Is there a future for spermatid injections?

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Microinjection of spermatids into oocytes has proven to be a successful assisted reproduction procedure in the animal model. In the human, low fertilization and cleavage to the 4-cell stage were reported after intracytoplasmic sperm injection (ICSI) with round spermatids. In comparison with a conventional ICSI–testicular sperm extraction (TESE) programme, the implantation rate after round spermatid injection is dramatically low. Different problems have been encountered during the development of the spermatid injection technique and they could be partially responsible for the lower outcome when using round spermatids. Compared with the round spermatid cells, spermatids in the elongation phase are easy to isolate and identify from other round cells present in a wet preparation. The morphological identification does not reveal anything about the viability or the genetic normality of the round spermatids. Severe testicular dysfunction may have consequences on the quality of the few spermatogenic cells present. Others factors, such as the pathology of the patient, play an important role in the successful treatment. Even if the results are extremely low, spermatid injection seems more favourable for men who have already proven their capacity to produce some spermatozoa. A spermatogenic block at the round spermatid level has led to early abortions, increasing the suspicion of the role of a genetic factor. In order for this technique to be safe for use in clinics, more intensive work is needed to improve the selection and handling of cells and to ascertain the genomic imprinting and gene expression necessary for embryonic development. Hence, when using immature cells for conception, the screening of the patient and the follow-up of the pregnancies and babies should be mandatory.

Key words: male infertility/oocyte activation/spermatid identification/spermatid injection/spermatogenic arrest

Introduction

Since the introduction of intracytoplasmic sperm injection (ICSI) in 1992 (Palermo et al., 1992), this technique has been used for patients with severe
oligoasthenozoospermia using spermatozoa retrieved from the epididymis or testis (Schoysman et al., 1993). In some men showing severe testicular dysfunction, no spermatozoa could be found either in the ejaculate or in the testicular tissue. In such cases, Edwards et al. (1994) found that spermatids isolated from men with spermatogenesis arrest at any stage of spermiogenesis could be used as substitutes for spermatozoa to fertilize human oocytes.

These suggestions followed the findings of Ogura and Yanagimachi (1993) and Ogura et al. (1993), who were the first to report that round spermatid nuclei injected into hamster oocytes form pronuclei which could participate in syngamy. Later, several publications reported that, in rodents, round spermatids or a single spermatid nucleus could participate in full embryo development if they were injected directly or electrofused with mature oocytes (Ogura et al., 1993, 1994; Ogura and Yanagimachi, 1995; Sofikitis et al., 1996; Kimura and Yanagimachi, 1995a). Normal offspring have been reported after injection of cryopreserved round spermatids in the mouse (Ogura et al., 1996).

After the encouraging results of these animal investigations, an attractive challenge was to apply the injection of spermatids collected from the ejaculate or from testicular material to the treatment of men with non-obstructive azoospermia. This new approach gives hope in cases of azoospermia which, until recently, were considered to be untreatable. Such new perspectives encouraged several centres to obtain viable embryos after injection of elongated spermatids (Fishel et al., 1995, 1997; Vanderzwalmen et al., 1995, 1997; Chen et al., 1996; Mansour et al., 1996; Tesarik et al., 1996; Antinori et al., 1997a; Araki et al., 1997; Kahraman et al., 1998; Sofikitis et al., 1998a) or round spermatids retrieved from semen (Tesarik et al., 1995, 1996) or from testis tissue (Hannay, 1995; Amer et al., 1997; Antinori et al., 1997a; Fishel et al., 1997; Vanderzwalmen et al., 1997; Yamanaka et al., 1997; Kahraman et al., 1998). An ongoing clinical pregnancy has also been obtained following the injection of frozen–thawed round spermatids (Antinori et al., 1997b).

Compared with the results of testicular sperm extraction (TESE), the overall fertilization and pregnancy rates after the injection of sperm precursor cells are much lower. Different factors, such as the type and the quality of injected cells (Fishel et al., 1997; Vanderzwalmen et al., 1997; Tesarik et al., 1998), and the severity of the testicular pathology (Vanderzwalmen et al., 1997; Amer et al., 1997), can affect the outcome of fertilization, embryo quality and conception.

The intention of this paper is to actualize our data, which were previously presented (Vanderzwalmen et al., 1997). With the growing experience of the different centres offering such microassisted technology, can we definitely consider the use of spermatids as a valuable therapy?

Selection of patients

The use of testicular haploid germ cells was considered in two groups of men according to the severity of the testicular dysfunction. In the first group (partial
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Spermiogenesis failure, spermatids were collected from 46 men with unexpected transient failure of spermiogenesis. In 25% of our TESE–ICSI attempts, no spermatozoa could be found in different testicular biopsies on the day of oocyte retrieval, despite finding spermatozoa in some seminiferous tubules during previous attempts (explorative biopsies or in previous TESE–ICSI attempts) (46/166, 28%).

The second group (complete permanent spermiogenesis failure), comprised 26 men suffering from azoospermia resulting from a primary testicular disorder caused by various aetiopathological factors. In this group, previous histological examination of the testis repeatedly showed absence of complete spermatogenesis.

Classification and identification of sperm cell precursors

One of the problems of spermatid injection is the need for an accurate morphological approach and for selective tools to identify the haploid cells (Vanderzwalmen et al., 1998). As advocated by Tesarik (1997) and Tesarik et al. (1998), it is vital to define as exactly as possible the different types of spermatids that were handled in order to avoid confusion. In fact, without a clear terminology for each stage of spermiogenesis, it is difficult to draw conclusions and to compare results.

Spermiogenesis, the final phase of spermatogenesis, is the cytodifferentiation of spermatids into spermatozoa. During this process, no cell division is involved and the haploid round spermatids differentiate into highly specialized cells for motility and fertilization. The studies of Clermont (1963), de Krester and Kerr (1969), and Holstein and Roosen-Runge (1981), using staining techniques under light or electronic microscopy, clarified the cytological changes which characterize spermiogenesis. They established a classification which makes an accurate distinction between the different developmental stages involved in the start of spermiogenesis (Sa, Sb1) (Golgi phase, cap phase and acrosome swelling) followed by the nuclear changes and development of the tail (Sb2, Sc, Sd1, Sd2).

Unfortunately, in situ, without cytological techniques and under a conventional light microscope, it is difficult to make a strict distinction between the different developmental stages. In wet preparations, under the Hoffman modulation contrast system, four categories of spermatid can be distinguished, according to the shape, the amount of cytoplasm and the size of the tail: (i) round spermatids; (ii) elongating spermatids; (iii) elongated spermatids; and (iv) mature spermatids, just before their release from the Sertoli cells. This last category, which can be observed during the delivery of spermatids from the germinal epithelium, is more difficult to include in the classification. According to Silber and Lenahan (1995), a mature spermatid can be defined as a cell which is still trapped between the Sertoli cells.

After enzymatic treatment of the testicular biopsy (Crabbé et al., 1997) or after longer dilaceration of the tubules, most male gametes are released from the Sertoli cells and can be used successfully in a conventional ICSI–TESE–
testicular sperm aspiration (TESA) programme, without distinction between the spermatozoon and the mature spermatid. Therefore, in order to avoid confusion when analysing the results, we think that on morphological basis, such spermatids should not be included in the results under the term ‘elongated spermatids’ (Vanderzwalmen et al., 1997, 1998; Kahraman et al., 1998) but, as suggested by Silber et al. (1996), as ‘mature spermatids’.

As well as some basic concerns regarding ICSI using spermatids, a major problem concerns the recognition and identification of live round spermatids from other types of cells present in the ejaculate or in the testicular tissue. Identification of round spermatids in wet preparations is not as easy as in stained preparations. In general, round spermatids are identified from other cells, e.g. spermatogonia, spermatocytes and polymorphonuclear, according to their smaller size. The diameter (6.5–8 μm) is approximately similar to that of erythrocytes (7.2 μm) and also to small lymphocytes. Round spermatids include the Golgi phase, the cap phase and the acrosome phase (movement of the nucleus towards a peripheral position). Rotating and observing the cell, the round spermatids contain a centrally located uncondensed nucleus with a smooth appearance (Tesarik et al., 1996; Souza et al., 1998) and we tried to ascertain the presence of a developing acrosomal structure as a bright spot or a small protrusion on one side of the cell adjacent to the spermatid nucleus. This helpful criterion for the selection of round spermatids is not observed in such cells immediately after the second meiotic division. Round spermatids in the Golgi phase are extremely difficult to identify and can be confused easily with small lymphocytes, as they have a similar size and shape. Residual bodies were identified as enucleated round cells of 4–5 μm diameter with a roughened surface. It is very rare to confuse round spermatids with a Sertoli cell nucleus, the injection of which into oocytes may result in the appearance of two pronuclei (Silber and Johnson, 1998). In fact, contrary to the round spermatid, which is a three-dimensional round cell, the Sertoli cell nucleus is very flat, transparent, with a prominent centrally or adjacent localized nucleolus.

It is clear that the methodology for round spermatid isolation and purification (Yamanaka et al., 1997; Aslam et al., 1998), is a major factor affecting the outcome of spermatid injection and that such morphological criteria are highly susceptible to intra- and extra-observer variation.

Therefore, in order to feel more confident and validate the identification of round spermatids using morphological characteristics of a wet preparation, we need tools to confirm our ability to select round spermatids. Aslam et al. (1998) reported that the fluorescent-activated cell sorting (FACS) method provides the most suitable technique for the isolation of spermatids with a minimum loss of viability.

Various tools are available which offer the embryologist direct control over the selection procedure. Mendoza and Tesarik (1996) evaluated the occurrence of round spermatids in the ejaculate of men suffering from non-obstructive azoospermia, by cytochemistry and immunocytochemistry using markers...
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(flouroescein-labelled *Pisum sativum* agglutinin binding and antiacrosin antiserum immunolabelling) that selectively or specifically recognize the germ cell line.

Another method consists of the isolation and identification of one round cell, based on morphometric parameters after using computer-assisted image analysis (Yamanaka *et al.*, 1997). In order to ascertain that the cells were round spermatids, they processed one part for transmission electronic microscopy and another for confocal scanning laser microscopy.

According to Verheyen *et al.* (1998), the acrosomal vesicle or cap of round spermatids can be identified with a high degree of accuracy using phase-contrast optics on an inverted microscope; this is much less clear under Hoffman modulation phase contrast. Another approach for identifying round spermatids was undertaken by Angelopoulos *et al.* (1997). They performed a rapid correlation of the morphological features of a single stained cell by staining it on a prestained slide. They reported that cells <7.5 μm should be selected, since larger cells are likely to be secondary spermatocytes or white blood cells. In order to confirm the numerical chromosomal constitution of the presumed round spermatids that were isolated by the techniques described previously, they processed them using fluorescence in-situ hybridization (FISH) (Mendoza and Tesarik, 1996; Angelopoulos *et al.*, 1997; Verheyen *et al.*, 1998).

**Injection of spermatids**

The technique of injecting spermatids into the ooplasm was similar to that of conventional ICSI using mature spermatozoa (Vanderzwalmen *et al.*, 1996) and has been described by Tesarik and Mendoza (1996) and Vanderzwalmen *et al.* (1997).

Briefly, round spermatids were aspirated in a injection pipette with an inner diameter of 6–7 μm. The injection pipette was pushed through the zona pellucida and the oolemma at the equatorial level. Prior to injecting spermatids, vigorous ooplasmic aspiration was performed until there was a rapid outflow of cytoplasm into the injection pipette.

The percentage of one and two pronuclei (1 and 2PN) and the presence of two polar bodies was determined 18–22 h after injection. After 24 h of culture *in vitro*, embryo quality was assessed, and the embryos were classified according to the number and form of the blastomeres and the percentage of fragments. Regular sized blastomeres without fragmentation were classified as grade A; 10–20% fragmentation was present in grade B embryos. Grade C embryos were considered as embryos with uneven blastomeres and more than 20% fragmentation. All fertilized oocytes which resulted in embryos were transferred 44 h after oocyte retrieval.

**Fertilization, embryo quality and pregnancies according to the type of injected cell**

Fertilization, embryo quality and pregnancy rates are affected by the type of male germ cells (round, elongating, elongated spermatids) found in the testicular
Table I. Fertilization, cleavage and pregnancy rates according to the different types of injected spermatid: mature spermatid, elongated spermatid, elongating spermatid and round spermatid

<table>
<thead>
<tr>
<th>Type of spermatid</th>
<th>No. (%) of cases</th>
<th>No. of oocytes</th>
<th>2PN %</th>
<th>No. of transfers</th>
<th>Embryos grade A and B</th>
<th>No. (%) of pregnancies ongoing</th>
<th>live</th>
<th>birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature spermatid</td>
<td>3 (100)</td>
<td>15</td>
<td>53</td>
<td>3</td>
<td>25</td>
<td>1 (100)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Elongated spermatid</td>
<td>10 (100)</td>
<td>81</td>
<td>53</td>
<td>9</td>
<td>31</td>
<td>1a^a</td>
<td>3</td>
<td>1a^a</td>
</tr>
<tr>
<td>Elongating spermatid</td>
<td>4 (100)</td>
<td>25</td>
<td>60</td>
<td>4</td>
<td>33</td>
<td>1b^b</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>17 (24)</td>
<td>121</td>
<td>54</td>
<td>16</td>
<td>31</td>
<td>6 (35)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Round spermatids</td>
<td>55 (76)</td>
<td>476</td>
<td>17</td>
<td>51</td>
<td>11</td>
<td>2 (4)</td>
<td>1</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

^a Trisomy 9 (47,XY+9): interruption of pregnancy.
^b Arnold chiai.
2PN = two pronuclear.

biopsies and injected into oocytes (Table I). Of the oocytes, 54% showed 2PN after injection of elongating, elongated and mature spermatids in comparison with 17% after injection in the round stage. This was confirmed by Fishel et al. (1997), who observed, using sibling oocytes, that the overall fertilization rate with round spermatids was significantly lower than that with elongated cells isolated from testicular tissue.

An average of 71% of normal fertilized oocytes cleaved and all the embryos (grades A, B and C) were transferred. A higher rate of good quality embryos was observed after injection of spermatids isolated from the more advanced group (31%) and only 11% of grades A and B were obtained after round spermatid injection. The implantation rate after injection of the more developed haploid cells reached 11%, but was only 2% after injection of the round spermatids. From a total of 72 spermatid injections (17 with elongated spermatids and 55 with round spermatids), fetal and heart activity was observed in six women (five with elongated stage, one with round spermatids).

Based on these results, the late spermatid stage should be more suitable for injection. Firstly, the normality of the centrosome of the round spermatid needs to be ascertained (Fishel et al., 1996). An abnormal centrosome and, consequently, abnormal spindle formation can explain arrest during mitotic cell cycle progression and anomalies arising in embryos after the use of round spermatids (Simerly et al., 1995). Secondly, injection of spermatids may be associated with problems related to incomplete nuclear protein maturation (Souza et al., 1998). The spermatid DNA devoid of protamine is not easily protected against cytostatic factor and metaphase-promoting factor which can drive the spermatid chromatin to an inappropriate metaphase block.

Moreover, as elongated spermatids are in the final step of spermiogenesis, histone–protamine transition and nuclear DNA processing have already begun, and more gene transcription steps are completed. According to Ariel et al. (1994), DNA methylation of mouse male germ cells continues during the transit of the spermatozoa in the epididymis. In mice, remethylation is part of the process of
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Table II. Fertilization and pregnancy rates according to the severity of the testicular pathology

<table>
<thead>
<tr>
<th>Patient’s history</th>
<th>Type of spermatid</th>
<th>No. of cases</th>
<th>No. of oocytes</th>
<th>2PN (%)</th>
<th>Transfers</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermiogenesis failure Partial</td>
<td>elongating-elongated-mature round</td>
<td>29</td>
<td>224</td>
<td>27</td>
<td>29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1 birth)</td>
</tr>
<tr>
<td>Complete round</td>
<td>26</td>
<td>252</td>
<td>8</td>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>24 transfers with two pronuclear (2PN) embryos, five with 2PN and 1PN embryos.
<sup>b</sup>15 transfers with 2PN embryos, five with 2PN and 1PN embryos, two with 1PN only.

sperm maturation which occurs in the epididymis. Thirdly, the ability to activate oocytes may be lower after injecting round spermatids rather than elongated spermatids.

After ICSI with mature spermatozoa, oocyte activation is an essential step and spermatozoa activated the majority of the oocytes by releasing a cytosolic oocyte-activating factor, oscillin (Parrington et al., 1996). Souza et al. (1996) and Yamanaka et al. (1997) showed that in human round spermatids, the activity of the oocyte-activating factor was sufficient to induce a Ca<sup>2+</sup> oscillation mechanism in the oocyte and that the oocyte-activating factor had already appeared or had been activated the first stage of spermiogenesis.

The fertilization rate following round spermatids injection is very low. We suggest that in cases of severe testicular disorder, round spermatids may lack an active oocyte-activating factor or that the cytoplasm of round spermatids is not mature enough in comparison with its nucleus and that, consequently, the Ca<sup>2+</sup> oscillation response is lacking. Oocyte activation can be improved by the use of artificial treatments: application of electrical pulses (Sofikitis et al., 1996), application of vigorous cytoplasm aspiration (Tesarik and Mendoza, 1996), injection of oscillin (Parrington et al., 1996) and incubation with the calcium ionophore A23187 (Tesarik and Souza, 1995; Hoshi et al., 1995; Vanderzwalmen et al., 1997). Finally, elongated spermatids are easier to recognize morphologically than round spermatid cells.

Types of spermatid present in the testicular tissue is a function of the testicular dysfunction

The presence of these different cell types depends on the severity of testicular pathology (Table II). We observed only cells in the elongation stage (elongating, elongated and mature) in the group with occasional failure of complete spermatogenesis. Out of 46 patients from this group, spermatids in the elongating phase were observed in 37%. Histo-pathological examination suggested a relationship between the probability of finding spermatids in the elongation phase and the percentage of seminiferous tubules showing some spermatozoa. Complete absence of spermatozoa in the testes reflects a deterioration of spermatogenesis with a
total inability to produce mature spermatozoa or, at best, the production of a few spermatozoa in a few seminiferous tubules. Severe testicular damage may have consequences, particularly on the number of ultimate haploid cells. Aslam et al. (1998), report that there is significantly greater cell degeneration during the maturation of round spermatids into elongating spermatids in non-obstructive azoospermic patients in comparison with obstructive azoospermia. We suggest that the degree of degeneration can be related to the severity of the testicular disorder.

Histological evaluation of testicular tissue in different disorders (concentration of follicle stimulating hormone, normal testis or atrophic testis) showed that the number of cases exhibiting extremely few elongating cells or spermatozoa is very low (personal observation). Even if most tubules show a well-defined block, sporadic tubules with more mature germ cells are often observed. In such situations, only round spermatids are found and the more advanced stages are seldom found, even after a long search. Histo-pathological examinations of testes exhibiting a complete failure of spermiogenesis have shown an extremely low percentage of tubules (<10%) containing a few round spermatids with a pycnotic nucleus in the degenerated or atrophic phase as well as some desquamated spermatids (van de Casseye, 1994).

The observation of round spermatids alone, may be due either to a quantitative problem or to spermatogenic arrest that can occur at any level of germ cell differentiation (Martin-du Pan and Campana, 1993). Such an arrest may be due to chromosomal aberrations or to a single gene mutation (Verhoeven, 1998).

**Fertilization and embryo quality according to the testicular dysfunction**

For patients with complete spermatogenesia arrest, fertilization rates are extremely poor (8%). In the group with previous active spermatogenesis in a few tubules, acceptable fertilization rates after spermatid injection were obtained (54% with elongating spermatids and 27% with round spermatids) (Table II). In the group with complete spermiogenesis failure, specific alterations induce an absolute inability to produce mature spermatozoa in few seminiferous tubules.

The morphological identification and isolation does not, however, reveal anything about the viability or the genetic normality of the round spermatids. Aslam et al. (1998) evaluated the viability of germ cells using the Trypan Blue exclusion test. It was shown that 97% of round spermatids are viable at the moment of collection. However, without staining or destroying the cell, it is currently impossible to distinguish living cells from dead cells, to identify spermatids undergoing apoptosis or to differentiate between genomically normal and abnormal spermatids. During spermatid injection, the only parameter for evaluating the viability of a round spermatid, is its survival after aspiration in the micropipette; when no lysis is observed the cells are considered to be alive.

The poor validity of the viability tests which are currently available can explain, in part, the inability of round spermatids to achieve fertilization in
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Table III. Percentage of fertilization after injection of round spermatids isolated from patients with complete or partial arrest of the spermiogenesis and from patients with normal spermatogenesis showing spermatozoa

<table>
<thead>
<tr>
<th>Round spermatid injection from patients with:</th>
<th>No. of cases</th>
<th>No. of oocytes</th>
<th>Two pronuclei (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal spermatogenesis</td>
<td>4</td>
<td>12</td>
<td>42</td>
</tr>
<tr>
<td>Partial spermiogenesis failure</td>
<td>29</td>
<td>224</td>
<td>27</td>
</tr>
<tr>
<td>Complete spermiogenesis failure</td>
<td>26</td>
<td>252</td>
<td>8</td>
</tr>
</tbody>
</table>

patients with very severe spermatogenesis defects. Perhaps in the future, in-vitro culture of round spermatids will be possible since Aslam and Fishel (1998) have shown that round spermatids can develop a flagellum under specific conditions. They reported that the development of flagella during the first 4–8 h of in-vitro culture provides a better way of identifying their viability.

It is possible that round spermatids isolated from testes with incomplete or defective spermiogenesis may not be wholly identical to those obtained from the testes with complete spermatogenesis. We suggest that the absence of an elongation stage in all the tubules, which negatively affects the quality of spermatid maturation, may have a genetic cause. In such severe situations, it is possible that round spermatids are in the final step of gamete development hampering the competence of the germ cell to reach the zygote stage. In such situations, histo-pathological examinations generally show round spermatids with pycnotic nuclei, in the degeneration phase and also some desquamated round spermatids (van de Casseye, 1994).

In a recent study, Lopez et al. (1997) showed that fragmented DNA could prevent the initiation or completion of decondensation leading to fertilization failure. DNA damaged in such a way could cause arrest during embryo cleavage. The incomplete histone–protamine transition in spermatids is also suspected to lead to arrest; round spermatids had a mean DNA fragmentation of 55% compared with ejaculate spermatozoa (15%) or testicular spermatozoa (27%).

Another factor that can affect the quality of the round spermatids is the status of the oocyte-activating factor. J.Tesarik (personal communication) observed no Ca²⁺ oscillation mechanism after injecting poor quality spermatids from patients with extremely severe spermatogenesis dysfunction.

An increase in the fertilization rate after round spermatid injection has been observed when a small number of oocytes (given with the consent of four patients) were injected with round spermatids isolated from men exhibiting a normal spermatogenesis (Table III). In this case, five oocytes out of 12 (42%) were normally fertilized.

We have shown previously (Vanderzwalmen et al., 1997) that the quality of the embryos was directly related to the type of haploid cells injected. In fact, the number of embryos of good quality selected for transfer was low when round spermatids were used and especially when the embryos originated from a man with complete spermiogenesis failure. Janny and Ménézo (1994) identified a
strong paternal effect on embryo development and blastocyst formation. As explained previously, abnormal centrosome and consequently abnormal spindle formation can explain an arrest during mitotic cell cycle progression and anomalies arising in embryos after the use of round spermatids (Simerly et al., 1995). It is possible that defects at the level of the DNA prevent the genome of the round spermatids from completing embryogenesis.

Pregancy rate after spermatid injection

Several ongoing pregnancies have already been reported after injection of elongated spermatids (Fishel et al., 1996; Mansour et al., 1996; Araki et al., 1997; Vanderzwalmen et al., 1997). However, the results obtained after injection of round spermatids remain dramatically lower. To date, the birth of only three babies has been reported after round spermatid injection (Tesarik et al., 1995, 1996; Vanderzwalmen et al., 1997). The lack of selection (identification of viable cell) methods can explain the low reproductive capacity when spermatids were recovered from patients showing very severe defects of spermatogenesis. It is possible that such spermatids may not be wholly identical to those obtained from testes with partial failure of spermiogenesis or with focal complete spermatogenesis.

Three babies were conceived after injection of round spermatids from men who had produced some spermatozoa in their ejaculates a few weeks prior to the procedure (Tesarik et al., 1996) or 1 year earlier during a previous TESE–ICSI attempt (Vanderzwalmen et al., 1997). In the cases of patients who showed a complete failure of spermiogenesis, only Antinori (1997a) obtained two pregnancies after round spermatid injection. According to our results, and those of Hannay (1995) and Amer et al. (1997), no ongoing pregnancy was reported when round spermatids were isolated from men in whom spermatozoa had never been detected either in the ejaculate or in testicular tissue.

Imprinting status

The data in the animal field reporting normal full-term pregnancies after round spermatid (Ogura et al., 1994; Sofikitis et al., 1996a; Kimura and Yanagimachi, 1995a) or secondary spermatocyte injection (Kimura and Yanagimachi, 1995b) have supported the possibility that the imprint may either be sufficiently consolidated at this stage or may achieve appropriate consolidation in the oocyte cytoplasm (Tycko et al., 1997). The excellent health of all of the offspring and their fertility potential suggest that such cells can provide the paternally imprinted genes needed for embryonic development and that the nuclei of round spermatids are genetically ready to participate in normal fertilization (Ogura and Yanagimachi, 1995).

In view of the recent effort to use spermatids, animal experiments are indicated
to test whether the imprint is adequately consolidated at the spermatid stage of gametogenesis. After erasure of the previous imprint, probably in the primordial germ cells or replicating spermatogonia, it seems likely that re-establishment of the imprint must begin at some point prior to the pachytene stage of the meiosis (Tycko et al., 1997) and consolidation of the imprint may well be ongoing in the spermigenesis step, seeing that methyltransferase is present in round spermatids (Tycko, 1997).

A better knowledge of the different imprinted genes, of their consolidation and the mechanism of reactivation of normally silent allele could reduce possible epigenetic risk factors. It remains mandatory that we should undertake careful investigations on gene expression and genomic imprinting when using prespermatozoaal cells in clinical applications. Since genomic imprinting is an epigenetic process, a disruption of normal imprinting may cause human genetic diseases and promote the development of malignant childhood tumours.

Conclusions

In conclusion, it now seems that the earlier enthusiasm to help men suffering from extremely severe testicular disorders by the use of spermatid injections, should be reconsidered. At the present, spermatid injection seems better indicated for azoospermic men who have previously proved their capacity to produce (albeit only a few) spermatozoa. However, in severe pathological cases, the usefulness of round spermatids has not yet been proved. These data suggest that spermatid injection should be considered as an option only in cases of unexpected absence of spermatozoa. In patients with a complete block of spermiogenesis, the use of round spermatids should not be proposed as a therapy.

Couples who want to undergo round spermatid injection should be advised about the risks and the extremely low efficacy of this procedure. The risk of genetic transmission of Y chromosome deletions and of genomic imprinting anomalies should not be overlooked and care should be taken to avoid the serious consequences of such pathologies. In order to obtain the full range of clinical benefits from this procedure, more intensive work is needed to improve the selection and handling of cells and to ascertain the genomic imprinting and gene expression necessary for embryonic and post-embryonic development. Therefore, when using immature cells for conception, screening of the patients is recommended. Clearly, this goes well beyond a simple karyotype analysis and demands further work on the genes of the Y chromosome.

If the use of round spermatids is to be considered as a treatment, in view of the actual results, further research is needed. Firstly, to evaluate the required in-vitro culture conditions needed to induce progression of spermatocyte I or spermatocyte II (extremely rare in humans) to the round spermatid stage and then to the elongated stage. Secondly, an alternative to improving the feasibility of this method should be a search for a viability selection test in the case of round spermatid injection.
Another possible avenue is offered by the results of culture attempts of round spermatids up to the appearance of a flagellum (Aslam and Fishel, 1998). J. Tesarik (unpublished) recently reported two pregnancies from seven attempts, after injection of cultured round spermatids developing a short tail, for patients deprived of spermatozoa and elongating cells. Both in mice (Kimura and Yanagimachi, 1995b), and in the human (Sofikitis et al., 1998b), pregnancies have been obtained after injection of secondary spermatocytes.

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References


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