle. The modified ICSI technique that we describe increases the intracellular concentration of free calcium ion in the injected oocyte compared with the standard ICSI technique.

On the basis of empirical findings and theoretical considerations, human oocyte activation appears to be controlled by a two-step mechanism that is driven by two sequentially acting factors called *trigger* and *oscillator* (3). The former factor is dependent on the interaction between the sperm and the oocyte surfaces during natural fertilization; in contrast, during ICSI, it is substituted by a micromanipulation-generated calcium influx called *pseudotrigger* (3). In natural fertilization and ICSI, the actions of the oscillator depend on specific compounds released from the spermatozoon to the oocyte cytoplasm. The oscillator is responsible for resetting

the components of the oocyte that are involved in calcium homeostasis (calcium channels and pumps in the plasma membrane and endoplasmic reticulum membrane) in a manner that facilitates the oscillatory response to an increase in the free intracellular calcium concentration (3).

The modified ICSI technique described here cannot alter the function of the oscillator component of the oocyte activation mechanism. However, this micromanipulation method strongly enhances the trigger intensity by facilitating entry of external calcium into the oocyte. None of the sperm-borne or oocyte-borne oocyte activation failures reported here could have been caused by a defect in the trigger function, which is not relevant to fertilization after ICSI (3); all of the cases must therefore have been caused by a deficiency of the oscillator. Hence, our data suggest a link between the trigger and the oscillator functions, in that artificial enhancement of the former may alleviate a deficiency of the latter, regardless of whether the deficiency is due to suboptimal signal output from the spermatozoon or to a failed response mechanism of the oocyte.

The normal pattern of oocyte activation events, including a series of autonomous calcium oscillations, appears to be restored by artificially increasing the oocyte calcium load at the time of ICSI. A single transient increase in calcium in human oocytes subjected to ICSI, with no subsequent oscillations in calcium level, is not sufficient for normal fertilization (14, 15). In our study, we did not directly test this hypothesis because of ethical concerns about destruction of human fertilized oocytes that such an analysis would necessitate.

Regardless of the underlying mechanism by which the micromanipulation technique described here overcomes oocyte activation failures, our findings may have an immediate clinical application in patients with repeated fertilization failures after ICSI suspected to be caused by insufficiency of sperm-borne oocyte activation factors or by a defective oocyte response to these sperm factors. Our technique represents an alternative to the use of calcium ionophores, which have been shown to have a similar effect in cases of sperm-borne oocyte activation failures (2, 6). The possibility of using a simple modification of the standard ICSI micromanipulation technique instead of ionophores alleviates concerns about the possible harmful effects on human embryos of these insufficiently tested drugs. The modified ICSI technique may also reassure patients who would benefit from assisted oocyte activation and facilitate institutional review board approvals of clinical studies.

References

1. Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. Hum Reprod 1994;9:2374–80.
2. Tesarik J, Sousa M. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial oocyte activation with calcium ionophore. Fertil Steril 1995;63:343–9.
3. Tesarik J, Mendoza C. In vitro fertilization by intracytoplasmic sperm injection. Bioessays 1999;21:791–801.
4. Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P, Dhont M. Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum Reprod 1996;11:2170–5.
5. Battaglia DE, Koehler JK, Klein NA, Tucker MJ. Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. Fertil Steril 1997;68:118–22.
6. Rybouchkin AV, Van der Straeten F, Quatacker J, De Sutter P, Dhont M. Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil Steril 1997; 68:1144–7.
7. Kim ST, Cha YB, Park JM, Gye MC. Successful pregnancy and delivery from frozen-thawed embryos after intracytoplasmic sperm injection using round-headed spermatozoa and assisted oocyte activation in a globozoospermic patient with mosaic Down syndrome. Fertil Steril 2001;75:445–7.
8. Tesarik J, Sousa M. Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. Fertil Steril 1995;64:770–6.
9. Ubaldi F, Nagy ZP, Rienzi L, Tesarik J, Anniballo R, Franco G, et al. Reproductive capacity of spermatozoa from men with testicular failure. Hum Reprod 1999;14:2796–800.
10. Rienzi L, Ubaldi F, Anniballo R, Cerulo G, Greco E. Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. Hum Reprod 1998;13:1014–9.
11. Liu J, Nagy Z, Joris H, Tournay H, Devroey P, Van Steirteghem A. Successful fertilization and establishment of pregnancies after intracytoplasmic sperm injection in patients with globozoospermia. Hum Reprod 1995;10:626–9.
12. Trokoudes KM, Danos N, Kalogirou L, Vlachou R, Lysiotis T, et al. Pregnancy with spermatozoa from a globozoospermic man after intracytoplasmic sperm injection treatment. Hum Reprod 1995;10:880–2.
13. Stone S, O'Mahony F, Khalaf Y, Taylor A, Braude P. A normal livebirth after intracytoplasmic sperm injection for globozoospermia without assisted oocyte activation: a case report. Hum Reprod 2000; 15:139–41.
14. Tesarik J, Sousa M. Comparison of Ca^{2+} responses in human oocytes fertilized by subzonal insemination and by intracytoplasmic sperm injection. Fertil Steril 1994;62:1197–204.
15. Tesarik J, Sousa M, Testart J. Human oocyte activation after intracytoplasmic sperm injection. Hum Reprod 1994;9:511–8.