Potential health risks associated to ICSI: insights from animal models and strategies for a safe procedure

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ART is considered a risk factor for different pregnancy complications such as high blood pressure, preeclampsia, growth retardation, bleeding or even premature births, and intrauterine death [reviewed in Ref. (4)]. Furthermore, multiple reports have associated more severe health problems such as abortions, congenital malformations, and imprinting disorders. In this review, we will discuss the possible implications of the technique per se on these adverse outcomes and highlight the importance of several experiments using mammalian models to truthfully test these implications and to uncover the molecular base that originates these health problems. We also dissect the specific hazards associated to ICSI and describe some strategies that have been developed to mimic the gamete selection occurring in natural conception in order to improve the safety of the procedure.

Keywords: ICSI, ART, IVF, DOHaD, animal models, sperm selection, transgenerational, imprinting

The great advances in artificial reproductive techniques (ART) over the last decades have fulfilled the dreams of millions of infertile couples. An estimated 3.75 million births have resulted from assisted conceptions (ESHRE 2010, ART fact sheet) and it has been estimated that 1.7–4% of all children born today in developed countries are conceived through the use of these techniques (1). Intracytoplasmatic sperm injection (ICSI) is currently the most commonly used ART, accounting for 70–80% of the cycles performed (2), and there is a trend toward an increase of its use worldwide (3), which highlights the importance of the study of the potential health risks associated with this technique.

The use of ICSI may pose a risk for the health of the mother and child. ARTs are considered a risk factor for different pregnancy complications such as high blood pressure, preeclampsia, growth retardation, bleeding or even premature births, and intrauterine death [reviewed in Ref. (4)]. Furthermore, the risk of premature birth rises between two and three times depending on the study (5, 6). The health of the assisted-conception children may be also compromised. A recent consensus opinion review from a group of diverse experts working at ART clinics (the Evian Annual Reproduction Workshop Group) concluded that IVF/ICSI children have lower birthweights and higher peripheral fat, blood pressure, and fasting glucose concentrations than controls (7). Furthermore, multiple reports have associated more severe health problems such as congenital malformation or the appearance of imprinting disorders with the use of IVF/ICSI. The extensive literature available about the increased risk for congenital malformation in IVF/ICSI compared with naturally conceived children has been summarized in several meta-analyses. An analysis of 19 publications selected by a quality score based on sample size and appropriateness of control group observed that major malformation rates ranged from 0 to 9.5% in IVF, 1.1 to 9.7% for ICSI, and 0 to 6.9% in naturally conceived children, leading to a statistically significant overall odd ratio of 1.29 (8). A more recent review of 56 studies selected based on appropriateness of control group yielded an estimation for congenital malformations following IVF/ICSI of 1.37 compared with naturally conceived children (9). Large scale epidemiologic analyses have also observed an increased risk for congenital malformations following IVF/ICSI. A particularly sizeable study conducted in Israel, where the national insurance policy covers all IVF procedures for the first two children, reported an adjusted odd ratio for congenital malformations of 1.45 for the comparison between the IVF/ICSI population (9,042 live births) and naturally conceived infants (213,737 live births) (10). The odd ratio of this study was adjusted for other significant interacting factors such as maternal age or gender of the child, limiting the chance of spurious relation.

Genomic imprinting is an epigenetic mechanism based on DNA methylation at imprinting control regions (ICR) that determines the monoparental expression of a subset of genes. These methylation marks are established during gametogenesis in a sex-specific manner and remain unaltered after syngamy, evading the global demethylation taking place during preimplantation development (11). However, ART may alter this special protection resulting into abnormal imprinting patterns that lead to transcriptional dysregulation of imprinting genes. The altered
transcriptional patterns of the imprinting genes leads to aberrant embryonic and placental development, ultimately manifested as imprinting syndromes in the offspring (12). Several of these syndromes, such as Beckwith–Wiedemann (BWS), Angelman (AS), Silver–Russell syndrome, and retinoblastoma have been associated with ART, but others negate this association [reviewed in Ref. (13)]. One comprehensive meta-analysis suggested that only three imprinting disorders BWS, AS, and maternal hypomethylation syndrome, all of which associated with hypomethylation at different maternal ICRs, have been consistently linked to the use of ART (14). Later epidemiologic observations regarding BWS and AS also agree with the notion that an ART-induced hypomethylation at ICR is responsible for the increased incidence of imprinting disorders in IVF/ICSI children. BWS and AS may have a genetic (i.e., mutations in the DNA sequence) or epigenetic (i.e., imprinting defect: alterations in the methylation patterns at ICRs) origin. It has been observed that 90–100% of the IVF/ICSI children with BWS had imprinting defects, in contrast with 40–50% of the naturally conceived children with BWS (15). Likewise, whereas only 5% of the spontaneously conceived children with AS had an epigenetic origin, 71% of the AS cases in IVF/ICSI children were attributed to imprinting defects (15). In a similar context, a lower DNA methylation has been reported in the placenta of children conceived in vitro compared to the control, although the reduction in CpG methylation affected equally imprinted and non-imprinted regions (16).

PUT THE BLAME ON ICSI?

Some of the adverse outcomes observed following ART may be due to the increased risk for health problems in couples pursuing ART, rather than the ART per se (9, 17, 18). The contribution of this parental factor to the health problems associated to the ART is difficult to disentangle from the technique itself in human studies, as the appropriate control group to establish a possible relation between ICSI and risk of pregnancy complications or birth defects should be babies naturally conceived by infertile couples. Also, the rarity of the some of the diseases associated to ART such as congenital malformations or imprinting disorders result in a very low statistical power (17). Unfertile couples often show an increased risk factor for pregnancy disorders. Women are on average older, which increases the proportion of low quality oocytes with chromosome abnormalities (19), however, even with donor oocytes, only 5% of fresh oocytes produce a baby (20). Furthermore, other potential risk factors such as cycle irregularities, uterine anomalies, or obesity – with mixed effects on oocyte quality or uterine receptivity (21) – are also more common in these patients (4). The higher occurrence of multiple pregnancies resulting from the transfer of more than one embryo constitutes another risk factor for pregnancy complications (4). On the paternal side, the spermatozoa from infertile male have been shown to display genetic and epigenetic alterations that can be linked to a reduced embryo development and the appearance of abnormal phenotypes in their offspring. In subfertile men, a higher incidence of DNA fragmentation (22, 23) and aberrant DNA methylation at ICRs (24) have been reported.

On the other hand, it is difficult to discern between the damage associated to ICSI and other ARTs associated to the procedure. The assisted reproduction treatment for ICSI is not limited to the injection of a sperm head into an oocyte. It also involves the hormonal induction to achieve supernumerary oocyte production, in vitro maturation (IVM) of the oocytes retrieved, in vitro culture (IVC) of the zygotes produced by ICSI, and cryopreservation of gametes and embryos, all of which may play a role in hampering optimal embryo development (25, 26) (Figure 1). In this context, IVF and ICSI share most of the procedures but the sperm injection, which evades the spermatozoa selection at the zona pellucida (ZP). The possible differential risk for adverse outcomes between both procedures remains controversial. Experiments conducted in animal models have observed several developmental alterations exclusively attributed to ICSI, as detailed below. Besides, an epidemiologic study reported a higher major malformation rate in babies obtained by ICSI with cryopreserved sperm compared with IVF (8.4 vs. 4.6%) (27). However, large meta-analyses of a mix of selected publications using fresh or frozen spermatozoa under different conditions did not observe a differential risk for birth defects between ICSI and IVF (8, 9).

NEGATIVE EFFECTS OF ICSI OBSERVED IN MAMMALIAN MODELS

Mammalian models constitute a valuable tool to study the adverse outcomes associated with ART. Studies in animal models provide a proper control group of healthy and fertile animals, reduce the environmental variations, and provide a pre-set experimental frame that avoids selective reporting. Furthermore, the molecular mechanisms behind the phenotypic alterations caused by ART are shared between mammalian species. A very well-known example of this is the so called "large offspring syndrome" in ruminants, caused by suboptimal IVC, remarkably similar in phenotype and molecular base to the human BWS (28).

Due to practical and technical limitations in ruminants or pigs, the rodents have been the most frequently used models to study the long-term effects of ICSI in the health of the offspring. In rodent models, ICSI has been reported to alter DNA decondensation (29) and calcium oscillation (30) in mouse zygotes compared with IVF, and to impair the active demethylation of the male pronucleus in rat zygotes (31). ICSI performed with fresh sperm has been reported to increase the appearance of abnormal chromosome segregation (ACS) at the first mitotic division in mouse (32). Half of these ACS embryos developed into morphologically normal blastocysts able to implant, but unable to develop to term, resulting in spontaneous abortions at E7.5 (32). However, less severely impaired, yet abnormal embryos produced by ICSI may be able to survive through pregnancy, resulting in long-term effects on the adult life in the context of the Developmental Origins of Adult Health and Disease (DOHaD).

In this perspective, several alterations have been described in the offspring, such as aberrant transcriptional aberrations spanning to the neonatal stage (33), alterations in glucose parameters in adult mice (34), and decreased testis weight, abnormal testicular tubule morphology, and increased testicular apoptosis (35). These long-term effects are usually manifested in a sex-specific manner, which can be explained by the widespread epigenetic sexual dimorphism observed in preimplantation embryos (36–38).
The situation is more severe when DNA-fragmented spermatozoa are used for ICSI (DFS-ICSI). The study of DFS-ICSI effects in animal models is particularly relevant because a significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa, which may be morphologically normal and thereby inadvertently used for ICSI (39). Sperm DNA fragmentation has been reported to affect embryo post-implantation development in ICSI procedures in humans, resulting in pregnancy loss (40). Studies in mice using DFS-ICSI, produced by freeze-thawing without cryoprotectants, have observed that DFS-ICSI induces epigenetic and genetic alterations in the embryo, resulting in detrimental effects in the offspring. On preimplantation mouse embryos, DFS-ICSI has been observed to delay male pronucleus demethylation, alter blastocyst gene expression, and modify the expression of imprinting genes (41). These early alterations may result in embryonic death or in aberrant phenotypes in the offspring, such as aberrant growth, premature aging, abnormal behavior, and a higher incidence of mesenchymal tumors (41). Furthermore, male offspring produced by DFS-ICSI has been reported to display a reduced fertility (42). The same study also observed that the offspring derived from DFS-ICSI displayed an increased chance for the appearance of abnormal phenotypes (kinky-tail) in the AxinFu mouse model of metastable epiallele, suggesting a transgenerational inheritance of the epigenetic alterations generated by DFS-ICSI (42). However, the heritability of other epigenetic changes has not been observed in other studies (43), so the transgenerational epigenetic inheritance may depend on the specific epigenetic alteration.

**ICSI-SPECIFIC HAZARDS: BYPASSING NATURAL BARRIERS**

While dissecting the ICSI-specific hazards, two are exclusive from this technique: the injection of the sperm head into the oocyte and the bypass of the natural spermatozoa selection mechanisms, which is partly shared with IVF.

The micromanipulation itself may harm the oocyte and the resulting embryo. Oocyte activation seems to be abnormal, as Ca\(^{2+}\) oscillations differ between mouse IVF and ICSI (30). The injection of a sperm head that has not undergone acrosome reaction increases the risk of vacuole formation in the oocyte. Although this effect, observed in mice, only happened when three or more intact spermatozoa were injected (44), removal of sperm plasma membrane improved embryo development in mice (45) and selection for spermatozoa with reacted acrosome improved implantation rates in humans (46) and mouse (29).

Although a large number of spermatozoa are present in the ejaculate, only a minority reach the fertilization place. On the journey of the sperm from the site of deposition to the site of fertilization, spermatozoa should pass through different barriers that ensure that only those with normal morphology and vigorous motility will have chances to fertilize the oocyte forming a healthy embryo (47, 48). Spermatozoa selection under natural circumstances is based on three different steps: (1) the female reproductive tract microenvironment, (2) the sperm–oviduct interactions at the caudal isthmus, and (3) the sperm–zone pellucida interaction (48) (Figure 2). ICSI bypasses this natural selection process of the fertilizing sperm (49), as it does not allow the sperm–oviduct interaction and other spermatozoa selection processes including ZP binding-penetration.

Spermatozoa DNA fragmentation is one of the most studied spermatozoa alterations generally excluded by these bypassed natural barriers. Furthermore, membrane altered spermatozoa have been reported to release endonucleases to the media that could induce DNA fragmentation in spermatozoa with unaltered membranes (50). Female genital tract seems to act as a selective barrier against DNA-fragmented spermatozoa. A study in the mouse model showed that natural mating with males showing an increased percentage of spermatozoa DNA damage – produced by scrotal heat treatment or irradiation – resulted in different percentages of DNA fragmentation according to the region of the female reproductive tract where the sample was collected. Particularly, those spermatozoa reaching the oviduct had lower DNA fragmentation compared with those situated on lower, more distant to the fertilization place, portions (51). Apart from this selection at the reproductive tract, the study also pointed spermatozoa binding to the ZP as a second barrier against DNA fragmentation. A study in the mouse model showed that natural mating with males showing an increased percentage of spermatozoa DNA damage – produced by scrotal heat treatment or irradiation – resulted in different percentages of DNA fragmentation according to the region of the female reproductive tract where the sample was collected. Particularly, those spermatozoa reaching the oviduct had lower DNA fragmentation compared with those situated on lower, more distant to the fertilization place, portions (51). Apart from this selection at the reproductive tract, the study also pointed spermatozoa binding to the ZP as a second barrier against DNA-fragmented spermatozoa, as the percentage of DNA fragmentation was lower for the sperm bound to the ZP than to the unattached spermatozoa (51).
Since it is unlikely that uterine or oviductal cells are able to assess sperm DNA quality per se, the selection needs to be based on sperm phenotype and function related to DNA integrity (52). Among the possible candidates, motility is the main selective factor on the trip (53), but it is not the only one, as other barriers such as leukocytic/phagocytic responses from the immune cells present in the uterine mucosa (54) may play a role, and binding to the oviduct has been correlated with chromatin stability in the pig model (55). Some studies have found a correlation between DNA integrity and sperm motility. Thus, a negative correlation was established between the computer-aided sperm analysis (CASA) percentage of motile sperms and DNA fragmentation index (DFI) (56). Likewise, negative correlations were observed between sperm DNA fragmentation assayed by TUNEL and sperm motility under natural conditions (57) or after H₂O₂ or alpha irradiation-mediated DNA damage (58). In agreement, the spermatozoa selection method swim-up, which enriches for motile spermatozoa, reduces the percentage of apoptotic spermatozoa (59).

On a similar context, the binding of spermatozoa to ZP selects those with progressive motility, normal morphology, and chromatin structure (60). Spermatozoa with single stranded or denatured DNA were reported to bind less or do not bind at all to the ZP (61). ZP binding may even exclude those with numerical chromosomal aberrations (62), which seem not to display impaired motility (63).

**STRATEGIES FOR A SAFER ICSI**

As the bypass of spermatozoa selection seems to be one of the most critical hazards when performing ICSI, several spermatozoa selection methods have been proposed. Density gradients commonly used in IVF are able to separate dead and alive spermatozoa and other techniques such as Swim-up allow the selection of motile spermatozoa, less prone to display DNA fragmentation (59). Similarly, novel methods for spermatozoa selection based on motility have been developed in microfluidics platforms, allowing sperm selection in oligozoospermic samples with high amounts of non-gamete cell contamination (64), and enriching the sample for sperm with intact chromatin and DNA integrity (65).

High resolution morphology has also been used to improve implantation rates (66). In this line, motile sperm organelle morphology examination (MSOME) allows grading spermatozoa based on the detection of vacuoles in the sperm heads (67). These vacuoles negatively affect implantation, pregnancy, and live birth rates following ICSI (68). However, the beneficial effects of applying morphological criteria for sperm selection before ICSI on implantation and pregnancy rates remain a controversial issue, with contrasting results obtained by different groups [reviewed in Ref. (69)]. Other techniques such as the use of sperm selection chambers (70), a peptide ligand based stain capable of binding damaged DNA structures (71), and Raman microspectroscopy (72) have been proposed to select spermatozoa with low DNA damage, whereas birefringency was used to select acrosome reacted spermatozoa (46). In a similar line, hyaluronic acid sperm selection or ZP binding before ICSI have been reported to be able to select for mature spermatozoa, reduce DNA fragmentation rate, and improve embryo quality and development (73–75). These binding-based techniques have been suggested to select against immature sperm that has not reached its final nuclear and cytoplasmic maturation (76).

Oocyte quality is another factor to take into account, as on one hand the oocyte itself may be the source for genetic or epigenetic alterations (77, 78) and on the other it may repair the genetic or epigenetic alterations of the spermatozoa. Oocytes have been suggested to be able to repair sperm DNA when the damage is <8% (79), but the DNA repair ability depends on oocyte quality and age (78). Thereby, improvements in IVM or ovarian stimulation may reduce the adverse effects of ICSI. In this sense, the use of low hormone doses may help toward a more stringent selection of oocytes (7).

**CONCLUDING REMARKS**

The current widespread use of ICSI together with the perspective of growth in its use urge for an analysis of the possible health risk associated with this technique. Epidemiologic studies have established associations between the use of ICSI and diverse health...
problems, ranging from premature births and metabolic complications in the offspring to abortions, congenital malformations, and imprinting disorders. Animal models provide an invaluable means to experimentally test these associations and to understand the molecular root behind the adverse outcomes of ICSI in the offspring, as an initial step to improve the safety of the technique. ICSI bypasses a series of biological barriers, but novel strategies based on gamete selection may mimic these barriers, restoring the natural selection process required for a flawless embryonic and fetal development.

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