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REVIEW

Failed fertilization after clinical intracytoplasmic sperm injection

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Abstract Intracytoplasmic sperm [illegible]jection (IC[illegible] has resulted in pregnancy and birth for many couples, including those with severe male factor infertility. Howeve[illegible]n after ICSI, [illegible]mplete failure of fertilization occurs in 1–3% of cycles. Most cases occur due to low number of mature oocyt[illegible], failu[illegible]f oocyte act[illegible]vation or non-availability of appropriate spermatozoa for injection. Given the significant emotional and [illegible]ancial invol[illegible]ment in assisted reproductive cycles, failure of fertilization in all mature oocytes is a distressful event. It is no[illegible]redictable. Since [illegible]llow-up ICSI cycles result in fertilization in 85% of cases, repeated ICSI attempts are suggested. Physician[illegible]should c[illegible]nsel patients experiencing repeated failure of fertilization after ICSI cycles about available options including donated [illegible]cytes/[illegible]bryos, donor sperm insemination, adoption or remaining childless if these choices are not acceptable due to religious or et[illegible] reasons. T[illegible]s review discusses the causes and remedies for failed fertilization after clinical ICSI.

KEYWO[illegible]S: faile[illegible]ertilizatio[illegible]nfertility; intracytoplasmic sperm injection; in-vitro fertilization

Introduction

When all other forms of assisted fertilization fail, intracytoplasmic sperm injection (ICSI) is the method of choice to overcome male factor infertility. The ICSI procedure allows direct injection of a single spermatozoon into the cytoplasm of an oocyte. Thus, fertilization is possible in cases in which sperm motility is impaired and inability to penetrate the zona pellucida is the major cause of infertility. ICSI is possible with spermatozoa obtained from ejaculation, microsurgical epididymal sperm aspiration, percutaneous epididymal sperm aspiration or testicular sperm extraction. In addition, indications for ICSI include idiopathic infertility and repeated conventional IVF failures (Benavida, et al., 1999).

Total failed fertilization (TFF) refers to failure of fertilization in all the mature oocytes and the term 'failed fertilization' refers to failure of fertilization in any mature oocyte. For all ages and with all the different sperm types,

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fertilization after ICSI is at about 70—80% (Palermo, et al., 2009). This suggests that, despite injecting spermatozoa into mature oocytes, failed fertilization still occurs. Given the considerable emotional and financial investment involved in a cycle of assisted reproduction, TFF is a distressing event for the infertile couple as well as the fertility professionals. TFF occurs in 5—10% of IVF cycles (Mahutte and Arici, 2003) and 1—3% of ICSI cycles (Flaherty, et al., 1998). TFF after ICSI cycles is mostly due to low number of mature oocytes (Flaherty et al., 1998) or oocyte activation failure (Ebner, et al., 2004). TFF is a rare event in cases with normal oocytes and spermatozoa (Mansour, et al., 2009). Some patients may face repeated TFF in spite of normal sperm parameters and good ovarian response (Tesarik, et al., 2002). In such cases, the primary reason for failed fertilization after ICSI is lack of oocyte activation, as more than 80% of these oocytes contain a spermatozoon (Flaherty et al., 1998). Considerable advances in artificial oocyte activation and recovery of spermatozoa from epididymis or testis that are suitable for ICSI help to avoid TFF. This review discusses the causes and remedies for failed fertilization after clinical ICSI.

Oocyte related factors

Oocyte morphology

Poor oocyte morphology is considered a major determinant of failed or impaired fertilization. Normal features of a healthy mature oocyte at metaphase II (MII) include: presence of a polar body, a round even shape, light colour cytoplasm with homogenous granularity, a small perivitelline space without debris and a colourless zona pellucida. In oocytes denuded for ICSI, the morphological structure and the nuclear maturity but not cytoplasmic maturity can be assessed in detail. The MII oocytes with apparently normal cytoplasmic organization may exhibit extracytoplasmic characteristics, such as increased perivitelline space, perivitelline debris and/or fragmentation of the first polar body, which have been suggested to reduce developmental competence of the oocyte involved (Xia, 1997). It is common for extra-cytoplasmic and cytoplasmic dysmorphisms to occur together in the same oocyte (**Figure 1**).

It has been suggested that dysmorphic phenotypes, which arise during meiotic maturation, may be associated with failed fertilization and aneuploidy, while those occurring later in maturation may cause a higher incidence of developmental [illegible] (Otsuki, et al., 2006; Van Blerkom and Henry, 2002).

Decreased fertilization rates with respect to some oocyte dysmorphisms have been reported (Xia, 1997), while others failed to observe that association (Ciotti, Nmarangelo, Morsclli-Labate, et al., 2004; De Santis, Cino, Rabellotti, et al., 2005; Meriano, Alexis, Visram-Zaver, et al., 2001; Mikkelsen and Lindenberg, 2001; Otsuki, et al., 2004). Meriano et al. (2001) reported lower pregnancy and implantation rates when the transferred embryos originated from cycles with more than 50% dysmorphic oocytes and the same dysmorphism repeated from one cycle to the other. The authors suggested that the repetitive organelle clustering was associated with an underlying adverse factor affecting the entire follicular cohort. The presence of a dark cytoplasm decreases the likelihood of obtaining good-quality embryos by 83% (Ten, Mendiola, Vioque, et al., 2007). However, an earlier study did not find any adverse impact of dark colour of the oocytes on the fertilization, embryo development and pregnancy rate (Esfandiari, Burjaq, Gotlieb, et al., 2006). In human oocytes, the cytoplasmic granularity can be homogeneous affecting the whole cytoplasm or concentrated in the centre with a clear peripheral ring giving a darkened appearance to the cytoplasm (Serhal, Ranieri, Kinis, et al., 1997). The abnormal changes in the cytoplasm of MII oocytes may be a reflection of delayed cytoplasmic maturation that is unsynchronized with nuclear maturity (Katz and Tur-Kaspa, 2006).

Normal fertilization, embryo development and births are achieved after ICSI in oocytes with dark zonae, abnormal morphology or repeated polyspermia following conventional IVF. The oocytes with extreme morphological abnormalities should not be discarded as ICSI may overcome the barriers to fertilization, cleavage and normal embryonic development (Esfandiari, Ryan, Gotlieb, et al., 2005b; Esfandiari et al., 2006). Zona-free oocytes may be fertilized normally after ICSI and develop to the blastocyst stage (Jelínková et al., 2001). Pregnancy in human (Stanger, Stevenson, Lakmaker, et al., 2007) and live birth in mouse (Naito, et al., 1992) and pig (Wu, Lai, Mao, et al., 2004) have been obtained after transfer of embryos resulting from zona-free oocytes.

Oocyte maturity

One of the major causes of TFF after ICSI is a low number of retrieved MII oocytes (Esfandiari, Javed, Gotlieb, et al., 2005a). About 20% of retrieved oocytes from ovarian stimulation cycles are immature, either at metaphase-I (MI) or germinal-vesicle (GV) stage in human IVF (Huang, Chang, Tsai, et al., 1999; Rienzi, Ubaldi, Anniballo, et al., 1998). Some of these oocytes may extrude the first polar body during in-vitro culture and may be used as a source of oocytes for sperm injection in ICSI cycles. However, the increase in the number of embryos derived from immature oocytes does not efficiently translate into pregnancies and live births. Therefore, the clinical significance of using immature oocytes in stimulated cycles needs further investigation (Shu, Gebhardt, Watt, et al., 2007).

The injection of MI oocytes immediately after denudation results in a high degeneration rate due to increased fragility of the oolemma. The fertilization rate of retrieved MI oocytes that remained MI at the time of ICSI is lower than the fertilization rate of retrieved sibling MI progressing to MII *in vitro* (25% compared with 62.2%, respectively). It is less than half when compared with the fertilization rate of retrieved sibling MII oocytes (69.5%). A high rate of multinucleated oocytes is also found in fertilized MI oocytes injected immediately after denudation (Shu et al., 2007).

In cases of poor responders and in patients with an unsynchronized cohort of follicles, where the presence of immature oocytes is frequent after stimulation (Smitz and Cortvrindt, 1999), the use of immature oocytes is important in order to increase the number of embryos obtained in each cycle. Based on the assumption that oocyte maturity is a prerequisite for obtaining normal fertilization, attempts

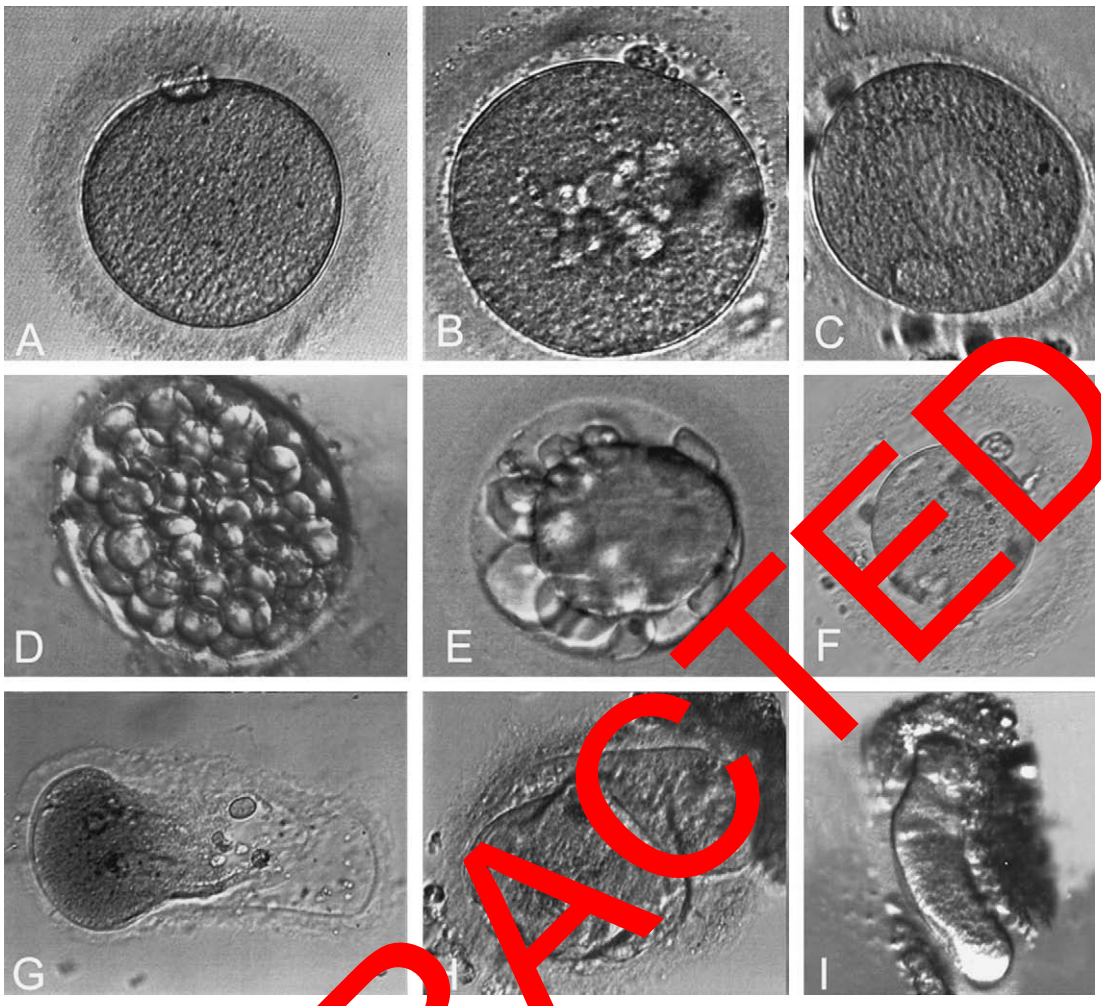

Figure 1 Normal and abnormal morphology oocyte (**A**) normal oocyte, (**B** and **C**) cytoplasmic vacuoles, (**D** and **E**) fragmentation, (**F**) perivitelline debris, and (**G**, **H**, and **I**) abnormal zona pellucida and cytoplasm.

have been made to mature GV and MI oocytes in-vitro (Huang et al., 1999). Despite the use of varying culture techniques and different stimulation protocols, such in-vitro matured oocytes consistently have lower fertilization rates, frequent cleavage blocks and overall retarded cleavage rate compared with sibling MII oocytes (Chen, et al., 2000; Huang et al., 1999). The limited number of transfer cycles makes it difficult to draw solid conclusions about the value of transferring these embryos. It should be noted that the immature oocytes collected in stimulated cycles have already been under stimulation with high doses of gonadotrophins and are exposed to human chorionic gonadotrophin before retrieval. The nuclear maturation, cytoplasmic maturation and ensuing developmental capacity of these oocytes may very well be different in comparison with immature oocytes collected from small antral follicles of unstimulated ovaries in the typical in-vitro maturation (IVM) procedure (Sun, et al., 2001).

Oocyte activation

Oocyte activation is a complex series of events that results in the release of the cortical granules, activation of membrane-bound ATPase, resumption of meiosis and, finally, the formation of the male and female pronuclei with the extrusion of the second polar body. The ovulated or retrieved oocyte is activated when the spermatozoon enters, by either natural fertilization or ICSI. Artificial means of oocyte activation have been used in cases of oocyte activation failure.

In mammals, ovulated oocytes should be arrested at MII if maturation has been completed. When one spermatozoon contacts the oolemma and penetrates into the ooplasm, intracellular calcium oscillation occurs (Yamano, et al., 2000). This increase in the concentration of calcium underlies oocyte activation and initiation of development. In mammals, growing experimental evidence supports the notion that, following fusion of the gametes, a factor from the spermatozoon is responsible for inducing calcium oscillations and stimulating inositol 1,4,5-trisphosphate (IP3) production (Miyazaki and Ito, 2006). Initial evidence stemmed from injection of cytosolic sperm extracts into oocytes that reproduced the calcium responses associated with fertilization regardless of the species of origin (Swann, 1990; Wu, et al., 1997). Subsequent biochemical characterization of the extracts revealed that the active component contained a protein moiety (Swann, 1990) that possessed phospholi-

pase C (PLC)-like activity capable of inducing production of IP3 (Jones, Cruttwell, Parrington, & Swann, 1998; Wu, Smyth, Luzzi, et al., 2001) and that the PLC activity was highly sensitive to calcium (Rice, Parrington, Jones, et al., 2000). A screen of expressed sequenced tags from testes identified a sperm-specific phospholipase C, PLC. The presence of PLCζ correlates with calcium activity in cytosolic sperm extracts (Saunders, 2002). Moreover, injection of oocytes with the recombinant protein (Kouchi, Fukami, Shikano, et al., 2004) or with the encoding mRNA induces fertilization-like oscillations (Saunders, 2002), whereas depletion of PLCζ from the extracts with specific antisera abrogates PLCζ activity (Kurokawa, Yoon, Alfandari, et al., 2007) and the calcium oscillatory activity of the extracts (Kurokawa et al., 2007; Saunders, 2002). The PLCζ is located in the equatorial region of human spermatozoa (Yoon, Jellerette, Salicioni, et al., 2008). Human whose spermatozoa are unable to initiate calcium oscillations consistently fail to fertilize following ICSI and lack PLCζ (Yoon et al., 2008). Nonetheless, whether PLCζ represents the sole calcium oscillation-inducing factor in mammalian spermatozoa and how its absence has an impact on male fertility has not been conclusively established (Yoon et al., 2008).

The process of natural fertilization encompasses the entry of the spermatozoon, oocyte activation and the first mitotic division resulting in a 2-cell embryo. Two steps are important for successful fertilization following ICSI, namely immobilization of the spermatozoon and rupture of the oolemma in order to facilitate the liberation of the cytosolic sperm factor responsible for the oscillator function (Vanderzwalmen, Bertin, Lejeune, et al., 1996).

Low fertilization rates after ICSI in patients with round-head spermatozoa, globozoospermia, is a result of reduced ability of round-head spermatozoa to activate the oocyte. In the literature, the success rates of ICSI in cases of globozoospermia are variable. Assisted oocyte activation with ICSI may overcome the infertility associated with globozoospermia. Normal healthy live birth without assisted oocyte activation has also been documented (Stone, O'Mahony, Khalaf, et al., 2000). Apart from low fertilization rates associated with the use of round-head spermatozoa, cleavage rates are also compromised and these spermatozoa may lack normal centrosomes (Battaglia, Koehler, Klein, et al., 1997). Assisted oocyte activation and ICSI restore fertilization, embryo cleavage and development for patients with globozoospermia (Heindryckx, Van der Elst, De Sutter, et al., 2005).

Assisted oocyte activation aims to mimic the action of sperm penetration (Nakada & Mizuno, 1998). Some assisted activation treatments such as strontium chloride (Cuthbertson, Whittingham, & Cobbold, 1981) and ionomycin (Loi, Ledda, Fulka, et al., 1998), promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores; others such as electrical stimulus promote influx of calcium from the extracellular medium and some treatments such as ethanol promote both effects (Loi et al., 1998).

A birth after oocyte activation by treatment with the calcium ionophore A23187 and ICSI was achieved in 1994 (Hoshi, Yanagida, Yazawa, et al., 1995). Human oocytes injected with round-head spermatozoa are activated following combination of calcium chloride injection and ionophore treatment. This activation was followed by an apparently normal completion of meiosis, male and female pronuclei formation, embryonic development and successful delivery of a healthy infant (Rybouchkin, Van der Straeten, Quatacker, et al., 1997). A combination of calcium ionophore A23187 with puromycin stimulates the unfertilized oocytes 20–68 h after ICSI. It resulted in an activation rate of 91.2% (31/34), a cleavage rate of 64.7% (22/34) and high-quality embryo rate of 44.1% (15/34). Nearly all activated embryos derived from 2PN/2PB had a normal set of sex chromosomes and developed normally (Lu, Zhao, Gao, et al., 2006). Although calcium ionophore A23187 and puromycin do not appear to be cytotoxic to oocytes and result in pregnancies and the birth of healthy babies when low concentrations are used, the possible teratogenic and mutagenic activity of calcium ionophore A23187 and puromycin need further investigation in animal models and in humans.

Treatment with 10 mmol/l strontium chloride for 60 min, approximately 30 min after ICSI, results in activation and fertilization of all injected oocytes (Kyono, Kumagai, Nishinaka, et al., 2008). Development of the embryos to the blastocyst stage and delivery in patients with repeated fertilization failure (Yanagida, Kimizumi, Katayose, et al., 2006). Physical and mental development of the children from birth to 12 months is normal (Kyono et al., 2008). However, further studies are required to substantiate the finding that strontium chloride treatment is an effective method of artificial oocyte activation.

An electrical field can generate micropores in the cell membrane of gametes and somatic cells to induce sufficient calcium influx through the pores to activate cytoplasm through calcium-dependent mechanisms (Ozil, 1990). Mouse oocytes injected with secondary spermatocytes or spermatids were fertilized when stimulated by electroporation and developed into normal offspring when the resultant embryos were transferred to a recipient (Kimura and Yanagimachi, 1995). Clinical pregnancy and delivery after oocyte activation by electrostimulation in combination with ICSI in previously failed-to-fertilize oocytes has been obtained (Yanagida, Katayose, Yazawa, et al., 1999). Electrical stimulation rescues human oocytes that failed to fertilize after ICSI and stimulates them to complete the second meiotic division, to form pronuclei and to undergo early embryonic development (Zhang, Wang, Blaszcyzk, et al., 1999). Although the fertilization rate was similar regardless of the number of electrical pulses applied, subsequent embryo development was dramatically improved in those oocytes that received three electrical pulses (Zhang et al., 1999). The embryo formation rate in the electrically activated group was 80% compared with 16% in the control group (Manipalviratn, Ahnonkitpanit, Numchaisrika, et al., 2006). Although the fertilization rate was significantly higher in the electroactivated group (68%) as compared with that of the control (60%), a higher miscarriage rate was reported in the electroactivated group (five of 15 pregnancies) compared with the control (three of 33) (Mansour et al., 2009).

Like any other new assisted reproductive procedure, the impact of electrical activation on oocyte and embryo health must be evaluated in larger studies before this procedure can be considered for routine clinical purposes. Ideally, karyotyping or fluorescent in-situ hybridization analysis should be performed to assess the incidences of aneuploidy and mosaicism in the resultant embryos.

Poor ovarian response

The definition of poor response in the literature is often based on a combination of factors, including the number of mature follicles, the number of oocytes retrieved and the peak oestradiol concentration (Saldeen, et al., 2007). The cut-off concentrations for the number of follicles or oocytes that define poor response vary widely from study to study. Some authors feel that the definition of poor response should also include the degree of ovarian stimulation used and that a low oocyte number is detrimental only when high total dose of FSH has been administered (Kailasam, et al., 2004).

Various endocrine and ultrasonographic markers and dynamic tests to assess ovarian reserve have been evaluated. Such tests include basal FSH on cycle day 3, clomiphene citrate challenge test, inhibin B, oestrogen, anti-Müllerian hormone, antral follicle counts and ovarian volume. The success of each test can be measured against ovarian response or live birth rate per cycle (Sun, Stegmann, Henne, et al., 2008). However, none of these tests has demonstrated a reliable predictive value and for many women poor ovarian response is not discovered until the first IVF cycle.

Poor response to gonadotrophin stimulation occurs more often in older women, but may also occur in young women, regardless of the endocrinological profile (Lashen, Ledger, Lopez-Bernal, et al., 1999). Poor responders have a significantly lower pregnancy rate per retrieval compared with normal to high responders in the same age group (Saldeen et al., 2007). Although it is possible to have normal embryo and pregnancy in younger poor responders, the fertilization rate and quality of embryos in older poor responders are always low and the chance of achieving pregnancy in such patients is low. Poor responders also have an increased cycle cancellation rate due to retrieval of few or no oocytes and/or TFF. One of the major contributing factors for TFF after retrieval of three or less MII oocytes retrieved (Esfandiari et al., 2005a). The rate of fertilization failure increases as the number of injected oocytes decreases (Yanagida, 2001). Melie, Adeniyi, Igbineweka, et al. (2003) have shown that there is a higher chance of having no embryos for transfer and significantly lower pregnancy rates when less than five oocytes are retrieved compared with cycles with more than five oocytes. Limited information is available on IVM of immature oocytes retrieved from poor responders in conventional stimulation IVF/ICSI cycles and IVM is not a viable alternative to cancellation of IVF cycles in such patients (Esfandiari et al., 2005a).

Fecundity significantly decreases with increasing maternal age (Dorfmann, Godoy, Smitz, et al., 1996). In a classic study of the Hutterite women, sterility increased from just over 10% at 30 years of age to over 85% by the age of 44 years (Tietze, 1957). In women, all germ cells are formed during fetal life. The population of germ cells appears to rise steadily from 600,000 at 2 months post conception, reaching a peak of 6,800,000 at 5 months. By the time of birth, the number declines to 2,000,000 of which 50% are atretic. Of the 1,000,000 normal oocytes in the newborn infant, only 300,000 survive to the age of 7 years (Baker, 1963). Continuous loss of oocytes occurs through the physiological process of follicular growth and atresia throughout life (Djahanbakhch, Ezzati, and Zosmer, 2007).

The incidence of TFF increases with increasing age (Esfandiari et al., 2005a). Older women are more likely to undergo multiple cycles, have a decreased number of oocytes retrieved and a lower number of embryos transferred (Shen, et al., 2003).

Sperm-related factors

Sperm motility and progression

Whether sperm movement is slow or rapid generally has no influence on ICSI results. However, injection of immotile spermatozoa usually results in impaired fertilization. In particular, where a non-viable immotile spermatozoon is injected into an oocyte, normal fertilization and pregnancy rarely occurs (Konc, et al., 2006; Wang, Lai, Wang, et al., 1997). In the case of an immotile sperm injection, it is possible that the spermatozoon may be dead. The most common practice to select viable non-motile spermatozoa for ICSI involves the hypo-osmotic swelling (HOS) test. However, preliminary results in animal experiments (mouse and rabbit) indicate that viability of injected spermatozoa is not an absolute prerequisite for fertilization. Embryos derived after injecting mouse oocytes with freeze-dried and thawed spermatozoa developed normally (Kusakabe, Szczygiel, Whittingham, et al., 2001). It appears that, provided the DNA integrity of the spermatozoa is maintained, embryos can be generated, at least in animal models, from severely damaged spermatozoa that are no longer capable of normal physiological activity.

In patients with 100% immotile spermatozoa, the HOS test is a useful method to examine sperm viability. It measures the functional integrity of the sperm membrane (Jeyendran, Van der Ven, Perez-Pelaez, et al., 1984). Upon exposure of the spermatozoa to hypo-osmotic conditions, the intact semi-permeable barrier formed by the sperm membrane allows an influx of water and results in swelling of the cytoplasmic space and curling of the sperm tail fibres. Only viable spermatozoa react to the HOS solution since dead spermatozoa are unable to maintain the osmotic gradient.

The sperm HOS test based on fructose and sodium citrate dihydrate is applied for identification of immotile spermatozoa for ICSI (Casper, Meriano, Jarvi, et al., 1996). A significantly greater fertilization and cleavage rate after injection of spermatozoa selected using the HOS test is achieved in contrast to injection of randomly selected spermatozoa. A modified HOS test based on NaCl solution further improves fertilization rate in patients with 100% immotile spermatozoa (Liu, Tsai, Katz, et al., 1997). In these procedures, approximately 200,000 spermatozoa are exposed to the HOS solution for 1 h at 37°C. A modified HOS test has been used for samples with a low sperm count such as testicular samples (Ahmadi and Ng, 1997). In this technique, an individual morphologically normal spermatozoon was aspirated by microinjection pipette and was exposed to HOS solution for a brief period to minimize the sperm membrane damage.

A mixture of 50% culture medium and 50% deionized grade water has the least delayed harmful effects on sperm vitality (Verheyen, Joris, Crits, et al., 1997). This mixture achieves similar implantation, pregnancy and ongoing preg-

nancy rates in the ejaculated and testicular non-motile sperm groups (Sallam, Farrag, Agameya, et al., 2001). It is a simple and practical procedure and achieves acceptable and comparable pregnancy rates.

Sperm origin

A new era in the field of assisted reproduction opened after the achievement of pregnancies and births after ICSI of human oocytes (Palermo, Joris, Devroey, et al., 1992). In special cases of long-standing male infertility, only a few functional spermatozoa are available. By means of ICSI, most subfertile men and even men previously considered sterile (those with azoospermia, extreme oligozoospermia or cryptozoospermia) can now father a child.

Azoospermia, is the most severe form of male factor infertility. The condition is currently classified as 'obstructive' or 'non-obstructive'. Obstructive azoospermia is the result of obstruction in either the upper or lower male reproductive tract. Sperm production may be normal but the obstruction prevents the spermatozoa from being ejaculated. Non-obstructive azoospermia is the result of testicular failure where sperm production is either severely impaired or nonexistent, although in many cases spermatozoa may be found and surgically extracted directly from the testicles (Proctor, Johnson, Van Peperstraten, et al., 2009).

Conflicting results for fertilization and pregnancy rates are available in the literature after the use of ejaculated or surgically retrieved spermatozoa. After ICSI, ejaculated or surgically extracted spermatozoa, when motile and morphologically normal, result in similar fertilization, implantation (Bulkumez, Yucel, Yarali, et al., 2001; Wennerholm, Bergh, Hamberger, et al., 2000) and clinical pregnancy rates (Bulkumez et al., 2001). The incidence of early or late spontaneous abortion, ectopic pregnancy or malformations is also similar (Wennerholm et al., 2000). However, after conventional IVF, even testicular or epididymal sperm with very good sperm concentration and motility generally achieve low fertilization and pregnancy rates (Silber, Nagy, & Liu, 1994).

The effect of cryopreservation of spermatozoa on ICSI outcome has been thoroughly studied. Current studies suggest that the use of fresh or frozen—thawed spermatozoa does not appear to affect ICSI outcomes (Lewis & Klonoff-Cohen, 2005). Testicular tissue and epididymal spermatozoa can be cryopreserved successfully without markedly reducing subsequent fertilization and implantation rates and repeated testicular biopsy can be avoided without the risk of any detrimental effect in the outcome (Matyas, Papp, Kovacs, et al., 2005).

Sperm maturity

Round spermatid nucleus injection (ROSNI) or round spermatid injection (ROSI) are methods in which precursors of mature spermatozoa obtained from ejaculated specimens or testicular sperm extraction are injected directly into oocytes. ROSNI has been proposed as a treatment for men in whom other more mature sperm forms (elongating spermatids or spermatozoa) cannot be identified for ICSI (Saremi, Esfandiari, Salehi, et al., 2002). It is not widely performed and not as successful as ICSI and it is still an experimental procedure. It should be applied only in the setting of clinical trial approval and overseen by a properly constituted institutional review board. Accurate identification of round spermatids is a technical challenge of ROSNI. It is difficult to distinguish haploid round spermatids from diploid spermatogenic precursors and somatic cells using the standard optics present in most clinical IVF laboratories. Mouse round spermatids have increased levels of DNA fragmentation (Jurisicova, Lopes, Meriano, et al., 1999) that may interfere with fertilization (Lopes, Jurisicova, & Casper, 1998). Increased DNA damage may occur because of deficient sperm nuclear protamine to histone replacement and decreased nuclear condensation in these immature spermatozoa allowing increased susceptibility to reactive oxygen species and other damaging agents in culture. Another major concern is genetic risk. Any genetic abnormality sufficiently severe to result in meiotic arrest during spermatogenesis may also have adverse effects on other normal cellular processes or other systemic manifestations. Occurrence of significant congenital anomalies in ROSI conceived pregnancies raises serious concerns (Zech, Vandervorst, Prapas, et al., 2000). ROSI should not be performed when more mature sperm forms (elongating spermatids or spermatozoa) can be identified and used for ICSI. Patients who may be candidates for ROSNI should receive careful and thorough pretreatment counseling to ensure they are clearly informed of the limitations and potential risks of the procedure (The Practice Committee of American Society for Reproductive Medicine, Practice Committee of Society for Assisted Reproductive Technology, 2008).

Sperm structural defects

Normal sperm ultrastructure correlates with positive IVF results (Malgorzata, Depa-Martynów, Butowska, et al., 2007). Single structural defects involving the totality of ejaculated spermatozoa are among rare cases of untreatable human male infertility. This form of infertility is of genetic origin and is generally transmitted as an autosomal recessive trait. Numerous defective genes are potentially involved in human isolated teratozoospermia but such defects have not been defined at the molecular level in most cases (Francavilla, Cordeschi, Pelliccione, et al., 2007). An in-depth evaluation of sperm morphology by transmission electron microscopy (TEM) can improve the diagnosis of male infertility and can give substantial information about the fertilizing competence of spermatozoa (Kupker, Schulze, & Diedrich, 1998; Yu & Xu, 2004). TEM evaluation of spermatozoa can also identify potentially inheritable genetic disorders (for example primary ciliary dyskinesia, Kartagener's syndrome), providing valuable information for couples contemplating ICSI (Lamb, 1999).

Acrosome agenesis is most often associated with a spherical shape of the head and is usually defined as 'round head defect' or globozoospermia. The underlying causes of the syndrome remain to be elucidated (Dam, Feenstra, Westphal, et al., 2007). Kullander and Rausing (1975) have postulated a genetic contribution. An additional case report (Kilani, Ismail, Ghunaim, et al., 2004) supports it. However, the gene responsible or the mode of inheritance remains ob-

scure. Globozoospermia results from perturbed expression of nuclear proteins or from an altered golgi–nuclear recognition during spermiogenesis. The spermatozoa show both gross and ultrastructural abnormalities, including the complete lack of an acrosome, abnormal nuclear membrane and midpiece defects. Depending on the severity of the defect, the fertilization rate after ICSI with round-head spermatozoa ranges from 0% to 37% (Battaglia et al., 1997; Rybouchkin et al., 1997). Successful pregnancies have been reported after ICSI in patients with globozoospermia with or without oocyte activation (Edirisinghe, Murch, Junk, et al., 1998; Rybouchkin et al., 1997; Stone et al., 2000). The most likely cause for failed fertilization after ICSI using round-head spermatozoa is inability of the spermatozoon to activate the oocyte. In some forms of globozoospermia, arrest of nuclear decondensation and/or premature chromosome condensation also causes fertilization failure (Edirisinghe et al., 1998).

Premature chromosomal condensation

When a cell with chromosomes in MII fuses with an interphase cell, the nuclear membrane of the cell in interphase dissolves and its chromatin condenses. This phenomenon is called premature chromosomal condensation (PCC; Johnson & Rao, 1970). Following penetration of the spermatozoon into an oocyte, oocyte activation is triggered, resulting in completion of meiosis and formation of both male and female pronuclei. Under some circumstances, although the spermatozoon is within the oocyte, fertilization fails to occur, the oocyte remains in the MII stage and the sperm head undergoes PCC separate from the oocyte chromosomes (Kalousek, & Yuen, 1994; Schmiady, Sperli[illegible]entenic et al., 1986). Chromatin analysis of human oocyte has revealed that sperm PCC is one of the prevalent causes of fertilization failure in both IVF and ICSI (Schmiady et al.,

It is not yet fully understood how the spermatozoon activates the oocyte. The failure of fertilization after ICSI may result from either the lack or deficiency of activating factors in the spermatozoon or from the lack of ooplasmic factors triggering sperm chromatin decondensation (Van Blerkom, Davis, & Merriam, 1994; Yanagida et al., 1999). Several pieces of evidence point to PLCζ being the physiological agent of oocyte activation and is detectable in different localities within the sperm head (the equatorial segment and acrosomal/postacrosomal region) (Grasa, Coward, Young, et al., 200

During normal spermiogenesis, 85% of histones are replaced with protamines (Balhorn, 1982), which results in sperm chromatin condensation. A spermatozoon with a condensed nucleus is in the G1 stage when entering a MII oocyte and is protected from PCC because an active maturation-promoting factor (MPF) is not capable of reacting with protamine-associated DNA. Once sperm nuclear decondensation factors from the ooplasm enter the spermatozoon, the sperm head swells and sperm-associated oocyte-activating factor is released. This results in MPF inactivation (Dozortsev, Qian, Ermilov, et al., 1997), the completion of meiosis II and the oocyte enters the G1 stage. During this time, protamines are slowly replaced by histones and cell cycle synchronization takes place. Under some circumstances, the oocyte fails to activate and remains arrested at MII. Because of the presence of an active MPF, sperm chromatin transforms into condensed chromatin. Sperm with excessive histones are prone to PCC.

Sperm PCC has been associated with the type of ovarian stimulation protocol. Some protocols, such as clomiphene citrate and human menopausal gonadotrophin stimulation, may tend to recruit immature oocytes with immature cytoplasm (Ma & Yuen, 2001). Immature cytoplasm is believed to make a spermatozoon susceptible to a high incidence of PCC after insemination because of the inability of the immature oocyte to undergo oocyte activation (Calafell, Badenas, Egozcue, et al., 1991). The incidence of sperm PCC reported in the literature ranges from 1.1% to 85% (Rosenbuch, 2000; Schmiady, Schulze, Scheib[illegible] et al., 2005), with higher values noted in cases of round-head sperm injection as they fail to activate the oocyte. Furthermore, other studies suggest a correlation between fertilization outcome post ICSI and percentage of spermatozoa with protamine deficiency (Nasr-Esfahani, [illegible], & Tavalaee, 2008). The effect of sperm protamine deficiency on fertilization rate emphasizes the need for accurate sperm selection during ICSI because protamine-deficient spermatozoa, in the form of slightly amorphous head, may have the chance of being injected due to inappropriate sperm selection (Nasr-Esfahani et al., 2008).

Sperm DNA damage

DNA damage in the male germ line is associated with poor fertilization rates following IVF, defective preimplantation embryonic development and high rates of miscarriage and morbidity in the offspring, including childhood cancer (Aitken & De Iuliis, 2007; Virro, Larson-Cook, & Evenson, 2004). Activation of embryonic genome expression occurs at the 4–8-cell stage in human embryos (Braude, Bolton, & Moore, 1988), suggesting that the paternal genome may not be effective until that stage. Therefore, a lack of correlation between elevated DNA strand breaks in spermatozoa and fertilization rates may occur before the 4–8-cell stage (Tesarik, Greco, & Mendoza, 2004; Twigg, Irvine, & Aitken, 1998). Many published articles indicate that DNA strand breaks are clearly detectable in ejaculated spermatozoa and their presence is heightened in the ejaculates of men with poor semen parameters (Irvine, Twigg, Gordon, et al., 2001; Sun, Jurisicova, & Casper, 1997). Nuclear DNA damage in mature spermatozoa includes single-strand nicks and double-strand breaks that can arise because of errors in chromatin rearrangement during spermiogenesis, abortive apoptosis and oxidative stress (Lopes et al., 1998; Sikora, Kempisty, Jedrzejczak, et al., 2006).

Two tests have been most commonly reported as indicators of sperm nuclear integrity: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and sperm chromatin structure assay (SCSA). The TUNEL technique labels single- or double-stranded DNA breaks, but does not quantify DNA strand breaks in a given cell. SCSA, a quantitative and flow cytometric test, measures the susceptibility of sperm nuclear DNA to acid-induced DNA denaturation *in situ*, followed by staining with acridine orange (Evenson, Larson, & Jost, 2002). SCSA accurately estimates

the percentage of sperm chromatin damage expressed as DNA fragmentation index (DFI) with a cut-off point of 30% to differentiate between fertile and infertile samples (Potts, Newbury, Smith, et al., 1999). A statistically significant difference was seen between the outcomes of ICSI versus IVF when DFI was more than 30% (Bungum, Humaidan, Axmon, et al., 2007). The biological explanation behind the superior results of ICSI in cases of high DFI needs to be elucidated. One possibility may be that women undergoing ICSI, on average, produce healthier oocytes with a better DNA repair capacity than women undergoing IVF, because infertility is mainly caused by male factor in the ICSI group.

Other tests of sperm nuclear DNA integrity include in-situ nick translation and the comet assay. The toluidine blue and sperm chromatin dispersion test are potential new assays (Spano, Seli, Bizzaro, et al., 2005). At present, there are two major strategies that may be considered for the treatment of men exhibiting high levels of DNA damage in their spermatozoa: (i) selective isolation of relatively undamaged spermatozoa; and (ii) antioxidant treatment (Aitken, De Iuliis, & McLachlan, 2009).

Procedural effects of the ICSI technique

The risk of oocyte damage by the ICSI procedure is low in humans and may be related to both the skill of the person performing the injection procedure itself and to the quality and quantity of the gametes used during the procedure (Palermo, Cohen, Alikani, et al., 1995). The embryologist conducting the ICSI procedure is a significant predictor of fertilization, while laboratory conditions (i.e. incubators, culture of oocytes individually versus grouped) do not affect the rates (Shen et al., 2003). When fertilization failure in most or all of the injected oocytes occurs with experienced practitioners using normal spermatozoa, a diagnosis to oocyte dysfunction, oocyte activation failure or inability of the spermatozoon to be decondensed and processed by the oocyte.

Although ICSI is now considered routine, it remains a very demanding technique to master, due partly to its inherent technical difficulty and partly to the heterogeneity of the cases. It is generally agreed that the ICSI procedure is subject to a learning curve (Shen et al., 2003) and that one common technical failure is not depositing the spermatozoon within the oocyte cytoplasm. In this situation, the oocyte membrane may not have been broken during attempts to aspirate the ooplasm into the ICSI needle. Thus, the spermatozoon is deposited next to the membrane so that when the oolemma returns to its original position, the spermatozoon is pushed out into the perivitelline space (**Figure 2A**), or is trapped inside a sac formed by the membrane (**Figure 2B**) (Esfandiari et al., 2005a). The spermatozoon may also adhere to the tip of the injection needle or remain within the injection needle and be inadvertently pulled out upon withdrawal of the needle from the cytoplasm. The degeneration of oocytes after ICSI is often a result of a fault in the ICSI technique, e.g. an injection pipette that is too large or not sharp enough. Aspiration of the ooplasm is always used to make sure that the oocyte membrane is broken during injection. However, if the ooplasm is aspirated too much, degeneration of the oocyte frequently results (**Figure 2C**).

Proper orientation of the polar body and spindle position are also important, since improper positioning can damage or disrupt the metaphase plate during needle entry. In addition, disturbance in the nuclear spindle may dispose oocytes to aneuploidy or fertilization arrest. Thus, perturbation of the cytoskeletal integrity of the oocyte may critically influence the fate of the embryo.

During ICSI, the location of the first polar body is commonly used as an indication of the spindle position, with the assumption that they are located in close proximity. To avoid damage to the spindle, oocytes are injected at the 3 o'clock position with the first polar body at the 6 or 12 o'clock position. However, with the aid of the computer-assisted polarization microscopy, some reports suggest that the location of the first polar body does not necessarily correspond to the spindle position (Wang, Meng, Hackett, et al., 2001a; Wang, Meng, Hackett, et al., 2001b). The reasons for the displacement of the spindle are not fully understood (Woodward, Montgomery, Hartshorne, et al., 2008).

Injection of motile spermatozoa without immobilization leads to poor fertilization rates (Vanderzwalmen et al., 1996). In such cases, a spermatozoon with a moving tail can be seen in the oocyte and spermatozoon—oocyte interaction is obstructed by the normal sperm plasma membrane. Damage to the sperm membrane is now generally considered necessary for successful oocyte activation following ICSI, as it induces gradual disruption of other parts of the sperm membrane allowing entry of sperm nucleus decondensing factor of the oocyte to induce initial swelling

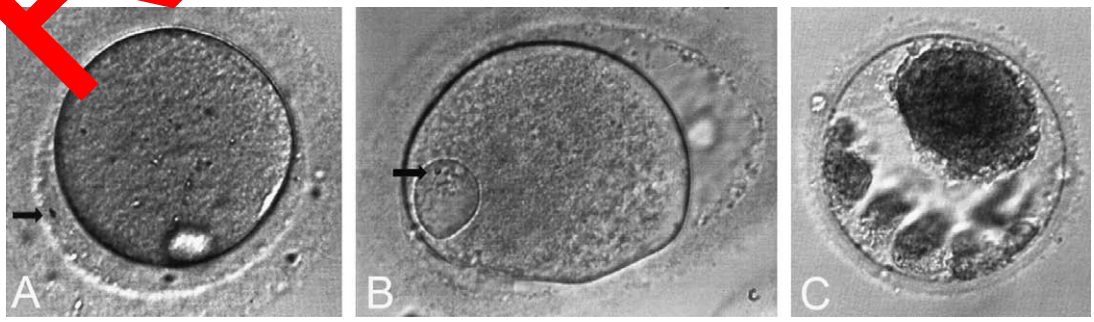

Figure 2 Failure of fertilization due to intracytoplasmic sperm injection technique: (**A**) a spermatozoon trapped in perivitelline space (arrow), (**B**) a spermatozoon trapped in a membrane fold (arrow), and (**C**) an atretic oocyte.

of the head. As a result of this swelling, the sperm plasma membrane ruptures and sperm-associated oocyte-activating factors are released into the ooplasm and induce oocyte activation. A modified ICSI technique is characterized by pushing the needle tip close to the membrane opposite the puncture site, aspirating the cytoplasm at this point and releasing the spermatozoon in the centre of the oocyte (Ebner et al., 2004). This modification improves fertilization in oocyte-dependent activation failure, but its routine application does not improve the overall results.

ICSI after previous ICSI cycle failure

Repeated ICSI treatment can be useful or necessary because there is a high possibility of achieving normal fertilization if a reasonable number of oocytes with normal morphology are available and motile spermatozoa can be found. If there are no motile spermatozoa present in the first ejaculate, a second sample should be required followed by percutaneous epididymal sperm aspiration or testicular sperm extraction to obtain motile spermatozoa. In this way, a sufficient number of motile spermatozoa for ICSI are usually found in most men with severe asthenozoospermia.

A history of failed fertilization may be related to some gamete abnormality that may be modified or corrected at the next cycle. It has been documented that fertilization results for a particular patient can be quite varied when followed through several ICSI cycles at the same centre (Moomjy, Sills, Rosenwaks, et al., 1998). The differences between fertilization rates are unexplained, although fluctuations in the gamete quality are probably contributory. Pretreatment endocrine assays and semen analyses prove to be of little value in forecasting failed fertilization. One-third of the patients with TFF achieved pregnancy with their own oocytes in a subsequent ICSI cycle (Esfandiari et al., 2005a). Since follow-up ICSI treatment has been shown to result in fertilization in 70% of cases, repeated ICSI attempts are suggested in TFF (Flaherty et al., 1998; Rouzi & Amarin, 2002).

Options for patients after repeated ICSI cycle failure

Physicians should counsel patients based on the best possible evidence available and allow the couple to make an informed choice. The adverse result of a failed ICSI cycle does not imply a hopeless prognosis for future ICSI treatment. Very subtle improvements in semen parameters and/or oocyte yield/quality may result in fertilization in a subsequent ICSI attempt (Moomjy et al., 1998). Otherwise, the options of donor sperm insemination, donated oocytes or embryos, adoption and remaining childless should be discussed with the couple (Wen, Walker, Léveillé, et al., 2004).

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