Review

Current and future perspectives on intracytoplasmic sperm injection: a critical commentary

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Abstract

Intracytoplasmic sperm injection (ICSI) is an increasingly popular means of treating infertility in couples who wish to conceive. However, there are many potential complications that can be faced by the clinician while performing ICSI. These complications and other related issues are discussed, with an emphasis on understanding how these issues are being resolved, or how they can be resolved in the future. Matters of sperm selection and injection are discussed, as well as the effect of ICSI on fertilization, embryonic growth and development, and the health of ICSI-conceived children. These aspects are viewed from various perspectives, including genetic, mechanistic, developmental and clinical. Since new studies on ICSI are published regularly, it is important that the established protocol is revised often, and that the role of ICSI in infertility therapy is continually re-evaluated.

Keywords: *assisted reproductive technology, DNA, fertilization, ICSI, intracytoplasmic sperm injection, male infertility*

Introduction

Since the first healthy child was conceived via intracytoplasmic sperm injection (ICSI) in 1992, the technique has become increasingly popular as a means of infertility therapy in the USA (Jain and Gupta, 2007). The most current report by the Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine found that ICSI represented 58.8% of all assisted reproductive techniques in the United States in 2001 (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine, 2007). Initially, ICSI was used exclusively in cases of male infertility, including problems of decreased sperm concentration, motility or morphology. Currently, however, ICSI is also being performed for infertile couples with normozoospermic males, even if the less-invasive IVF would likely produce a similar outcome (Price *et al*., 2007). Some clinics even utilize ICSI in all instances of treatment with IVF. This could be problematic, since several studies have advised against routinely using ICSI for all cases of IVF treatment (Ola *et al*., 2001, Oehninger and Gosden, 2002).

An extensive report on ICSI cycles found that there was a 94% oocyte survival rate after ICSI. Normal fertilization was achieved in 75% with ejaculated spermatozoa, and 69.8% with surgically obtained spermatozoa (Palermo *et al*., 2001). Pregnancy rates after ICSI were reported as 42.8% with ejaculated spermatozoa and 48.8% with surgically retrieved spermatozoa (Neri *et al*., 2004). However, complete failure to fertilize following ICSI was shown to be quite uncommon. In cases of fertilization

failure this could be the result of oocyte activation failure, or abnormal sperm parameters (Ebner *et al.*, 2004). Fortunately, researchers and clinicians are working towards producing a more effective ICSI protocol, from improving sperm selection techniques, to evaluating how appropriate ICSI may be for an infertile couple. For example, spindle view (PolScope) systems have been developed which reveal useful information about oocyte maturity at the time of ICSI, thereby increasing the likelihood of successful, normal fertilization (Rienzi *et al.*, 2003). Also, it was found that removing the outer plasma membrane and acrosome cap from the spermatozoa before injection for ICSI significantly decreased the time between sperm injection and oocyte activation by spermatozoa, thereby improving the quality, health and development of the resulting embryo (Morozumi *et al.*, 2006).

Prenatal issues with ICSI

Contrary to conventional IVF, the natural means of selecting spermatozoa for fertilization, such as zona-pellucida penetration by spermatozoa, as well as fusion of spermatozoa to the oolemma, are bypassed with ICSI. Hence, spermatozoa with abnormal morphology and physiology, as well as abnormal chromatin in many cases, can fertilize oocytes even though they would naturally fail to fertilize using IVF (Hardy et al., 2002). The occasional, though unintentional, use of spermatozoa with DNA fragmentation in ICSI is seemingly unpreventable as of now, and it may have a negative impact on the health and normality of the resulting embryos. This has sparked much concern and debate over the current value of ICSI as a common procedure in infertility management, since the long-term effects of ICSI on the resulting children are largely unknown (Fatehi *et al.*, 2006).

DNA fragmentation in male or female gametes is believed to be a primary cause for fertilization failures (Bosco *et al.*, 2005) as well as failed sperm chromatin decondensation (Razavi *et al.*, 2003). Using a bovine model, Fatehi *et al.* (2006) demonstrated that damaged gamete DNA does not affect embryo development until after the first cleavage, when embryonic gene expression begins. This is due to the fact that before the first cleavage, the mRNA still present from the cytoplasm of the gametes is being translated instead of waiting for the embryonic DNA to be transcribed. However, when embryonic gene expression is first initiated during the second or third cleavage, one can observe the formation of an abnormal mitotic spindle. DNA fragmentation also occurs at that point, and it can generate severe developmental problems as the embryo attempts to form a blastocyst (Fatehi et al., 2006).

Studies have found that parental germ-cell mutation transmission to offspring occurs at similar rates when comparing children conceived naturally with ICSI- or IVF-conceived children (Walter *et al.*, 1998). However, one could reason that ICSI should result in increased paternal mitochondrial DNA (mtDNA) disease transmission, since oligozoospermic males have an increased likelihood of mtDNA mutations, and a whole sperm cell with its mitochondria is injected into the oocyte. Marchington *et al.* (2002) attempted to find paternal mtDNA within the embryonic and extraembryonic tissue of ICSI-conceived children. Solid-phase mini-sequencing was utilized in the study, enabling them to detect paternal mtDNA in concentrations as low as 0.001% among the mtDNA from the mother. However, no paternal mtDNA was found, refuting the idea that there should be increased paternal mtDNA disease transmission when ICSI is utilized (Marchington *et al.*, 2002). This finding supports the theory that the paternal mitochondria are tagged with ubiquitin shortly after fertilization, effectively eliminating them from the cytoplasm (Reynier *et al.*, 2001).

There is evidence to suggest that de-novo deletions of the Y chromosome are responsible for abnormal spermatogenesis and male infertility, and the deletions are always passed to male offspring (de Vries *et al.*, 2001). This helps to explain approximately 10–14% of male infertility cases (Aitken and Sawyer, 2003). Due to this high incidence of de-novo Y deletions, Aittomaki *et al.* recommended that both karyotype analysis and Y-chromosome microdeletion screening should be performed on males with nonobstructive azoospermia, or oligospermia below 5×10^6 spermatozoa/ml, since studies show that most men with a higher sperm count do not have the deletion (Aittomaki *et al.*, 2004).

Post-natal issues with ICSI

Several studies have been published to determine the normality of babies conceived using IVF or ICSI, and conflicting evidence has been found. One collaborative European study compared the mental and motor development of 511 children conceived through ICSI with that of 424 children conceived through IVF and 488 naturally conceived children. Among these groups, no significant differences were found regarding the children's verbal, performance or full-scale IQ scores (Ponjaert-Kristoffersen *et al.*, 2005). However, an investigation of the neuromotor development of children who were ICSI singletons found that minor neurological dysfunction was more common among ICSI singletons (66.3%, $n = 87$) than children who were conceived naturally (50.6%, $n = 85$), but these results were not significant after adjusting for parity and maternal age (Knoester *et al.*, 2007).

Several studies have been published which describe the increased risk of various birth defects with ICSI. For example, one large study demonstrated a significantly increased risk of hypospadias for boys conceived through ICSI when compared with naturally conceived boys (Kallen *et al.*, 2005). Another large study found that boys who were conceived by ICSI had reduced serum levels of testosterone and an increased LH: testosterone ratio. The findings were still significant after excluding boys of irregular gestational age or birth weight from the statistical analysis, indicating that ICSI most likely does directly affect hormone levels in children (Mau Kai *et al.*, 2007).

A number of published reports found increased genetic and chromosomal defects in children born by ICSI (Bonduelle *et al.*, 2002). Specifically, there are many studies and reports published on the various imprinting disorders that seem to be prevalent among ICSI conceived children. Two cases have been reported of ICSI conceived children who developed Angelman's syndrome, a severe neurogenetic disorder, due to imprinting errors (Cox *et al.*, 2002). Likewise, it was reported that children conceived through IVF or ICSI are six times more likely to have Beckwith–Wiedemann syndrome, a

disease where there is loss of imprinting of the *H19* gene on the chromosome 11p15.5. *H19* is a tumour-suppressing gene that is maternally expressed, though not translated. The resultant RNA inhibits the expression of the carcinogenic, insulin-like growth factor-II (IGF-II) (Wilkin *et al*., 2000). Beckwith–Wiedemann syndrome patients with loss of imprinting tend to exhibit hypermethylation of *H19*, resulting in an underexpression of *H19* and therefore hyperactivation of IGF-II, leading to tumour growth (DeBaun *et al*., 2003). While there are many accounts of imprinting disorders among ICSI-conceived children, a recent Dutch study found no significant increase in imprinting disorders among such children after correcting for the parents' fertility problems (Doornbos *et al*., 2007).

Interestingly, a different study found no significant increase in imprinting errors of spermatogonia from infertile men, despite the above evidence, which suggests that an increase should be observed (Hartmann *et al*., 2006). A second study also shows no increase of global methylation defects in males with abnormal protamine levels, which are indicative of defective spermatogenesis and male infertility (Aoki *et al*., 2006). To help discover the real relationship between imprinting errors and male infertility, differentially methylated regions are being analysed quantitatively and qualitatively in various ways to reveal critical information regarding methylation (Wong, 2006). For example, if quantitative data are needed regarding the methylation of bisulphite-modified DNA sequences, then the combined bisulphite restriction analysis (COBRA) technique can be utilized (Eads and Laird, 2002). For qualitative methylation information, methylation-specific polymerase chain reaction is commonly utilized. It amplifies both the unmethylated and the methylated forms of a bisulphite-modified DNA sequence, which can then be used for comparative analysis. This is done by utilizing two distinct primer sets for the methylated and unmethylated forms, which can then anneal to the cytosines, which may or may not be methylated (Price *et al*., 2007).

Issues in sperm selection

Most current research being conducted to improve the ICSI protocol is exploring various methodologies of selecting genetically healthy spermatozoa from a pool of subnormal cells. Spermatozoa are naturally selected for fertilization with IVF or natural conception based on how a spermatozoon interacts with the zona pellucida. However, since this selection mechanism is bypassed with ICSI, sperm selection is instead based on the embryologist's visual morphological assessment. Therefore, exploring the relationship between sperm morphology and fertilizing capacity is of critical importance to the success of ICSI procedures (Celik-Ozenci *et al*., 2004).

Since structurally abnormal human spermatozoa do not necessarily contain an abnormal chromosome constitution (Viville *et al*., 2000), it is not surprising that many normal babies have been born after ICSI using deformed spermatozoa. Examples include the birth of babies after ICSI using round-headed spermatozoa without acrosomal caps (Zeyneloglu *et al*., 2002), stump-tail spermatozoa (Stalf *et al*., 1995), and immotile spermatozoa of men with axonemal defects (Okada *et al*., 1999). Spermatozoa from some men may be immotile due to low intracellular concentration of cAMP. 'Awakening' spermatozoa from such samples using a phosphodiesterase inhibitor known as pentoxifylline before performing ICSI has resulted in healthy children (Terriou *et al*., 2000). It has also been found that some immotile spermatozoa are still viable for ICSI use, as indicated by functional sperm tail membrane integrity. This integrity was tested by aiming a pulse of diode laser at the tail. A viable spermatozoon would curl its tail in response to the laser. ICSI outcomes are significantly improved when spermatozoa are selected through diode laser, as opposed to a hypo-osmotic swelling sperm selection technique (Aktan *et al*., 2004). Still, one should not dismiss the negative correlation between sperm deformation and genetic health, since the levels of reactive oxygen species, DNA fragmentation, and chromosomal aberrations are all increased significantly in men with oligozoo/asthenozoo/teratozoospermia (Griffin *et al*., 2003; Aitken and Baker, 2006; Strassburger *et al*., 2007). Even though sperm morphology is still considered to be the best predictor of fertilization for the natural method, the conventional IVF method and the intrauterine insemination method of conception, many studies have found no correlation between sperm morphology and success with ICSI (Bartoov *et al*., 2002). On the other hand, many studies have also found the opposite to be true (Berkovitz *et al*., 2005). Celik-Ozenci *et al*. (2004) expanded on these studies by examining the correlation between chromosomal aberrations and sperm morphology using a variety of methods, including fluorescence in-situ hybridization, objective morphometry and Kruger strict morphology. They reported that while the risk of chromosomal aberrations is somewhat increased for amorphous spermatozoa, such aberrations can be present in most any sperm cell, regardless of morphology. Ultimately, it is understood that a more direct and accurate measure of DNA damage in sperm cells is needed for clinicians performing ICSI (Celik-Ozenci *et al*., 2004). One potential solution could involve the novel electrophoretic method, which has been reported to isolate spermatozoa without DNA damage, using cell size and electronegative charge as selection criteria (Ainsworth *et al*., 2005). The group recently established their first pregnancy using this method for a couple suffering from extensive DNA damage in spermatozoa (Ainsworth *et al*., 2007).

A study was recently conducted by the Cleveland Clinic on the use of magnetic cell sorting (MACS) to separate apoptotic spermatozoa from non-apoptotic spermatozoa in a sample. It was found that removing the apoptotic spermatozoa resulted in improved sperm quality as well as increased capacity to penetrate an oocyte, and lessened DNA fragmentation. Hence, implementing MACS in the current IVF protocol has obvious advantages. Unfortunately, the benefits of using MACS for ICSI are much less certain, since reducing apoptosis and DNA fragmentation did not seem to affect the ability of spermatozoa to complete the vital chromatin decondensation step. While using MACS for ICSI would certainly improve the genetic health of the early and late embryos that are produced, the fertilization rate and initial embryo quality would not necessarily be improved. Before MACS could be implemented into ICSI protocol, further studies would have to be performed to illustrate that MACS could improve the fertilization rate (Said *et al*., 2006).

It has been suggested that complications with ICSI and IVF may result from fine morphological abnormalities in spermatozoa. For example, one study by Wittemer *et al*., (2006) analysed the impact of abnormal fine sperm morphology on the outcomes of IVF and ICSI. They found that when high-

magnification evaluation (×12,500) revealed that less than 8% of spermatozoa in a sample were of normal fine morphology, the successful fertilization rate for IVF was significantly lower than that of ICSI (Wittemer *et al.*, 2006). It was also found that subtle malformations in the sperm nucleus, which would not be observed by morphologists or embryologists in the routine sperm selection process, cause poorer ICSI outcomes. In one prospective study, a motile sperm organellar morphology examination (MSOME) was utilized to explore the relationship between the morphological normalcy of sperm nuclei and the potential to achieve fertilization and pregnancy after ICSI is conducted. A positive correlation was found for both fertilization and pregnancy potential (Bartoov *et al.*, 2002). Additionally, it was shown that the presence of large vacuoles housed in the sperm nucleus is more indicative of nuclear DNA damage than the size or shape of the nucleus. A significant negative correlation was noticed between DNA integrity and vacuole size by using the sperm chromatin structure assay. The implantation, pregnancy, and abortion rates of ICSI were then compared with those of intracytoplasmic morphologically selected sperm injection (IMSI). IMSI is a technique similar to ICSI, except MSOME is utilized in the sperm selection procedure, ensuring that the selected spermatozoa have morphologically normal nuclei. It was found that using IMSI resulted in significantly increased rates of both implantation and pregnancy, while the abortion rate was significantly decreased (Berkovitz *et al.*, 2005). Even though using MOSME would greatly increase the success rate of ICSI procedures, the prohibitively high cost of the required microscopy system and video enhancement will surely prevent its widespread use in most ART clinics.

Hyaluronic acid (HA) is a linear polysaccharide thatenhances the long-term motility and velocity of spermatozoa in normozoospermic, oligozoospermic and frozen–thawed sperm samples. Additionally, HA-selected spermatozoa have no DNA degradation or active caspase-3, which greatly contributes to the abnormal apoptosis process associated with some immature spermatozoa (Cayli *et al.*, 2004). HA-based sperm processing also shows better post-thaw Computer Assisted Semen Analyser velocity parameter scores when compared with density gradient and other methods (Bhattacharyya *et al.*, 2005). Another interesting observation was that when HA-migrated spermatozoa were washed and suspended in IVF culture media, a considerable sperm fraction of normozoospermic samples exhibited hyperactivation-like motility patterns. Moreover, the HA selected spermatozoa had improved DNA integrity when compared with neat semen, as observed by acridine orange staining (AC Varghese *et al.*, unpublished observation).

Jakab *et al.* (2005) reported a non-invasive strategy for incorporating HA sperm selection into the current ICSI protocol, which should result in a significant decrease in apoptosis and aneuploidies. The method is based on the discovery that HA-binding sites on spermatozoa are regulated in a very similar manner to zona-pellucida-binding sites during plasma membrane remodelling and spermatogenesis. As they predicted, they found a similar frequency of chromosomal aberrations between HA-selected spermatozoa and zona-pellucida-selected spermatozoa, regardless of the average frequency in the sperm sample. Hence, the widespread clinical use of HA-mediated sperm selection could ultimately solve the pertinent problem of aneuploidies and DNA fragmentation when ICSI is performed with immature sperm samples (Jakab *et al.*, 2005).

Another potential method of sperm selection is using a polarizing microscope to detect normal birefringence in sperm heads, since birefringence can be disturbed in spermatozoa whose internal protoplasmic structures are pathologically altered. Significantly higher fertilization and implantation rates were achieved when comparing the results of ICSI with birefringence selected spermatozoa and conventionally selected spermatozoa (Crippa *et al.*, 2006). Likewise, polarizing microscopy can be utilized to ensure full oocyte maturity before spermatozoa are injected, since it can detect the presence and position of a birefringent meiotic spindle in a metaphase II (MII) oocyte (**Figure 1**). It has been shown that absence or misalignment of the spindle at the time of ICSI is a good predictor of oocyte immaturity, potentially leading to fertilization failure or abnormalities (Rienzi *et al.*, 2003). Studies of fertilization and embryo quality indicated that if ICSI is to be performed using an in-vitro matured oocyte, it is best to wait 2–4 h following the first polar body extrusion. It was then postulated that after nuclear maturation, the oocyte still needs time to reach full cytoplasmic maturity before it can produce a viable embryo (Hyun *et al.*, 2007).

Issues in sperm injection

One study on mice found that removing the outer plasma membrane and acrosome cap from the spermatozoa before injection for ICSI significantly decreased the time between sperm injection and oocyte activation by spermatozoa, thereby improving the quality, health and development of the resulting embryo (Morozumi *et al.*, 2006). With natural conception, the acrosome and its contents are removed before a spermatozoon enters the oocyte, exposing the perinuclear material (theca) of the spermatozoon. Once the spermatozoon enters, the oocyte is immediately activated by sperm-borne oocyte activating factor, which is bound to the now exposed perinuclear theca of the spermatozoon (Morozumi *et al.*, 2006). This activation is essentially the onset of Ca^{2+} oscillations within the oocyte. Removing the outer plasma membrane and acrosome cap before ICSI activates oocytes in the same manner as in natural conception (Morozumi *et al.*, 2006).

When typical ICSI protocol is utilized, the whole spermatozoon is injected, including its outer plasma membrane and acrosome cap. Therefore, enzymes within the ooplasm must break down the sperm plasma membrane and acrosome before sperm-borne oocyte activating factor can activate the oocyte (Morozumi *et al.*, 2006). This delay of oocyte activation often results in an extension of the normal sperm head remodelling process. Consequently, an asymmetry in sperm chromatin decondensation develops due to the persistence of perinuclear theca at the base of the acrosome. This delay in chromatin decondensation apparently leads to a delay in subsequent nuclear remodelling processes including the recruitment of nuclear pore constituents, and also leads to a delay in DNA replication and first cell cycle following fertilization (Ramalho-Santos *et al.*, 2000). When DNA replication in the early embryo is inhibited or delayed as such, it can lead to significantly decreased transcription and translation (Memili and First, 1999). All of these factors obviously are not fatal or critical to offspring health, since ICSI has been performed successfully in both humans and mice (Morozumi *et al.*, 2006).

Oocyte activation failure following sperm injection is a major problem encountered during ICSI, and it is caused by

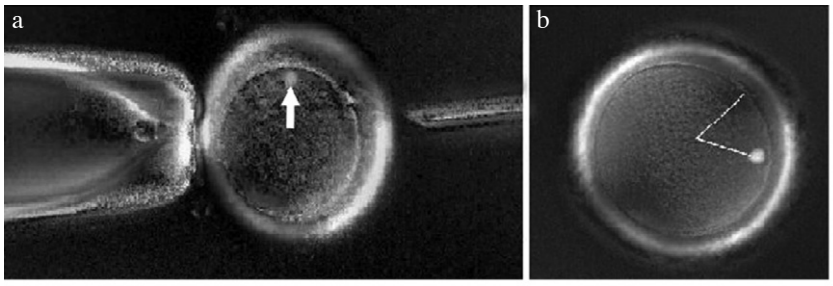

Figure 1. Oocyte spindle as viewed with a polscope. (**a**) Normal, (**b**) deviated from normal position. Image captured at ×200 total magnification using a Nikon T2000 and Cri Oosight™ Imaging system (Woburn, MA, USA)..

the inability of a sperm cell to trigger Ca^{2+} oscillations within the oocyte. A spermatozoon may not be able to induce Ca^{2+} oscillations if it undergoes premature chromatin condensation, or fails to undergo decondensation at the proper time (Rawe *et al*., 2000). In one case report, Ca^{2+} ionophore was utilized to rescue an oocyte that failed to activate, and a healthy child was born (Murase *et al*., 2004). In a recent prospective randomized study comprising ejaculated, epididymal and testicular spermatozoa, it was shown that implantation rates after ICSI improved when artificial oocyte activation using Ca^{2+} ionophore was performed with testicular spermatozoa. However, artificial oocyte activation had no effect when ejaculated or epididymal spermatozoa were used (Iaconelli Junior *et al*., 2006).

Recently a sperm-specific cytosolic phospholipase C (PLCz) has been found to induce Ca^{2+} oscillations within mouse eggs (Saunders *et al*., 2002). It was then shown that if complementary PLCz RNA was injected into oocytes that failed to fertilize after IVF or ICSI, then Ca^{2+} oscillations were induced. Greater concentrations of PLCz resulted in greater oscillation frequency. At low concentrations, PLCz was shown to activate the oocytes and lead to fertilization, cleavage and healthy blastocyst formation. Therefore, PLCz treatment could be utilized for oocytes that failed to activate during ICSI or IVF (Rogers *et al*., 2004). It was found that globozoospermatic spermatozoa can have deficient production or release of PLCz, and result in failure to activate the oocyte. It was then postulated that PLCz injection with such spermatozoa could greatly improve the rate of oocyte activations and fertilization (Schmiady *et al*., 2005). PLC offers an alternative means by which failed activation may be restored with a more physiological stimulus than ionophore.

Another consequence of injecting spermatozoa with intact acrosomes is that the hydrolytic acrosomal contents are released into the oocyte instead of outside it, as happens with natural conception. It was reported that both cholesterol in the sperm plasma membrane and hydrolysing contents within the acrosome are potentially harmful to the resulting embryo, and can lead to chromatin remodelling and DNA damage (Tateno and Kamiguchi, 2007)., The majority of healthy cells have some natural degree of defence against such enzymes, but if and how the enzyme activity is suppressed upon contact with the ooplasm remains a mystery (Hewitson, 2004). It has been expected that infertile males will show abnormal levels of hydrolytic acrosomal enzymes, either increased or decreased. It has also been deemed possible that infertile women may possess oocytes which are more sensitive to hydrolytic acrosomal enzyme activity, making ICSI a challenge for the clinician, and likely resulting in abnormal embryo growth, or death (Morozumi and Yanagimachi, 2005).

Returning to the study of acrosome removal for ICSI on mice, the chances of producing a live, healthy offspring were greatly improved in mice when the acrosome and plasma membrane were removed prior to conducting ICSI. However, any similar improvements in human ICSI protocol would have to account for the fact that human sperm plasma membranes are much more stable than that of mice and many other animals. The greatest results achieved during the mouse study were obtained by removing the plasma membrane for each individual spermatozoon, and then immediately conducting ICSI. To remove the membrane, lysolecithin, a membrane disruptor and a product of plasma membrane phospholipid hydrolysis, was used on the spermatozoa at a concentration of 0.02% for a full minute (Morozumi *et al*., 2006).

One interesting study compared the fertilization rates of ICSI using fresh versus cryopreserved spermatozoa, and noted that the rates of fertilization by cryopreserved spermatozoa were consistently higher than that of fresh spermatozoa. They postulated that this was caused by the plasma membrane damaging properties of freeze–thaw cycles. When cryopreservation is used, the sperm plasma membrane weakens and ruptures under the stress of osmotic pressure and the formation of ice crystals. This could allow sperm chromatin to be released from the sperm cell quicker upon entering the ova through ICSI, and result in greater fertilization rates (Wald *et al*., 2006).

It was recently reported that when oocytes did not undergo complete denudation, which is cumulus cell removal, before execution of ICSI, both embryo quality and blastocyst development were greatly improved. However, injecting a sperm cell into an oocyte in the proper location is very difficult if there are cumulus cells that block the desired point of injection. In that case, one must either find an alternate point of injection, or pierce a cumulus cell in the process, potentially compromising the oocyte's potential to fertilize (Ebner *et al*., 2006). One remedy may be to use a laser beam to specifically remove cumulus-corona cells from the injection pole after holding the complex with a holding pipette (Rienzi *et al*., 2001).

Revising the current ICSI protocol

Figure 2 illustrates a means of selecting spermatozoa for IVF. This mode of selection procedure simulates in-vivo selection pressures faced by human spermatozoa. ICSI could surely benefit from utilizing this IVF procedure, since spermatozoa selected for ICSI do not normally face these important selection pressures. The spermatozoa that pass this type of selection process were demonstrated to be acrosome reacted and capacitated, with improved motility, morphology, and capacity to bind to zona-pellucida (Hong *et al.*, 2004). Initial experiments have also indicated that spermatozoa which are migrated through the straw column containing 5 mg/ml HA and 25 mg/ml bovine serum albumin (Tang *et al.*, 1999) in Medicult flushing media (Medicult, Copenhagen, Denmark),and then collected in to the culture media, possess very good motility kinematics (AC Varghese *et al.*, unpublished observation). It also showed a very high fraction of hyperactivated spermatozoa as assessed by Sperm Class Analyser (Microptic SL, Barcelona, Spain).

Figure 3 presents prototype of a selection and injection model for ICSI. Sperm cells from an oligo/oligo-astheno/percutaneous epididymal sperm aspiration (PESA)/testicular sperm aspiration (TESA) sample are incubated with follicular fluid and cumulus cells in order to specifically augment competent, acrosome-reacted sperm cells. The high magnification objectives (like recently described IMSI) can select morphologically normal spermatozoa, which could potentially be injected into the oocyte. **Figure 3a** depicts a model to select zona-bound and presumably normal spermatozoa for ICSI. Samples having adequate progressive motility to interact with zona may be ideal candidates for this procedure. Frozen donor MI or unfertilized MII oocytes with a normal zona pellucida, could be thawed on the day of ICSI and patient spermatozoa incubated with these oocytes along with some cumulus cells. The spermatozoon that first bound to the zona pellucida would then be removed from it and microinjected into the patient's oocyte. One study found that spermatozoa that can bind to the zona pellucida are robust, acrosome-reacted, and almost always possess normal, double-stranded chromatin, as determined by acridine orange fluorescence. Therefore, this added selection step should theoretically help to improve ICSI fertilization rates (Liu and Baker, 2007). A non-denuded or partially denuded oocyte would be held with a holding pipette and a laser would be utilized to remove the necessary cumulus cells before microinjection. The study by Ebner *et al.* (2006) showed the beneficial effect of using partially denuded oocytes for the ICSI procedure. It was shown to increase the quality of subsequent embryo and blastocyst development by enhancing the cytoplasmic and nuclear maturation of retrieved oocytes (Ebner *et al.*, 2006). Finally, the oocyte would be cultured with other autologous cumulus cells and then transferred to the uterus. That process is called cumulus assisted embryo transfer, and this was shown to increase pregnancy rates (Parikh *et al.*, 2006). We would like to indicate that these revised protocols are proposed models that require well-controlled animal and human studies before they can be incorporated into the current ICSI protocol.

Defining the role of ICSI in infertility management, and where do we go from here?

Ultimately the question arises: if a sperm sample reveals that IVF or ICSI can be used for infertility therapy, which technique should be utilized? Though ICSI has been recommended as the method of choice for teratozoospermic samples and samples having <4% normal morphology according to strict criteria, recent reports show that fertilization through IVF yields similar or greater pregnancy rates than ICSI (Check *et al.*, 2007; Keegan *et al.*, 2007). It has been suggested that even when a male's spermatozoa show subnormal morphology according to strict Kruger's criteria, ICSI may not always be the proper treatment, since it consumes much of the embryologist's time, as well as the patients' and clinic's monetary resources. Additionally, it was found that utilizing ICSI for such patients resulted in significantly greater fertilization rates, but significantly lower rates of implantation, pregnancy and delivery compared with conventional insemination (Check *et al.*, 2007). If neither IVF nor ICSI prove to be the superior technique over the course of many years and published studies, it might be advantageous simply to treat half of the retrieved oocytes in each infertility cases with IVF and half with ICSI. This would hopefully lessen any potentially negative effect of IVF or ICSI on the human gene pool. A well-defined embryo scoring strategy and embryo transfer technique are also major determinants for a better pregnancy outcome with IVF/ICSI cases (Scott, 2003; Terriou *et al.*, 2007; Visschers *et al.*, 2007).

Since mature spermatozoa contain little cytoplasm and are not yet known to synthesize proteins, it was always believed that a sperm cell only contributed its genetic content to the resulting embryo (Ainsworth, 2005). However, this concept is fast changing in the genomic era, and it is now known that sperm cells deliver RNAs that are vital in directing early embryo development. Still, these RNAs are not necessarily used for protein translation within the embryo, since they play a role in controlling embryonic stem cell differentiation and gene activity, such as regulating gene imprinting (Ostermeier *et al.*, 2004). Additionally, there has been much evidence suggesting that the phenotypic characteristics of offspring are influenced by sperm RNA (Miller and Ostermeier, 2006). In order to improve the effectiveness of ICSI and clinical infertility management in general, research should be conducted which analyses the RNA profiles of infertile males who undergo procedures like ICSI.

Conclusion

For couples who do not find success with natural conception or IVF, ICSI can truly be a blessing. However, due to the invasive nature of the procedure, there is great potential for complications of all degrees of severity. Additionally, the long-term effects of ICSI on the resulting children remain largely unknown, since the first successful ICSI-conceived child was born in 1992, and most long-term ICSI studies have not yet completed data collection. Efforts to conceive using ICSI can be frustrated by a number of factors, including poor embryonic genetic health, and failure of germ cells to properly execute vital biological events such as fertilization. In some instances, success with ICSI is compromised because of the technical limitations of

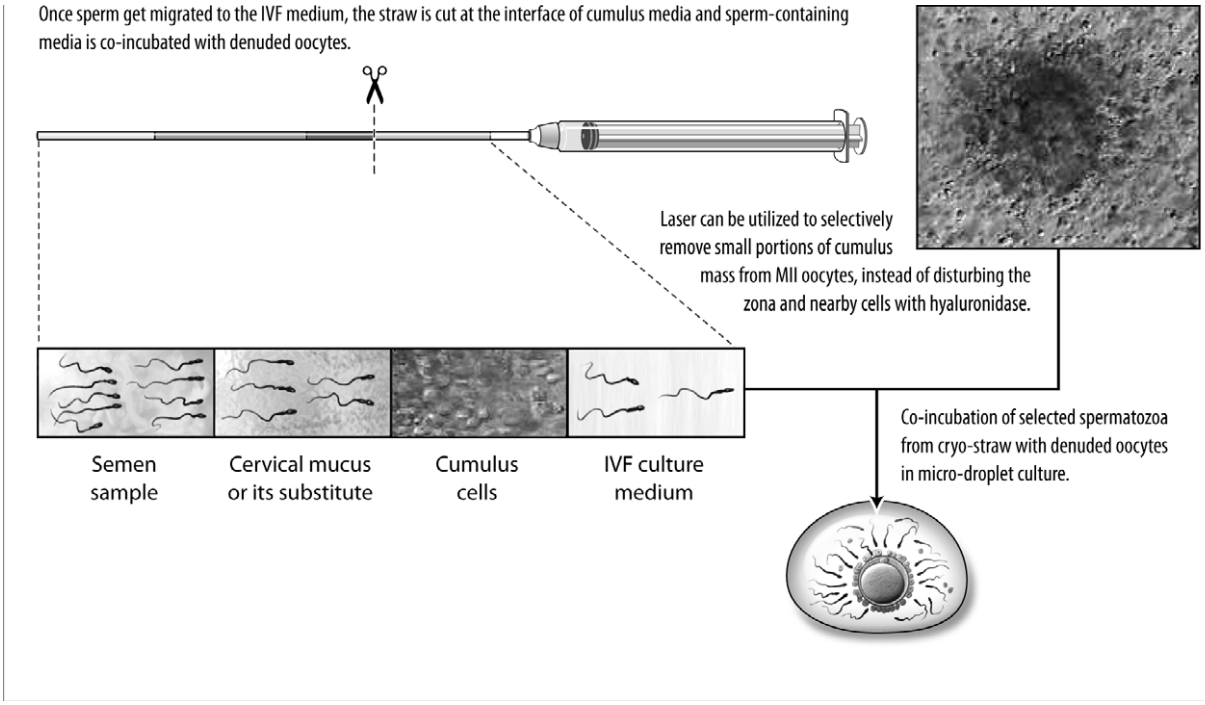

Figure 2. A physiologically oriented sperm selection and IVF procedure for subnormal semen samples having moderate-to-good motility. MII = metaphase II.

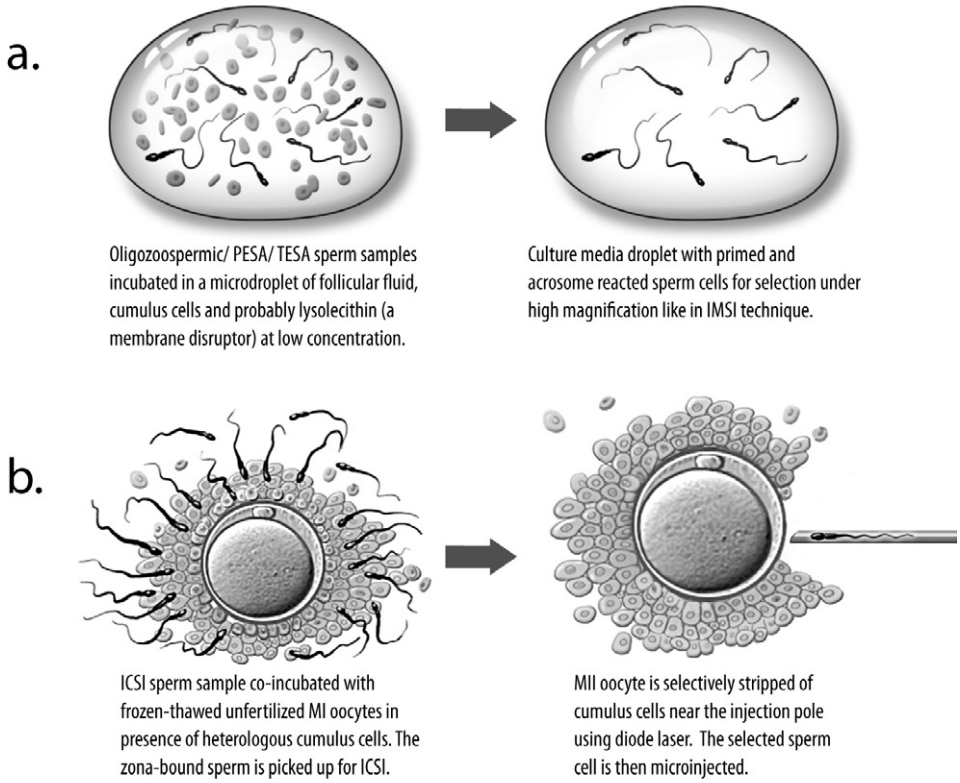

Figure 3. (**a**) An in-vitro priming of sperm cells combined with induction of acrosome reaction and selection of morphologically normal cells by high-magnification objectives. IMSI = intracytoplasmic morphologically selected sperm injection; PESA = percutaneous epididymal sperm aspiration; TESA = testicular sperm aspiration. (**b**) Zona-bound spermatozoa selection for intracytoplasmic sperm injection (ICSI) and selective removal of cumulus from metaphase II (MII) oocytes before microinjection using diode laser.

ICSI instruments, clinicians and laboratory technicians. In other failed cases, solutions cannot be proposed until more knowledge is gained on the underlying pathology of infertility. As new studies on the subject continue to be published, it is important that the established protocol of ICSI is revised on a regular basis, and that its role in infertility therapy is continually re-evaluated.

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