Can we justify spermatid microinjection for severe male factor infertility?

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During 1995 and 1996 the first spermatid pregnancies were announced with both round spermatid (ROSI) and elongated spermatid (ELSI) injections. These publications were flanked by live births from ROSI in a number of animal species, with resulting offspring appearing normal, healthy and fertile. However, the live births in humans heralded a scientific and ethical debate on the clinical use of this technology; and in a number of countries nationwide moratoria prohibiting spermatid microinjection were enjoined. Concerns surrounded the biological condition of spermatids and clinical implications of utilizing an immature spermatozoon for conception. Nevertheless, case reports and a few scientific studies on human spermatid conception have been published in recent years, and further polemic on testicular histopathology and prognosis has ensued. This paper reviews the current arguments on the clinical use of ROSI and ELSI, and evaluates the biology of the main contribu-

tory components of a spermatozoon to the subsequent embryo, namely the genetic material, the microtubular organizing complex and the putative oocyte activating factor. We also consider the relevant testicular histopathology and likely outcome in the context of the current birth rate from ROSI and ICSI. We conclude by considering the way forward for infertile men who require this technology to become genetic fathers, and whether the time is now appropriate to consider clinical trials.

Key words: assisted conception/male infertility/microinjection/spermatids

Spermatid injection and clinical trials

In many non-obstructive azoospermic patients enough spermatozoa can be recovered to achieve in-vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI), but in a few patients spermatozoa cannot be recovered even if minute foci of complete spermatogenesis exist. In these patients, if spermatids were retrieved from testicular biopsy or the ejaculate, then spermatid microinjection is the only option for them to have their own genetic offspring. However, the live births in humans heralded a scientific and ethical debate on the clinical use of this technology; and in a number of countries nationwide moratoria prohibiting spermatid microinjection were enjoined. Concerns surrounded the biological condition of spermatids and clinical implications of utilizing an immature spermatozoon for conception. Nevertheless, case reports and a few scientific studies on human spermatid conception have been published in recent years, and further polemic on testicular histopathology and prognosis has ensued. This paper reviews the current arguments on the clinical use of ROSI and ELSI, and evaluates the biology of the main contribu-

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**Spermatogenesis in different pathological conditions**

The process of spermatogenesis, from multiplication of spermatogenic precursor cells to the formation of mature spermatozoa, is tightly synchronized and integrated. Various pathological conditions can result in spermatogenic abnormalities with resultant subfertility, and in extreme conditions, sterility (Barratt, 1995). Sertoli cell-only syndrome, maturation arrest, post-cryptorchidism tubular atrophy, post-chemotherapy testicular atrophy, post-mumps orchitis and Klinefelter syndrome represent extreme conditions where no spermatozoa are found in the ejaculate (azoospermia). In spermatogenic arrest the morphogenetic programme may be halted at any stage; primary spermatocyte arrest is most common followed by the arrest at the spermatid level and, least commonly, spermatogonial arrest. Arrest at the primary spermatocyte stage can be incomplete, so that a few secondary spermatocytes or spermatids are observed (Girgis et al., 1969). The aetiological factors that can cause maturation arrest remain unclear. Not all cases of maturation arrest are due to genetic mutations and could be due to epigenetic factors such as stress and injury caused by infections, hormonal, thermal or toxic factors (Martin-du-Pan and Campana, 1993). In some cases inadequate gonadotrophic stimulation may be the cause of this condition (Franchimont et al., 1972). In Sertoli cell-only syndrome the tubules demonstrate a total absence of germ cells, a decrease in size and the presence of Sertoli cells only. A variable degree of tubular fibrosis is present (Glezerman, 1982). This syndrome, at least in a partial form, seems to occur rather frequently. In a series of 1294 cases of male infertility this condition was observed in 2.7% of patients (Dubin and Amelar, 1971). Among azoospermic patients, this condition has been shown to account for approximately one-third of cases (Chandley et al., 1976). Not all cases of Sertoli cell-only syndrome are due to genetic factors and could be due to combined radio- and chemotherapy, long-term oestrogen therapy, cryptorchidism and/or idiopathic aetiology (Schulze, 1984). Sertoli cell-only syndrome can be partial and unless a considerable number of sites are biopsied bilaterally, it is possible to misdiagnose a partial Sertoli cell-only syndrome as complete. Deficient spermatogenesis in these conditions cannot be stimulated by any known conventional mode of therapy. All efforts to stimulate spermatogenesis in such patients using hormones or other treatments have failed to demonstrate any effectiveness. But most of these men have small foci in their seminiferous tubules where spermatogenesis is active (Silber et al., 1995) to at least early stages of spermiogenesis and the presence of round spermatids.

**Spermatid recovery from azoospermic patients and its correlation with histopathology**

Testicular sperm extraction and intracytoplasmic sperm injection (TESE–ICSI) was first introduced for treatment of obstructive azoospermia (Schoysman et al., 1993). TESE–ICSI’s latter use for patients with non-obstructive azoospermia (Silber et al., 1995) was based on quantitative analysis of spermatogenesis in testicular biopsy specimens (Silber and Rodriguez-Rigau, 1981). In many cases of non-obstructive azoospermia, an occasional spermatozoon or spermatid was noted in the testicular biopsy despite being categorized as absent spermatogenesis. Although the qualitative histology of non-obstructive azoospermic cases undergoing TESE–ICSI has been reported to be highly variable (Tourmaye et al., 1996), the technique was found to be equally successful in azoospermia caused by either incomplete maturation arrest or by incomplete Sertoli cell-only (Devroe et al., 1995). It was also noted that in most of the maturation arrest cases, the spermatogenic defect was in meiosis and round spermatids were not seen. Whenever meiosis was complete and round spermatids were found, elongated spermatids and spermatozoa could also be observed (Silber et al., 1996). Recent data demonstrate that men with non-obstructive azoospermia caused by germinal failure (Sertoli cell-only syndrome, maturation arrest, cryptorchidism or post-chemotherapy azoospermia) had a mean of 0–6 elongating spermatids/semiferous tubule seen on a diagnostic testicular biopsy, compared to 17–35 elongating spermatids/tubule in men with normal spermatogenesis and obstructive azoospermia. It was also noted that more than half of azoospermic patients (26/45) with germinal failure had minute foci of spermatogenesis which, although insufficient to produce spermatozoa in the ejaculate, had detectable spermatids present in the diagnostic biopsy (Silber et al., 1997).

Immature germ cells may also be present in ejaculates of subjects with a normal sperm count (Tomlinson et al., 1992), oligozoospermia (Tomlinson et al., 1993), or azoospermia (Kurilo et al., 1993) and the presence of immature germ cells increases as the sperm count decreases (Sperling and Kaden, 1971). Mendoza and Tesarik (1996) observed round spermatids in ejaculates of 69% (86/124) of azoospermic patients. The presence and quantity of round spermatids in the ejaculate did not appear to be related to serum follicle stimulating hormone (FSH) concentration; even
FSH concentrations higher than 3-fold normal did not predict the absence of round spermatids (Mendoza and Tesarik, 1996). These high FSH concentrations predict Sertoli cell-only syndrome or early maturation arrest (Hargrave and Jequier, 1978). In fact, persisting foci of continuing spermatogenesis are present in the testis despite markedly elevated serum FSH concentrations (Gil-Salom et al., 1995).

Various methods of isolation of spermatids are practised by different groups. Most commonly used is the mechanical method in which a small piece of testicular tissue is torn apart using needles (Fishel et al., 1995; Angelopoulos et al., 1997; Antinori et al., 1997a; Araki et al., 1997). Although with this method spermatids can be obtained for immediate use, it is not possible to obtain large populations of purified spermatids to cryopreserve them for subsequent treatment cycles. Enzymatic digestion of the human testicular tissue results in a mixture of all types of cells present in testis including the whole series of spermatogenic cells (Salzbrunn et al., 1996) from which spermatids can be isolated manually under the microscope. Enzymatic digestion of testicular tissue has the advantage that several biopsies can be concentrated into one pellet of testicular cells. Different enzyme combinations are advocated by different groups including collagenase with or without elastase (Crabbe et al., 1997), trypsin in combination with DNase I (Aslam et al., 1998) and even the use of erythrocyte lysing buffer treatment has been recommended (Nagy et al., 1997). Discontinuous Percoll centrifugation gave satisfactory purification during isolation of the spermatids from the ejaculate (Tesarik and Mendoza, 1996; Zavos et al., 1997) or testicular biopsies (Vanderzwalmen et al., 1997; Aslam et al., 1998). Our clinical experience has shown that spermatids retrieved from testicular biopsies yield a higher incidence of fertilization as compared to spermatids retrieved from ejaculate (Fishel et al., 1997). Therefore, we developed a method to isolate large homogeneous spermatid populations which can be used fresh for microinjection and cryopreserved for subsequent treatment cycles. Trypsin and DNase I gave satisfactory dissociation of the testicular biopsies and sedimentation velocity under unit gravity technique in combination with discontinuous Percoll centrifugation gave satisfactory purification of the spermatids. Similar purification was also achieved with a fluorescent activated cell sorter (Aslam et al., 1998). Although the methods used in our studies have value for basic research, homogeneous populations will have a role in routine clinical practice, cryopreservation and in-vitro culture (see below). For this purpose enzymatic digestion is advantageous compared to mechanical disassociation of the testicular tissue, and we recommend digestion with trypsin and DNase I during clinical practice (Aslam et al., 1998).

Recently Silber and Johnson (1998) reported that they were not able to find round spermatids in the testicular biopsies of 205 azoospermic patients in the absence of mature sperm or elongated spermatids. Our experience with spermatid isolation from testicular biopsies also supports their observation. Our initial work with human spermatids centred on evaluating the methodology for isolation of large homogeneous populations of spermatids from testicular biopsies of obstructive and non-obstructive (hypospermatogenesis, maturation arrest and Sertoli cell-only syndrome; diagnosed by previous diagnostic biopsies) azoospermic patients. Although, at least once in the past, presence of mature spermatids or elongated spermatids had been demonstrated in the ejaculate or diagnostic testicular biopsy of non-obstructive azoospermic patients in our study, even then the yields from these patients of primary spermatocytes, round spermatids and elongating spermatids were significantly decreased ($P < 0.001$) as compared to obstructive azoospermic patients (Aslam et al., 1998).

**Identification of spermatids**

With the usual ICSI set-up (with Hoffman or Nomarski optics) it is not easy to identify and distinguish immature spermatogenic cells, especially early round spermatids, with absolute certainty. There is definitely a problem of identification of round spermatids by many practitioners, and we believe small lymphocytes can mistakenly be identified as round spermatids. Different identification methods are employed by different groups but the most commonly used method is based on the morphological characteristics of the round spermatid (Tesarik and Mendoza, 1996; Angelopoulos et al., 1997). Briefly, an early round spermatid (phases 1–3) was identified as a 7–8 µm diameter spherical cell with smooth outline. In both Golgi and cap phases of spermiogenesis, the acrosomal vesicle or cap was identified as a granule or crescent-shaped protrusion on one side of the cell. The nucleus was generally round and centrally located with smooth appearance. In more mature stages, the nucleus appeared slightly oval when it made contact with the cytoplasmic membrane on the side of the cell. In early stages a small smooth rim of cytoplasm was present all around the nucleus. Phase 1 spermatids had no flagellum, but in phase 2 spermatids the developing flagellum was seen protruding from the cell. Elongated spermatids (phases 4–6) were asymmetric, with an elongated nucleus at one side of the cell and with a large cytoplasmic region at the other side surrounding the flagellum. Although it was difficult to differentiate very imma-
ture spermatids from secondary spermatocytes the remainder of spermatogonial cells (primary spermatocytes, spermatogonia). Sertoli cells and Leydig cells were distinguishable from the round spermatids because of their relatively larger size. Lymphocytes had roughly the same diameter as spermatids but distinguishable from them mainly by a larger nucleus:cytoplasm ratio. Lymphocyte nuclei usually appeared slightly kidney-shaped or round and eccentric, and were dense. Lymphocytes had a very thin and discontinuous pink cytoplasmic zone. Neutrophils were distinguished from spermatids by the nuclei with interconnected lobes and the granular cytoplasm. These observations were in agreement with previously published results obtained by immunocytochemical visualization of proacrosin with the monoclonal antibody 4D4 combined with autosomal DNA fluorescence in-situ hybridization (FISH) of ejaculated germ cells from patients with defective spermiogenesis. The proacrosin immunoreactivity was detected in cells of round spermatid size presenting a haploid FISH figure as well as in larger cells whose ploidy corresponded to primary and secondary spermatocytes. These data showed that cell size is the main criterion to be used for the identification of round spermatids, whereas the presence of the developing acrosome represents only an auxiliary criterion (Mendoza et al., 1996).

To overcome the problem of round spermatid identification we recommend testicular biopsy dissociation with enzymatic digestion to obtain a large number of testicular cells. A few round spermatids can be identified with certainty observing the morphological criteria, even with Hoffman modulation system from such a suspension (Aslam et al., 1996). In the cellular suspension of such testicular tissue digestion we identified primary spermatocytes as the largest cells with a diameter of 16–18 µm. Round spermatids were spherical cells and had a diameter of 6–8 µm with clear cytoplasm and smaller but distinctive round nucleus. This was centrally situated in the early round spermatids and peripheral in maturing spermatids. The acrosomal cap could be visualized only in the cap phase spermatids but frequently this required manual rotation under visual inspection. Elongating spermatids had a diameter of 4–6 µm and were asymmetric cells. Spermatids that had just begun the condensation and elongation processes had a protruding nucleus with a forming acrosome at one pole and the bulging cytoplasmic lobe at the other. These cells were larger than the more fully condensed late stage spermatids, which often did not retain their cytoplasmic lobes and resembled the head of a mature spermatozoon (elongated spermatid). Occasional binucleated or trinucleated cells were also observed. These were identified as symplasts (round spermatids joined together). Multiple developing acrosomes and prominent nucleoli were the basis of identification. Residual bodies were identified as enucleate round cells with roughened surface and diameter of 4–5 µm. Leydig cells had shape and diameter resembling round spermatids but were identified on the basis of their highly refractile surface which is due to their lipid and cholesterol intracytoplasmic inclusions. Sertoli cells were flat-shaped with an irregular cell boundary in contrast to the spherical shape of all other cell types (Aslam et al., 1998).

Another advantage of enzymatic digestion of testicular tissue was demonstrated by growth of flagella by early round spermatids during in-vitro culture of mixtures of testicular cells were cultured in vitro (Aslam and Fishel, 1998). We are certain that in-vitro flagellar growth by early round spermatids will remove problems relating to the recognition of round spermatids from other types of round cells in the mixture, and by virtue of a spontaneous positive selection of viable spermatid the risk of injecting a dead spermatid will also be removed.

**Clinical successes with spermatid microinjection**

Edwards et al. in 1994 stressed the need for clinical use of spermatids for the treatment of severe male factor infertility. The first report of human fertilization with spermatid microinjection was by Vanderzwalmen et al. (1995). They injected only one oocyte with a ‘late stage spermatid’ which resulted in normal fertilization. The first human pregnancy with spermatid microinjection was reported by our group utilizing an elongated spermatid (ELSI) (Fishel et al., 1995). Both histologically and clinically this, at that time, was a departure from the usual histologically confirmed spermatozoon derived from seminiferous tubule. Although some may wish to redefine the distinction between the elongated spermatid and the spermatozoon obtained from testicular tissue (Silber and Johnson, 1998), the classical histology should not be redefined. Here we support the clinical use of histological classification suggested by Tesarik (1997).

The first live birth with round spermatid microinjection (ROSI) was reported by Tesarik et al. (1995). The latter report utilized spermatids retrieved from the ejaculate. The reported pregnancy by our group resulted in the first live birth with an elongated spermatid extracted from the seminiferous tubules (Fishel et al., 1996). Since these initial reports a number of infertility clinics have reported success with the spermatid microinjection.

Analysis of data from these reports shows that in 93 cases 712 oocytes were injected with round spermatids (ROSI) resulting in 233 fertilized oocytes (32.7%) out of which 165
(70.8%) cleaved. Embryo transfers resulted in 10 pregnancies and nine normal deliveries (Tesarik and Mendoza, 1996; Tesarik et al., 1996; Antinori et al., 1997a,b; Fishel et al., 1997; Vanderzwalmen et al., 1997; Kahraman et al., 1998). Table I shows the complete cycle histories which are available for 80 cycles from the published reports on round spermatid microinjection. In these 80 cycles the incidence of fertilization was 31.3%, and 75 cycles resulted in an embryo transfer. The incidence of clinical pregnancy per embryo transfer was 12.5% and 13.3%, respectively, with a implantation rate of 6.5% per embryo (Tesarik and Mendoza, 1996; Tesarik et al., 1996; Antinori et al., 1997a; Araki et al., 1997; Vanderzwalmen et al., 1997; Sofikitis et al., 1998; Kahraman et al., 1998).

Although spurious to compare uncontrolled data from different laboratories, at a glance two particular aspects of the data require comment. Despite a high pregnancy rate with ELSI, both ROSI and ELSI have a low implantation rate per embryo compared with conventional IVF or ICSI. Consequently, the incidence of multiple pregnancy in these data is low, apparently even when ELSI is utilized. However, this comparison has a very preliminary value, especially with the few cases available for analysis. Further interpretation of these clinical studies also suggests that the outcome of spermatid microinjection depends upon the previous presence of these clinical studies also suggests that the outcome of spermatid microinjection depends upon the previous presence of spermatogenesis showing spermatozoa or at least elongated spermatids in the diagnostic testicular biopsy (Vanderzwalmen et al., 1997; Amer et al., 1997; Silber and Johnson, 1998; Tesarik et al., 1998; Vanderwalzmen et al., 1998). Vanderzwalmen et al. (1997) showed that patients with a positive preliminary biopsy had significantly more two-pronuclear development (33%) when compared to those with a severe spermatogenic dysfunction and in whom no spermatozoa were found (11%) (P < 0.05). Similarly, outcome of sperm microinjections is better when spermatozoa are recovered from obstructive azoospermic patients as compared to non-obstructive azoospermic patients. Indeed, prognosis is poor when in non-obstructive azoospermic patients only spermatids are present and are utilized for microinjection (Fahmy et al., 1996). Even in cases of severe spermatogenic defects, microinjection with elongated and elongated spermatids provide acceptable fertilization and implantation rates (Fishel et al., 1995; Araki et al., 1997; Vanderzwalmen et al., 1997; Kahraman et al., 1998; Sofikitis et al., 1998). On the other hand, in cases with severe spermatogenic defects, the efficiency of round spermatids in achieving fertilization and implantation rates is disappointing (Hannay, 1995; Tesarik et al., 1996; Amer et al., 1997; Antinori et al., 1997a,b; Vanderzwalmen et al., 1997; Yamanka et al., 1997). Fertilization analysis also shows that testicular spermatids are more efficient compared to spermatids retrieved from the ejaculate (Fishel et al., 1997). However, despite the inefficiencies so far experienced the most encouraging aspect of all these reports is that all live births were healthy and normal with no chromosomal or congenital abnormalities reported.

Table I. Round spermatid microinjection (ROSI) and elongating spermatid microinjection (ELSI) outcome for published complete cycle histories

<table>
<thead>
<tr>
<th>Type of cells injected</th>
<th>ROSI</th>
<th>ELSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cycles</td>
<td>80</td>
<td>55</td>
</tr>
<tr>
<td>No. of oocytes injected</td>
<td>648</td>
<td>426</td>
</tr>
<tr>
<td>No. of oocytes fertilized (2PN) (%)</td>
<td>203 (31.3)</td>
<td>245 (57.5)</td>
</tr>
<tr>
<td>No. of embryo transfers (%)</td>
<td>75 (93.7)</td>
<td>55 (100)</td>
</tr>
<tr>
<td>No. of transferred embryos (%)</td>
<td>155 (76.3)</td>
<td>198 (80.8)</td>
</tr>
<tr>
<td>No. of clinical pregnancies</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>% of pregnancies/cycle</td>
<td>12.5</td>
<td>21.8</td>
</tr>
<tr>
<td>% of pregnancies/embryo transfer</td>
<td>13.3</td>
<td>21.8</td>
</tr>
<tr>
<td>% of transferred embryos</td>
<td>6.5</td>
<td>6.1</td>
</tr>
<tr>
<td>No. of live births</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>% of births/cycle</td>
<td>11.3</td>
<td>20.0</td>
</tr>
<tr>
<td>% of births/embryo transfer</td>
<td>12.0</td>
<td>20.0</td>
</tr>
<tr>
<td>% of transferred embryos delivered</td>
<td>5.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*See text for details and references.

2PN = two-pronuclear.

In 72 cases 518 oocytes were injected with elongating and/or elongated spermatids (ELSI) resulting in 269 fertilized oocytes (51.9%), 199 (73.9%) oocytes cleaved and embryo transfers resulted in 11 normal deliveries (Fishel et al., 1995, 1996, 1997; Tesarik and Mendoza, 1996; Tesarik et al., 1996; Antinori et al., 1997a; Araki et al., 1997; Vanderzwalmen et al., 1997; Kahraman et al., 1998; Sofikitis et al., 1998). Table I shows the complete cycle histories which are available for 55 cycles from the published reports on elongating spermatid microinjection. In these 55 cycles the incidence of fertilization was 57.5%, and 55 cycles resulted in an embryo transfer. The incidence of clinical pregnancy and delivery per cycle was 21.8% each, with an implantation rate of 6.1% per embryo (Fishel et al., 1995, 1996; Tesarik and Mendoza, 1996; Tesarik et al., 1996; Antinori et al., 1997a; Araki et al., 1997; Vanderzwalmen et al., 1997; Sofikitis et al., 1998; Kahraman et al., 1998).
Clinical concerns regarding spermatid microinjection

Although results of our studies and analysis of published reports indicate that the spermatid might be a competent male gamete, these reports also show the highly variable and unpredictable outcome of human spermatid conception attempts, particularly in terms of failure of implantation and pregnancy losses (Hannay, 1995; Fishel et al., 1997; Yamana et al., 1997). This has led to many concerns, such as DNA immaturity, genomic imprinting, normality of the centrosome, presence of sperm-derived oocyte activation factor, methodology of separation and isolation of spermatids and effects of in-vitro culture and cryopreservation (Aslam and Fishel, 1996; Fishel et al., 1996; Tesarik, 1996, 1997; Sousa et al., 1998). Many of the concerns are valid and careful consideration is required to reveal why spermatid microinjection, and ROSI in particular, from patients who showed no evidence of complete spermatogenesis in the past, have poor outcome. For this we have to look at the contributions of the spermatid during the fertilization process and their comparison with the spermatozoon.

Sperm contributions in fertilization

For normal fertilization three essential elements are contributed by the spermatozoon: (i) the genetic material; (ii) the centrosome; and (iii) a putative factor in the cytoplasm of the spermatozoon to initiate oocyte activation.

The genetic material

The genetic material is the combination of DNA and the nucleoproteins. Similar to mature spermatozoa, spermatids contain a haploid set of chromosomes. Therefore, the developing spermatozoa even at the round spermatid stage contain exactly the same amount of DNA as is carried by the mature spermatozoa. However, the development of spermatids into spermatozoa is marked by salient changes in the composition of nuclear proteins, the histones being progressively removed and replaced with protamines (whereas an inverse protamine–histone transition occurs in the male nucleus after fertilization) (Perreault, 1990). The question arises about what will happen when an early spermatid nucleus, with DNA still associated with histones rather than protamines, is exposed to oocyte cytoplasmic factors. Recent work in amphibians has suggested that complete removal of sperm-specific histone variants is not necessary for the post-fertilization sperm nucleus remodelling (Itoh et al., 1997). Moreover, unlike most mammalian species in which virtually all nuclear histones are replaced with protamines during maturation (Meistrich, 1989), ~10% of the DNA of mature human spermatozoa remains bound to histones (Choudhary et al., 1995). It is thus possible that the removal of spermatid histones is not necessary for the development of the male pronucleus after the spermatid injection into the human oocyte.

An important epigenetic modification of genes during spermatogenesis is genomic imprinting. Paternal and maternal genomes do not play an identical role during mammalian embryogenesis. The former appears to be preferentially needed for the development of extra-embryonic tissues, and the latter for preimplantation development and embryogenesis (Barton et al., 1984). In addition, chromosomes of both parental origins are needed for development to proceed to term (McGrath and Solter, 1984). While homologous chromosomes are spatially segregated during oogenesis and spermatogenesis, they are subjected to modifications in the genes prior to fertilization that subsequently evoke differential expression of the modified genes at later events throughout development (Surani, 1986). DNA methylation has been postulated to play a role in this specific modification of gene activity (Cedar, 1984). The risk of genomic imprinting abnormalities represents a major concern. For example, the maternally inherited mouse H19 allele is transcriptionally active, while the paternally inherited allele is silent (Bartolomei et al., 1991). The reactivation of the normally silent (paternal) allele has been reported in some Wilms’ tumour samples (Rainier et al., 1993). The recent work in our laboratory on the methylation of the H19 gene in the mouse and human, from the spermatid stage through to the mature spermatozoa, indicates that genomic imprinting of spermatogenic cells is completed in the testes before the second reductional (meiotic) division (Aslam et al., 1996). This is in support of the biological data of Ogura et al. (1994). The studies of Kimura and Yanagimachi (1995) in rodents, and the clinical results discussed already, indicate that genomic imprinting of the genes in spermatids is completed in the testes before the second meiotic division. Therefore, on this basis we can consider spermatid microinjection as a safe procedure.

Several genes have been suggested as causative factors in human spermatogenic disorders. Two candidate genes, Y chromosome RNA recognition motif (YRRM) gene (Ma et al., 1993) and DAZ (deleted in azoospermia) (Reijo et al., 1995), have been characterised and mapped to the long
arm of the human Y chromosome, to a region called AZF (azoospermia factor). Tiny deletions or mutations in the AZF region have been shown to be associated with some cases of azoospermia (Qureshi et al., 1996). In’t Veld et al. (1997) found 5% azoospermic patients have AZF deletion associated with DAZ gene. AZF/IDAZ region deletions have also been demonstrated to be transmitted from father to son (Vogt et al., 1996). It is clinically known that men with DAZ region deletions are mostly azoospermic and that spermatogenesis is variable, ranging from a pattern of Sertoli cell-only to the presence of condensed spermatids in tubules (Reijo et al., 1995). It is not known in what proportion of azoospermic patients with Y chromosome microdeletions spermatids can be recovered for assisted reproduction. However, if such microdeletions are present in the spermatids used for treatment, this does not jeopardize the fertilization performance of these spermatids or subsequent embryonic and fetal development (Mulhall et al., 1997). In fact, this kind of genetic abnormality, though affecting sperm differentiation and function, does not appear to affect embryo viability, although it may be transmitted to male progeny after ICSI.

Hence the spermatid’s genetic material contributed at fertilization, exclusive of the nucleoprotein associations, is similar to mature spermatozoa. On this basis there is no evidence to presume that the genetic material is related to the poor outcome of round spermatid microinjection.

Centrosome

In the process of fertilization the spermatozoon contributes the centrosome, the microtubule organizing centre (MTOC). Although it was suggested nearly a century ago that the centrosome is contributed by the spermatozoon (Boveri, 1901), the maternal inheritance observed in mice confused the picture (Schatten et al., 1991). It is now known that in humans the centrosome has paternal inheritance (Simerly et al., 1995). A centrosome consists of two morphologically distinct centrioles: a pair of cylinders enclosed in a complex and asymmetrical arrangement and the pericentriolar material from which spindle microtubules are generated (Palermo et al., 1994). At the end of the second meiotic division newly formed spermatids possess a pair of centrioles, a proximal and a distal one. The distal centriole gives rise to flagellum and the proximal centriole develops a cylindrical prolongation, known as the centriolar adjunct. In early round spermatids the pair of centrioles and the base of the flagellum move towards centre and become closely associated with the caudal pole of the spermatid nucleus at the implantation fossa. The distal centriole, after giving rise to flagellum, undergoes profound changes and ultimately disappears as a distinct entity (de Kretser and Kerr, 1994). Consequently, the mature spermatozoon cell has only one centriole. This spermatozoon-derived MTOC is responsible for the organization of zygote microtubules including those of the mitotic spindles employed in subsequent cleavage divisions (Palermo et al., 1994; Simerly et al., 1995).

During our isolation studies (Aslam et al., 1998) using enzymatic dissociation of the testicular tissue, both the developing flagella and some of the cytoplasmic lobes were severed from the elongating spermatids. Microscopic studies showed that loss of flagellum was not complete as some of the flagellar stump was seen protruding out of the cell body. In spermatids the flagellum arises from the distal centriole, whereas the proximal centriole which forms the centriolar adjunct in elongating spermatids and gives rise eventually to the centrosome (Sathananthan et al., 1996) is attached to the implantation fossa at the basal plate. Hence, even loss of part of the flagellum during enzymatic digestion and spermatid preparation poses no danger of losing the centriole. On the other hand loss of cytoplasmic lobes during preparation is indistinct from shedding of the cytoplasmic lobe during spermatiation, i.e. cytoplasmic lobes attached to the flagellum are lost with no detachment of cytoplasm from the head of the maturing spermatid. Therefore, the spermatid isolation procedures appear functionally safe and do not effect the spermatid contributions during fertilization.

Oocyte activation factor

It is generally agreed that intracellular calcium (Ca^{2+}) release is the universal signal for triggering oocyte activation (Vitulo and Ozil, 1992; Homa et al., 1993; Tesarik et al., 1994), but it is not clear how a spermatozoon activates this. Cytosolic sperm extracts microinjected into unfertilized hamster eggs stimulated a series of Ca^{2+} increases and mimicked fertilization, suggesting that a sperm cytosolic factor transferred into the egg at the time of fertilization causes oocyte activation (Swann, 1990). These findings were also confirmed in human oocytes (Tesarik et al., 1994; Dozortsev et al., 1995). Electron microscopic analysis of human oocytes that failed to display signs of fertilization by ICSI suggested that this failure after ICSI is basically a failure of oocyte activation (Sousa and Tesarik, 1994). The effect of oocyte activation on the success rate of spermatid microinjection was established by Kimura and Yanagimachi (1995). The highest rate (77%) of normal fertilized oocytes with spermatid microinjection was obtained when the oocytes were first electro-stimulated, and then injected. In human, the successful pregnancies and deliveries with spermatid injection indicate that a putative
sperm cytosolic factor required for oocyte activation is also present in the spermatid and that spermatid injection can activate the oocyte (Tesarik et al., 1995).

However, these observations were obtained with spermatids from men with complete and normal spermatogenesis. In a recent study Tesarik and his associates assessed the function of the oocyte activating factor of spermatids from patients with complete spermatogenic failure, in whom spermatids did not develop beyond very early stages (non-obstructive azoospermia). Briefly, oocytes were injected with spermatids avoiding a rapid oocyte activation of vigorous ooplasmic aspiration. When Ca$^{2+}$ oscillations were examined 30–60 min after the ionophore challenge (A23187), 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 (unpublished results, personal communication). This is not in agreement with their previous data on the occurrence of Ca$^{2+}$ oscillations after injection of spermatids from fertile men, 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 more than 2 h (Tesarik and Sousa, 1994; Tesarik et al., 1994; Sousa et al., 1996b). These differences suggest that an oocyte-activating factor is deficient in most round spermatids retrieved from men with spermatogenic failure.

These data may explain the poor clinical outcome of round spermatid microinjections in cases of severe spermatogenic disorders. Ca$^{2+}$ oscillations generated after fertilization influence the developmental processes far beyond the reactivation of the zygote’s cell cycle and pronuclear development. In the mouse, Ca$^{2+}$ oscillations regulate embryonic growth and differentiation throughout preimplantation development (Stachecki and Armanit, 1996), and the duration and amplitude of these oscillations regulate the progression of embryonic cell division and differentiation during both preimplantation and early post-implantation 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). Therefore, the suboptimal activity of the oocyte activating factor in the round spermatids retrieved from non-obstructive azoospermic patients may have developmental consequences reaching far beyond fertilization and be responsible for the low implantation and the high early abortion rates after transfer of spermatid-derived embryos. Perhaps an externally applied stimulus might complement the spermatid deficient in a suitable cytosolic factor. This was suggested by animal studies (Kimura and Yanagimachi, 1995), and, when Vanderzwalmen et al. (1997) used Ca$^{2+}$ ionophore A23187 to initiate oocyte activation after round spermatid microinjection, this resulted in improvement in the fertilization rate — 36% (nine cases) compared to 16% (23 cases) without Ca$^{2+}$ inophore activation — and one live birth. In our opinion oocyte activation with Ca$^{2+}$ ionophore should be applied half an hour prior to spermatid microinjection. Oocyte activation will cause the resumption of meiotic division of the oocyte resulting in decreased maturation promoting factor in oocyte cytoplasm, and this will protect the spermatid chromatin from premature chromosome condensation and will bring the spermatid and oocyte cell cycle in balance (Edwards et al., 1994; Fishel et al., 1996).

Conclusion

With the present state of knowledge, it may be argued that it is premature to use spermatids for human conception in vitro. However, the introduction of ICSI required a leap into the dark, and more so than spermatid microinjection, given the current level of of information based on animal, molecular and clinical studies. Ironically, provided a spermatozoon exists this can be utilized for ICSI even if it is grossly abnormal or resulted from an anomalous genetic condition, such as globozoosperma, Kartagener syndrome, immotile sperm, pin-head sperm, tail-stump syndrome etc. Current and future case reports on spermatid conception add little to the body of knowledge and understanding with respect to the efficacy and efficiency of ROSI and ELSI. Additional information gained only from animal studies (especially when no suitable animal model equivalent to human non-obstructive azoospermic condition is available) cannot confidently be extrapolated back to the human parallel, now an axiom of ICSI per se. We would argue that the time is now right to support controlled clinical trials using spermatids for conception in the human, under due professional care, as the only way forward. This must include assessing the parents and monitoring the offspring after obtaining genuine informed consent. We believe this should be done in limited centres of excellence and regulated, monitored and audited by the HFEA in the UK, and by similar organizations elsewhere.

References


