Fertilization of oocytes by injecting spermatozoa, spermatids and spermatocytes

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The feasibility of fertilization by injecting spermatozoa into oocytes has increased significantly the possibilities for treatment of severe male infertility. However, the rapidity of human application has raised some concern about potential health hazards for the progeny. Human pregnancies and births have also occurred with the use of immature spermatozoa and spermatids, and normal offspring have been born after the injection of secondary spermatocytes into mouse oocytes. This short review deals with the problems that may arise from the injection technique and from the use of deficient or immature sperm cells for fertilization, with particular attention to human applications. Tests for screening parents and follow-up of children are suggested to control the main suspected risk factors.

The first successful fertilization of a mammalian oocyte by a direct intra-ooplasmic transfer of a spermatozoon was reported in hamsters 20 years ago (Uehara and Yanagimachi, 1976). In rabbits and cattle, embryos obtained with the use of this method were transferred to recipient mothers, resulting in the birth of normal offspring (Iritani, 1991). The importance of this pioneering work for subsequent human application needs to be acknowledged. However, the success rate of this method was very low until the early 1990s when an improvement of the micromanipulation technique, performed on human gametes, led to a spectacular increase in fertilization rates and first childbirths (Palermo et al., 1992). Since then, the method, known as ICSI (for ‘intracytoplasmic sperm injection’), has been used throughout the world as a treatment of last chance for men with extremely severe oligo- and asthenozoospermia but also as a ‘more efficient’ treatment in less severe cases in which the standard in vitro fertilization could also be envisaged. Owing to a strong clinical demand, the human application of ICSI has advanced animal studies significantly, and experimental work on human oocytes donated by patients for research purposes has also lagged behind clinical applications mainly performed on a purely empirical basis.

The use of a microinjection technique to transfer the spermatozoon into the oocyte has rendered unnecessary many systems required for normal fertilization in which spermatozoa undergo a functional maturation during the passage of spermatozoa through the male genital tract (for example the motility apparatus, zona pellucida receptors, acrosome) so that ICSI has become a treatment of choice for patients with obstructive azoospermia in whom spermatozoa can be obtained by epididymal aspiration or testicular biopsy (for example Silber, 1994). Moreover, animal experiments showing that viable embryos can develop from oocytes fertilized with round spermatids, the youngest male germ cells to have a set of haploid chromosomes (Ogura et al., 1994; Sofikitis et al., 1994), have encouraged attempts at fertilizing human oocytes by injecting spermatids from patients with nonobstructive azoospermia. Successful fertilization with round and elongated spermatids in humans has been reported from a few centres (Fishel et al., 1995; Tesarik et al., 1995; Vanderzwalmen et al., 1995). These efforts led to the first childbirth after injection of round spermatids into human oocytes (Tesarik et al., 1995) and to the establishment of other ongoing pregnancies (Fishel et al., 1995; Tesarik et al., 1995). Kimura and Yanagimachi (1995) demonstrated that normal mice can also develop from oocytes injected with secondary spermatocyte nuclei, showing that the problem of ploidy associated with the use of the early stages of spermatogenic cells that have not yet completed meiosis can be resolved by the use of an appropriate technique.

Questions relating to the injection technique

The injection technique brings about two biologically relevant effects that are common to all methods using intra-ooplasmic injection for fertilization, regardless of the quality and maturity of the injected sperm cell. These effects are disruption of the plasma membrane and elimination of the normal interaction between the surfaces of both gametes preceding gamete fusion.

Mammalian cells do not survive disruptions to the plasma membrane unless an efficient resealing mechanism is rapidly set in motion after injection. This mechanism implies an accumulation and fusion with the wounded plasma membrane of one or more cytoplasmic membrane compartments at the disruption site and is triggered by the disruption-provoked Ca2+ influx down an approximately 104-fold gradient between the extracellular and the intracellular milieu (Miyake and McNeil, 1995).

The contact and interaction between the surfaces of both gametes before fusion is believed to be involved in triggering activation of mammalian oocytes; the most popular theory explaining this event implies the binding of as yet unidentified molecules on the sperm surface to receptors on the oocyte surface, leading to activation of a G-protein–phosphoinositide messenger system (Jaffe, 1990). In normal fertilization, this mechanism appears to be complemented by another oocyte-activating mechanism that relies on the release from the fertilizing spermatozoon after sperm–oocyte fusion of a cytosolic factor (reviewed in Tesarik and Sousa, 1994). Both mechanisms...
produce an increase in the free cytoplasmic Ca^{2+} concentration which is important for pronuclear formation and cleavage and which has an oscillatory character in mammals (Swann and Ozil, 1994). The first sperm-induced Ca^{2+} increase has been shown to occur at least 7 s after sperm–oocyte membrane fusion in sea urchins (McCulloh and Chambers, 1992). A soluble sperm protein, termed oscillin and existing as an oligomer with a subunit of M, 33 K, capable of activating mammalian oocytes by triggering a characteristic series of Ca^{2+} oscillations has been cloned and shown to have sequence identity of 53% to a glucosamine-6-phosphate isomerase isolated from Escherichia coli (Parrington et al., 1996). Experience with ICSI, in which the normal interaction between gamete surfaces is abolished, suggests that this cytosolic factor alone can ensure all of the oocyte activation events. It appears that the Ca^{2+} influx at the puncture site is essential for oocyte activation after ICSI and that it performs, by an as yet determined mechanism, the role of the surface component of the normal oocyte activation system (Tesarik and Sousa, 1995). This Ca^{2+} influx is facilitated by hydrodynamic forces provoked by the aspiration of the oocyte cytoplasm just before sperm expulsion from the microinjection needle (Tesarik and Sousa, 1995) and is important for resealing the damaged plasma membrane as well as the subsequent oocyte activation.

It may be relevant that elimination of the normal gamete surface interaction leads to a slight modification of the oocyte Ca^{2+} response to the fertilizing spermatozoon (Tesarik and Sousa, 1994). The pattern of these Ca^{2+} signals can be modified by the injection technique (Tesarik and Sousa, 1995). Because the fertilization-induced Ca^{2+} signal may act as an epigenetic factor influencing embryo quality (Swann and Ozil, 1994), the injection technique must be considered as a possible source of abnormalities of oocyte activation that may consequently affect the embryonic genome by provoking cell cycle irregularities (Tesarik, 1995).

Last but not least, the injection technique increases the risk of unintentional introduction of foreign DNA into the oocyte. Thus, all materials and media coming into contact with the gametes used for ICSI should be tested for the absence of DNA contamination, and the precautions normally taken in a molecular biology laboratory should be taken during all steps of the manipulation.

**Questions relating to sperm cell deficiency**

**Genetic factors**

It is known that gross abnormalities of sperm morphology and function can be due to genetic factors. Microdeletions on the long arm of the Y chromosome have been detected in some patients with azoospermia and severe oligozoospermia (Kobayashi et al., 1994). Some of these microdeletions are associated with spermatogenic arrest at the spermatid stage (Reijo et al., 1995). In other cases, oligo(asthenoterato)zoospermia may be related to X-linked androgen receptor insensitivity syndrome (McPhaul et al., 1993) or to hereditary elliptocytosis (Patrizio, 1995). Some cases of the congenital absence of vas deferens are caused by cystic fibrosis mutations (Patrizio, 1995). Without previous diagnosis, ICSI is likely to increase the risk of transmission of these abnormalities to progeny; some of these concern only fertility but others have a complex pathology which may affect the duration and quality of human life.

**Epigenetic factors**

In addition to the male genome, the fertilizing spermatozoon delivers to the future embryo a number of epigenetic factors that are no less essential for normal development. These factors comprise a cytosolic oocyte-activating factor (see above), the microtubule organizing centre (centrosome), and a male-specific imprint on certain developmentally regulated genes. In the absence of the normal gamete surface interaction, the cytosolic sperm factor is the principal agent responsible for oocyte activation after ICSI (Tesarik and Sousa, 1994). Later, during embryo cleavage, Ca^{2+}-releasing activity becomes associated with blastomere nuclei (Kono et al., 1995), where it may have other specific functions. The sperm-derived centrosome is necessary for the development of the microtubule organizing activity of human embryonic cells (for example Sathananthan et al., 1991) as is the case in most mammals studied. The paternal specific mark (imprint) on the sperm-derived alleles of certain genes is implied in transcriptional control and is required for normal development. All these factors may be deficient in abnormal spermatozoa, although direct experimental evidence for such an association is lacking.

**Questions relating to sperm cell immaturity**

When considering the use of immature spermatogenic cells for fertilization, the problem of ploidy, associated with the use of pre-meiotic male germ cells, has long been thought insurmountable. However, an elegant study by Kimura and Yanagimachi (1995) has shown that this problem can be resolved by manipulating the time at which oocyte activation is triggered. In the absence of oocyte activation, the injected cell nucleus is exposed to metaphase-promoting factor (MPF) and cytostatic factor (CF), which are active in the oocyte cytoplasm and maintain the chromosomes of mature mammalian oocytes in metaphase of the second meiotic division. After being translocated to the oocyte periphery, the injected nucleus thus reacts to MPF and CF in the same way as does the oocyte nucleus and enters metaphase (Fig. 1). When an artificial oocyte-activating signal is subsequently delivered, MPF and CF are inactivated and both the oocyte chromosomes and the chromosomes of the injected nucleus enter anaphase, which leads to the haploidization of both chromosomal sets (Fig. 1). Thus, the premature chromosome condensation (PCC) occurring in the injected nucleus in the absence of oocyte activation is essential for achieving fertilization with secondary spermatocytes (Kimura and Yanagimachi, 1995). However, the same phenomenon of PCC presents a risk of aneuploidization when haploid spermatid nuclei are injected. Mature spermatozoa appear to be protected from PCC by their special chromatin arrangement preventing a rapid reaction to MPF and CF.

Regardless of ploidy, all the questions concerning sperm cell deficiency raised in the previous section also apply to sperm cell immaturity. As for genetic factors, the X-linked form of Kallman syndrome (Prager and Braunstein, 1993) must also be taken into account when the gonadotrophin concentrations are extremely low and, in the absence of spermatogenesis, the injection of
sperm precursor cells is envisaged. As for epigenetic factors, the birth of normal progeny after intra-ooplasmic injection of secondary spermatocytes (Kimura and Yanagimachi, 1995) and round spermatids (Ogura et al., 1994) in mice and of round spermatids in humans (Tesarik et al., 1995) supports the contention that genomic imprinting status at the respective stages in the two species can be compatible with normal development. However, this limited experience does not mean that the risk of genomic imprinting abnormalities linked to the use of immature sperm cells is totally excluded. Moreover, this uncertainty has not yet been overcome even for the use of more mature stages, such as testicular and epididymal spermatozoa, because experiments conducted in mice show that reprogramming of certain sperm genes continues in the epididymis (Ariel et al., 1994).

**Questions relating to human application**

The application of any new therapy to humans is normally preceded by testing its safety in animal models. It must be admitted that, for the reasons explained above, ICSI has been applied with minimal previous animal experience. Animal experiments showing normal development of progeny after fertilization with spermatids (Ogura et al., 1994; Sofikitis et al., 1994) did precede the human application; but the question of whether this preliminary work was sufficient to justify human application can be posed. Although the available data do not suggest an increased risk of health problems for ICSI babies, it must be taken into account that this application is recent and the long-term effects have yet to be evaluated. The human experience with the use of spermatids for fertilization is even more limited.

The risk of producing abnormal babies can be reduced by appropriate examinations of both partners of an infertile couple before their inclusion into the treatment group. Many of the potential health hazards associated with the use of abnormal or immature sperm cells can now be controlled (Table 1). The examination of the male partner for Y chromosome microdeletions, X-linked androgen receptor insensitivity and Kallman syndrome, and of both partners for hereditary elliptocytosis can predict the risk of transmission of these diseases to progeny. The examination of both partners for the mutations associated with cystic fibrosis will reveal the cases in which both partners are carriers and in which embryos should not be transferred without previous preimplantation diagnosis to avoid the clinical manifestation of this disease. Testable abnormalities of known

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**Fig. 1.** Schematic representation of the mechanism by which the oocyte can reduce the chromosomes of an injected male germ cell. (a) Mature oocyte with female chromosomes (orange) maintained in metaphase configuration by the action of metaphase promoting factor (MPF) and cytostatic factor (CF), just after the injection of a male germ cell nucleus (red). The first polar body is not represented. (b) The injected male germ cell nucleus is translocated to the oocyte periphery. (c) In the absence of oocyte activation, the persisting MPF and CF have induced premature chromosome condensation (PCC) in the injected male nucleus, resulting in the formation of a second metaphase plate. (d) The delivery of an oocyte-activation signal leads to the elimination of the MPF and CF activities leading to the entry of both the female and the male chromosomes into anaphase followed by the extrusion of the respective polar bodies.
imprinted genes (Table 1) should be screened for in babies resulting from the injection of immature spermatoza (epididymal and testicular) and spermatids to evaluate the risk associated with the use of these cells for reproduction. Centrosomal defects can be detected in oocytes that fail to fertilize to avoid unnecessary repetitions of unsuccessful treatment cycles (Table 1).

Finally, as with any other new method, there is a certain degree of unpredictable risk that cannot be controlled by any of the available methods. Pretreatment medical counselling and the informed consent form should include adequate information about the existence of this risk.

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