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Using the Male Gamete for Assisted Reproduction: Past, Present, and Future

Andrology Lab Corner*

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Since the discovery of the male gamete, numerous techniques have been developed to assist its function in order to allow fertilization in different cases of infertility. These techniques were employed in various pathological situations concerning the male, the female, or both in which the physical rapprochements and/or interactions of sperm and oocyte were compromised. Problems of ejaculation and other abnormalities disturbing sperm deposition in the fornix of the vagina; abnormal sperm count, movement, or morphology; functional sperm abnormalities impeding the binding to, or penetration of, the female gamete; and the presence of sperm autoantibodies interfering with sperm passage through the female genital tract or with sperm–egg interaction are some examples of the male pathologies concerned. Female indications for assisted reproduction include problems of patency of the fallopian tubes; cervical hostility, sometimes associated with the presence of sperm-reactive isoantibodies; oocyte-borne abnormalities of the composition of the oolemma and the zona pellucida; and premature or physiological menopause (where oocyte donation is required). This is a brief overview of the progression of the state of the art in this field.

From the Discovery of Spermatozoa to Artificial Insemination

When originally discovered by Antonij van Leeuwenhoek and his assistant, Ludwig Hamm, in 1677 in the city of Delft (Leeuwenhoek, 1678), spermatozoa were considered to be parasites of the male genital tract. The concept of fertilization and the role the spermatozoon has in it was not realized until the 19th century. This knowledge was being acquired in a stepwise manner, owing to investigations conducted by Prevost and Dumas, Peltier, and Dujardin between 1820 and 1840. Anyway, the reproductive potential of semen was known before the formal recognition of the spermatozoon as the oocyte fertilizing agent. The first documented successful attempt at artificial insemination (AI) was performed in the dog by Spallanzani (1784), and undocumented tales mention that Arabic horse breeders used AI as early as the 13th century. The first attempts at human AI were made in 1785 by John Hunter (1728–1793), a Scottish surgeon, resulting in the birth of a child in the same year (Seibel, 1997). At its beginnings, AI was performed with crude, untreated semen samples. Because the introduction of seminal plasma into the uterus may cause infection and produces painful spasms (Mastroianni et al, 1957; Allen et al, 1985), semen samples were inseminated to the vagina or the cervical canal rather than the uterine cavity.

From AI to In Vitro Fertilization

After the observation, in the early 1950s, that motile spermatozoa are not fertile immediately after release from the male genital tract but require a species-dependent amount of time to acquire fertilizing ability (Austin, 1951; Chang, 1951), the time-dependent acquisition of sperm fertilizing ability has been termed ''capacitation'' (Austin, 1952). In vivo spermatozoa are capacitated during their ascent through the female genital tract toward the site of fertilization, but these changes can be easily reproduced in vitro by sperm incubation in appropriate media (Yanagimachi, 1994). The optimal conditions for sperm in vitro capacitation differ from species to species, and an understanding of them was a necessary prerequisite for the development of in vitro fertilization (IVF).

Most of the knowledge required to successfully conduct human IVF was at hand in the early 1970s. This knowledge was essentially derived from animal research primarily aimed at the development of IVF as a tool in animal production (Brackett et al, 1982). However, IVF and preimplantation embryo development, followed by term pregnancy after embryo transfer to a recipient female, could not be obtained in any mammalian species until the 1970s. In fact, IVF was successful in the hamster (Yanagimachi and Chang, 1964), but the resulting embry-

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os did not develop beyond the 1- to 2-cell stage, which also was the case for mice and rats. Rabbit embryos could be grown in vitro (Daniel, 1971), but IVF was not successful in this species because of special requirements for sperm in vitro capacitation. In domestic and agricultural animals, similar constraints were encountered. Compared to most animal species studied, the development of human IVF was relatively straightforward. Human IVF was introduced in 1969 (Edwards et al, 1969), and growth of in vitro–fertilized human embryos to the blastocyst stage was accomplished only 2 years later (Steptoe et al, 1971).

The idea of applying IVF in the treatment of human infertility initially met with much concern and adversity. From the technical point of view, in addition to the optimization of laboratory conditions for human IVF and early embryo culture (composition of culture media and gas phase, concentration of spermatozoa in the insemination medium, and evaluation of fertilization outcomes and embryo quality), it was necessary to develop efficient clinical protocols for controlled ovarian stimulation, oocyte recovery, and embryo transfer (Steptoe and Edwards, 1970). The first pregnancy achieved in the human by IVF was an extrauterine one (Steptoe and Edwards, 1976). Nevertheless, the birth of a healthy girl was achieved 2 years later by the same team of workers using the same methodology (Steptoe and Edwards, 1978). In the following years, human IVF was applied rapidly worldwide (eg, Trounson et al, 1981; Jones et al, 1984).

The finding that chicken sperm could be successfully frozen in solutions containing glycerol (Polge et al, 1949) was at the origin of the development of sperm cryopreservation protocols to be employed in different mammalian species and also in humans (Sherman, 1954). IVF with cryopreserved spermatozoa has been successful since 1985 (Cohen et al, 1985; Naz et al, 1985). In comparing IVF treatment attempts with fresh and cryopreserved spermatozoa from fertile donors, it was found that similar pregnancy rates were obtained between the 2 methods (Morshedi et al, 1990). Pregnancy rates with fresh and cryopreserved sperm from infertile patients were also similar in spite of slightly lower fertilization rates with cryopreserved sperm (Morshedi et al, 1990).

The clinical efficacy of IVF was further potentiated by the introduction of embryo cryopreservation. Because protocols of controlled ovarian stimulation usually lead to the recovery of numerous oocytes, embryo cryopreservation makes it possible to optimally benefit from the cohort of embryos available by transferring part of the embryos in the fresh state and cryopreserving supernumerary embryos for later transfer. The first successful cryopreservation protocols for human embryos used dimethyl sulfoxide as the cryoprotectant for embryos at early cleavage stages (Trounson and Mohr, 1983) and glycerol for blastocysts (Cohen et al, 1986). Later studies have shown that 1,2-propanediol is a better cryoprotectant than dimethyl sulfoxide for human early-cleaving embryos (Lassalle et al, 1985; Testart et al, 1986), and solutions containing this agent continue to be used for the cryopreservation of human embryos on days 2 or 3 after fertilization.

Laboratory and clinical experience achieved during IVF trials also helped improve the efficacy of more ancient assisted reproduction techniques, namely AI. The development of in vitro sperm washing methods made it possible to safely deliver adequate numbers of spermatozoa directly to the uterine cavity. In the 1980s, together with the rapid development of IVF, sperm isolation techniques combining the removal of seminal plasma with the selection of highly motile sperm subpopulations were applied to AI, leading to a substantial improvement in success rates (Marrs et al, 1983; Kerin et al, 1984; Sher et al, 1984; Byrd et al, 1987). With the use of these sperm preparation techniques, there is a wide consensus that intrauterine insemination (IUI) is superior to other AI techniques (Byrd et al, 1990; Patton et al, 1992; Wainer et al, 1995; Matorras et al, 1996).

Further improvement of IUI results was brought about by the introduction of ovarian stimulation (Kemman et al, 1987; Serhal et al, 1988; Chaffkin et al, 1991; Guzick et al, 1999). Compared to natural cycles, the inclusion of ovarian stimulation allows a better timing of the insemination procedure and the correction of subtle ovulatory disorders that might complicate the prediction of spontaneous ovulation (Dodson et al, 1987; Wallach, 1991). However, ovarian stimulation also increased the risk of multiple pregnancies after IUI (Navot et al, 1991; Farhi et al, 1996), which, however, can be minimized by the application of mild stimulation protocols, the careful monitoring of ovarian follicular development by vaginal ultrasonography, and the cancellation of treatment if too many follicles are recruited. Nevertheless, the optimal number of large preovulatory follicles to maintain acceptable cycle fecundity and minimize the risk of multiple pregnancy is not known. Nowadays, AI still has its place as a simple and inexpensive method in the treatment of certain types of infertility, in spite of the existence of more high-performance techniques.

The cryopreservation of sperm has further facilitated the use of donor sperm for both AI and IVF. The efficacy of using frozen sperm in donor AI was reported to be somewhat lower than in fresh sperm (Richter et al, 1984), but other studies did not find any significant differences (Bordsen et al, 1986; Keel and Webster, 1989).

Compared to AI, IVF represented a real breakthrough in the treatment of human infertility and strongly stimulated further development in this field. From the clinical viewpoint, it constituted an efficient treatment option for women suffering from tubal infertility, which was the

originally advocated indication for this technique. Soon after its introduction, however, it became clear that the application sphere of IVF was much wider, including endometriosis and other types of female infertility, idiopathic infertility, and, particularly, various types of male infertility (Mahadevan et al, 1983).

A modification of IVF, whereby spermatozoa and oocytes were mixed together in vitro and then deposited in the fallopian tube, was also designed (Shettles, 1979). It was first successfully performed in 1982, during an attempt at microsurgical reconstruction of fallopian tubes, resulting in the birth of a healthy child (Tesarik et al, 1983). This technique was later adapted to be performed laparoscopically and became popularized under the term of ''gamete intrafallopian transfer—GIFT'' (Asch et al, 1984).

From the scientific viewpoint, the most important advancement resulting from the clinical application of IVF was doubtlessly the possibility of direct observation of human sperm–egg interaction. In addition to providing a number of new pathophysiological insights with numerous diagnostic implications, it became a new and powerful stimulus for further development of assisted reproduction techniques.

Development of In Vitro Pharmacological Treatments to Assist Sperm–Egg Interaction

The clinical application of in vitro sperm capacitation revived the interest in the behavior of spermatozoa in these conditions. In particular, characteristics of sperm movement were no longer considered static parameters merely reflecting the quality of fresh or cryopreserved sperm samples, but they began to be analyzed as evolutive variables, subject to functionally relevant modifications in the period between ejaculation and fertilization. The development of computer-assisted systems for the analysis of sperm motion characteristics on a single-cell basis represented a significant technical impetus for this approach. It became increasingly evident that not only motility in its conventional sense (the percentage of sperm that move) but also the speed and quality of movement of each motile spermatozoon were important determinants of fertility.

These studies made it possible to determine the normal speed range and the characteristics of the trajectory of individual human spermatozoa at different time periods between ejaculation and fertilization as well as the effects of different components of the female genital tract on these parameters (Mendoza and Tesarik, 1990; Tesarik et al, 1990). It was also noted that abnormal sperm movement characteristics are associated with reduced fertilizing ability in vivo (Barratt et al, 1993) and in vitro (Jeulin et al, 1986; Liu et al, 1991). In many of these cases, sperm movement was enhanced and the fertilization rate was improved by sperm in vitro pretreatment with pentoxifylline, an inhibitor of phosphodiesterase that increases the intracellular concentration of cyclic adenosine monophosphate, before its use for IVF (Yovich et al, 1988, 1990; Rizk et al, 1995; Tarlatzis et al, 1995). Pentoxifylline has been shown to stimulate sperm velocity and the development of a special, capacitation-related change in sperm movement pattern, called ''hyperactivation'' (Tesarik et al, 1992b). Moreover, when used at adequate concentrations (around 1 mg/mL) and with appropriate incubation times (around 10 minutes), pentoxifylline also enhanced the response of spermatozoa to acrosome reaction–inducing stimuli (Tesarik et al, 1992a; Carver-Ward et al, 1994; Kay et al, 1994) and was used with success in patients with acrosome reaction insufficiency (Tesarik and Mendoza, 1993; Tasdemir et al, 1993). One study has also reported a beneficial effect of in vitro treatment with pentoxifylline on sperm binding to the zona pellucida in the hemizona assay (Yogev et al, 1995), but the mechanism of this effect is not known. Premature acrosome reaction may also occur spontaneously in some men with reduced fertility, and this condition can be alleviated by sperm pretreatment with egg yolk, which probably acts by stabilizing the abnormally fragile sperm plasma membrane in these patients (Tesarik and Mendoza, 1995).

After the advent of micromanipulation-assisted fertilization (see below), the pharmacological enhancement of sperm function lost much of its importance in attempts using freshly obtained ejaculated spermatozoa. However, it still maintains its place in assisted reproduction with poorly motile sperm samples, such as testicular spermatozoa from men with nonobstructive azoospermia (NOA) or cryopreserved sperm samples with poor prefreeze quality (Esteves et al, 1998). Pentoxifylline was also used with success in sperm preparation for IUI (Negri et al, 1996).

Development of Micromanipulation Techniques to Assist Sperm–Egg Interaction

The development of micromanipulation-assisted fertilization was motivated by the observation that failures of IVF were mostly associated with a failure of sperm–zona pellucida binding or zona pellucida penetration. From the technical point of view, it was facilitated by the increasing availability of good-quality cell micromanipulators and growing experience with their use in research studies with animal gametes. It was initially thought that problems of sperm–zona pellucida interaction could be treated by the injection of spermatozoa through the zona pellucida into the perivitelline space (Laws-King et al, 1987).

These techniques, however, were burdened by the unpredictability of sperm–oolemma fusion in these artificial conditions, leading to a high incidence of both fertiliza-

tion failure and polyspermic penetration. In fact, contrary to what was theoretically expected, most acrosome-reacted motile human spermatozoa proved incapable of fusing with the oolemma after subzonal insemination (Tesarik and Mendoza, 1994). It thus became clear that an efficient micromanipulation-assisted fertilization technique should not only ensure sperm passage through the zona pellucida, but it also should sever the oolemma as well in order to deposit a single spermatozoon directly in the oocyte cytoplasm.

Attempts at direct intracytoplasmic sperm injection (ICSI) into human oocytes were first reported in 1988 (Lanzendorf et al, 1988), but the first term pregnancy after the transfer of embryos resulting from ICSI was achieved only 4 years later (Palermo et al, 1992). The technique then evolved rapidly and soon achieved the highest fertilization rates among all micromanipulation-assisted fertilization techniques (Van Steirteghem et al, 1993). The other types of manipulation were then progressively abandoned.

Evolution of ICSI and Development of ICSI-Derived Techniques Using Immature Male Gametes

Extension of ICSI Indication—Originally developed to be used for assisted reproduction in cases of severe male infertility, ICSI has progressively become a treatment option for other indications, too. These include not only male but also female infertility. This development was essentially based on high and relatively stable fertilization rates with ICSI (Tournaye et al, 2002) and on the independence of results on different sperm and oocyte functions, such as sperm–zona pellucida recognition and binding, acrosome reaction, sperm–oolemma binding and fusion, and the presence of antibodies on gamete surfaces (Hamberger et al, 1998). Moreover, fertilization and pregnancy rates after ICSI with ejaculated spermatozoa are not influenced by sperm cryopreservation, even in patients with poor sperm quality (Kuczynski et al, 2001). By making it possible even for spermatozoa carrying various functional defects, which otherwise would not be able to penetrate the egg vestments, to get access to the oocyte cytoplasm, ICSI helped reveal sperm and oocyte functional abnormalities that were not known before. Among these, the failure of oocyte activation received the most attention.

Oocyte activation is a complex, sperm-initiated process whereby the mature oocyte, physiologically arrested at metaphase of the second meiotic division until fertilization, reactivates the cell cycle, completes the second meiosis, and, after nuclear syngamy, starts mitotic cell divisions of the early embryo. This process is essentially regulated by repetitive increases in free cytoplasmic Ca^{2+} concentrations (Ca^{2+} oscillations) that are triggered by the fertilizing sperm (Miyazaki et al, 1986). It has been shown that Ca^{2+} oscillations, similar to those observed after spontaneous sperm penetration into the oocyte (Taylor et al, 1993), also occur after ICSI (Tesarik et al, 1994). However, the mechanism whereby Ca^{2+} oscillations are triggered after ICSI is slightly different from normal (Tesarik and Mendoza, 1999). Because ICSI is usually performed with abnormal sperm samples, the content and quality of sperm factors responsible for oocyte activation can also be altered. Globozoospermia (round-headed sperm syndrome) is the best-known example of this deficiency (Rybouchkin et al, 1996; Battaglia et al, 1997), but it is known that spermatozoa from some men consistently fail to activate the oocyte without presenting the typical round-head morphology. Moreover, repeated failures of oocyte activation after ICSI can also be due to anomalies of oocytes rather than spermatozoa in some infertile couples (Tesarik et al, 2002b). To improve ICSI results in cases of repeated oocyte activation failure, oocyte activation can be boosted by calcium ionophores (Rybouchkin et al, 1997; Kim et al, 2001), which were shown to substantially increase the fertilization rate (Tesarik and Sousa, 1995) and to potentiate the sperm-induced Ca^{2+} oscillations (Tesarik and Testart, 1994) of sperm-injected human oocytes. However, a simple modification of the ICSI technique has recently been developed for cases of sperm- and oocyte-borne repeated oocyte activation failures without requiring recourse to the use of potentially toxic chemical agents (Tesarik et al, 2002b).

ICSI With Immature Sperm—The independence of fertilization by ICSI on sperm movement or on the presence of mechanisms required for oocyte penetration was at the origin of an increasing use of immature spermatozoa (epididymal and testicular) and even of sperm precursor cells for fertilization.

The association of ICSI with surgical sperm retrieval has revolutionized the treatment of azoospermic men. In cases of obstructive azoospermia (OA), sufficient numbers of spermatozoa for ICSI can usually be retrieved by epididymal sperm aspiration, whereas cases of NOA mostly require open testicular biopsy. Even with this more invasive approach, spermatozoa fail to be recovered in about a half of the cases of NOA in tissue samples obtained by multiple-site testicular biopsy (Kahraman et al, 1996; Westlander et al, 1999; Friedler et al, 2002). Unfortunately, a reliable test predicting successful sperm retrieval from the testes of men with NOA is still lacking.

There is a wide consensus that serum concentrations of follicle-stimulating hormone (FSH) do not predict sperm retrieval from men with NOA (Gil-Salom et al, 1995; Mulhall et al, 1997; Tournaye et al, 1997). Serum inhibin B levels, reflecting Sertoli cell function, were suggested to predict sperm retrieval by testicular biopsy from NOA patients (Ballesca et al, 2000; Brugo-Olmedo et al, 2001), but other studies (Von Eckardstein et al, 1999; Vernaeve

et al, 2002) failed to corroborate this hypothesis. In the absence of more reliable criteria, the strongest indicator for finding sperm for ICSI in NOA patients is testicular histopathology (Tournaye et al, 1997; Mercan et al, 2000; Vicari et al, 2001; Friedler et al, 2002) and histochemistry (Anniballo et al, 2000), together with the detection of at least a few round spermatids in the ejaculate with the use of immunocytochemistry with antibodies to specific germline marker antigens (Ezeh et al, 1998). In some cases in which spermatozoa fail to be recovered, fertilization with sperm precursor cells can be attempted.

Epididymal Sperm—The first birth after fertilization with epididymal spermatozoa from a patient with OA was achieved with the use of conventional IVF (Silber et al, 1990). However, the application of ICSI substantially improved fertilization and pregnancy rates (Silber, 1994; Tournaye et al, 1994), which currently are similar to those for ICSI with ejaculated sperm (Van Steirteghem et al, 1998).

Epididymal sperm can also be cryopreserved for later use in ICSI. Comparing the efficacy of fresh and frozen-thawed epididymal sperm in ICSI, most authors report similar fertilization and pregnancy rates (Silber et al, 1997; Friedler et al, 1998; Tournaye et al, 1999; Janzen et al, 2000; Cayan et al, 2001), although one study reported a significantly lower clinical pregnancy rate with cryopreserved vs fresh epididymal sperm in spite of similar fertilization rates (Palermo et al, 1999).

Testicular Sperm—Since the first successful attempts at ICSI with testicular sperm (Devroey et al, 1995), the technique has been perfected to give fertilization rates that are close to those obtained by ICSI with ejaculated sperm (Van Steirteghem et al, 1998). However, several studies have reported a dependence of ICSI outcomes on the underlying etiology of azoospermia. The ESHRE ICSI Task Force, analyzing data from the time period 1993–95, reported a lower fertilization rate in patients with NOA than in those with OA but no differences in the pregnancy rate, delivery rate, and perinatal outcome between the 2 groups of patients (Tarlatzis and Bili, 1998). Similar fertilization and pregnancy rates for patients with OA and NOA were also reported by others (Silber et al, 1997; Vicari et al, 2001). However, several authors have noted lower fertilization (Mansour et al, 1997; Palermo et al, 1999), pregnancy (Kahraman et al, 1996; Aboulghar et al, 1997; Mansour et al, 1997; Ghazzawi et al, 1998), and implantation (Ubaldi et al, 1999) rates and a higher abortion rate (Vicari et al, 2001) in ICSI attempts using testicular sperm from men with NOA compared to ICSI with epididymal or testicular sperm from men with OA.

No differences were found in fertilization and pregnancy rates with fresh and cryopreserved testicular sperm from patients with OA or NOA (Ben-Yosef et al, 1999; Habermann et al, 2000; Friedler et al, 2002).

Sperm Precursor Cells—The first birth after fertilization with elongated spermatids was reported in 1995 (Fishel et al, 1995). The ability of elongated spermatids to act as male gametes after injection into human oocytes (elongated spermatid injection [ELSI]) was further confirmed by different groups (Araki et al, 1997; Vanderzwalmen et al, 1997; Bernabeu et al, 1998; Kahraman et al, 1998; Sofikitis et al, 1998; Al-Hasani et al, 1999; Sousa et al, 1999; Tesarik et al, 2000). However, most of these reports did not mention the exact phase of spermiogenesis of the elongated spermatids that were used. Accordingly, there was an overlap between what some authors reported as ELSI and others as ICSI with testicular spermatozoa (Tesarik, 1997). Deeper analysis of the evolution of spermatid developmental potential during spermiogenesis (Sousa et al, 1999) showed that the chance of obtaining a normal embryo is relatively acceptable with spermatids at late stages of spermiogenesis (Sd1 and Sd2), but it drops markedly when less mature stages are used.

The original enthusiasm inspired by reports of births (Tesarik et al, 1995; Vanderzwalmen et al, 1997; Barak et al, 1998; Gianaroli et al, 1999) and ongoing pregnancies (Antinori et al, 1997a,b) after fertilization with round spermatids was subsequently tempered by the low success rates obtained in a larger series of round spermatid injection (ROSI) attempts (Al-Hasani et al, 1999; Tesarik et al, 2000). On the basis of a meta-analysis of data reported by different groups (255 treatment attempts), the overall fertilization, pregnancy, and confirmed birth rate after ROSI were 30.5%, 5.1%, and 2.0%, respectively (Tesarik et al, 2000). The results of ROSI were deceiving, especially as to the low birth rate, which resulted not only from the low implantation potential of embryos developing from oocytes fertilized with round spermatids but also from the unusually high rate of early wastage of spermatid-derived pregnancies (Amer et al, 1997).

Some improvement of ROSI success rates was achieved by the application of germ cell in vitro culture (see below), but the clinical efficacy still remains well below that of ELSI with late elongated spermatids or ICSI with testicular spermatozoa.

There also is an isolated report on a pregnancy achieved with embryos conceived by intracytoplasmic injection of secondary spermatocytes into human oocytes (Sofikitis et al, 1998). However, this experience has not been further confirmed either by the same or by another group.

Germ Cell In Vitro Culture and its Clinical Applications—Several early studies described the occurrence of spermatogenic events during in vitro culture of pieces of animal and human testicular tissue (reviewed in Tesarik et al, 2000). However, these attempts acquired potential clinical importance only in the last decade, when micro-

manipulation methods capable of delivering poorly motile sperm and sperm precursor cells to the interior of the oocyte became available. Currently, in vitro culture methods are used for 2 different purposes: to overcome in vivo maturation arrest and to select against spermatozoa carrying DNA damage.

Overcoming In Vivo Maturation Arrest by In Vitro Culture—It has been shown that spermatids from men with normal spermatogenesis can be stimulated to undergo isolated spermatogenic events (nuclear condensation and protrusion as well as flagellar growth) at a highly accelerated speed when cultured in vitro (Aslam and Fishel, 1998; Tesarik et al, 1998b; Cremades et al, 1999). The presence of high concentrations of FSH and testosterone in culture media further enhances the differentiation rate (Tesarik et al, 1998b).

When in vitro culture conditions were applied to germ cells obtained from men with in vivo maturation arrest, a reactivation of spermiogenesis, presenting the same morphological patterns, was observed in some but not all men with postmeiotic arrest at the round spermatid stage, and the development of late elongated spermatids (Sd stage) was achieved even in some men with meiotic arrest at pachytene, but not at earlier stages, of the first meiotic division (primary spermatocyte stage) (Tesarik et al, 1999a, 2002a). These in vitro–developed spermatids were able to fertilize human oocytes, and term pregnancies were achieved after the transfer of the resulting embryos, including the first birth after fertilization with germ cells from a man with in vivo maturation arrest at the primary spermatocyte stage (Tesarik et al, 1999a).

Selection Against Sperm Carrying Damaged DNA—A high incidence of DNA fragmentation in round spermatids from men with complete maturation arrest at the round spermatid stage (Tesarik et al, 1998a; Jurisicova et al, 1999) is suspected to be at the origin of the high prevalence of assisted reproduction failure when round spermatids are used for fertilization (Tesarik et al, 2000). However, a high incidence of DNA fragmentation is also often found in mature spermatozoa from some patients with complete spermatogenesis (Gandini et al, 2000; Muratori et al, 2000). Given the observation that in vitro culture facilitates the selection against spermatids with fragmented DNA (Tesarik et al, 1999b), the same approach has been suggested to improve ICSI outcomes in such cases. A subsequent pilot study confirmed this working hypothesis (Tesarik et al, 2001a). Consequently, sperm samples from men with repeated failure of assisted reproduction can now be tested for DNA fragmentation, and ICSI with in vitro cultured sperm can be proposed in an attempt to increase the chance of success. However, a testicular biopsy is needed to recover whole portions of seminiferous tubules for in vitro culture, even in cases in which spermatozoa are present in the ejaculate.

Safety Concerns About Micromanipulation-Assisted Fertilization Techniques

Concerns About the Use of ICSI for Fertilization—An analysis of the obstetric outcomes of 904 pregnancies after ICSI (Wisanto et al, 1996) did not suggest any increase in the risk of pregnancy loss, clinical abortion, pregnancy complications, and chromosomal aberrations (on the basis of a prenatal diagnosis of 64.4% of the clinical pregnancies) related to the use of the ICSI technique. However, one report suggested an increase in the incidence of sex chromosomal abnormalities among the ICSI babies (In't Veld et al, 1995). A slight but significant increase in chromosomal abnormalities (1.2%) compared to the general newborn population, partly due to de novo abnormalities, was confirmed by a larger prospective follow-up study of children born after ICSI (Bonduelle et al, 1996).

Several hypotheses have been formulated to explain the increased risk of chromosomal abnormalities after ICSI. In the first place, the prevalence of chromosomal abnormalities in couples undergoing ICSI was higher than in general population and, somewhat surprisingly, this concerned not only the males but also the female partners of the couples (Meschede et al, 1998). An increased incidence of numerical chromosomal abnormalities was also found in spermatozoa from patients treated by ICSI (Macas et al, 2001). The prevalence of sperm chromosomal abnormalities was found to be linked with gonadal failure (high serum FSH levels) in men undergoing ICSI (Levron et al, 2001). Surprisingly, chromosomal analysis of spontaneous abortions did not reveal any difference in the incidence of chromosomal abnormalities between ICSI and conventional IVF (Causio et al, 2002). Also, the ICSI technique itself has been suspected to contribute to the development of de novo chromosomal abnormalities in ICSI-derived embryos (Tesarik, 1995). This possibility remains to be further examined.

In addition to chromosomal abnormalities, the risk of mild delays in development (Bowen et al, 1998) and major birth defects (Ludwig and Katalinic, 2002) also appears to be slightly elevated after ICSI compared with spontaneously conceived children. However, another recent study has shown that the twofold increased risk of major birth defects compared with naturally conceived infants was not strictly related to the ICSI technique but concerned births after conventional IVF as well (Hansen et al, 2002).

A recent report on 2 children conceived after ICSI who developed Angelman syndrome (Cox et al, 2002) has suggested an increased risk of imprinting defects, but the possible relationship between this type of epigenetic anomaly and the ICSI technique remains to be evaluated.

Concerns About the Use of Immature Sperm for Fertilization—In spite of the fact that all postmeiotic germ

cells, from round spermatids to ejaculated spermatozoa, have the same nuclear DNA content, the form of DNA organization undergoes essential changes during postmeiotic maturation. It was pointed out that incomplete nuclear condensation in immature spermatids makes DNA more vulnerable to damage by nucleases and other factors (Tesarik et al, 1998c). In agreement with this concept, high frequencies of spermatids carrying apoptosis-related DNA fragmentation were found in patients suffering from maturation arrest at the round spermatid stage (Tesarik et al, 1998a; Jurisicova et al, 1999). This finding can at least partly explain the high incidence of implantation failure and early pregnancy wastage after ROSI (Tesarik et al, 2000).

The possible risk associated with genomic imprinting anomalies in the immature germ cells used for fertilization has also been discussed (Tesarik and Mendoza, 1996), but this risk remains purely hypothetical because no children with anomalies attributable to genomic imprinting have yet been born in these cases.

All babies resulting from ROSI were normal and healthy. This contrasts with a report on 4 pregnancies after the transfer of embryos resulting from ELSI with late elongated spermatids, 2 of which developed major fetal malformations (hydrocephalus associated with trisomy 9 and Arnold Chiari Syndrome type II, respectively; Zech et al, 2000). However, as mentioned earlier in this paper, many centers report cases of ELSI with late elongated spermatids similar to those described by Zech et al (2000) as ICSI with testicular sperm (Tesarik, 1997). Consequently, this isolated report on a small patient group must be interpreted with caution. A multicenter study employing exact staging of germ cells used for fertilization is needed to evaluate the real risks of ELSI and ICSI with testicular sperm.

Future Challenges

Spermatogenesis in a Host Testis—Pioneering experiments from Ralph Brinster's laboratory showed that mouse spermatogonial stem cells can repopulate the seminiferous tubules of the host mouse testis and subsequently undergo complete spermatogenesis (Brinster and Zimmermann, 1994; Russell et al, 1996), that the spermatozoa having developed in the host testis are fertile (Ogawa et al, 2000), and that even cryopreserved mouse spermatogonial stem cells are able to generate spermatogenesis in recipient seminiferous tubules (Avarbock et al, 1996). Further studies were aimed at the modification of this system to be used for heterospecific transplantations. These experiments attempted to achieve rat spermatogenesis (Clouthier et al, 1996; Russell and Brinster, 1996), hamster spermatogenesis (Ogawa et al, 1999), rabbit and dog spermatogenesis (Dobrinski et al, 1999), and boar, bull, and stallion spermatogenesis (Dobrinski et al, 2000) after the transplantation of spermatogonial stem cells from each of these species to the mouse testis. Spermatogenesis after these xenotransplantations led to the formation of mature spermatozoa in the rat-to-mouse system (Clouthier et al, 1996; Russell and Brinster, 1996) but was more or less incomplete in the other donor–host combinations.

Recolonization of seminiferous tubules with transplanted spermatogonial stem cells was also achieved in a nonhuman primate (cynomolgus monkey), but spermatogenesis progressed only to a very limited extent in this system (Schlatt et al, 1999). Xenogeneic transplantation of human spermatogonia to the mouse testis failed to ensure survival and differentiation of the transplanted cells (Reis et al, 2000). However, a recent study has demonstrated that an alternative approach, based on grafting tissue from immature testes under the back skin of castrated immunodeficient animals, can be successful in achieving complete xenogeneic spermatogenesis of phylogenetically (relatively) distant species (pig and goat) in the mouse host (Honaramooz et al, 2002).

Fertilization With Haploidized Somatic Cells—Mammalian metaphase II oocytes have an extraordinary capacity to rapidly drive any injected cell nucleus to metaphase, irrespective of the actual cell cycle phase of the cell from which the nucleus has been removed (Tesarik, 2002). Owing to this capacity, if a nucleus that has not yet undergone the DNA synthetic phase is introduced into a metaphase II oocyte, it is driven to a premature metaphase without previous DNA replication, thus bypassing a step that otherwise is obligatory in any mammalian cell type. This makes it possible to reduce the DNA content of injected cell nuclei by half. This phenomenon has been called ''somatic cell haploidization'' and has been suggested as a tool for replacing gametes with somatic cells in the fertilization process (Tesarik, 2002).

Somatic cell haploidization was initially suggested as a means for oocyte reconstruction in women with ovarian failure (Tsai et al, 2000). Being able to achieve chromosomal segregation between the reconstituted oocyte that resulted from the injection of a somatic cell (cumulus cell) nucleus into a previously enucleated metaphase II donor oocyte and a pseudo-second polar body during subsequent oocyte activation led to the development of 4-cell stage embryos that resulted from fertilization by ICSI of these oocytes has been documented recently (Tesarik et al, 2001b). A similar approach, but one using the somatic cell (fibroblast) nucleus as a substitute for the male gamete genome, has been tried in the mouse model, resulting in fertilization and early cleavage divisions in some of the treated oocytes (Lacham-Kaplan et al, 2001).

Because the techniques using somatic cells as substitutes for the male gamete must also circumvent the lack of sperm as the oocyte-activation trigger and the source

of the microtubule organizing center, they are expected to represent a more difficult challenge than the use of somatic cells for oocyte reconstitution (Tesarik and Mendoza, 2003). Notwithstanding, this technique represents an exciting challenge to future research because it could be used in yet untreatable cases of total germline absence (complete Sertoli cell–only syndrome) and maturation arrest at the early stages of spermatogenesis.

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