Spermatids as gametes: indications and limitations

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The feasibility of achieving viable embryos, developing to term after transfer into the uterus, by fertilizing oocytes with spermatids has been demonstrated both in animal studies and in preliminary human clinical trials. Here we review the current clinical indications of spermatid conception and discuss the predictable success rates associated with each of these indications. Potential health hazards relating to the use of spermatids for conception are updated taking into account the risk of abnormal or incomplete epigenetic modifications of newly discovered human imprinted genes. We also add new experimental data showing the occurrence of spermatids in patients lacking spermatozoa and demonstrating that round spermatids recovered from patients with complete spermiogenesis failure (no elongated spermatids or spermatozoa ever detected in the patient’s history) are often deficient in the factor(s) responsible for oocyte activation. The possible consequences of this deficiency for the occurrence of abnormal fertilization patterns and for the impairment of further preimplantation and post-implantation development are discussed. It is concluded that the development of diagnostic tests to assess the intrinsic quality of spermatids, with regard to their ability to act as gametes, is urgently needed as part of pre-treatment diagnosis before infertile couples are included in a spermatid conception programme. Centres wishing to use spermatids in human assisted reproduction should also be prepared to offer adequate diagnostic methods to control genomic imprinting abnormalities in the progeny.

\textit{Key words:} fertilization/genomic imprinting/medical counselling/spermatid conception/spermiogenesis failure

Introduction

Spermatids are haploid cells of the spermatogenic line from which spermatozoa are subsequently formed through a series of morphological and molecular
modifications collectively termed spermiogenesis. Since most of these modifications were supposed to provide the male gamete with an ability to negotiate the oocyte vestments and to penetrate into ooplasm, it was postulated that spermatids might be used as gametes in patients with spermiogenesis block, provided that they are introduced into the oocyte by an appropriate micromanipulation technique (Edwards et al., 1994). The feasibility of this concept was indeed corroborated both by experiments with oocyte fertilization by round spermatids conducted in mice (Ogura et al., 1994; Kimura and Yanagimachi, 1995) and rabbits (Sofikitis et al., 1994, 1996a), resulting in births of normal young, and by preliminary human clinical trials leading to pregnancies and births after uterine transfer of embryos obtained by fertilizing oocytes with round (Tesarik et al., 1995, 1996; Tesarik and Mendoza, 1996a) and elongated spermatids (Fishel et al., 1995, 1996). The first pregnancies and births in the mouse model were achieved by electrofusing oocytes with whole round spermatids previously injected into the perivitelline space (Ogura et al., 1994). Subsequently, round spermatid nucleus injection (ROSNI) into oocyte cytoplasm was demonstrated to be more efficient in the mouse (Kimura and Yanagimachi, 1995) and was also applied in humans (Yamanaka et al., 1997). Notwithstanding, most clinical studies have used intracytoplasmic injection of whole spermatids, i.e. round spermatid injection (ROSI) and elongated spermatid injection (ELSI) as described by Tesarik and Mendoza (1996a). These data show clearly that viable embryos can be obtained by fertilizing oocytes with spermatids. Nevertheless, the significance of this finding for the treatment of human infertility still remains to be evaluated.

The highly variable and unpredictable outcomes of human spermatid conception attempts, in terms of fertilization, implantation, fetal development and pregnancy loss (Hannay et al., 1995; Tesarik et al., 1995, 1996; Tesarik and Mendoza, 1996a; Mansour et al., 1996; Araki et al., 1997; Yamanaka et al., 1997; Antinori et al., 1997a; Amer et al., 1997), remain a major problem. Moreover, the completeness of the meiotic reduction in spermatids does not necessarily mean that all epigenetic (nuclear and cytoplasmic) modifications have attained a degree compatible with the behaviour of spermatids as fully developmentally competent gametes. This has led to some concern about the possible health hazards for children resulting from the use of spermatids for assisted reproduction (Tesarik, 1996; Tesarik and Mendoza, 1996b).

In this study, we performed a critical analysis of the actual state of the art of spermatid conception with particular reference to its practical application in the treatment of human sterility. We also add some new experimental data suggesting that the fertilization potential of spermatids is highly dependent on the type of pathology underlying the spermatogenic defect. These findings are discussed with regard to the attitudes to be taken by physicians and other health professionals who may be confronted with the complex problem of spermatid conception in their counselling, diagnostic and treatment practice.

Spermiogenesis block: what is it and how frequent is it?

Traditionally, spermatogenic block has been supposed to occur mainly at the entry of germ cells to the first meiotic division, i.e. at the primary spermatocyte
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stage (maturation arrest). However, recent data have suggested that, in cases of severe spermatogenic disorder, some germ cells can escape the primary spermatocyte arrest and continue further to be arrested at the spermatid stage. In most of these cases, a partial block at the primary spermatocyte stage does occur, as evidenced by accumulation of primary spermatocytes seen in tissue samples recovered by testicular biopsy. By meticulous examination of such samples, however, infrequent round or elongated spermatids can be identified.

The aetiological factors that can arrest spermatogenesis at the spermatid stage remain to be determined. In some cases, genetic abnormalities impairing the function of germ cells or of Sertoli cells can be at the cause. For instance, c-kit proto-oncogene, encoding a transmembrane receptor tyrosine-kinase (Yarden et al., 1987), appears to be involved in mouse spermatid elongation (Albanesi et al., 1996). In addition, the c-kit receptor and the corresponding ligand, for which different terms have been used (stem-cell factor, steel factor, mast-cell growth factor), play an important role in the partitioning of spermatogonial stem cells of the testis between the renewal, the apoptotic and the meiotic differentiation pathways (reviewed in Dym, 1994). Mutations in the W (white spotting) locus of the mouse, encoding the c-kit receptor (Chabot et al., 1988; Geissler et al., 1988) or in the Sl locus, encoding the c-kit ligand (Huang et al., 1990; Anderson et al., 1990), may thus be responsible for spermatogenesis problems both at pre-meiotic stages and at an early spermatid stage. An incomplete pre-meiotic block, leaving some germ cells to develop up to the spermatid stage, is a frequent observation in human spermatogenic disorders. Similarly, mutations in the genes encoding retinoic acid receptor α (Akmal et al., 1997) and retinoic X receptor β (Kastner et al., 1996) are likely to cause spermiogenesis arrest. In fact, transgenic mice carrying a mutation in the retinoid X receptor β gene showed a highly abnormal Sertoli cell-germ cell interaction with abnormal spermatid differentiation (Kastner et al., 1996). A research into homologous human genes and their function in men with spermiogenesis arrest is warranted. In addition to these genetic causes, a number of non-genetic factors, such as radio- and chemotherapy, long-term oestrogen therapy, nutritional factors, heat, infections, endocrinopathies, cryptorchidism and idiopathic aetiology, can also be involved in spermiogenic block (Creasy and Foster 1991; Martin-du-Pan and Campana, 1993).

Interestingly, spermatids can often be found in the ejaculate of patients suffering from spermiogenesis arrest, and the actual presence or absence of spermatids does not appear to be related to serum follicle stimulating hormone (FSH) concentrations (Mendoza and Tesarik, 1996). The massive release of spermatids to the ejaculate is likely to result from an abnormal relationship between spermatids and Sertoli cells in these patients. A similar detachment of round spermatids, accompanied by spermiogenesis arrest, can be produced experimentally in rats by testosterone withdrawal (O’Donnell et al., 1996). Testosterone has been shown to promote the adhesion of rat round spermatids to Sertoli cells in an in-vitro co-culture (Cameron and Muffly, 1991; Cameron et al., 1993), but a previous priming of the Sertoli cells with FSH appears to be required to render them ‘binding competent’ (Muffly et al., 1994). These observations
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point out the possibility of several additional genetic aetiologies of human spermiogenesis arrest. These include abnormalities of the FSH and testosterone receptor and of steroid conversion pathways, in addition to the possible non-genetic aetiologies such as local deficiencies of Leydig cell distribution and function in the testis or abnormalities of testicular minivasculature leading to locally reduced concentrations of FSH in certain parts of the testis.

The frequent finding of round spermatids in the ejaculate of patients with non-obstructive azoospermia prompted us to perform a prospective study comparing the occurrence of round spermatids in the ejaculate and in the testicular biopsy samples obtained at the same day from patients undergoing a spermatid conception attempt. The patients were asked to produce a semen sample early on the day of the planned testicular biopsy. When no spermatids were found in this sample, another semen sample was requested 1–2 h later. Bilateral open testicular biopsy was then performed. Samples from at least three different locations in each testis were taken. The biopsy was done even in those cases in which spermatids were identified in the ejaculate, for two reasons. Firstly, when round or early elongating spermatids are the most differentiated spermatogenic cells seen to be present in the ejaculate, there is still some hope of recovering a few late elongated spermatids or spermatozoa from the testis (Amer et al., 1997). Secondly, the percentage of viable spermatids recovered from the testis is usually superior as compared with the ejaculate. The biopsy material was disintegrated mechanically with the use of microscope slides and inspected in the native state. Thereafter, the samples were fixed, and spermatids were identified by cytochemical visualization of acrosin using two different methods, a one-step staining with fluorescein-conjugated Pisum sativum agglutinin (Mendoza and Tesarik, 1996) and a two-step immunocytochemical method with 4D4 anti-proacrosin monoclonal antibody (Mendoza et al., 1996). We have previously confirmed by fluorescent in-situ hybridization (FISH) the haploid status of cells of round spermatid size staining positive for acrosin with 4D4 monoclonal antibody (Mendoza et al., 1996). This antibody is also suitable for identification of those round spermatids that have not yet developed a morphologically distinct acrosomal granule because it reacts with the protein core of the proacrosin molecule even in spermatocytes and Golgi-phase spermatids (Mendoza et al., 1996). Thus, this immunocytochemical method has a higher sensitivity, and at least the same specificity, as the recently suggested method for round spermatid identification using an inverted microscope-associated computer-assisted image analysis system (Yamanaka et al., 1997). It can be expected to give similar results as transmission electron microscopy with which the Golgi phase of the acrosomal development can also be identified (Yamanaka et al., 1997).

This prospective study is still under way. Data available nowadays indicate that only round spermatids, lacking any signs of elongation, are present in about one half of these men, whereas the other half either lack spermatids at all or present different stages of spermatid elongation. We use the term complete spermiogenesis failure (Amer et al., 1997) for those cases in which no more
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Table I. Comparison of round spermatid (ROS) identification data in two successive ejaculates and in a testicular biopsy sample obtained on the same day from 66 patients with non-obstructive azoospermia

<table>
<thead>
<tr>
<th>First ejaculate</th>
<th>Second ejaculate*</th>
<th>Biopsy sample*</th>
<th>No. (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS present</td>
<td>Not requested</td>
<td>ROS present</td>
<td>24 (36)</td>
</tr>
<tr>
<td>ROS present</td>
<td>Not requested</td>
<td>ROS absent</td>
<td>10 (15)</td>
</tr>
<tr>
<td>ROS absent</td>
<td>ROS present</td>
<td>ROS present</td>
<td>6 (9)</td>
</tr>
<tr>
<td>ROS absent</td>
<td>ROS present</td>
<td>ROS absent</td>
<td>17 (26)</td>
</tr>
<tr>
<td>ROS absent</td>
<td>ROS absent</td>
<td>ROS present</td>
<td>9 (14)</td>
</tr>
</tbody>
</table>

*The search for spermatids was first performed in unstained preparations of living cells with the use of Hoffman optics. In all cases, these data were later confirmed by analysis of fixed cell smears stained with Pisum sativum agglutinin and anti-proacrosin antibody as described by Mendoza and Tesarik (1996) and Mendoza et al. (1996).

than round spermatids are present, whereas spermiogenesis failure is considered as incomplete when signs of elongation are seen in at least some spermatids.

With the use of the above approach and terminology, we identified 66 patients with complete spermiogenesis failure; data concerning the presence or absence of round spermatids in the two semen samples and the testicular biopsy sample are presented in Table I. These data show that only in 30 cases (45%) spermatids were found both in the ejaculate and the testicular tissue. In the remaining cases, the findings obtained with the ejaculate and with the testicular biopsy sample were discordant. In nine cases (14%) no spermatids were found in the ejaculate although there still were some present in the testis. In as many as 27 patients (41%) at least a few round spermatids were identified in the ejaculate but not in the testicular biopsy samples (Table I). This latter finding is interesting for several reasons. First, the biopsy samples analysed in the present study were taken from multiple testicular locations, as is usual when the biopsy is performed with the aim to obtain gametes for assisted reproduction treatment. Spermatids were not found and yet they were present in the ejaculate of the same day. This suggests that these patients had a spatially very restricted focus, or a few foci, in which spermatogenesis proceeded to the round spermatid stage, while spermatogenesis was absent or blocked at earlier stages elsewhere. The production of round spermatids in these isolated foci, that were not detected by testicular biopsy, was active enough for the spermatids to reach the ejaculate. Alternatively, it is also possible that spermatid production in such residual spermatogenic foci has a cyclic character so that the finding of spermatids in the ejaculate, reflecting past spermatogenic activity, is not necessarily in agreement with the findings in testicular biopsy samples although these may include the same tissue that was responsible for the production of the ejaculated spermatids. Finally, spermatocytes also undergo a premature release from Sertoli cells in men with non-obstructive azoospermia and can be identified in the ejaculate (Mendoza et al., 1996). It cannot be excluded that some of such prematurely released spermatocytes may develop into spermatids during epididymal transit although this possibility appears highly improbable. Even though the idea that transmeiotic differentiation of
human spermatogenic cells may occur without Sertoli cell support is weakly compatible with the traditional view of spermatogenesis control, Sertoli cell-independent differentiation of bovine secondary spermatocytes into spermatids capable of fertilizing oocytes with subsequent development to the blastocyst stage has been achieved in vitro, in the absence of any additional cellular support (Goto et al., 1996). If spermatids are found both in the ejaculate and in a testicular biopsy sample, the latter can be expected to be of higher biological quality because of the likely oxidative damage having occurred as spermatids aged during the epididymal transit.

In spite of the frequent failure of spermatid recovery from testicular biopsy samples, we still believe that testicular biopsy is worth doing in any ROSI attempt. When compared with ejaculated spermatids, testicular spermatids usually have a better viability, and their fertilizing ability may still be increased by in-vitro culture which is much more problematic for the more aged ejaculated spermatids. In most patients, ejaculated spermatids are also more frequently prone to apoptosis in comparison with testicular spermatids (J.Tesarik, unpublished observation). Cryopreservation of testicular tissue may avoid the need for repeated biopsy for future ROSI attempts.

Irrespective of the underlying biological mechanism, these data show that the finding of round spermatids is a frequent situation in cases in which spermatozoa fail to be found. It is thus important to perform a deeper analysis of these cases in order to establish additional criteria for prediction of success rates and health hazards of the use of spermatids obtained in these particular situations for assisted reproduction. On the other hand, numerous primary spermatocytes are usually present in those cases in which only very few spermatids can be obtained. If in-vitro culture methods supporting the development of primary spermatocytes into spermatids are available, the treatment of such cases would obviously be rendered much easier.

Factors affecting success and failure of spermatid conception

Complete and incomplete spermiogenesis failure

We have recently used the term complete spermiogenesis failure for those cases in which the round spermatid is the latest stage of spermatogenesis ever detected in the patient’s history (Amer et al., 1997). Spermatid conception was attempted in 59 such cases, but ongoing clinical pregnancies were only achieved in two out of three cases in which late elongated spermatids were unexpectedly recovered by testicular biopsy and injected into oocytes (Amer et al., 1997). Hence, the spermiogenesis failure was in fact incomplete in these successful treatment cycles which thus bore much resemblance to the successful spermatid conception cycles described previously by ourselves (Tesarik et al., 1995, 1996; Tesarik and Mendoza, 1996a) and others (Fishel et al., 1995; Araki et al., 1997). A similar difference in ROSNI success rates between patients with complete and incomplete
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Spermiogenesis failure has been reported recently by Vanderzwalmen et al. (1997). These clinical data are in agreement with a previous experimental study showing that the reproductive capacity of round spermatids selected from animals with primary testicular damage was lower than that of round spermatids recovered from healthy animals (Sofikitis et al., 1996b).

Notwithstanding, our fertilization and cleavage rates obtained in patients with complete spermiogenesis failure (Amer et al., 1997) were not dissimilar to those we reported previously for cases in which spermiogenesis failure was incomplete (Tesarik and Mendoza, 1996a). Moreover, four biochemical pregnancies were established with round spermatids from patients with complete spermiogenesis failure; of these, however, none developed into a clinical pregnancy (Amer et al., 1997). This means that the factors responsible for the failure of spermatid conception in cases of complete spermiogenesis failure act mainly around the time of implantation and in the early post-implantation period rather than at fertilization and during early preimplantation development. These factors, largely unknown for the time being, may be related to genetic anomalies or to various cytological, biochemical and cell-signalling disorders most of which may impact on embryonic gene dosage and activity and can thus be characterized as epigenetic factors.

Genetic anomalies

Several genes have been suggested as causative factors in human spermatogenic disorders. Three candidate genes, \textit{YRRM1} and \textit{YRRM2} (Y-specific genes with RNA recognition motif) (Ma et al., 1993) and \textit{DAZ} (deleted in azoospermia) (Reijo et al., 1995), have been characterized. They all map to the long arm of the human Y chromosome, to a region called AZF (azoospermia factor) because the association of major deletions (detectable in karyotype) in this region with severe spermatogenic disorders suggested that it contained one or several loci (AZF loci) responsible for spermatogenesis (Tiepolo and Zuffardi, 1976). It cannot be excluded that other yet unidentified genes can be localized in this Y-chromosome region. In addition, an autosomal homologue of \textit{DAZ}, called \textit{DAZLA} (DAZ-like autosomal) has been discovered on the human chromosome 3 (Yen et al., 1996). Tiny deletions (microdeletions) or mutations in the AZF region, which are not detectable in the karyotype but can be revealed by polymerase chain reaction, have been shown to be associated with some cases of azoospermia or severe oligoasthenoteratozoospermia (for recent reviews, see Qureshi et al., 1996; Pryor et al., 1997).

A complex interplay between different genes involved in spermatogenesis is supposed to control individual stages of this process. The polygenic control of human spermatogenesis may explain why deletions of the same size and chromosomal location can be associated with different gravity of the spermatogenic disorder in different patients. It is not known in what proportion of azoospermic patients with Y-chromosome microdeletions spermatids can be recovered for assisted reproduction. No analysis of the Y chromosome integrity,
beyond routine karyotype examination, has been performed in any of the spermatid-injection treatment cycles yet reported in the literature. However, if such microdeletions were present in the spermatids used for treatment, this would be unlikely to jeopardize the fertilization performance of these spermatids or subsequent embryonic and fetal development. In fact, this kind of genetic abnormality, though affecting sperm differentiation and function, does not appear to affect embryo viability and can be transmitted to progeny when fertilization is assisted by appropriate micromanipulation techniques (Mulhall et al., 1997). Consequently, if the failure of embryonic or fetal development after fertilizing oocytes with spermatids is due to genetic factors, the gene(s) responsible for developmental failure are unlikely to be identical with the known genes responsible for spermatogenic failure and still remain to be identified. Alternatively, the factors responsible for developmental failure in these cases may be of epigenetic rather than genetic origin.

**Epigenetic disorders**

**Nuclear proteins**

The development of spermatids into spermatozoa is marked by salient changes in the composition of nuclear proteins, histones being progressively removed and replaced with protamines, basic proteins rich in S–S bonds responsible for nuclear condensation, whereas an inverse protamine–histone transition occurs in the male nucleus after fertilization (reviewed in Perreault, 1990). A question arises about what happens when an early spermatid nucleus, with DNA still associated with histones, is exposed to oocyte cytoplasmic factors regulating the S–S bond reduction, protamine–histone transition and the male pronuclear development (Tesarik and Kopecny, 1989a) without the previous passage through the protamine-associated configuration. It has already been proven that the timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulphide bond content (Naish et al., 1987). A relatively rapid (<10 h) appearance of pronuclei has been reported after human ROSI (Tesarik and Mendoza, 1996a) and ROSNI (Yamanaka et al., 1997). Recent work in an anuran amphibian, *Rana catesbeiana*, has suggested that a complete removal of sperm-specific histone variants is not necessary for the sperm nucleus remodelling into somatic chromatin that takes place after fertilization (Itoh et al., 1997). Moreover, unlike most mammalian species in which virtually all nuclear histones are replaced with protamines during spermiogenesis (Meistrich, 1989), ~10% of the DNA of mature human spermatozoa remains bound to histones (Tanphaichitr et al., 1978; Gatewood et al., 1987; Choudhary et al., 1995). It is thus possible that a complete removal of spermatid histones does not necessarily occur during the development of the male pronucleus after human ROSI. However, the unusual respective dosage of histones of the male and of the female origin, along with the presence of a variable amount of protamines, may alter the normal sequence of early post-fertilization chromatin functions, including the
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early transcriptional activity detected in human pronuclear zygotes (Tesarik and Kopecny, 1989b, 1990). Such irregularities may be responsible for the abnormalities in the pronuclear development observed after human ROSI, such as the persistence of a very small dense nucleus or a prolonged appearance of a single syngamic nucleus after previous development of two normal-sized pronuclei (Tesarik and Mendoza, 1996a).

Centriole

Spermatids possess a pair of centrioles, proximal and distal, of which the latter disappears in late elongated (Sd₂) spermatids (De Kretser and Kerr, 1988). Consequently, the mature sperm cell has only one centriole, associated with a microtubule-organizing centre (MTOC); this sperm-derived MTOC is responsible for the organization of zygote microtubules including those of the mitotic spindles employed in the forthcoming cleavage divisions (Sathanthan et al., 1991; Palermo, et al., 1994; Simerly et al., 1995). It is not known whether the spermatid’s distal centriole is also associated with an MTOC that can become active within the oocyte cytoplasm. If so, this might lead to the creation of multipolar mitotic spindles and thus cause an irregular distribution of chromosomes at anaphase resulting in numerical chromosomal abnormalities. An abnormal function of the sperm centriole is one of the mechanisms suspected to be involved in the development of chromosome abnormalities after intracytoplasmic sperm injection (ICSI) (Tesarik, 1995), and this concern is even more substantiated in the case of the use of spermatids. The examination of the karyotype of all babies resulting from spermatid conception is thus strongly recommended.

Oocyte-activating factor

As in other mammalian species, sperm-induced activation of human oocytes at fertilization is mediated by a special form of Ca²⁺ signal, consisting of a series of repetitive sharp and short increases (spikes) of intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) referred to as Ca²⁺ spiking or Ca²⁺ oscillations (Taylor et al., 1993; Tesarik and Sousa, 1994). The sperm-induced Ca²⁺ oscillations also develop after ICSI (Tesarik and Sousa, 1994; Tesarik et al., 1994) and are supposed to be dependent on the action of an oocyte-activating factor contained in the cytoplasm of the injected spermatozoon. A recently characterized protein, termed oscillin (Parrington et al., 1996), is a candidate for this function. Even though an increase in [Ca²⁺]ᵢ is regularly produced by the ICSI procedure itself, and this artificial [Ca²⁺]ᵢ increase has been shown to potentiate the action of the oocyte-activating factor (Tesarik and Sousa, 1995), Ca²⁺ oscillations do not develop in the absence of sperm (Tesarik and Testart, 1994; Tesarik et al., 1994; Tesarik and Sousa, 1995). Because Ca²⁺ oscillations are believed to be required for normal oocyte activation in mammals (reviewed in Swann and Ozil, 1994), it is important to know whether the oocyte-activating factor is already present in spermatids and whether it has reached its full biological activity by this stage.
Table II. The ability of round spermatids from patients with complete spermiogenesis failure to support Ca$^{2+}$ oscillations in oocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Injected</th>
<th>Oscillating at 30–60 min</th>
<th>Oscillating at 1–2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>2</td>
<td>1*</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*This oocyte was one of the two that oscillated at the 30–60 min observation interval.

To answer this question, surnumerary human oocytes, donated by consenting patients, were injected with different stages of spermatogenic cells or with polymorphonuclear leukocytes, and the subsequent Ca$^{2+}$ response was evaluated by confocal microscopy (Sousa et al., 1996a). Data obtained showed that Ca$^{2+}$ oscillations, similar to those following the injection of a mature spermatozoon, developed after the injection of a round spermatid but not after the injection of a primary or secondary spermatocyte or of a polymorphonuclear leukocyte (Sousa et al., 1996a). Hence, electrical stimulation is not required for normal activation of spermatid-injected human oocytes (Tesarik et al., 1995), similarly as for cynomolgus monkey oocytes (Ogura et al., 1997), whereas it is important for optimal oocyte activation after ROSI and ROSNI in the mouse (Kimura and Yanagimachi, 1995) and rabbit (Sofikitis et al., 1996a).

However, the above results were obtained with spermatids from men who produced apparently normal mature spermatozoa. Here we performed additional experiments in which we used the same materials and methods (Sousa et al., 1996a) to assess the function of the oocyte-activating factor of spermatids from patients with complete spermiogenesis failure, in whom spermatids did not develop beyond the Sa stage according to the classification of de Kretser and Kerr (1988). Briefly, oocytes were injected with spermatids without performing the vigorous ooplasmic aspiration, thus avoiding a rapid oocyte activation, and subsequently loaded with the Ca$^{2+}$ indicator fluo-3. The oocytes were challenged with 10 μM ionophore A23187 to boost activation and examined in a confocal laser scanning microscope for the subsequent development of Ca$^{2+}$ oscillations. Results of these experiments are shown in Table II. When [Ca$^{2+}$]$_i$ was monitored 30–60 min after the ionophore challenge, only six out of 36 oocytes injected (17%) showed Ca$^{2+}$ oscillations, and only two oocytes (6%) oscillated when examined 2–3 h after ionophore treatment (Table II). This contrasted with our previous data on the occurrence of Ca$^{2+}$ oscillations after injection of spermatids from men capable of completing spermiogenesis, showing the development of Ca$^{2+}$ oscillations in 13 out of 17 oocytes (76%) injected with round spermatids (Sousa et al., 1996a), as well as with the duration of the Ca$^{2+}$-oscillation period in human oocytes injected with mature spermatozoa, which was superior to 2 h (Tesarik and Sousa, 1994; Tesarik et al., 1994; Sousa et al., 1996b). The existence
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of these differences suggests that, in spite of the presence of oocyte-activating factor in round spermatids from men with normal spermatogenesis, this factor may be deficient in many round spermatids recovered from men with complete spermiogenesis failure.

These data may explain why the fertilization rates reported after ROSI in patients with severe disorders of spermiogenesis is lower than for ICSI with mature spermatozoa (Tesarik et al., 1995; Tesarik and Mendoza, 1996a; Fishel et al., 1997), although at least one study (Yamanaka et al., 1997) reports fairly high oocyte activation rates after ROSNI in patients with complete spermiogenesis failure. The reason of these differences is not known. Moreover, the suboptimal activity of the oocyte-activating factor in patients’ spermatids may have developmental consequences reaching far beyond fertilization and can also be responsible for the low implantation rate and the high early-abortion rate after transfer of spermatid-derived embryos that were observed in cases of complete spermiogenesis failure (Amer et al., 1997). It is now clear that the quality of Ca\(^{2+}\) signals generated in freshly fertilized oocytes influences developmental processes far beyond the reactivation of the zygote’s cell cycle and the pronuclear development. In the mouse model, transient [Ca\(^{2+}\)]\(_i\) increases were shown to regulate embryonic growth and differentiation throughout preimplantation development (Stachecki and Armant, 1996), and the form of Ca\(^{2+}\) signals in mouse zygotes and early embryos was related to the progression of embryonic cell division and differentiation during both preimplantation and early postimplantation development (Bos-Mikich et al., 1997). Similarly, human preimplantation embryos were shown to generate Ca\(^{2+}\) signals that appeared to be related to the blastomere cell cycle and viability (Sousa et al., 1996b). It is thus possible that the quality of the initial fertilization-induced Ca\(^{2+}\) signal may affect the forthcoming Ca\(^{2+}\) signalling events controlling further development of human embryos. Further improvement of ROSI success rates in patients with complete spermiogenesis failure will thus depend on our ability to mimick the normal pattern of fertilization-associated and embryonic Ca\(^{2+}\) signals in the absence or relative insufficiency of the oocyte-activating factor in the patients’ spermatids.

Potential health hazards

Health hazards relating to the use of spermatids in human assisted reproduction have been largely discussed previously (Tesarik, 1996; Tesarik and Mendoza, 1996b). Besides the risk of transmission of infertility genes, which does not appear to be higher than for ICSI with mature spermatozoa in cases of severe spermatogenic defects, the risk of genomic imprinting abnormalities represent a major concern (Tesarik and Mendoza, 1996b). This concern is based on the uncertainty of whether the process of genomic imprinting, taking place during an unknown period of gametogenesis, is actually completed in immature spermatogenic cells, although minor abnormalities may be repaired after fertilization (Howlett and Reik, 1991). In addition to the three imprinted genes known
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Table III. Human imprinted genes associated with pathological phenotypes*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome mapping</th>
<th>Normal expression</th>
<th>Abnormal expression</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2</td>
<td>11p15</td>
<td>Monoallelic paternal</td>
<td>Biallelic</td>
<td>Wilms' tumour Adenocortical carcinoma Rhabdomyosarkoma Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>p57KIP2</td>
<td>11p15</td>
<td>Monoallelic paternal</td>
<td>None</td>
<td>Wilms’ tumour Adenocortical carcinoma Rhabdomyosarkoma Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>H19</td>
<td>11p15</td>
<td>Monoallelic paternal</td>
<td>Biallelic</td>
<td>Wilms’ tumour</td>
</tr>
<tr>
<td>SNRPN</td>
<td>15q11-13</td>
<td>Monoallelic paternal</td>
<td>None</td>
<td>Wilms’ tumour</td>
</tr>
<tr>
<td>PEG1/MEST</td>
<td>7q31-34</td>
<td>Monoallelic paternal</td>
<td>None</td>
<td>Prader–Willi syndrome</td>
</tr>
</tbody>
</table>

*pUpdated version of a previously published overview (Tesarik and Mendoza, 1996b) including data from references newly added in the present article (see main text).

To be potentially involved in human diseases, IGF2, H19 and SNRPN, that have been discussed previously (Tesarik and Mendoza, 1996b), two other human imprinted genes whose abnormal expression causes pathological phenotypes, p57KIP2 (Matsuoka et al., 1996; Hatada et al., 1996) and PEG1/MEST (Kobayashi et al., 1997), have now been characterized.

p57KIP2 belongs to the same gene cluster on human chromosome 11 as IGF2 and H19, encodes a cyclin-dependent kinase inhibitor and, as with the two neighbouring genes, abnormalities of its expression appear to result in altered cell proliferation and differentiation leading to phenotypes characterized as Beckwith–Wiedemann syndrome, including predisposition to certain rare childhood neoplasias such as Wilms’ tumour, adrenocortical carcinoma, rhabdomyosarcoma and hepatoblastoma (Matsuoka et al., 1996; Hatada et al., 1996; Zhang et al., 1997).

PEG1/MEST (paternally expressed gene 1/mesoderm-specific transcript) is the first imprinted gene mapped to human chromosome 7 and has been proposed as a candidate for a gene responsible for primordial growth retardation including Silver–Russel syndrome (Kobayashi et al., 1997). An updated overview of human imprinted genes whose anomalies can be responsible for known pathological phenotypes is shown in Table III.

The actual state of imprinting of a gene is known to be closely reflected by a
specific pattern of DNA methylation, concerning CpG dinucleotides, in the gene itself or its promoter (Brandeis et al., 1993). However, all methylated CpG sites are not involved in genomic imprinting. Even though the specific sites involved in imprinting are not yet known for all human imprinted genes, knowledge in this field is emerging rapidly (Jinno et al., 1996). Once the specific methylated sites are definitely identified, it will be possible to check the imprinting status of the respective genes by evaluating their methylation pattern by analysis of the size of DNA fragments after digestion with methylation-sensitive restriction enzymes. Methods for the distinction of monoallelic versus biallelic gene expression, using reverse transcription followed by polymerase chain reaction, are also known and can be used to check the parent-specific allele repression of imprinted genes, although the expression studies also have not yet been fully conclusive (Szabo and Mann, 1995a,b), and much remains to be done. Hopefully, these methods will be available in the near future to analyse the five imprinted human genes (Table III) in lymphocytes of children resulting from spermatid conception. We believe that the availability of this screening should be ensured in any future spermatid-conception clinical programme because the eventual detection of imprinting abnormalities can call attention to children at a higher risk of disease, to whom a special clinical surveillance is to be offered in order to ensure early diagnosis to propose efficient treatment in case of a developing pathology.

Practical recommendations

Quality assurance

It is important to stress that, many times in cases of non-obstructive azoospermia, spermatids are far from being predominant cells in patients’ ejaculate and testicular biopsy samples, even when methods for preparation of spermatid-enriched fractions are used. Thus, the recognition of living round spermatids from other round cells requires considerable skill. This skill can be best acquired by a person who is involved not only in spermatid injection trials but also in the diagnostic procedures aimed at the identification of individual stages of germ cells in patients’ samples. In this way, the student of spermatid morphology is first confronted with a relatively easy task of identifying germ cells with the use of cytological stains and more-or-less specific markers. The recently described methods for identification of round spermatids by a selective staining of acrosin contained in the developing acrosome with the use of fluorescein-labelled Pisum sativum agglutinin (Mendoza and Tesarik, 1996) or a more specific method visualizing proacrosin with the use of a monoclonal antibody (Mendoza et al., 1996) are examples of this approach. The latter method can be successfully combined with the detection of haploid cells by FISH (Mendoza et al., 1996) and is also suitable for the identification of spermatocytes and Golgi-phase round spermatids (stage 1) that still do not possess a distinct acrosomal vesicle. Among
the positively reacting cells, the distinction between spermatocytes and spermatids can then be made on the basis of cell size (Mendoza et al., 1996). This background knowledge is very useful for passing to the much more difficult task which is to identify round spermatids in fresh, unstained cell preparations (Tesarik and Mendoza, 1996a). The identification of elongated spermatids is easier as compared to round spermatids. However, individual stages of elongated spermatid development should be distinguished, at least on the basis of classical histology, and the morphology of the spermatids used for injection should be described in the greatest detail possible to avoid confusion of data interpretation when clinical results obtained with different patients are compared (Tesarik, 1997).

The injection of spermatids also requires additional skill as compared with ICSI (Tesarik and Mendoza, 1996a) and should be learned with animal models (mouse or hamster oocytes) before going to human application. The timing of pronuclear development after fertilization with spermatids is often different from ICSI and requires more frequent inspection of the injected oocytes. The special features of spermatid-derived zygote development, particularly the frequent development of a large syngamy nucleus (Tesarik and Mendoza, 1996a), have also to be taken into account.

Spermatid and spermatocyte cryopreservation: an obligatory step in cases of testicular sperm recovery failure

The failure of sperm recovery from testicular biopsy samples on the day of planned ICSI treatment is a relatively frequent complication. In many such cases, spermatids or spermatocytes can be identified. Moreover, it appears that it is just this situation of unexpected sperm absence which predicts better results of spermatid conception as compared to cases in which no spermatozoa have ever been detected (see above). However, if previous informed consent applicable to this situation has not been obtained, the use of spermatids in the present treatment cycle is impossible. Because the couple may demand a spermatid conception attempt at a later date, and in order to avoid the need for a new surgical intervention on that occasion, we recommend cryopreserving all the testicular biopsy samples for eventual future use. In fact, a human pregnancy has been achieved after fertilizing oocytes with frozen–thawed round spermatids (Antinori et al., 1997b).

Furthermore, many testicular biopsy samples lack both spermatozoa and spermatids but contain relatively high numbers of primary spermatocytes. Although the technique of fertilization with primary spermatocytes has not yet been developed, it is likely to emerge in the near future, possibly making use of transmeiotic in-vitro development of primary spermatocytes into spermatids or of injection of primary spermatocytes into germinal vesicle oocytes allowing both the male and the female nucleus to undergo simultaneous reduction divisions within the oocyte. Because a rapid degradation of spermatogenesis, progressing from maturation arrest to Sertoli cell-only syndrome, is not an uncommon finding, cryopreservation of testicular tissue containing primary spermatocytes is highly
recommendable to preserve the individual’s reproductive potential for the time at which an adequate assisted reproduction method will be available.

**Medical counselling**

When discussing the predictable success rates of spermatid conception with concerned couples, it is absolutely necessary to avoid drawing general conclusions from the small series of cases yet published. Nevertheless, it seems that prognosis is better when at least a small number of mature spermatozoa has been identified by previous patient’s examinations (in the ejaculate or in a testicular biopsy sample). If spermatozoa have never been found in the patient’s history, the prognosis of spermatid conception is poor and is often further compromised by the wife’s age. As an example, in our consultation, we inform patients that, with the actual state of the art, the predictable take-home-baby rate for a spermatid conception attempt is 5–10% in cases with past sperm production in which the wife’s age is <35 years. This prediction drops to 1–5% in cases of suspected complete spermiogenesis failure and when the wife’s age approaches 40 years. Such a realistic estimation, accompanied by a detailed explanation of the potential health hazards the method can create (see above), makes part of pre-treatment counselling sessions after which couples decide whether they wish to enter the spermatid conception programme.

In all cases, it has to be recalled to the couples that there is a rapid increase of knowledge in the field so that success rates are likely to improve in the near future. This will be possible partly because of a progress in diagnostic methods, thus avoiding the treatment of those couples in which successful conception is impossible due to genetic or epigenetic factors that cannot be therapeutically overcome, partly because of improvements of the laboratory techniques used for the introduction of male germ cells into oocytes and subsequent oocyte activation. However, the time for which couples can wait is limited by the wife’s age. Unfortunately, couples in whom we are urged to rush because of the wife’s age are often those in which the poor quality of oocytes is an additional adverse factor. No straightforward lead can be formulated as to how to manage these situations whose solution depends on the free decision of each couple, based on complete information about the actual and predictable future state of the art, the probable success rate, motivation of both partners and their social background.

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**References**


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Clinical use of spermatids


Clinical use of spermatids


