

Intracytoplasmic sperm injection: state of the art in humans

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Abstract

Among infertile couples, 25% involve both male and female factors, while male factor alone accounts for another 25% due to oligo-, astheno-, teratozoospermia, a combination of the three, or even a complete absence of sperm cells in the ejaculate and can lead to a poor prognosis even with the help of assisted reproductive technology (ART). Intracytoplasmic sperm injection (ICSI) has been with us now for a quarter of a century and in spite of the controversy generated since its inception, it remains in the forefront of the techniques utilized in ART. The development of ICSI in 1992 has drastically decreased the impact of male factor, resulting in millions of pregnancies worldwide for couples who, without ICSI, would have had little chance of having their own biological child. This review focuses on the state of the art of ICSI regarding utility of bioassays that evaluate male factor infertility beyond the standard semen analysis and describes the current application and advances in regard to ICSI, particularly the genetic and epigenetic characteristics of spermatozoa and their impact on reproductive outcome.

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Background

Infertility is defined as the failure to conceive after one year of unprotected intercourse and affects approximately 15% of couples of reproductive age worldwide (Palermo *et al.* 2014a). Therefore, the use of ART to treat couples unable to conceive has increased steadily, representing 1.5% of all infants born in the United States (Sunderam *et al.* 2015). Among all indications, male factor infertility is responsible for approximately 50% of couples who are unable to conceive.

Many procedures were developed in the 1980s to address fertilization failure due to dysfunctions of the male gamete, notably zona drilling (Gordon & Talansky 1986), zona softening (Gordon *et al.* 1988, Kiessling *et al.* 1988) or partial zona dissection (Cohen *et al.* 1988) with the most efficient process being subzonal injection of a single spermatozoon into the perivitelline space (Laws-King *et al.* 1987, Palermo & Van Steirteghem 1991, Palermo *et al.* 1992a). Indeed, it was during the performance of subzonal injection of an oocyte that the oolemma was accidentally breached and the spermatozoon was delivered into the ooplasm, subsequently establishing the development of intracytoplasmic sperm injection (ICSI) as it is performed still today in humans (Palermo *et al.* 1996a).

To date, ICSI (Palermo *et al.* 2014d) has been responsible for over two million babies worldwide

(ESHRE 2012, Sullivan *et al.* 2013) and has supplanted prior assisted fertilization techniques due to its ability to successfully bypass zona pellucida irregularities and circumvent the presence of antisperm antibodies, sperm acrosome dysfunction and sperm kinetic defects (Palermo *et al.* 1996c).

As previously mentioned, ICSI involves the injection of a single sperm cell directly into the ooplasm. The treatment capabilities of ICSI range from the utilization of spermatozoa with poor progressive motility to those gametes microsurgically collected from the epididymis and the testis of azoospermic patients (Palermo *et al.* 1995, 1999, Wallach *et al.* 1996). Beyond male factor, an additional application for ICSI is cases with low oocyte yields. Indeed, ICSI has been used in European countries, such as Germany and Italy, to comply with restrictive laws that limit the number of eggs to be inseminated (Ludwig & Diedrich 1999, Benagiano & Gianaroli 2004). ICSI is also very useful for fertilization of oocytes that were previously cryopreserved (Porcu *et al.* 1997) as cryostress can lead to a premature exocytosis of cortical granules and zona hardening, hindering spermatozoa from penetrating naturally (Johnson 1989, Schalkoff *et al.* 1989, Vincent *et al.* 1990, Van Blerkom & Davis 1994). ICSI is the preferred insemination method to avoid polyspermy, fertilize a high number of oocytes and generate a maximal cohort of embryos. Additionally, the selection of a single spermatozoon significantly reduces



the chance of transmission of HIV, HBV and HCV, among others. Indeed, the eventual presence of viruses in semen or accompanying cells may be reduced by the removal of seminal fluid by density gradient preparations and by retrieval of sperm cells directly from a viscous medium just prior injection (Vitorino *et al.* 2011), electing ICSI as the preferential method of insemination for patients at risk for HIV (Peña *et al.* 2002, Sauer & Chang 2002, Mencaglia *et al.* 2005).

ICSI is also unaffected by the immaturity of the male gamete, such as spermatozoa retrieved directly from the epididymis or testis, which are often characterized by an incomplete flagellum and an underdeveloped cell membrane (Palermo *et al.* 1996c, 1999). Successful pregnancies from the use of these spermatozoa has pressed the boundaries of the application of ICSI to the most extreme aspect of male infertility, often encountered in cryptozoospermia, virtual azoospermia or of men with absolute azoospermia where surgical retrieval is required (Ron-El *et al.* 1997).

In regard to the popularity of ICSI, a cross-sectional survey of ART procedures performed in 60 countries during 2010 by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), reported that 63.0% (455,845 of 723,855) of all cycles utilized ICSI (Dyer *et al.* 2016) ranging from a prevalence of 58.4% in Asia to a virtual totality of 98.4% in the Middle East (Dyer *et al.* 2016). Another recent publication, which analyzed ART trends in the United States between 1996 and 2012, reported an increase in the use of ICSI from 36.4% in 1996 to 76.2% in 2012 (Boulet *et al.* 2015). Indeed at our center, there has also been a progressive increase in ICSI utilization starting at 32.2% in 1993 rising to 48.8% in 1995, 73.6% by 2002 and 79.29% in 2016 (Palermo *et al.* 2015b, Dyer *et al.* 2016).

The high utilization of ICSI at our center is partly related to the tertiary nature of the clinic and the fact that we are highly integrated with the services of male reproductive urology and surgery. This being the reason why our patients are referred from other clinics, often with a history of several failed ART cycles in terms of fertilization and/or pregnancy. As a note of caution, this should not encourage the indiscriminate utilization of ICSI for cases of non-male factor infertility and should mainly focus on cases with male reproductive dysfunction.

The ability of ICSI to achieve fertilization independently of any observable characteristics of the spermatozoon, although puzzling initially, has guided research into the processes involved in successful fertilization, particularly in cases where dysfunctional spermatozoa have been delivered into the ooplasm. The disparity between the success of ICSI and classic semen parameter thresholds has induced the development of new bioassays aimed at qualifying the male gamete from a genetic and epigenetic point of view. In this review, we describe such pertinent bioassays that investigate the

effects on clinical outcome in relation to chromosomal aneuploidy, chromatinic integrity, perinuclear PLCZ responsible for triggering oocyte activation and inducing the initial step of fertilization, the role of the centrosome as a scaffold for the segregation of the first embryonic cleavage, and finally the recently discovered presence of small RNA, that appear imperative post-fertilization to guide embryo development prior to the activation of the embryonic genome. We will also describe the clinical achievements of ICSI throughout the last quarter of a century in regard to the use of spermatozoa of various sources, quality and status considering safety implications on the offspring generated.

Male gamete bioassays

The semen analysis is the first test that reproductive physicians consult to gain initial information on the male partner's fertility. Semen analysis is carried out according to the WHO guidelines (WHO 2010) and while the assay measures individual parameters such as semen volume, concentration, motility and morphology of the spermatozoa present, frequent variability among ejaculates in individuals is a recurrent issue (Schwartz *et al.* 1979, Mallidis *et al.* 1991). Moreover, the assessment of semen parameters are subjective and so may appear inconsistent across laboratories (Neuwinger *et al.* 1990, Cooper *et al.* 1992, Matson 1995). It should be noted that the range of normal values published by the WHO are not evidence based and therefore are difficult to interpret in relation to their diagnostic value, resulting in a blanket diagnosis of unexplained infertility that nonetheless can be identified by use of assays with a higher sensitivity (such as the acrosome reaction, antisperm antibody and PLCZ tests). The converse is also true when gametes with suboptimal semen parameters display normal function (Irvine *et al.* 1986, Glazener *et al.* 1987). Due to this disparity between the subjectivity of the semen analysis and WHO standards, it would be advisable for each individual laboratory to identify their own 'normal' semen profile.

High magnification sperm morphology

Traditionally the morphological assessment of spermatozoa has been considered a valuable element (MacLeod & Wang 1979) to predict the fertility potential of infertile men undergoing ART (Kruger *et al.* 1986). However, the introduction of ICSI (Palermo *et al.* 1992b) has diminished the relevance of semen parameters in this procedure due to their inability to predict fertilization and pregnancy outcomes in male factor cases (Nagy *et al.* 1995).

Although there is no apparent correlation with clinical outcome, ICSI has shifted the focus from evaluating blanket semen parameters to the observation of each individual male gamete, with the aim of identifying

a spermatozoon with normal morphology implying inherent competence in achieving fertilization and supporting embryo development. Selection of individual spermatozoa under high magnification, defined as intracytoplasmic morphologically selected sperm injection (IMSI), is a method used to select spermatozoa that have the choicest morphology in couples with the most severe male factor. IMSI has been proposed in patients with recurrent implantation failure or spontaneous abortions (Lo Monte *et al.* 2013) attributing these pregnancy failures to a sperm defect. However, recent studies have challenged this view, arguing that IMSI does not improve outcomes for couples undergoing a repeated ART attempt (Oliveira *et al.* 2011, Gatimel *et al.* 2016).

In human spermatozoa, it is known that irregular vacuolization of the head is almost ubiquitous (Watanabe *et al.* 2011) and seems to be a paraphysiologic finding (Bedford *et al.* 1973, Tanaka *et al.* 2012), not a sign of incompetency. Irregularities of the sperm head surface occur during spermiogenesis and may have a disparate prevalence in male gametes retrieved from different sites of the male genital tract, and in addition, the appearance and size of the vacuoles may be related to the different stages of capacitation (Kacem *et al.* 2010). Indeed, previous studies have demonstrated that abnormal semen profiles are associated with a modest increase in the frequency of sperm chromosomal abnormalities and that spermatozoa with aberrations in their shape and head contours may be carriers of chromatinic defects (Lewis-Jones *et al.* 2003, Sun *et al.* 2006). However, vacuoles identified on the sperm head are not necessarily related to DNA fragmentation or aneuploidy and therefore, their influence on embryo development remains unclear (Watanabe *et al.* 2011). Moreover, the issue of safety remains paramount to address, indeed infants born from this procedure tend to have a higher occurrence of low birth weight (<2500 g) (Junca *et al.* 2010).

Although different high-power magnification techniques corroborated by video-generated magnification have been suggested to deselect dysmorphic spermatozoa, these methods are inherently limited by the clarity of the image, the time required for image analysis and the risk of variable spermatozoa head-positioning during imaging due to the asymmetry of the head contour (Palermo *et al.* 2011).

While the current concept of IMSI is still being debated and further augmented, the interest in the meticulous assessment of the spermatozoon, its contour and all its facets remains an intriguing quest. To address these observational issues, a study was performed at our center aimed at eliminating the aforementioned limitations on high magnification sperm morphology, in which an image-tracking software was used to capture serial photographs of spermatozoa from recorded videos. The images were automatically extracted from each

digital frame using enhanced correlation coefficient maximization; the general shape of the spermatozoa was then extracted via space-carving. The reconstructed image was rotated to permit viewing from any vantage and the final image was rendered via interpolation. This method yielded images that enable noninvasive, 3-D, real-time, *in vitro* assessment of sperm surface morphology that is easily automated and required little equipment, presumably available in most embryology laboratories. From this study, we observed that although a spermatozoon may appear to be morphologically acceptable by IMSI standards, when rotated, the head of the cell revealed a vacuole, suggesting that even spermatozoa selected via high magnification for injection may still possess the same characteristics as the spermatozoa deselected in the IMSI procedure (Levine *et al.* 2015).

While the selection of the sperm is undoubtedly the proper approach, it is clear that physical characteristics do not provide any specific information on the health of the genome and epigenome of the male gamete. Therefore, further screening needs to be employed to truly quantify the competence of the spermatozoa.

Sperm aneuploidy assessment

High fetal wastage in humans is commonly attributed to aneuploidy. Most aneuploid pregnancies do not survive, with the majority of losses occurring during the first few weeks of uterine life. In general, autosomal trisomies constitute the large majority of aneuploid embryos, with 16, 18 and 21 having a maternal origin and sex chromosomal aneuploidies (45X, 47XXY, 47YY) often originating from the paternal origin (Hassold & Hunt 2001, Neri *et al.* 2014a). While meiotic errors that lead to fetal aneuploidy can originate from either the male or female gamete, the occurrence is lower in spermatozoa (9%) when compared to oocytes (20%) (Hassold & Hunt 2001, Neri *et al.* 2014a). Regardless, assessment of male gamete ploidy is an important aspect of pre-fertilization genetic diagnosis.

The assessment of sperm chromosome abnormalities has increased over the years with the popularity of ICSI, especially after it was recognized that infertile men possess an increased frequency of sperm aneuploidy despite having a normal peripheral karyotype (Martin 2006). It was previously demonstrated that men with suboptimal semen parameters have an increased frequency of sperm chromosomal abnormalities (Colombero *et al.* 1999a). Furthermore, chromosomal evaluation of the aging male gamete has become of particular interest and is crucial in determining the extent of meiotic errors, which can affect the conceptus' health. In studies performed on ejaculated spermatozoa, the occurrence of chromosome 18 disomy has been found to be significantly higher in men over 50 years of age (Griffin *et al.* 1996). There was also a linear increase in

chromosome 9 disomy with respect to age (Bosch *et al.* 2003). Similar results were reported for chromosome 21 (McIntosh *et al.* 1995), where a higher frequency of disomy 21 was identified in men more than 60 years of age (Rousseaux *et al.* 1998). Chromosome 1 disomy was reported to increase with advancing paternal age, while the assessment of gonosomal disomy observed an increase in disomy YY (Martin *et al.* 1995). There was also a significant relationship established between increasing paternal age and XY disomy (Asada *et al.* 2000).

These findings, however, were not always confirmed by other studies. For instance, in a separate study, there was no correlation found between paternal age and chromosome 12, XX or XY disomy (Martin *et al.* 1995). Even more contradictory, chromosome 13 and 18 disomy have both been found to increase in younger men instead (Steiner *et al.* 2015). Furthermore, another study found that although aneuploidy of the gonosomes is more common than that of autosomes, neither occur more frequently with increasing paternal age (Donate *et al.* 2016).

Although fluorescent *in situ* hybridization (FISH) enables the detection of chromosomal abnormalities, the indications for FISH on sperm are currently not fully established (Funaro & Paduch 2014). At our center, patients with a history of recurrent ART failure or recurrent pregnancy loss are assessed for sperm aneuploidy. This assessment is particularly stressed for azoospermic patients who are undergoing epididymal and testicular retrieval. However, with FISH, only a limited number of chromosomes can be analyzed and only specific regions of interest labeled by the fluorescent probe may be visualized (Fig. 1) (Neri *et al.* 2014a). Twenty-four chromosome FISH on spermatozoa may be a more advantageous method to determine the overall aneuploidy in a particular specimen (Hu *et al.* 2011). Furthermore, a more accurate aneuploidy assessment can be achieved by determining specific copy number variants of sequenced DNA in individual spermatozoa.

Sperm chromatin assessment

During late spermiogenesis in the testis, male gametes undergo a complex and sensitive process of chromatin condensation, in which DNA strands are severed in order to allow tight supercoiling around protamines, owing to the action of testis-specific serine kinase 6 (TSSK6) (Palermo *et al.* 2014c). The chromatin packing of sperm cells is markedly different from that in somatic cells, primarily differentiated by the substitution of protamines for DNA compaction rather than histones (Ward 2010). Said protamines are introduced through an exchange with histones, regulated by the H1 histone family, member N, testis specific (H1FNT) to allow for a tighter compaction of the chromatin. The spermatozoa's DNA is densely wrapped for its protection, rendering the spermatozoa transcriptionally inactive and resilient to

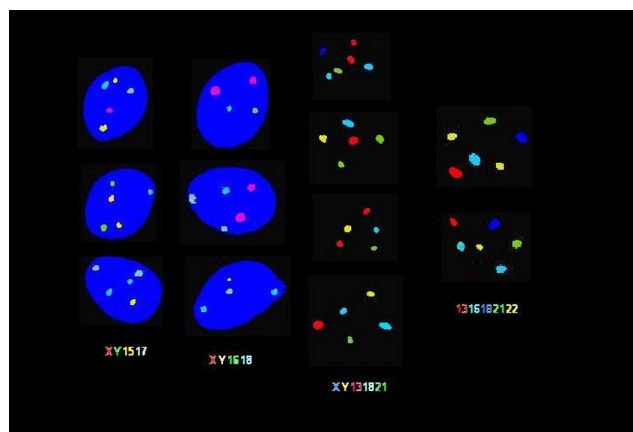


Figure 1 Fluorescent *in situ* hybridization (FISH) analysis of ejaculated human spermatozoa. FISH analysis was carried out using 4 different probe sets. In the 2 columns on the left, sperm chromatin stained with 4',6-diamino-2-phenylindole (DAPI) appears in blue. As indicated from left to right: spermatozoa were assessed by probe sets for chromosomes X/Y/15/17, X/Y/16/18, X/Y/13/18/21 and 13/16/18/21/22 in various colors. As depicted in each cell, disomy is indicated by the appearance of multiple fluorescent signals in the same color. Spermatozoa exhibiting various occurrences of gonosomal and autosomal disomy are shown.

damage during transport through the male genital tract and subsequently, the female genital tract. In addition to providing protection while in transit through the uterine environment, nuclear compaction results in a hydrodynamicity of the head, allowing for better sperm mobility and penetration through the zona pellucida of the ovum (Dadoune 2003).

Although the majority of spermatozoon DNA is tightly bound around protamines, between 2% and 15% of the chromatin is bound in histone linker sections (Dadoune 2003, Hammoud *et al.* 2009b) that can be found throughout the genome, specifically at gene promoter regions (Wykes & Krawetz 2003). The family of genes involved in embryo development has been observed to preferentially persist on residual histone regions in human spermatozoa (Hammoud *et al.* 2009b). This finding demonstrates that histones, rather than being distributed haphazardly in the sperm genome, are linked to specific genes and compose conserved linker regions with high nuclease sensitivity between each protamine-bound toroid (Sotolongo *et al.* 2003). It is these histone linker regions that are actually assessed by the majority of chromatin status tests (Noblanc *et al.* 2013).

Understanding this unique process of chromatin packing is essential in the development of tests for male infertility and the assessment of sperm chromatin characteristics, which may have distinct consequences on ART outcomes (Evenson & Wixon 2006, Zini & Sigman 2009). Several studies have suggested that fertile men with normal semen analyses generally have lower levels of DNA breakage than infertile men, in particular those with compromised semen parameters. However, up to

8% of infertile men with compromised DNA integrity may present with normal parameters for concentration, motility and/or morphology (Zini *et al.* 2001).

Thus, the origin of DNA damage in the male gamete seems to be caused by a multitude of inherent and external factors. Protamine deficiencies and DNA packaging defects (Hammoud *et al.* 2009a) comprise just a small number of the inherent factors that indicate the potential for DNA damage of this cell.

Separately from innate defects that can affect chromatin integrity, advanced age in men has been found to be associated with a higher incidence of sperm DNA damage (Plastira *et al.* 2007, Sakkas & Alvarez 2010, Zanko *et al.* 2014). In addition, environmental factors such as cigarette smoking, (Künzle *et al.* 2003) genital tract inflammation, varicoceles (Saleh *et al.* 2003) and hormone deficiencies (Xing *et al.* 2003) are correlated with increased DNA damage, as described in both human and animal studies (Mathur & D'Cruz 2011, Lewis *et al.* 2013).

It is postulated that spermatozoon DNA integrity is closely associated with sperm quality, male fertility potential and pregnancy outcomes (Brewer *et al.* 2002). Specifically, an abnormal DNA fragmentation index (DFI) is thought to have an inverse relationship with male fertility success (Zini *et al.* 2001) and if pregnancy does occur, these conceptuses are thought to be at an increased risk of miscarriage (Kumar *et al.* 2012).

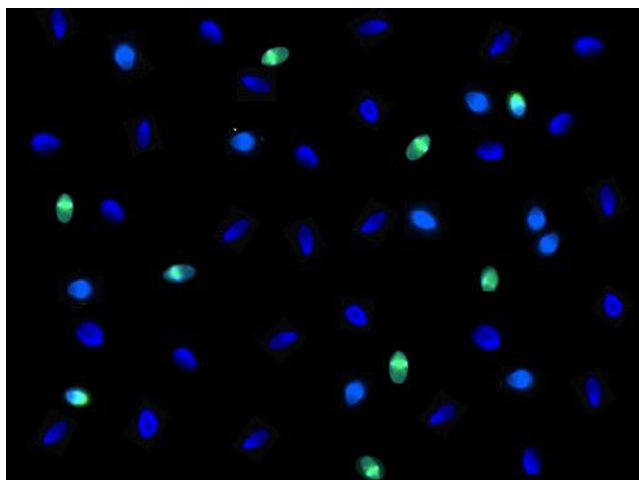


Figure 2 Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) immunofluorescent staining of human spermatozoa for detection of DNA fragmentation. Sperm cells which fluoresce green indicate the presence of DNA fragmentation that occurs during the late stages of apoptosis, detected by the action of the TdT enzyme. Spermatozoa are considered TUNEL positive if approximately 40% or more of the head is fluorescent. Spermatozoa without compromised chromatin integrity are shown in blue using DAPI counterstain. Five hundred sperm cells per sample are assessed using fluorescent microscopy in order to determine the sperm chromatin fragmentation, with a threshold of $\leq 15\%$ TUNEL positivity considered normal.

The most popular techniques for evaluating sperm DNA integrity include the sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling assay (TUNEL) (Fig. 2), sperm chromatin dispersion test (SCD) and the comet assay (Funaro & Paduch 2014). Although there has been little standardization within chromatin integrity assays and a high variability among index thresholds per assay and per testing laboratory, SCSA, TUNEL and SCD have been found to have comparable predictive values for DNA fragmentation (Chohan *et al.* 2006). Although these tests provide invaluable information regarding the chromatin integrity of an individual spermatozoon, it is important to note that these assays are consumptive; in other words, once the sample is used for any of the aforementioned assays, it cannot be used for the treatment of the couple (Funaro & Paduch 2014). Thus, in current clinical practice, it is difficult to isolate spermatozoa with a high level of chromatin integrity for use at the time of insemination (Funaro & Paduch 2014) as characteristics such as an abnormal motility or morphology of the spermatozoon in question, does not directly signify impaired chromatin integrity (Chen *et al.* 2011, Palermo *et al.* 2015a).

With that being said, DNA fragmentation as assessed by the most popular assays, does correlate with semen parameters of motility and morphology *en masse* (Younglai *et al.* 2001, Tang *et al.* 2010); however, this correlation is not predictive of pregnancy outcomes in relation to ICSI insemination as corroborated by Evenson and Wixon (Evenson & Wixon 2006) and by Zini (Zini 2011). ICSI insemination is *sui generis* for sperm transport, eliminating the effects of sibling spermatozoa. Thus, the selection of the best-looking, progressively and regularly motile spermatozoon may account for the lack of correlation between DFI values and pregnancy rates, as it has been found in several studies that utilization of the motile portion of ejaculated spermatozoa with *in vitro* insemination methods curtails DNA-damaged spermatozoa from generating a conceptus (Zini *et al.* 2008, Aitken *et al.* 2009, Simon & Lewis 2011, Palermo *et al.* 2014c).

With that being said, a recent meta-analysis of 16 IVF studies and 24 ICSI studies using TUNEL, SCSA, COMET or SCD did suggest that there is an effect of DNA fragmentation on IVF and ICSI outcomes (Simon *et al.* 2017). However, the majority of the studies included relating DNA fragmentation to ICSI did not control for confounding female factors. These include advanced maternal age that may result in oocytes with a compromised ability to repair DNA damage of spermatozoa (Sakkas & Alvarez 2010, Meseguer *et al.* 2011). Additionally, it was noted in another recent meta-analysis assessing the four most popular chromatin integrity assays mentioned above that, while the TUNEL and COMET tests were of fair predictive power in regard to clinical pregnancies with IVF and ICSI once

compared to SCSA and SCD, the specificity of their predictive power was low suggesting that even levels of DNA fragmentation below threshold did not guarantee a successful pregnancy (Cissen *et al.* 2016). Considering these factors, while a valuable understanding of the effects of DNA fragmentation is beginning to form, widely accepted standards for each assay need to be assigned before we can more accurately evaluate the impact of DNA fragmentation on ART procedures.

PLCz

Despite the widespread success of ICSI treatments for male factor infertility, cases of complete fertilization failure continue to persist. Their occurrence is emotionally devastating for couples, with significant expenditure of logistic and financial resources to no avail. Therefore, understanding the etiology of fertilization failure is of critical importance to counsel patients and devise a successful treatment protocol.

At our center, ICSI with ejaculated spermatozoa yields a fertilization rate well over 70%, irrespective of sperm characteristics. It seems the only requirement for fertilization is that the spermatozoon displays some level of motility and certainly maintenance of viability (Palermo *et al.* 2009). With that being said, couples undergoing ICSI can still experience a total lack of fertilization, with a frequency of occurrence just below 3% (Palermo *et al.* 2015a). Complete fertilization failure can be due to various etiologies such as unsuccessful immobilization and breaching of the spermatozoal membrane, a lack of decondensation of the sperm nucleus or a failure of the oocyte to undergo activation (Sousa & Tesarik 1994, Flaherty *et al.* 1995, Palermo *et al.* 1996c, Yanagida 2004), although most often, the rationale can be attributed to an ooplasmic dysmaturity (Pereira *et al.* 2016b). Nonetheless, in a very small proportion of cases, recurrent failed fertilization is a result of the inability of the male gamete to activate an oocyte due to a lack of an activating cytosolic factor (Swann *et al.* 2004, Kashir *et al.* 2010, Neri *et al.* 2014b).

Our team has been among many that attempted to identify and correct for a sperm cytosolic oocyte-activating factor (Palermo *et al.* 1997, Wolny *et al.* 1999), attributing its absence to the reason why certain infertile men fail to fertilize their partner's oocytes (Neri 2010, Neri *et al.* 2010).

Over a period of 23 years at our center, ICSI was performed in 19,757 couples, of which 2.6% experienced a complete failure of fertilization. In a small portion of these couples, the absence of an oocyte-activating factor in the spermatozoa was reported by PLCZ (phospholipase c-zeta) assessment (Neri *et al.* 2010), distinguishing cases with an oocyte-activating factor deficiency vs an ooplasmic dysmaturity (Neri *et al.* 2014a,b, Pereira *et al.* 2016b).

Oocyte activation is induced by a sizeable influx of calcium into the cell, stimulating the different pathways required for proper fertilization and prevention of polyspermy (Tosti & Ménéz 2016). It has been recently demonstrated in animal studies that PLCZ found in the perinuclear theca of the spermatozoa is the factor responsible for oocyte activation in mammals. A study in mice used the CRISPR-Cas9 system in order to create *Zfy1/2* double-knockout mice, significantly decreasing PLCZ expression and producing mice with spermatozoa that failed to fertilize oocytes after ICSI (Nakasuji *et al.* 2017). In a second study also using CRISPR-Cas9 gene editing technology, investigators were able to create *Plcz1*-null mice and tested their fertilizing capability using IVF and ICSI (Hachem *et al.* 2017). When compared to the wild type, *Plcz*-null mice gametes had significantly higher events of polyspermy in IVF and significantly lower instances of normal fertilization and embryo development after ICSI (Hachem *et al.* 2017). Therefore, in the event of recurrent fertilization failure, it would be useful to screen male patients for the presence of PLCz in spermatozoa. Couples that have experienced fertilization failure as a result of low expression of PLCz in the male partner's gametes then have the option to undergo assisted oocyte activation (AOA) (Neri *et al.* 2010). Although the prevalence of such cases using AOA are low and often result in little success, the use of calcium ionophore treatment in these patients can improve fertilization results and even progress safely to pregnancy (Chi *et al.* 2004, Sugaya 2010, Neri *et al.* 2014b).

Centrosome

During the process of fertilization, fusion of the two parental haploid chromosomal complements allows for the formation of the diploid genome of the conceptus. In humans, the mature oocyte contains the supporting elements capable of sustaining the development of the embryo, while a primary contribution of the spermatozoon is to provide the centrosome, the scaffold that generates the first mitotic spindle and ordains a correct chromosomal segregation for this new entity. In one of the early studies at our center, we were able to define the contribution of the male gamete in supplying the centrosome to the oocyte for proper embryonic cleavage (Palermo *et al.* 1994). The centrosome consists of a pair of centrioles carried at an angle from each other, the proximal centriole, comprising nine triplets of microtubules located at the base of the sperm head, and the distal centriole, representing the main framework for the development of the flagellum (Sathananthan *et al.* 1991, 1996, Neri *et al.* 2010). The use of immunological techniques allows for the identification of proteins that are intimate components of this microtubule organizing center (Kimble & Kuriyama 1992, Palermo *et al.* 1997, Colombero *et al.* 1999b, Neri *et al.* 2011). Indeed, a

centrosomal defect results in embryonic aneuploidy or mosaicism resulting in the inability of the conceptus to undergo the first embryonic cleavage effectively (Moomjy *et al.* 1999). In cases where syngamy fails to occur after ICSI or a chaotic distribution of the chromosomes within the conceptus is identified, the spermatozoon centriole can be labeled with anti-centrin antibodies to assess for its presence and estimate its integrity by measuring the angle that the proximal centriole creates with the distal centriole of the flagellum (Neri *et al.* 2011). Indeed, the angle appears to be unique to the centriolar geometry within the human spermatozoon. Several studies have identified cases of the centrosome being severely altered or absent in infertile men, plagued by abnormal midpieces and stump tails originating in the testis and often resulting in complete immotility of the spermatozoa in the ejaculate (Baccetti *et al.* 1989, Barthelemy *et al.* 1990, Stalf *et al.* 1995, Toyama *et al.* 2000, Gambera *et al.* 2009, Neri *et al.* 2011). Although there is a high rate of centrosomal abnormality in these ejaculate specimens, the use of ICSI with these immotile spermatozoa can result in normal fertilization and pregnancies (Stalf *et al.* 1995, Barros *et al.* 1997). This confirms the importance of the male gamete to contribute in generating an euploid conceptus, and therefore, the availability of an assay capable of estimating centrosomal integrity and function is undeniable.

Recent studies have identified that variants in maternal *PLK4*, normally responsible for mediating centriole duplication and embryo cleavage, result in aneuploidy within the embryo (McCoy *et al.* 2015). Furthermore in a recent study, assessment of a cohort of infertile women evidenced that a common variant rs2305957 of the *PLK4* gene yielded a significantly lower blastocyst rate in comparison to a control; this variant was particularly pronounced in a subset of patients with early recurrent miscarriage (Zhang *et al.* 2017).

The understanding of the role of the centrosome in the human male gamete, made possible by a shift in focus to the competence of a single spermatozoon induced by ICSI, has heightened the study of the spermatozoon as a contributor not only of the paternal genome, but as a vector of an important organelle that ordains the chromosomal segregation during the first mitotic division.

Sperm small RNA

The recent discovery of RNA present in human spermatozoa has raised several interesting questions regarding its role in male fertility (Krawetz *et al.* 2011, Jodar *et al.* 2013). Analyses of spermatozoal RNA transcripts have shown to contain remnants of prior events in spermatogenesis as well as highlight potential genes that may be critical for fertilization and embryo development (Krawetz *et al.* 2011, Jodar *et al.* 2013). In addition to mRNA, human spermatozoa

carry small non-coding RNAs (sncRNAs), in which the distribution in ejaculated specimens is as follows: 65% repeat-associated small RNAs, 17% Piwi-interacting piRNAs, 11% quiescent RNAs and 7% microRNAs (Krawetz *et al.* 2011). Evidence of sncRNAs suggests a role of spermatozoal transcripts in post-fertilization development and further designates them as an emerging biomarker of male infertility (Krawetz *et al.* 2011).

The first specific mRNA identified in human spermatozoa was *C-MYC* mRNA (Kumar *et al.* 1993), thereafter several studies utilizing RT-PCR (reverse transcriptase PCR) or ISH (*in situ* hybridization) identified specific transcripts encoding protamines, progesterone and estrogen receptors, *CYCLIN B1*, *STAT4*, *DAZL*, *SRY* and *PLCZ* (Dadoune 2009). Additionally, ISH was used in studies to pinpoint the localization of such RNA at the periphery of the nucleus beneath the nuclear envelope, revealing an interior component of the nuclear matrix previously unexplored (Hamatani 2012). The presence of such mRNAs consistently persisting in ejaculated spermatozoa suggests a purposeful conservation of these transcripts post-spermiogenesis (Ostermeier *et al.* 2002, Miller & Ostermeier 2006). Indeed, several conserved transcripts in spermatozoa between mammalian species have been recently identified, suggesting an important role of these RNA in the spermatozoon's contribution to early embryonic development (Schuster *et al.* 2016). An example of such transcripts contributing to the oocyte is the identification of RNA encoding the previously discussed *PLCZ*, which upon isolation and injection into a mouse oocyte, triggered calcium oscillations and activation of the oocyte (Sone *et al.* 2005).

An important role attributed to spermatozoal RNA is the epigenetic reprogramming of the sperm chromatin (Miller *et al.* 2005), acting as a stabilizer for the interaction between the nuclear envelope and the small regions of histone-bound DNA, as well as mediating the selective escape of these histone-bound sequences from tight packing around protamines, therefore influencing the balance between protamine- and histone-packaged DNA (Hammond *et al.* 2009a,b). Epigenetic studies are foreseen to play an increasingly important role in the etiology of human infertility (Carrell 2012) as epigenetic regulation is becoming apparently more useful in quantifying the impact of environmental factors on the male gamete (Furrow *et al.* 2011).

Gene expression is regulated post-transcriptionally via small non-coding RNA entities as well as miRNA, responsible for fine-tuning cell differentiation through translational regulation during spermatogenesis (McIver *et al.* 2012). Further, antisense RNA has been identified in the human male gamete and includes small antisense RNAs, PIWI-interacting RNAs (piRNAs), MIWI and germline-specific argonaute proteins involved in RNA silencing (Ostermeier *et al.* 2005a,b, Girard *et al.* 2006, Grivna *et al.* 2006, Kim 2006). This small antisense RNA carried by the sperm cells suggest that paternal

ribonucleic acid may help regulate embryonic gene expression (Biermann & Steger 2007, Boerke *et al.* 2007). Further, non-coding RNA have been found to be involved in the X-inactivation occurring in the spermatocyte during spermatogenesis, thus representing a large complex in ejaculated human spermatozoa that may also orchestrate gene expression upon fertilization (Royo *et al.* 2015).

There have already been preliminary efforts into classifying fertile and infertile men via the analysis of their respective spermatozoal transcriptomes. Microarray analysis comparing oligozoospermic infertile men to normozoospermic fertile men revealed a remarkable down-regulation of genes relating to germ cell anti-apoptotic mechanisms (*PRN2*, *SP2-1*, *STATA-4*, *MRA-1*, *CREM*), a reduced expression of DNA repair (*NIPBL*), oxidative stress regulation (*PARK-7*), histone modification (*DDX3X*), spermatogenesis and sperm motility (Montjean *et al.* 2012). RNA profiling by microarray was also carried out in infertile men with normal semen parameters reporting a several-fold reduction in the expression of 136 genes (Garrido *et al.* 2009). In another study assessing infertile men with teratozoospermia in comparison to a cohort of men that had previously fathered a child, teratozoospermic men were found to lack RNA involved in the ubiquitin-proteasome pathway as well as other transcripts involved in acrosomal development and oocyte activation (Platts *et al.* 2007).

Furthermore, transcriptome analysis is already being applied to studying clinical outcomes in infertile men. A study of a small cohort of patients undergoing their first cycle of intrauterine insemination (IUI) treatment screened for over 19,000 transcripts, reporting that the cohort supporting a pregnancy had 756 overexpressed genes while only 194 of these common transcripts were overrepresented in couples that did not achieve a pregnancy (Garcia-Herrero *et al.* 2010). Additionally, expression of 741 transcripts was identified as exclusive to the fertile cohort. These findings are also in concordance with a more recent study evidencing that the absence of certain sperm RNA elements (SREs), as assessed in 96 couples with idiopathic infertility, were predictive of clinical outcome, reducing timed intercourse and IUI outcome success rates from 73% to 27% (Jodar *et al.* 2015).

In men with unexplained infertility, supplementary tests are pivotal to gaining insight into the paternal contribution to the zygotic genome. Profiling men via RNA sequencing to supplement the standard semen analysis may aid in the diagnosis and management of couples with recurrent ART failures. Although further research on spermatozoa-borne transcripts is needed, based on findings to date, screening men for an epigenetic imbalance of sncRNA and lncRNA may provide crucial information on the etiology

of unexplained infertility and overall reproductive capacity of the infertile male.

25 years of ICSI

In this section, we will recount our experience at our center using ejaculated, epididymal and testicular spermatozoa with ICSI over the last quarter of a century; data have been updated from studies by Palermo and coworkers (Palermo *et al.* 2012, 2014b). To summarize our overall clinical data from ICSI, of a total of 35,065 cycles, 15,646 cycles presented with a positive β hCG (44.6%), resulting in losses of 2694 biochemical (17.2%) and 861 blighted ova (5.5%). Among the 11,548 cycles that progressed to clinical pregnancy as defined by the observation of a fetal heartbeat, 1343 resulted in additional losses from miscarriage or therapeutic abortion. These overall cases resulted in a pregnancy and delivery rate of 32.9% per retrieval (11,548/35,065) and 38.1% per embryo replacement (11,548/30,289), resulting in the birth of 12,719 neonates from 9572 deliveries, consisting of 6230 females and 6315 males. The frequency of multiple deliveries including 2711 twins was (28.3%), 212 triplets (2.24%), and 4 quadruplets (0.04%).

Ejaculated spermatozoa

Between September 1993 and April 2017, a total of 31,723 ICSI cycles using ejaculated spermatozoa were performed. To provide an overview, a total of 262,659 MII oocytes were injected; 75.7% fertilized normally while 2.5% and 3.8% were 1PN and 3PN respectively, with no fertilization observed in 14.4%. The clinical pregnancy rate per oocyte retrieval was 36.4% and per embryo transfer procedure was 42.1%.

The advantages ICSI has provided over other ART techniques for severe male factor patients have been well documented in the literature over the past 25 years. Indeed, a study assessing a cohort of men with asthenoteratozoospermia reported a pregnancy rate of just 8% with IUI, which increased to 29% with ICSI (Mangoli *et al.* 2008).

Additionally, oligozoospermic patients with a count less than 5 million spermatozoa/mL in the neat sample have been found to have a pregnancy rate as low as 4% with intrauterine insemination (Mangoli *et al.* 2008). Our patient population includes many severely oligozoospermic men with a concentration less than 1×10^6 /mL spermatozoa, therefore signifying a primary indication for ICSI. In 1969 ejaculates where the initial specimen presented with a count less than 1 million/mL, a high-speed centrifugation was carried out, resulting in an average sperm density of $0.85 \pm 2.6 \times 10^6$ /mL and a motility of $29.8 \pm 29\%$. Subsequent use with ICSI yielded a fertilization rate of 61.1% and a clinical pregnancy rate of 40.0% (Table 1).

Table 1 ICSI outcomes in men with severe oligozoospermia ($<1 \times 10^6/\text{mL}$ spermatozoa).

| Parameter | Value |
|--|----------------------|
| Cycles | 1969 |
| Mean initial concentration (10^6 per mL \pm s.d.) | 0.2 \pm 0.2 |
| Mean initial motility (% \pm s.d.) | 17.8 \pm 21.8 |
| Mean morphology (% \pm s.d.) | 0.7 \pm 1 |
| Fertilization (%) | 11,036/18,067 (61.1) |
| Clinical pregnancy (%) | 788 (40.0) |

Surgically retrieved spermatozoa

The value of ICSI chiefly lies in the ability to establish a successful pregnancy with surgically retrieved specimen from azoospermic men. Prior to the implementation of ICSI, such situations were resolved with the use of donor spermatozoa. Azoospermia presents in less than 1% of men and in about 10–15% diagnosed with infertility, either as obstructive (OA) or non-obstructive (NOA) azoospermia (Hamada *et al.* 2013). Obstructive azoospermia can be due to a congenital bilateral absence of the vas deferens (CBAVD) often linked to a defect in the CFTR gene associated with cystic fibrosis (Patrizio & Zielenski 1996), trauma, infection, or vasectomy (Hamada *et al.* 2013). Specimen to be utilized for ICSI may be aspirated from the epididymis (MESA) or retrieved percutaneously (PESA) (Kahraman *et al.* 1996, Schlegel *et al.* 1997, Schlegel & Li 1998). In men with non-obstructive azoospermia in which spermatogenesis is scant, a testicular sperm extraction (TESE) or a micro-TESE (mTESE) can yield adequate spermatozoa while maintaining anatomical integrity (Schlegel 2009). Additionally, epididymal and testicular sampling can also be an effective alternative in males with extreme oligoasthenoteratospermia (OAT) (Palermo *et al.* 1992b, 2014a, Nyboe Andersen *et al.* 2009). Furthermore, men with cryptozoospermia that undergo a surgical retrieval have resulted in improved clinical outcomes compared to their previous cycle with ejaculated specimen (Bendikson *et al.* 2008, Ketabchi 2016).

The use of spermatozoa sourced from different areas within the male genital tract has led to an increased scrutiny in regard to fertilizing ability and clinical outcome. Over the past 25 years, our center has carried out 1140 cycles using epididymal spermatozoa and 1713 cycles with testicular spermatozoa. Table 2 summarizes the patient demographic and embryologic data of couples inseminated with different spermatozoa sources, chiefly ejaculated, epididymal, and testicular. Epididymal spermatozoa yielded the highest number of fertilized oocytes per cycle, followed by the ejaculate and testicular spermatozoa (Fig. 3). Although having a lower fertilization rate, testicular spermatozoa demonstrated a significantly higher pregnancy rate compared to the ejaculated specimen (Fig. 4), which has also been confirmed in other studies (Esteves *et al.* 2015, Babuccu *et al.* 2017).

Table 2 Outcomes using ejaculated, epididymal and testicular spermatozoa. Data are presented as mean \pm s.d.

| Parameter | Ejaculated | Epididymal | Testicular |
|---------------------------------------|----------------------------|---------------------------|---------------------------|
| Maternal age (years) | 37.7 \pm 5 ^a | 35.3 \pm 5 ^b | 33.8 \pm 6 ^c |
| Cycles | 31,723 | 1140 | 1713 |
| Oocytes retrieved | 10.3 \pm 6 ^d | 12.0 \pm 7 ^e | 12.5 \pm 7 ^f |
| Oocytes injected | 8.3 \pm 5 ^g | 9.6 \pm 5 ^h | 10.1 \pm 5 ⁱ |
| Oocytes fertilized | 6.3 \pm 4 ^j | 6.9 \pm 5 ^k | 5.2 \pm 4 ^l |
| Clinical pregnancy rate per cycle (%) | 11,536 (36.4) ^m | 576 (53.2) ⁿ | 687 (40.1) ^o |

a vs b vs c: ANOVA, 2 *df*, effect of sperm source on average maternal age, $P < 0.0001$; d vs e, f: *t*-test, 1 *df*, effect of sperm source on number of oocytes retrieved, $P < 0.0001$; g vs h, i: *t*-test, 1 *df*, effect of sperm source on number of oocytes injected, $P < 0.0001$; j vs k vs l: ANOVA, 2 *df*, effect of sperm source on number of oocytes fertilized, $P < 0.0001$; m vs n vs o: χ^2 , 3 \times 2, 2 *df*, effect of sperm source on clinical pregnancy rate, $P < 0.0001$.

Implantation rates were congruent with the clinical pregnancy rates by sperm source, with epididymal specimens yielding the highest implantation rate, followed by testicular, and finally ejaculated specimen. In our experience, utilization of fresh testicular spermatozoa with ICSI yielded a more consistent zygote development and clinical pregnancy rate in comparison to the cryopreserved counterpart (Table 3), which appears to be discordant with the findings of other studies that did not see a significant difference (Habermann *et al.* 2000, Ohlander *et al.* 2014, Schachter-Safrai *et al.* 2017). Although reaching comparable fertilization rates, thawed epididymal spermatozoa had impaired motility and lower pregnancy outcomes than the fresh counterpart (Table 3). It must be noted that this analysis is purely academic, due to the fact that spermatozoa retrieved from different sources address different clinical indications.

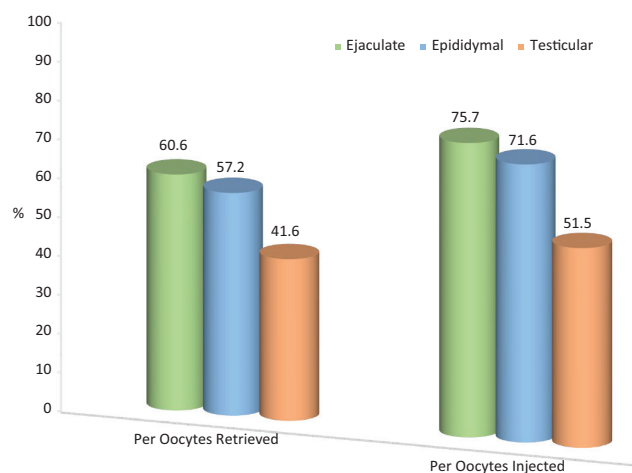


Figure 3 Comparison of fertilization rates according to spermatozoa source. Ejaculated specimens yielded a fertilization rate comparable to the epididymal and both were superior to testicular spermatozoa per oocytes retrieved (χ^2 , 2 \times 3, 2 *df*; $P < 0.0001$). A similar pattern was observed once the fertilization rate was calculated based on the number of metaphase II oocytes injected (χ^2 , 2 \times 3, 2 *df*; $P < 0.0001$).

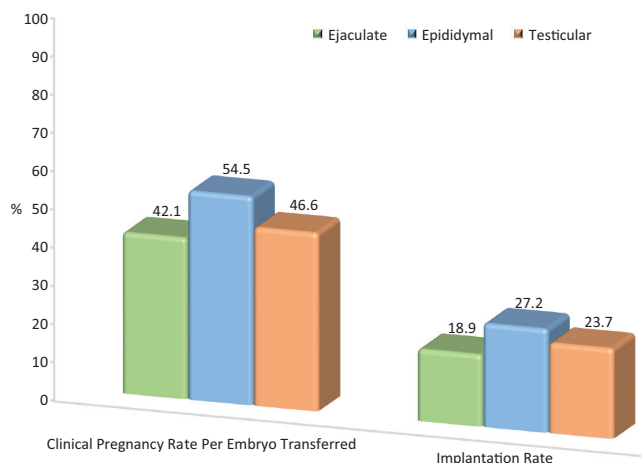


Figure 4 Comparison of pregnancy and implantation rates according to spermatozoa source. Embryos generated from epididymal spermatozoa had the highest pregnancy rate followed by testicular and ejaculated spermatozoa (χ^2 , 2×3 , $2df$; $P < 0.0001$). Embryo implantation rate was highest with epididymal, followed by testicular and ejaculated specimen (χ^2 , 2×3 , $2df$; $P < 0.0001$).

Overall, the implementation of surgical retrievals in conjunction with ICSI cycles has aided many men with no chance of natural conception to have their own child. However, further observation and follow-up studies need to be carried out as the cohort of reproductive aged offspring from these procedures increases.

Extreme ICSI cases

When very few to no spermatozoa are seen even after high-speed centrifugation, an extensive search is performed prior to ICSI in order to identify cells for injection. In 986 ICSI cycles, as updated from Palermo *et al.* (Palermo *et al.* 2014d), a search for injectable spermatozoa that required at least 30 min was carried out in ejaculate and testicular biopsy specimens. Ejaculated spermatozoa demonstrated a significantly higher fertilization rate in comparison to TESE specimen without however, affecting clinical pregnancy rates, which remained comparable between the two sources (Fig. 5). A similar study of patients with virtual

azoospermia found an improved clinical pregnancy and implantation rate when using surgical vs ejaculated specimen (Ketabchi 2016).

Although scant spermatozoa can occasionally be found in ejaculated and testicular samples of these aforementioned cases, 40–60% of NOA patients that undergo a TESE/micro-TESE fail to retrieve spermatozoa (Bernie *et al.* 2013, Vloeberghs *et al.* 2015). The high occurrence of failed TESEs in NOA patients has prompted research into alternative approaches for these men to conceive a child of their own. In the event that no spermatozoa are identified in a testicular sample, studies have reported that in patients identified as having scant spermatogenesis and even maturation arrest, the use of round spermatids for injection (ROSI) has the ability to fertilize an oocyte (Vanderzwalmen *et al.* 1997). It should be noted however, that this procedure has incited vehement assertion that cases where round spermatids are identified always contain elongating spermatids as well (Silber *et al.* 2000). Nevertheless, a recent study using ROSI has now claimed to have yielded 14 babies as of 2015 from a patient cohort that failed their first micro-TESE (Tanaka *et al.* 2015). Although these data seem encouraging regarding patients that did not appear to have spermatozoa upon surgical extraction and further evaluation, injection of these round cells did not consistently induce oocyte activation without the aid of electric stimulation. From what we have learned thus far about the role of paternal RNAs in embryogenesis, these observations could indicate a lack of necessary transcripts available to the oocyte once a round spermatid is injected, possibly due to its spermiogenic block. As a result, close follow-ups of the offspring should be conducted before any clinical value can be assumed from the procedure.

ICSI safety

The issues related to the safety of intracytoplasmic sperm injection will be discussed in further detail following this chapter.

The general adoption of ICSI and its success has not been without some concern that this procedure bypasses

Table 3 Spermatozoal parameters and intracytoplasmic sperm injection outcome according to retrieval sites and specimen condition.

| No. of items | Spermatozoa | | | |
|-------------------------------------|----------------------------|--------------------------|---------------------------------|-------------------------------|
| | Epididymal | | Testicular | |
| | Fresh | Frozen/thawed | Fresh | Frozen/thawed |
| Cycles | 364 | 776 | 1158 | 555 |
| Density (10^6 per mL \pm s.d.) | 37.9 \pm 44 | 21.1 \pm 26 | 0.3 \pm 2.6 | 0.3 \pm 1.8 |
| Motility (% \pm s.d.) | 19.0 \pm 17 ^a | 3.9 \pm 9 ^a | 2.9 \pm 7 | 1.4 \pm 5 |
| Morphology (% \pm s.d.) | 1.7 \pm 2 | 1.3 \pm 2 | 0 | 0 |
| Fertilization (%) | 2775/3829 (72.5) | 5072/7126 (71.1) | 6418/12,220 (52.5) ^c | 2477/5059 (49.0) ^c |
| Clinical pregnancy (%) | 221 (60.7) ^b | 353 (45.5) ^b | 500 (43.2) ^d | 187 (33.7) ^d |

^aStudent's *t*-test, two independent samples, effect of epididymal cryopreservation on sperm motility, $P < 0.0001$; ^b χ^2 , 2×2 , $1df$, effect of epididymal cryopreservation on clinical pregnancy rate, $P < 0.0001$; ^c χ^2 , 2×2 , $1df$, effect of testicular cryopreservation on fertilization rate, $P = 0.02$; ^d χ^2 , 2×2 , $1df$, effect of testicular cryopreservation on clinical pregnancy rate, $P < 0.0001$.

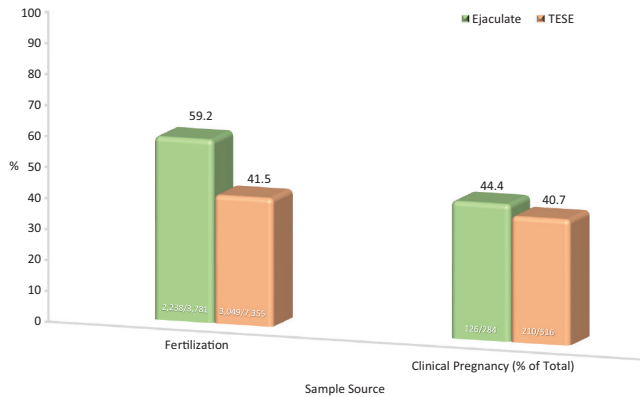


Figure 5 Comparison of fertilization and clinical pregnancy rates in cases with few spermatozoa identified. In 986 cycles, extremely few spermatozoa were seen after high-speed centrifugation. Samples were searched for injectable spermatozoa in drops under oil for up to several hours by multiple embryologists until all oocytes were injected. Oocytes injected with ejaculate spermatozoa demonstrated a higher fertilization rate compared to those injected with testicular specimen (χ^2 , 2×2 , 1 df; $P < 0.0001$). Clinical pregnancy rates remained comparable between the two sperm sources.

natural sperm selection (Cummins & Jequier 1994, de Kretser 1995, De Rycke *et al.* 2002, Edwards & Ludwig 2003) bringing into question the growth, cognitive development and postnatal well-being of the offspring as well as the impact on their future reproductive capacity (Bowen *et al.* 1998, Schieve *et al.* 2004).

Several surveys of children born through ART evidenced an increased rate of neonatal malformations (Hansen *et al.* 2002), lower birth weights (Schieve *et al.* 2002), the prevalence of imprinting errors (DeBaun *et al.* 2003, Gicquel *et al.* 2003, Maher *et al.* 2003, Orstavik 2003, Halliday *et al.* 2004), and even some forms of cancer (Moll *et al.* 2003). However, these studies did not link the cases of imprinting disorders or childhood neoplasia to the ICSI procedure itself (Edwards & Ludwig 2003).

Nevertheless, concerns related to the utilization of these less than ideal spermatozoa may still be the reason for genetic and congenital abnormalities (Ludwig & Katalinic 2002). Contrary to these concerns, we observed that the rate of malformation from ICSI was no higher than naturally conceived offspring reported in New York State (Palermo *et al.* 1996b). Additionally, a study of 14,211 ART children determined that the malformation rate with ICSI is comparable to that with IVF (Palermo *et al.* 2013).

In fact, the follow-up literature assessing ICSI offspring from neonates to adolescents have shown satisfactory physical and psychological development (Belva *et al.* 2007, 2011, 2012, Basatemur & Sutcliffe 2008, Knoester *et al.* 2008, Leunens *et al.* 2008, Goldbeck *et al.* 2009, Basatemur *et al.* 2010, Carson *et al.* 2013).

Among all epigenetic diseases, Beckwith–Wiedemann Syndrome (BWS) is the only disorder that

has been unequivocally linked to ART (Sutcliffe *et al.* 2006) although not related to a specific reproductive technique. A more recent study assessed the methylome in cord blood of children generated through standard *in vitro* insemination, ICSI, and compared to a naturally conceived offspring (El Hajj *et al.* 2017). The initial analysis failed to evidence any particular effect of the ART on the DNA methylation patterns. A confirmatory analysis pointed at some small differential methylation of two specific genes (*ATG4C* and *SNORD114*). However, in consideration of several confounding factors, the authors were not able to distinguish between the effect of a specific insemination method or the contribution of a male factor. The authors also acknowledged that this study, carried out by a non-clinically validated assay, appears inconclusive until a larger study is carried out. Separately from the insemination procedure, long-term blastocyst culture has been associated with gene expression imbalances (Basatemur & Sutcliffe 2008, Rivera *et al.* 2008), furthermore, there is presently no clear evidence that resort to ICSI predisposes offspring to gene expression disorders, in animals or humans (Wilson *et al.* 2007).

In summary, the most prevalent factor that may contribute to adverse postnatal outcomes in children conceived by ART stems from high order-gestation (Pereira *et al.* 2016a), a common consequence of assisted reproduction. Indeed, the adoption of single embryo transfer procedures has helped to diminish this issue. Additional complications such as prematurity, low birth weight, perinatal mortality, as well as congenital malformations have been indiscriminately linked to ART, though the primary factor responsible appears to be inherent in the infertility indication itself. While ICSI is not a causation of long-term neurodevelopmental defects or cancer, further follow-up studies into adulthood should be continued to better inform our understanding of assisted reproduction and more comprehensively answer these questions (Palermo *et al.* 2013).

In consideration of the foregoing, the first studies on the oldest ICSI cohort have been recently published, assessing the male and female offspring independently compared to a naturally conceived control. A survey of male reproductive hormones in young ICSI men proved to be comparable to naturally conceived peers (Belva *et al.* 2017a). A second study in a similar cohort of ICSI men focused assessing their spermatogenesis by measuring the semen characteristics in comparison to a control. This study demonstrated lower semen parameters in the ICSI cohort, although all metrics were still considered above threshold according to the WHO criteria (WHO 2010, Belva *et al.* 2016). The fact that these ICSI offspring have semen parameters within the normal range, and therefore supports the notion of their potential ability to conceive, is somewhat reassuring, considering they were generated from fathers afflicted by male infertility incapable of procreating naturally

or by standard *in vitro* insemination. Alike, the most recent follow-up study on young women born from ICSI has shown encouraging results in relation to their fertility status, demonstrating a comparable hormonal profile to naturally conceived girls (Belva *et al.* 2017b). Nonetheless, as these cohorts assessed are relatively small, further multicenter studies would be welcome in order to confirm these preliminary findings.

Conclusions

In spite of the fact that ICSI was developed almost by chance, and earlier conclusions that the intracytoplasmic approach was too invasive and unreliable, its value has been affirmed in a variety of challenging situations, particularly for severe male factor couples wishing to have their own genetic child. Not least, over these last 25 years, ICSI has made possible the utilization of immature forms of the male gamete such as epididymal and testicular spermatozoa.

At our center, ICSI is additionally used in all cases using cryopreserved donor and husband spermatozoa samples in order to compensate for poor survival upon thawing. The utilization of a single spermatozoon has been instrumental to permit proper fertilization of oocytes prior to cryopreservation and in cases with a low egg yield, as often seen in couples with advanced maternal age or in poor responders to ovarian superovulation.

The advantages of an insemination technique that leveled the equivalency between a single male and female gamete have been indispensable in helping to explain specific aspects of sperm–oocyte interaction, such as understanding the acrosomal function in relation to the stability of the spermiolemma and its inherent connection to sperm motility as well as validating the mechanism of inheritance of the sperm centrosome.

Failure to achieve fertilization with ICSI has stimulated research into the mechanisms behind oocyte activation and has conversely indicated that a dysmature ooplasm is not receptive even to a fully competent spermatozoon.

The introduction of assays, particularly the assessment of chromatin integrity of sperm cells has evidenced that DNA fragmentation can influence the reproductive outcome of couples, running the gamut of assisted reproductive techniques from programmed intercourse and IUI, to standard *in vitro* insemination, and rarely intracytoplasmic injection.

It appears that ICSI will continue to play a role in the immediate and distant future of assisted reproductive technology and remain of paramount importance in cases involving mitochondrial therapies of micro-manipulated oocytes or eventually, for use of sperm cells generated through *in vitro* spermatogenesis and even neo-gametogenesis from stem cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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