

Intracytoplasmic spermatid injection and in vitro maturation: fact or fiction?

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Intracytoplasmic injection with testicular spermatozoa has become a routine treatment in fertility clinics. Spermatozoa can be recovered in half of patients with nonobstructive azoospermia. The use of immature germ cells for intracytoplasmic injection has been proposed for cases in which no spermatozoa can be retrieved. However, there are low pregnancy rates following intracytoplasmic injection using round spermatids from men with no elongated spermatids or spermatozoa in their testes. The in vitro culture of immature germ cells to more mature stages has been proposed as a means to improve this poor outcome. Several years after the introduction of intracytoplasmic injection with elongating and round spermatids, uncertainty remains as to whether this approach can be considered a safe treatment option. This review outlines the clinical and scientific data regarding intracytoplasmic injection using immature germ cells and in vitro matured germ cells.

KEYWORDS: Round Spermatid; Elongating Spermatid; Elongated Spermatid; ICSI; In Vitro Maturation.

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■ INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has constituted a breakthrough in the treatment of severe male-factor infertility (1). This technique was initially introduced as a treatment for severe oligoasthenoteratozoospermia using ejaculated sperm. In 1993, the first successful pregnancy using spermatozoa that had been directly extracted from the testis of an azoospermic man was achieved (2). Azoospermia is present in 1% of the general male population and in 10 to 15% of infertile men. There are two major etiologic categories of azoospermia: obstructive azoospermia (OA) and nonobstructive azoospermia (NOA). In OA, complete spermatogenesis is observed during histological analysis, whereas in NOA, either germ cell aplasia (a Sertoli cell-only pattern), maturation arrest or tubular sclerosis and atrophy is revealed by histological analysis. The histology of the latter three may or may not show focal spermatogenesis. The most mature stage of the male gamete at the end of spermiogenesis is the elongated spermatid. After spermiation, spermatozoa are released into the tubular lumen. These spermatozoa become functional during the passage through the epididymis. Testicular sperm retrieval is successful in virtually 100% of

patients with OA, and sufficient numbers of spermatozoa can be obtained for ICSI and/or cryopreservation. However, the recovery of fully elongated spermatids or spermatozoa fails in approximately 50% of NOA men (3). The only hope for these patients to father their own genetic children is the use of more immature germ cells for ICSI. Spermatids are the earliest cells in the male germ cell lineage with a haploid number of chromosomes. In various studies using mouse, hamster and rabbit models, two techniques, elongated spermatid injection (ELSI) and round spermatid injection (ROSI), have been successfully employed to fertilize a mature oocyte with such immature germ cells, resulting in the delivery of healthy offspring (4-7). Although the fertilization, pregnancy and live birth rates were low in these animal models, the results demonstrated the potential of spermatids to contribute to normal fertilization and embryonic development. Based on these observations, Edwards suggested the use of spermatids for ICSI in humans when sperm at more mature stages were not available (8). In humans, the injection of spermatids leading to fertilization and early cleavage was reported by Vanderzwalmen (9). The first reported successful births were by Tesarik using round spermatids from the ejaculate (10) and by Fishel using elongated spermatids extracted from the testis (11). The first reports of human pregnancies following round spermatid injection involved round spermatid nucleus injection (ROSNi), but all of these pregnancies ended in spontaneous abortions (12). In the mid-nineties, several IVF centers used testicular spermatids for ICSI, and most of the reported pregnancies were achieved using late spermatids. When round spermatids were used, the pregnancy rate was much lower. More than 15 years after

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the first live birth achieved with ROSI, fewer than ten children have been born worldwide using this technique.

The in vitro culture of immature germ cells to more mature stages of spermatogenesis has been suggested as an approach to improve the poor live birth rate associated with ROSI and as a tool to improve both the identification and the selection of spermatids used for ICSI.

Despite a number of reports of the delivery of healthy offspring in humans following the use of immature germ cells for ICSI, several ethical concerns have been raised. In some countries, e.g., the United Kingdom, government regulations have banned the use of spermatids for ICSI.

■ BASICS OF SPERMATOGENESIS AND SPERMIOGENESIS

The term “spermatogenesis” refers to all of the processes and events involved in the production of mature gametes that occur within the seminiferous tubules of the testis. Spermatogenesis begins with the division of a testicular stem cell and ends with the formation of a mature spermatozoon, and it can be divided into three major stages (13):

- 1) The mitotic proliferation and differentiation of diploid germ cells (i.e., **spermatogonia**) into diploid (2n) primary spermatocytes.
- 2) The meiotic division of tetraploid germ cells (i.e., **spermatocytes**) into four haploid germ cells called **spermatids**; the first meiotic division produces two secondary spermatocytes, which are separated into haploid (1n) spermatids during the second meiotic division. The secondary spermatocytes contain a set of haploid chromosomes in duplicate form. The meiotic process is a critical event during gametogenesis because it involves the recombination of genetic material, a reduction in the chromosome number and the development of spermatids.
- 3) The transformation of the haploid germ cells (spermatids) into testicular sperm (i.e., **spermiogenesis**). Spermatids are mitotically inactive round cells that undergo a remarkable and complicated transformation leading to the ultimate production of differentiated elongated spermatids and spermatozoa. This transformation includes the condensation and structural shaping of the cell nucleus, the formation of the flagellum and the expulsion of a large portion of the cytoplasm. The overall process is termed “spermiogenesis.” When spermatogenesis is completed, the cytoplasmic connections between the sperm and the Sertoli cells are broken, followed by the release of the sperm from the germinal epithelium into the tubular lumen. This process is referred to as **spermiation**.

The coordinating mechanism behind the processes of human spermatogenesis and spermiogenesis remains unclear. When maturation arrest occurs at the primary spermatocyte stage, the germ cells still contain a diploid number of chromosomes and are therefore not suitable for ICSI. When maturation arrest occurs at the round spermatid stage, the germ cells contain a haploid number of chromosomes and theoretically possess all of the genetic information needed to fertilize mature oocytes by ICSI.

■ ICSI WITH SPERMATIDS: CLINICAL EXPERIENCE

Following the reports of results in animals, several groups have described the use of round or elongated spermatids, retrieved from either the ejaculate or testicular tissue, to

treat men with NOA when no testicular spermatozoa could be retrieved. Either intact round spermatids (ROSI), round spermatid nuclei (ROSNI) or elongated spermatids (ELSI) are injected into the oocyte. Although ELSI, ROSI and ROSNI were introduced more than 15 years ago, the number of reported pregnancies has remained extremely limited.

ELSI

A 2002 review summarized the literature on ICSI with elongated spermatids (ELSI) (14). Elongated spermatids isolated either from ejaculate or directly from testicular tissue have been successfully used. An analysis of the studies using late spermatids for injection indicated that this technique was associated with a low fertilization rate (48.4%) but an otherwise acceptable pregnancy rate (28.9%) (14). The overall results of ICSI using testicular elongated spermatids were comparable to those using testicular spermatozoa (14). The retrospective nature of most of the studies and the presence of patient selection bias and even publication bias may explain these results. Furthermore, some of the ELSI studies also included men who exhibited normal spermatogenesis.

ROSI

The first successful reports of ROSI in humans described seven azoospermic men who had round spermatids but no mature spermatozoa in their ejaculate (10). Round spermatids were used for ICSI and produced two viable pregnancies.

Sousa et al. reviewed reports of ICSI with round spermatids isolated from either ejaculate or testicular tissue. Compared with ELSI, the success rates of ROSI are dramatically lower; the latter approach appears to be clinically inefficient, with a 21.8% fertilization rate and a 2.8% clinical pregnancy rate (14). Since 2002, no clinical pregnancies have been reported following ROSI (15-20). In summary, seven clinical pregnancies have been reported overall after the use of ROSI: three with spermatids from the ejaculate and four with spermatids extracted from the testis. Antinori is the only researcher to have reported three clinical pregnancies after ROSI in the absence of spermatozoa in the preliminary ejaculate or in biopsies (21,22). After the publication of Antinori's work, no pregnancies have been reported following the use of round spermatids from patients with a complete absence of elongated spermatids or spermatozoa in preliminary ejaculate or diagnostic testicular biopsies. Based on the above results, it may be concluded that ROSI is an inefficient approach for treating infertility in azoospermic men with primary testicular failure who exhibit no spermatozoa in testicular biopsies.

ROSNI

On the basis of animal experiments, the ROSNI technique has been suggested to overcome some of the disadvantages of ROSI: (1) the injecting micropipettes used for ROSNI have a smaller diameter, which reduces the risk of oocyte damage, and (2) the presence of a large amount of cytoplasm around the spermatid nucleus (in ROSI) may impede the transformation into the male pronucleus (23). Human experiments, however, have revealed that oocyte degeneration after ROSI is not adversely affected by the use of a larger microinjection pipette nor by the presence of a cytoplasmic layer surrounding the spermatid nucleus; the rupture of the cytoplasmic membrane and the nuclear



envelope is rapidly achieved after microinjection (14). Sousa reported a low oocyte degeneration rate (9%) in 33 ROSI cycles following the injection of 200 oocytes (14).

Most human pregnancies reported after ROSNI ended in spontaneous abortion (12). To our knowledge, only one publication has reported live births (three) following ROSNI (23,24).

Secondary spermatocyte injection (SECSI)

There have also been isolated reports of the successful use of human secondary spermatocytes for ICSI; including the delivery of a healthy boy (25). However, limited clinical benefits can be expected from this approach because most NOA men with secondary spermatocytes in their testicular biopsies also have more mature germ cells (23). Furthermore, no additional reports have confirmed the success of this approach in humans.

As a result of the overall disappointing results of spermatid injection (ROSI, ROSNI and ELSI) following its initial hype, most IVF programs stopped using spermatid injection in the mid-nineties. Consequently, there have been no published reports of spermatid injection since that time period.

■ ICSI WITH SPERMATIDS: IDENTIFICATION IS CRUCIAL

In most of the reports of ICSI with spermatids, the spermatid stage that was used is unclear identified. The adoption of a clear terminology is needed to avoid confusion regarding success rates, so that reliable conclusions can be drawn and results can be compared between different centers (26).

A critical problem in the use of spermatids for ICSI is the identification of spermatids within a heterogeneous population of round cells that have been obtained from either testicular tissue or the ejaculate. In an unstained wet preparation under an inverted microscope, haploid spermatids can be divided into four categories according to their shape, amount of cytoplasm and tail size: round (Sa, Sb1), elongating (Sb2), elongated (Sc, Sd1), and fully elongated (Sd2) spermatids (27). Elongating or elongated spermatids are easy to recognize, but the identification of round spermatids is more difficult. The presence of pathological conditions makes this identification even more difficult because the cells are retrieved from patients with abnormal spermiogenesis. Tesarik and Mendoza (28) have described a method to distinguish round spermatids from other round cells in the ejaculate (leucocytes, lymphocytes, monocytes, erythrocytes, Sertoli cells, spermatogonia and primary and secondary spermatocytes) according to the shape and size of the cell and its nucleus. A developing acrosomal granule can be recognized as a bright spot adjacent to the spermatid nucleus; however, this bright spot may be easily confused with a vacuole (29). Misidentification can lead to the injection of round cells that are not round spermatids, which might explain the low success rates of ROSI. Using the Hoffman modulation contrast microscopy, which is employed by most centers for the ICSI procedure, the identification of round spermatids is extremely difficult (29,30). Optimal identification techniques should be developed and applied in everyday clinical practice, allowing correctly selected round spermatids to be injected. Although staining procedures may clearly reveal the presence of

round spermatids, this approach has diagnostic value only; it is not feasible in clinical practice (31,32). Yamanaka et al. have characterized round spermatids using confocal scanning laser microscopy (33); however, this equipment is expensive and therefore not accessible to all laboratories. The use of phase-contrast optics on an inverted microscope is a simple and reliable method of identifying round spermatids (29).

■ ICSI WITH SPERMATIDS: IS THERE A TARGET GROUP?

ROSI has been proposed as an ART technique for males with primary testicular failure resulting from complete maturation arrest. However, maturation arrest at the level of the round spermatid is rare; Shulze et al. (34) reviewed 1,418 stained biopsies from 766 azoospermic men and identified late maturation arrest (i.e., round spermatid arrest) in only seven men (0.9%). Silber and Johnson (35) reviewed 125 stained biopsies from men with unexplained NOA and found no evidence of arrest at the round spermatid stage. In biopsies where round spermatids were observed, elongated spermatids or even spermatozoa were invariably present as well. Furthermore, in our experience, an extensive search of wet preparations revealed identifiable round spermatids only in patients for whom the more mature stages of development were also present (29). Therefore, round spermatids may be observed in the testicular biopsies of only a limited number of patients from whom no spermatozoa or elongated spermatids can be recovered.

■ IN VITRO MATURATION AND ART

In vivo spermatogenesis is a long and complex process of germ cell development within the seminiferous tubules and is regulated not only by gonadotropins but also by interactions between spermatogenic cells and somatic Sertoli cells (36,37). In theory, in vivo arrest may be overcome by in vitro culture, either by co-culturing isolated germ cells on a somatic cell monolayer or by culturing isolated segments of seminiferous tubules. In vitro culture can target either the meiotic or postmeiotic maturation (spermiogenesis) of germ cells. Therefore, in vitro culture has been proposed to improve the selection against spermatids or spermatozoa carrying DNA damage and to overcome in vivo maturation arrest (38).

Postmeiotic differentiation via the in vitro culture of round spermatids to the elongating stage may be a way to replace ROSI with ELSI, as the latter is technically much simpler and yields a superior outcome (28). Aslam and Fishel (39) observed flagellar growth in 22% of round spermatids during the first 4-8 h of culture in modified Eagle's minimum essential medium with no hormonal supplementation. Follicle-stimulating hormone (FSH)-independent rapid flagellar growth in vitro by round spermatids has also been reported by others (40,41). In contrast to flagellar growth, the processes of nuclear condensation, the peripheral migration and protrusion of the spermatid nucleus, as well as the differentiation of the acrosome, are strictly dependent on the presence of FSH in the culture medium (41). Cremades et al. demonstrated that round spermatids could mature into elongating and elongated spermatids in vitro after seven days of culture at 32 °C in microdrops of Vero cell-conditioned



medium and that these cells could successfully fertilize oocytes to form blastocysts (42).

The in vitro culture of germ cells may be useful for overcoming the inadvertent use of apoptotic spermatids in assisted reproduction and may therefore increase the fertilization rate (43). However, many authors have reported that both developmental capacity and clinical outcomes remain extremely poor after the use of in vitro-cultured round spermatids and have concluded that in vitro culture offers no clinical benefit apart from improving the fertilization rate (27,44,45).

The births of two healthy babies have been reported following ICSI with elongated spermatids obtained after the in vitro culture of round spermatids from a patient with complete maturation arrest at the round spermatid stage (46,47). Later, the birth of two healthy babies (twins) was achieved by the same group using an in vitro culture system that supported the postmeiotic differentiation of round spermatids from patients with incomplete arrest of spermiogenesis and a high frequency of apoptosis among postmeiotic germ cells (48).

Arrest at the primary spermatocyte stage is the most common type of maturation arrest in men with NOA (49). To overcome this type of arrest, the in vitro transmeiotic differentiation of primary spermatocytes has been proposed.

Testicular biopsies of patients with obstructive azoospermia and normal spermatogenesis were cultured in vitro for 24 h by Tesarik et al. (41). Whole segments of the seminiferous tubules and Sertoli cells were cultured in the presence or absence of recombinant FSH and testosterone (T) at a temperature close to that of the human testis. The presence of recombinant FSH increased the proportions of primary and secondary spermatocytes undergoing meiotic progression and postmeiotic differentiation (41). The addition of testosterone enhanced the effects of FSH on meiosis and spermiogenesis by preventing the apoptosis of Sertoli cells in culture, whereas no effect was observed for the addition of T without FSH (50). Compared with the normal kinetics of the human germinal epithelium (51), a surprisingly rapid maturation (two days) was observed when culturing primary and secondary spermatocytes to elongated spermatids (50).

Tanaka et al. were the first to report the in vitro development of four round spermatids derived from a single primary spermatocyte using co-culture with a Vero cell line (52). They observed in vitro meiotic division that was independent of the addition of FSH and T, in contrast to earlier reports by Tesarik et al. (50). The newly divided cells were confirmed to be round spermatids using chromosomal analysis (52). Although co-culture with Vero cells could replace the use of Sertoli cells as a support for in vitro meiosis, the Vero cells appeared unable to support postmeiotic differentiation to later stages, i.e., elongated spermatids or spermatozoa (52).

The same in vitro culture conditions that had been previously recommended by Tesarik were used to culture testicular cells from five men with maturation arrest at the primary spermatocyte stage. Spermatids from two of the men were cultured after two days, and the injection of these in vitro cultured elongated spermatids successfully fertilized oocytes and resulted in normally developing embryos in both cases and healthy twin in one case (46). Later Tesarik et al. reported on the birth of a third healthy child after the

injection of in vitro matured elongated spermatids from men with maturation arrest at the primary spermatocyte stage (53).

These findings provide strong evidence that premeiotic arrest at the primary spermatocyte stage can be overcome in vitro under optimized culture conditions and can result in haploid cells with full reproductive capacity.

■ ART WITH SPERMATIDS: A SAFE OPTION?

Apart from its low overall success rate, the safety of ICSI with immature haploid germ cells has been questioned. One of the major concerns about any reproductive technology is the possibility of genetic and epigenetic risks to the offspring.

There are concerns related to genomic imprinting following spermatid injection. Although genes are expressed equally from the two parental alleles, a small subgroup of genes are differentially expressed depending on whether they were inherited maternally or paternally. Genes that display inhibited expression when derived from the maternal germline are termed "maternally imprinted," and genes with inhibited expression when transmitted by the father are termed "paternally imprinted." The differential expression of the paternal and maternal alleles of imprinted genes is related to differential DNA methylation patterns in these genes. Genomic imprinting primarily occurs during gametogenesis and may be incomplete or defective in immature gametes or in gametes that have matured under abnormal conditions, e.g., in an in vitro culture (54).

During spermatogenesis, the histone-to-protamine transition ensures protection from mutation of the spermatid DNA. However, in a knock-out mouse model with abnormal spermiogenesis, the round spermatids displayed increased levels of DNA damage caused by a deficiency in the histone-to-protamine transmission (55). In NOA men, a high frequency of DNA damage in round spermatids has been reported in patients with complete spermiogenesis failure (54), whereas abnormal chromatin packing in elongated spermatids has been reported (56). Spermatids differ from mature sperm in their chromatin structure, and this difference may affect the epigenetic behavior of the paternal genome (57). The strict control of DNA methylation in the preimplantation embryo is necessary for normal development.

Preimplantation genetic diagnosis (PGD) has been proposed to increase both the implantation rate and safety of ROSI. Benkhalifa et al. reported the results of preimplantation genetic diagnosis (PGD) performed on embryos obtained by ROSI (20). Their data indicated that the failure of ROSI to produce pregnancy and live births occurs at both the prezygotic and postzygotic stages and is primarily caused by aneuploidy. Although Benkhalifa et al. did not report any increases in chromosomal or other genetic abnormalities in the rare pregnancies that occurred, they concluded that ROSI should not be used in ART programs.

Additional concerns about ICSI with immature haploid germ cells are related to the immaturity of the cytoplasm. The cytoplasm of male gametes contains two factors that are important for normal embryonic development: the centrosomes and oocyte-activating factor (OAF). Abnormal or damaged centrosomes due to spermatid immaturity may cause abnormal spindle formation and may explain the arrest, mosaicism and anomalies observed in embryos that



develop after spermatid injection (58). The injection of human round spermatids into oocytes produces normal fertilization, which indicates that human spermatogenic cells have developed OAF- and calcium oscillation-inducing capabilities by the round spermatid stage (49), whereas the round spermatids of rodents require oocyte activation after injection (60). Aneuploidy may result from either immature centrosomes (which prevent the formation of normal microtubule-organizing centers) or abnormal oocyte activation.

In 2000, Zech et al. reported two major congenital malformations in four pregnancies achieved using ELIS: one fetus exhibited the Arnold-Chiari malformation, and another fetus displayed hydrocephaly combined with trisomy 9 (47, XY,+9) in all of the amniotic cells (61). There have been no further published reports of abnormalities caused by imprinting disorders related to the use of immature germ cells. Epigenetic failure in germline cells is of great concern because it may only manifest later in life (such as through an increased predisposition to cancer) and/or be silently transmitted to the next generation (62).

Finally, there are some concerns about the accelerated speed of the in vitro development of germ cells from men with in vivo maturation arrest. There are suspicions of a possible negative effect on DNA when some of the essential checkpoints of in vivo development are bypassed (47).

AUTHOR CONTRIBUTIONS

Vloeberghs V participated in the acquisition, analysis and interpretation of the data, as well as the drafting and final approval of the manuscript. Verheyen G participated in the analysis and interpretation of the data, as well as the revision and final approval of the manuscript. Tournaye H participated in the conception and design of the manuscript, as well as the analysis and interpretation of the data and the revision and final approval of the manuscript.

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