Intracytoplasmic sperm injection: current perspectives and future development

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Submitted: 17 October 2008
Accepted: 22 November 2008

Arch Med Sci 2009; 5, 1A: S109–S114

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Abstract

Intracytoplasmic sperm injection (ICSI), a novel technique rapidly gaining popularity among infertile couples, alleviates the burdens posed by male factor infertility by offering infertile couples a chance to conceive. Although numerous studies suggest success rates comparable to traditional *in vitro* fertilization, ICSI is not without fault. Potential associated risks include, but certainly are not limited to, birth defects, DNA fragmentation, de novo Y-chromosome microdeletions, paternal mtDNA disease transmission, chromosomal aberrations, and genomic imprinting disorders. Fortunately, there has been ongoing research to develop a more effective ICSI protocol. This paper explores potential risks associated with prenatal and postnatal development in ICSI offspring, as well as modern technical advances in the ICSI protocol.

Key words: intracytoplasmic sperm injection, sperm selection, male infertility.

Introduction

Within one decade of the dramatic introduction of *in vitro* fertilization (IVF), another novel technique, intracytoplasmic sperm injection (ICSI) came as a boon for male factor infertility. This new technology came as a rescue for those males who would otherwise never father a child, in an era when the global trend of declining sperm quality came into play. Presently ICSI is the norm for fertilization in most ART clinics. However, concerns regarding the safety of this invasive technique also have been raised.

In recent years, researchers have been working to produce a more effective ICSI protocol, from introducing novel sperm selection techniques to evaluating how appropriate ICSI may be for an infertile couple.

Prenatal and postnatal issues in intracytoplasmic sperm injection

Although ICSI is increasing in popularity among many infertile couples, this procedure may be considered slightly unethical due to its ability to bypass natural selection mechanisms, such as spermatozoal penetration of the zona-pellucida and the spermatozoa-oocyte fusion events. Suffice to say, ICSI provides a method not observed in standard IVF for spermatozoa with deficient parameters, such as poor motility, abnormal

morphology, or damaged chromatin to fertilize oocytes. Unfortunately, determining spermatozoa DNA integrity solely based on morphologic assessment is difficult. Seemingly normal sperm may in actuality suffer from DNA damage, thereby adversely affecting the future development of the embryo. With its first successful conception in 1992, ICSI is still considered a relatively novel approach in ART, making it difficult for us to document the largely unknown long-term effects.

Studies indicate that DNA fragmentation is a leading cause in fertilization failure and in abnormalities in embryonic development [1]. Three current theories attribute DNA fragmentation to oxidative stress, defective DNA repair mechanisms, and abortive apoptosis [2]. Oxidative stress, which is triggered by an imbalance between reactive oxygen species (ROS) and their scavenging antioxidants, induces lipid peroxidation of the spermatozoal membrane, thereby inflicting mitochondrial and genomic damage [3]. Alternatively, a defect in topoisomerase II activity may result in failure to repair DNA strands or abnormal chromatin structure, also contributing to DNA fragmentation [4]. Occasionally, an abortive apoptosis may render the cell's homeostatic function ineffective, allowing for an increased frequency of abnormal spermatozoa [3].

Within the 15% of couples who experience infertility [5], 10 to 14% of male infertility cases result from de-novo Y chromosome microdeletions [6]. Implications of *de-novo* Y-chromosome microdeletions include abnormal spermatogenesis and conditions resulting in low or no sperm count, such as oligozoospermia and azoospermia, thereby promoting male infertility [7].

Studies reviewing the relationship between de-novo Y-chromosome microdeletions and ICSI produced confounding data. In a study examining 32 ICSI patients with a 12.5% overall microdeletion frequency and their 33 male offspring, two cases presented vertical transmission, two cases presented greater microdeletion than their fathers, and seven cases showed de novo deletions. This indicates ICSI treatment not only produced *de novo* deletions but also expanded deletions. In another study, Buch et al. shows no occurrences of *de novo* Y-microdeletions in ICSI conceived male offspring of infertile men characterized by severe oligospermia and non-obstructive azoospermia, concluding that *de novo* Y deletions are extremely rare [8].

Since ICSI involves the injection of the whole sperm, including the head, midpiece, and tail, the question of whether this procedure increases the risk of paternal mtDNA disease transmission has been raised [9]. Current studies on paternal mtDNA transmission provide controversial data at best. In one study, Marchington et al. used an adaptation of solid-phase mini-sequencing to detect paternal mtDNA in concentrations as low as 0.001% in a milieu of maternal mtDNA. The authors were not able to find any paternal mtDNA, thereby validating the role of ubiquitin in sperm mitochondrial degradation via proteolysis [10]. In a different study, St. John et al. used a combination of restriction enzymes and nested polymerase chain reaction (PCR) to detect paternal mtDNA in 32 human polyploidy embryos, defined as occurrence of three or more different pronuclei during fertilization, in ICSI and IVF patients. The study reported presence of paternal mtDNA in the embryo past the eight-cell stage [11]. Sutovsky et al. suggests that the process of paternal mtDNA transmission is made possible, especially in ICSI, due to the resistance provided by the plasma membrane surrounding the mitochondrial sheath [12].

Skepticism regarding ICSI-related births is common, considering this is a method intended for infertile males with poor sperm parameters. As a result, some studies explore this matter to determine the normalcy of ICSI- conceived offspring relative to naturally conceived offspring. Knoester et al. compared the neuromotor development of 5- to 8-year-old ICSI children with children conceived naturally and by IVF, using various tests examining parameters such as reflex abnormalities and coordination. The authors concluded that the prevalence of minor neurological dysfunction in ICSI-singletons (66.3%, n=87) was higher than that of naturally conceived children (50.6%, n=85); however, after adjustment for maternal age and parity, the results were not statistically significant [13]. A more recent follow-up study evaluating short- and long- term health of ICSI- conceived offspring indicated a significantly higher prematurity prevalence for ICSI singletons when compared with naturally conceived singletons (P=0.014). This study also suggested poorer perinatal outcomes associated with ICSI as opposed to natural conception; however, there were no other unfavorable health outcomes linked to ICSI [14]. In a follow-up study conducted by Leunen et al. the cognitive and motor development of 10-year-old ICSI singletons was compared with that of singletons spontaneously conceived. This study indicated that despite earlier reports of higher intelligence outcomes in 8-year-old ICSI- conceived children due to higher maternal education, the intelligence levels observed in 10-year-old ICSI singletons seemed to have converged with that of 10-year-old spontaneously conceived children, and no difference in cognitive and motor development was observed [15]. Cognitive development is very complex, however, and many demographic factors, such as age, ethnicity, social class, maternal and paternal education levels, and family environment,

can influence the data. Overall, current studies suggest no impairment in cognitive or motor development in association with ICSI.

Current literature indicates an increase in an array of birth defects associated with ICSI. In a Danish study, Mau Kai et al. showed a statistically significant decrease in serum testosterone levels of ICSI-conceived children (2.4 nmol/l) in comparison with the testosterone levels of naturally conceived offspring (3.3 nmol/l, P<0.001) [16]. Another study indicated an increase in genitourinary tract abnormalities, especially hypospadias and hypogonadism, in children conceived not only by ICSI but with other ART procedures as well [17].

Much of the current literature suggests an increase in genetic and chromosomal aberrations, especially in genomic imprinting, amongst ICSI singletons [18]. Angelman syndrome (AS), a rare neurogenetic disorder associated with mental retardation and absence of speech, is an archetypal genomic imprinting disorder characterized in ICSI-conceived offspring. AS is the product of a mutation or deletion of the *UBE3A* gene in chromosome 15 or, in more infrequent cases, a hypomethylation of the SNRPN allele [19]. One study examined two cases of AS triggered by hypomethylation, suggesting a possible link between ICSI and increased imprinting defects [20]. Beckwith-Wiedemann syndrome (BWS) is another model imprinting disorder identified by macroglossia, abdominal wall defects, and a predisposition to embryonic tumor development [21]. Beckwith-Wiedemann syndrome involves several maternally expressed genes (*CDKN1C*, a cyclin-dependent kinase inhibitor, and *H19*, a tumor-suppressing gene) and paternally expressed genes (*IGF-2*) [22]. Abnormal methylation of the *H19* gene may result in tumor growth due to hyperactivation of IGF-2 [23]. Despite numerous reports associating a higher risk of imprinting disorders with ICSI, some studies suggest otherwise. One study surveying 47 children from Central England and the Republic of Ireland showed no cases of AS and only 1 case of BWS, implying a very minimal (<1%) absolute risk of imprinting disorders [21].

Examining broader epigenetic characteristics of ICSI, Fulka et al. did not identify any differences in global methylation patterns of *in vivo*, *in vitro*, and ICSI-fertilized mouse zygotes [24]. However, global methylation patterns do not necessarily account for loci-specific imprints. In one study comprised of 97 infertile men, the authors found an abnormal paternal methylation imprint in 14.4% of the patients and an abnormal maternal methylation imprint in 20.6% of the patients. The results suggested a possible increased risk of transmitting abnormal primary imprints to offspring in infertile men [25].

Sperm selection and injection

Sperm selection is not only an integral aspect of ICSI procedure but also highly subjective, relying heavily on morphology. Unfortunately, morphology does not necessarily indicate good chromatin integrity. Therefore, establishing an alternative, effective method for sperm selection is essential. Many ongoing studies are exploring various sperm isolation techniques.

A recent technique called magnetic cell sorting (MACS) uses paramagnetic microbeads in conjunction with annexin V proteins to separate non-apoptotic spermatozoa from apoptotic spermatozoa. Externalization of the phospholipid phosphatidylserine (PS) to the outer plasma membrane surface is considered to be an apoptotic marker. Annexin V, which has a high affinity for PS, tends to bind to the plasma membrane of cells expressing externalized PS. As a result, apoptotic sperm can be filtered out with annexin V-conjugated microbeads, yielding annexin-positive (apoptotic) and annexin-negative (non-apoptotic) sperm. However, MACS does provide an advantage to sperm cell sorting when used with routine sperm preparation techniques such as density gradient centrifugation since MACS does not filter out other seminal plasma elements such as leukocytes and debris [26].

Hyaluronic acid (HA), or hyaluronan, is a linear polysaccharide known to improve motility and velocity by binding to sperm membrane receptors that may control ATP production or sperm motility [27]. Hyaluronic acid binding receptors, which form during plasma membrane remodeling in spermiogenesis, are mainly found in mature spermatozoa [28]. Furthermore, HA-bound viable mature spermatozoa are characterized by the absence of active apoptotic marker caspase-3, persistent histones, cytoplasmic retension, and DNA degradation [28]. As a result, selecting sperm based on HA binding can provide a way to separate mature, viable spermatozoa from spermatozoa with poor parameters. Many studies suggest HA sperm selection is an efficient method for selecting good quality mature, viable spermatozoa for ICSI [28-31]. Nasr-Esfahani et al. investigated the efficacy of routine sperm selection as opposed to HA sperm selection by examining the fertilization rate, embryo development, implantation and pregnancy rates in 50 infertile couples undergoing ICSI for the first time. The results showed significant improvement in fertilization rates with HA sperm selection (P=0.020); however, there was no significant difference in day 2 and day 3 cleavage and embryo quality [29]. Since HA-selected sperm have a frequency of chromosomal aberrations comparable to that of traditional zona pellucida-selected sperm [30], HA sperm selection can render

the transmission of the paternal genome to the embryo more efficiently.

Sperm morphology evaluation is integral to sperm selection in ICSI procedures due to its strong predictive role in ICSI outcome. Bartoov et al. developed a new method for evaluating sperm morphology using high magnification (6300×) to analyze various subcellular parameters – neck, tail, mitochondria, acrosome, chromatin content, nucleus shape. A recent study using MSOME criteria revealed a positive association between morphological normalcy of sperm nuclei and fertilization and pregnancy outcomes; however, no correlation was found between morphological normalcy of the entire sperm cell and pregnancy rates. The authors suggested that the embryologist may be unable to discern subcellular malformations using routine ICSI sperm selection protocol, thereby providing a possible explanation for the affected pregnancy rate [32].

A prospective randomized trial explored the advantages of intracytoplasmic morphologically selected sperm injection (IMSI) over ICSI by examining pregnancy, miscarriage, and implantation rates. Intracytoplasmic morphologically selected sperm injection, a protocol mirroring ICSI except with MSOME criteria for the sperm selection procedure, provided significantly higher pregnancy and implantation rates and significantly lower miscarriage rates [33].

Longitudinally arranged protein filaments along with anisotropic qualities of the protoplasm in mature sperm account for spermatozoal birefringence characteristics, which can be detected with a polarized microscope. Since birefringence presence is associated with good spermatozoal health, embryologists are able to use polarized microscopy in association with ICSI protocol for efficient sperm selection. A recent prospective randomized trial, examining the application of polarized microscopy as a potential sperm selection technique for ICSI, involved a clinical study group (n=112), which utilized polarized microscopy in addition to ICSI, and a control group (n=119), which was treated with the routine ICSI procedure. The data revealed similar pregnancy and implantation outcomes between the study and control groups among patients with less severe male factor conditions such as normospermia and oligoasthenoteratospermia with progressive motility. More severe conditions such as TESE showed a significant difference between the study and control groups, favoring treatment with polarized microscopy [34].

With an electric charge, the zeta potential, of –16 to –20 mV, mature sperm tend to adhere to glass or polystyrene centrifuge tubes during sperm processing procedures, such as density gradient centrifugation. By raising the charge of the tubes to +2 to +4 kV, embryologists can separate mature spermatozoa from debris and lesser charged sperm that are associated with inferior quality. As a result the novel zeta method, which uses electrostatic charge properties to separate sperm, is simple, quick, and inexpensive for sperm processing.

One study evaluated the efficiency of the zeta potential method for sperm selection by analyzing sperm parameters in a study group using the zeta method in comparison to a control group using density gradient centrifugation. The data showed significant improvements in forward progressive motility, hyperactivation motility, strict normal morphology, and DNA integrity; however, there was no significant difference in total sperm motility between the two methods. Zeta potential is a promising technique, but further studies are needed to address its limitations such as its low recovery rate (–8.8%) [35].

DNA damage is often associated with decreased ICSI success rates [36]. Electrophoretic sperm isolation is a technique that minimizes DNA damage by separating cells according to size and charge, while incurring no physical trauma. Electrophoretic processing takes advantage of the electronegative nature of spermatozoa to attract sperm to the positively charged anode terminal while avoiding unnecessary components of the seminal ejaculate, such as leukocytes and immature germ cells. One recent study revealed significantly improved morphology and significantly reduced DNA damage of spermatozoa in comparison to the excluded population ($P<0.05$). By eliminating DNA damage normally incurred during alternative sperm selection techniques and minimizing procedure time and ROS generation, electrophoresis shows great potential in the near future as a novel sperm isolation method [37].

One major consequence of injecting spermatozoa with intact acrosomes is that the hydrolytic acrosomal enzymes are released into the oocyte, unlike in natural conception or conventional IVF. Cholesterol in the sperm plasma membrane and hydrolyzing contents within the acrosome have been reported to be potentially harmful to the preimplantation embryo and can lead to chromatin remodeling [38]. The oocytes from young patients have some natural degree of defense against such enzymes, but if and how the enzyme activity is suppressed upon contact with the ooplasm remains a mystery [39]. Infertile males are expected to show abnormal levels and activities of hydrolytic acrosomal enzymes. Infertile women may possess oocytes that are more sensitive to hydrolytic acrosomal enzyme activity, making sperm microinjection a challenge for ART labs and likely resulting in abnormal embryo growth or death [40].

Conclusions

Intracytoplasmic sperm injection undoubtedly can be a blessing for those men suffering from severe male factor infertility. However, due to the invasive nature of the procedure, there is great potential for complications of all degrees of severity. Additionally, the long term consequences of this procedure on the resulting embryos and 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. A number of factors could affect the ICSI outcome, 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 ICSI instruments, clinicians, and laboratory technicians. In other failed cases, solutions cannot be proposed until more knowledge is gained about the underlying pathology of infertility. As new studies on the subject especially on sperm physiology and molecular biology continue to be published, it is important that the established protocol for ICSI is revised on a regular basis and that its role in infertility therapy is continually reevaluated.

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