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The clinical potential for fertilization was examined by using the human sperm–hamster oocyte assay system after microinjection of round (RS), elongating (ES) or elongated (EtedS) spermatids retrieved from obstructive and non-obstructive azoospermic patients. Freshly isolated, in-vitro cultured and cryopreserved spermatids were utilized. For each category of microinjected spermatids, we demonstrated that the more mature the injected spermatid, the higher the incidence of fertilization (for freshly isolated spermatids, P < 0.006 and P < 0.008, for in-vitro cultured spermatids, P < 0.007 and P < 0.007 and for cryopreserved spermatids, P < 0.006 and P < 0.007 for obstructive and non-obstructive azoospermic patients respectively). Short term in-vitro culture of the spermatogenic cells did not improve the incidence of fertilization. However, cryopreservation significantly decreased (P < 0.001) the incidence of fertilization when each corresponding spermatogenic cell stage was compared. The incidence of fertilization was not statistically different when corresponding stages of spermatogenic cells were compared from obstructive and non-obstructive patients.

Key words: cryopreservation/hamster oocytes/in-vitro culture/male infertility/spermatids

Introduction

Spermatogenesis is a complex cellular differentiation process which starts at puberty and continues throughout the reproductive life of man (de Kretser and Kerr, 1994). 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 these extreme conditions where no spermatozoa are found in the ejaculate (azoospermia). Spermatids are the first haploid cells in the complex spermatogenic cell series and appear to contain the relevant functional constituents necessary to contribute to successful fertilization after microinjection (Fishel et al., 1996). The first human pregnancies and births by spermatids retrieved from testicular tissue (Fishel et al., 1995b) and ejaculate (Tesarik et al., 1995) generated interest and opportunity for patients suffering from intractable male factor infertility.

Since these initial reports, a number of clinics have reported limited success with the spermatid microinjection. However, these reports also show the highly variable and unpredictable outcome of human spermatid conception attempts, particularly in terms of failure of implantation and pregnancy loss (Hannay, 1995; Fishel et al., 1997; Yamanaka et al., 1997). Concerns relating to methodology of separation, isolation and identification of spermatids; immaturity of the genetic material; genomic imprinting; normality of the centrosome; presence of sperm-derived oocyte-activation factor; and the effects of short-term in-vitro culture and cryopreservation of spermatids have been noted (Aslam and Fishel, 1996; Fishel et al., 1996). We have demonstrated that homogeneous populations of round and elongating spermatids can be separated from testicular biopsies of azoospermic men (Aslam et al., 1998a), and these spermatids can be short-term in-vitro cultured and cryopreserved successfully (Aslam and Fishel, 1998). Studies in the mouse confirmed that differential DNA methylation of parental genes (genomic imprinting) is complete at the spermatid stage (Aslam et al., 1996).

However, clinical results indicate a persistently low incidence of fertilization with spermatid microinjection (Tesarik and Mendoza, 1996; Fishel et al., 1997; Vanderzwalmen et al., 1997; Kahraman et al., 1998). Apart from the problems of identification (Aslam et al., 1998b) a biochemical explanation might relate to incomplete transition of nuclear proteins from histone to protamine. Histone to protamine transition is a continuous process throughout spermatid maturation (Grimes, 1986; Poccia, 1986) and lack of protamine exposes the spermatid nucleus to the maturation promoting factors (M-phase promoting factors) of the oocyte. Consequently, premature chromosome condensation (PCC) can occur (Tesarik, 1996) causing failed fertilization. Successful in-vitro culture of round spermatids might support further nuclear maturation improve the incidence of fertilization, and provide a means for securing identification.

As the success rate with spermatid microinjection is low (Tesarik and Mendoza, 1996; Fishel et al., 1997), repeated testicular biopsies for subsequent treatment cycles can further compromise severely deficient but spermatogenically active seminiferous tubules. Therefore, there is a need to examine the effects of cryopreservation and subsequent use of spermatids for fertilization.

In this paper, we report the effects of short-term in-vitro culture and cryopreservation of human spermatids on the incidence of fertilization after microinjection into hamster oocytes.
**Materials and methods**

**Patients and consent**

During 1 1/2 years, from January 1995 to June 1996, testicular biopsies were obtained from 57 azoospermic patients (these patients had shown, at least once in the past, presence of spermatozoa, either in the ejaculate or on a testicular biopsy) who were offered treatment at the Department of Obstetrics and Gynaecology assisted conception unit (Nottingham University, UK), all of whom consented for part of the samples to be used for research purposes. Azoospermic patients were admitted for epididymal aspiration (EA) but on the day of treatment, sperm recovery failed and testicular biopsies were taken. Seventeen patients were suffering from obstructive azoospermia and in all these sperm recovery was successful. Forty patients were suffering from non-obstructive azoospermia (hypospermatogenesis, maturation arrest and Sertoli cell only syndrome, confirmed on previous diagnostic biopsies) and in 38 patients sperm recovery was successful. In two patients no spermatozoa were obtained for clinical use. Biopsies were taken in enriched Krebs–Ringer bicarbonate medium (EKRB). EKRB medium was also used during spermatid isolation procedures (Aslam et al., 1998a) because viability and integrity as determined by dye exclusion, oxygen consumption and morphology at the light and electron microscope levels improved when a complete medium such as EKRB was utilized throughout the isolation procedures (Romrell et al., 1976).

**Preparation of spermatozoa for microinjection**

Motile spermatozoa for control studies were prepared from semen of healthy human donors who gave written consent. An aliquot 250 µl of liquefied semen was diluted with 3 volumes of BWW (Biggers, Whitten, Whittingham) (Irvine Scientific, Santa Ana, USA) medium in a 15 ml Falcon tube and centrifuged at 500 g for 10 min. The supernatant was discarded and 1 ml BWW medium containing 0.45% human serum albumin (HAS) (Sigma, Poole, UK) was very carefully layered on top of the pellet. The tube was incubated at 37°C for 1 h. From the top of the medium 500 µl was carefully removed and transferred to a clean tube. After mixing, the sperm density and motility were assessed before the spermatozoa were used for microinjection.

**Isolation of spermatids**

After enzymatic digestion with trypsin (type III, Sigma) and DNase I (Sigma), purified populations of round spermatids (RS) and elongating spermatids (ES) were isolated from testicular biopsies of obstructive and non-obstructive azoospermic patients, using either velocity sedimentation under unit gravity (VSUG) combined with discontinuous Percoll centrifugation (DPC) technique or fluorescent-activated cell sorter (FACS) as described previously (Aslam et al., 1998a). Elongated spermatids (EtedS) were collected manually from mixed cell suspensions of testicular biopsies from obstructive azoospermic patients and non-obstructive azoospermic patients. Viability of isolated spermatids was examined using the Trypan Blue exclusion test (Aslam et al., 1998a). Morphological identification of RS, ES and EtedS has been discussed elsewhere (Aslam et al., 1998a).

**In-vitro culture of spermatids**

Prepared semen samples, mixed cell suspensions and isolated fractions of spermatids from obstructive and non-obstructive azoospermic patients were cultured in vitro by the method described previously (Aslam and Fishel, 1998). Spermatozoa (showing motility), round spermatids (with growing flagella), elongating spermatids and elongated spermatids excluding Trypan Blue after 42–48 h of in-vitro culture were collected manually and washed twice through BWW medium to remove excess dye.

**Cryopreservation**

Prepared semen samples, mixed cell suspensions and isolated fractions of spermatids from obstructive and non-obstructive azoospermic patients were cryopreserved by the method described previously (Aslam and Fishel, 1998). Cryopreserved germ cells were examined for their viability on different days (2 days to 3 months after cryopreservation) and only spermatozoa showing motility and round, elongating and elongated spermatids excluding Trypan Blue were collected manually and used for microinjection after two washes through BWW medium to remove excess dye.

**Media for oocytes**

BWW medium (Irvine Scientific) supplemented with streptomycin sulphate (0.1 mg/ml) (Sigma), penicillin (K+ salt) (100 IU/ml) (Sigma) and HAS (3.5 mg/ml) (Sigma) was used to culture hamster oocytes before and after microinjection. Similar ingredients were mixed in HEPES-buffered BWW medium to prepare microinjection medium.

**Collection of hamster oocytes**

Oocytes were collected from oviducts of superovulated female golden Syrian hamsters (2–4 months old). Superovulation was achieved by injecting 0.2 IU of pregnant mare’s serum gonadotrophin (PMSG; Foliglon; Intervet, Cambridge, UK) per g body weight followed by 0.2 IU of human chorionic gonadotrophin (HCG; Chorulon, Intervet) 48 h later. The animals were killed between 15 and 17 h after administration of HCG (Kamiguchi and Mikamo, 1986) and their ovaries were removed. Oocyte–cumulus complexes were collected by tearing ovarian tissue with needles under the microscope and then oocytes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (type IV-S) (Sigma) in HEPES-buffered BWW medium for 2 min at 37°C. Cumulus-free oocytes were washed and kept in culture medium droplets under oil at 37°C and 5% CO2 in air in a humidified atmosphere until microinjection was performed.

**Micromanipulation procedures**

All micromanipulations were performed using a TDU 500 micromanipulator (Research Instruments, Cornwall, UK) mounted on an inverted microscope (Olympus IMT 2). Screw-actuated pneumatic pumps (SAS 10/2, Research Instruments) were used to generate positive or negative pressures through holding and microinjection needles. Holding micro-pipettes of 100 µm outer and 30 µm inner diameter were prepared and the intracytoplasmic micro-pipettes were purchased commercially (Humagen; Fertility Diagnostics Inc., USA). The injection needle had an inner diameter of 7 µm, and a 25° bend 25 µm from the tip which incorporated a spike.

Intracytoplasmic injections of hamster oocytes were done with freshly isolated spermatozoa, RS, ES, EtedS or after short-term in-vitro culture or cryopreservation. The ratios of spermatid types from obstructive and non-obstructive azoospermic patients injected into hamster oocytes are shown in Tables I, II and III. All the microinjections were performed on mature M-II hamster oocytes. For microinjection, one single motile spermatozoon was aspirated from the spermatozoa droplet and was transferred to PVP droplet where its tail was permanently damaged using the microinjection pipette (Fishel et al., 1995a). The sperm was re-aspirated into the microinjection pipette. The oocyte was held by the holding pipette at the 9 o’clock position with respect to the polar body either at the 6 or 12 o’clock position. Then the injection needle was pushed through the zona pellucida and into the oocyte cytoplasm at the 3 o’clock position under negative pressure. Puncture of the oolemma was identified by sudden flow of cytoplasm into the injection needle and loss of tension in the oocyte membrane. The pressure in
Table I. Incidence of fertilization (2PN) after the microinjection of the freshly isolated spermatogenic cells into hamster oocytes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Type of cells injected</th>
<th>No. of oocytes injected</th>
<th>No. of oocytes survived (%)</th>
<th>1PN (%)</th>
<th>2PN (%)</th>
<th>3PN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>80</td>
<td>76</td>
<td>11</td>
<td>53a</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>80</td>
<td>(19.5)</td>
<td>(4.4)</td>
<td>(69.7)</td>
<td>(6.57)</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>200</td>
<td>186</td>
<td>23</td>
<td>45b</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>141</td>
<td>16</td>
<td>43c</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>96</td>
<td>12</td>
<td>39d</td>
<td>5</td>
</tr>
<tr>
<td>Non-obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>120</td>
<td>111</td>
<td>15</td>
<td>78e</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>120</td>
<td>114</td>
<td>15</td>
<td>00</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>300</td>
<td>276</td>
<td>43</td>
<td>64f</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>142</td>
<td>22</td>
<td>40g</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>95</td>
<td>12</td>
<td>36h</td>
<td>4</td>
</tr>
</tbody>
</table>

PN: pronuclear formation; RS: round spermatid; ES: elongating spermatid; EtedS: elongated spermatid.

ab, ac and ad P < 0.001, bc = NS, bd P < 0.006, cd = NS, ef, eg and eh P < 0.001, fg = NS, fh P < 0.008, gh, bf, cg and dh = NS.

Table II. Incidence of fertilization (2PN) after the microinjection of the short-term in-vitro cultured spermatogenic cells into hamster oocytes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Type of cells injected</th>
<th>No. of oocytes injected</th>
<th>No. of oocytes survived (%)</th>
<th>1PN (%)</th>
<th>2PN (%)</th>
<th>3PN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>80</td>
<td>74</td>
<td>12</td>
<td>52a</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>80</td>
<td>(92.5)</td>
<td>(16.2)</td>
<td>(70.2)</td>
<td>(5.9)</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>200</td>
<td>181</td>
<td>21</td>
<td>48b</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>144</td>
<td>18</td>
<td>47c</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>97</td>
<td>13</td>
<td>42d</td>
<td>5</td>
</tr>
<tr>
<td>Non-obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>120</td>
<td>113</td>
<td>14</td>
<td>79e</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>120</td>
<td>(94.16)</td>
<td>(12.3)</td>
<td>(69.9)</td>
<td>(5.3)</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>300</td>
<td>277</td>
<td>41</td>
<td>72f</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>143</td>
<td>17</td>
<td>45g</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>94</td>
<td>12</td>
<td>39h</td>
<td>6</td>
</tr>
</tbody>
</table>

PN: pronuclear formation; RS: round spermatid; ES: elongating spermatid; EtedS: elongated spermatid.

ab, ac and ad P < 0.001, bc < 0.279, bd P < 0.007, cd P < 0.122, ef, eg and eh P < 0.001, fg P < 0.284, fh P < 0.007, gh P < 0.150, bf P < 0.987, cg P < 0.932, dh P < 0.915.

the injection needle was quickly reversed and the needle was slowly removed once its contents were deposited in the ooplasm at the periphery opposite to the point of entry.

Round spermatids were difficult to aspirate into the injection needle due to their diameter being larger than that of the internal diameter of the injection needle. When suction pressure during round spermatid aspiration was increased gradually, this resulted in the distortion of the spermatid membrane as it was sucked into the injection needle. However, the spermatids were tightly fitted inside the injection needle and their expulsion into the oocyte was difficult. To overcome this difficulty, round spermatids were repeatedly aspirated and expelled in the PVP droplet (generally four or five times) through the injection needle. This caused damage to their cell membranes and the spermatids turned into droplets of cytoplasm in which nucleus was present. The entire content of the spermatid (nucleus and loose cytoplasm) was aspirated into the injection needle and deposited into the ooplasm, as described above for the spermatozoon.

Elongating and elongated spermatids had diameters smaller than the internal diameter of injection needle, so they were easily aspirated. Elongating and elongated spermatids were aspirated individually after damaging whatever tail was visible with the microinjection needle in the PVP droplet and injected into the oocyte as described above.

After microinjection, oocytes were washed four times with culture medium and were left to incubate overnight at 37°C under a humidified
Hamster oocyte sperm injection assay

Table III. Incidence of fertilization (2PN) after the microinjection of the cryopreserved spermatogenic cells into hamster oocytes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Type of cells injected</th>
<th>No. of oocytes injected</th>
<th>No. of oocytes survived (%)</th>
<th>1PN (%)</th>
<th>2PN (%)</th>
<th>3PN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>80</td>
<td>(91.25)</td>
<td>9</td>
<td>42 a</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>80</td>
<td>(95)</td>
<td>11</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>200</td>
<td>(92)</td>
<td>21</td>
<td>24 b</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>(96)</td>
<td>18</td>
<td>26 e</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>(95)</td>
<td>13</td>
<td>26 f</td>
<td>5</td>
</tr>
<tr>
<td>Non-obstructive azoospermia</td>
<td>Spermatozoa</td>
<td>120</td>
<td>(95)</td>
<td>14</td>
<td>65 g</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>120</td>
<td>(94.16)</td>
<td>16</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td></td>
<td>RS</td>
<td>300</td>
<td>(90.66)</td>
<td>36</td>
<td>33 h</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>150</td>
<td>(93.3)</td>
<td>17</td>
<td>23 i</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>EtedS</td>
<td>100</td>
<td>(94)</td>
<td>11</td>
<td>23 j</td>
<td>5</td>
</tr>
</tbody>
</table>

PN: pronuclear formation; RS: round spermatid; ES: elongating spermatid; EtedS: elongated spermatid.

ab, ac and ad P < 0.001, bc = NS, bd P < 0.006, cd = NS, ef, eg and eh P < 0.001, fg = NS, fh P < 0.007, gh, bf, cg and dh = NS.

5% CO₂ atmosphere. They were observed the next day after (16–18 h) for the presence of pronuclei. Fertilization was considered successful when two pronuclei (with two polar bodies) were seen under the microscope.

Statistical analysis
Difference in the incidence of fertilization between spermatid groups was evaluated using the χ²-test for comparison between groups.

Results

Microinjections of freshly isolated spermatogenic cells
Table I shows the incidence of fertilization (2PN formation) after the microinjection of freshly isolated spermatogenic cells isolated from obstructive and non-obstructive azoospermic patients into hamster oocytes. When different maturation stages of spermatogenic cells were compared, results showed that the incidence of fertilization increased with the increased maturity of the injected cells (P < 0.006 when round spermatid microinjections were compared with elongated spermatids for obstructive azoospermic patients and P < 0.008 for non-obstructive azoospermic patients).

Microinjections of in-vitro cultured spermatogenic cells
Table II shows the incidence of fertilization (2PN formation) after microinjection of in-vitro cultured spermatogenic cells isolated from obstructive and non-obstructive azoospermic patients into hamster oocytes. When different maturation stages of in-vitro cultured spermatogenic cells were compared, the incidence of fertilization increased with the increased maturity of the injected cells (P < 0.007 when round spermatid microinjections were compared with elongated spermatids for obstructive azoospermic patients as well as for non-obstructive azoospermic patients).

Microinjections of cryopreserved spermatogenic cells
Table III shows the incidence of fertilization (2PN formation) after the microinjection of cryopreserved spermatogenic cells isolated from obstructive and non-obstructive azoospermic patients into hamster oocytes. When different maturation stages of cryopreserved spermatogenic cells were compared, the incidence of fertilization increased with the increased maturity of the injected cells (P < 0.006 when round spermatid microinjections were compared with elongated spermatids for obstructive azoospermic patients and P < 0.007 for non-obstructive azoospermic patients).

Discussion

This study demonstrated that fertilization (2PN) can be achieved after microinjection of human spermatids into hamster oocytes. The results also demonstrated that the more mature the injected spermatid, the higher the incidence of fertilization. In each category of microinjected cells, i.e. freshly isolated, in-vitro cultured and cryopreserved spermatids, the incidence that fertilization rate was not statistically different when each stage of spermatogenic cells isolated from obstructive azoospermic patients was compared with its counterpart stage isolated from non-obstructive azoospermic patients. The results also showed that short term in-vitro culture of the spermatogenic cells did not improve the incidence of fertilization. However, cryopreservation significantly decreased (P < 0.001) the incidence of fertilization when each freshly isolated injected spermatogenic cell stage was compared with its corresponding cryopreserved stage.

The direct injection of a spermatozoon into the cytoplasm of the oocyte (ICSI) is not a recent innovation. Sperm injection, subsequent oocyte activation and fertilization have been used for many years as a tool in echinoderm (Hiramoto, 1962) and
amphibian research (Brun, 1974). Although efforts to produce hamster young from in-vitro produced zygotes were hampered by difficulty in culturing hamster zygotes (Whittingham and Bavister, 1974; Schini and Bavister, 1988), the hamster system became the most commonly used model for sperm microinjection studies because its oocytes can tolerate rather harsh microsurgical operation and show satisfactory preimplantation development when cultured in BWW medium after micro-injection (Uehara and Yanagimachi, 1976). Furthermore, the behaviour of sperm nuclei injected into hamster oocytes and the mechanisms involved in the pronucleus formation are known (Perreault, 1990). After microinjection into hamster oocytes, heterologous spermatozoa (e.g. guinea-pig, human) have been shown to undergo transformation to pronuclei (Yanagimachi, 1972; Uehara and Yanagimachi, 1976; Usui and Yanagimachi, 1976) and initiate DNA synthesis (Naish et al., 1984). Trans- formation of female nucleus into female pronucleus starts after oocyte activation. Nearly 50% of oocytes complete their second meiotic division 2 h after microinjection (Nagy et al., 1994; Payne et al., 1997) to make available their chromosomes for syngamy. So, by the time female chromosomes are available for syngamy, the spermatic chromosomes will already have developed PCC resulting in failed fertilization. Under these circumstances, nuclei of round spermatids are more vulnerable than those of elongated spermatids because advanced histone to protamine transition in elongated spermatids will protect them from ooplasmic MPF. This might explain why in this study, the incidence of fertilization was significantly better with elongated spermatids as compared to round spermatids for each category.

The timing of male pronucleus formation is controlled tightly by the MPF present in the oocyte cytoplasm. MPF gradually disappears from the oocyte cytoplasm after activation and is no longer detectable 2 h after activation. Complete remodelling of male nuclei with reconstitution of pronuclei is thus possible only during a short period between metaphase II and telophase II of meiosis (Szollosi et al., 1988). Transformation of female nucleus into female pronucleus starts after oocyte activation. Nearly 50% of oocytes complete their second meiotic division 2 h after microinjection (Nagy et al., 1994; Payne et al., 1997) to make available their chromosomes for syngamy. So, by the time female chromosomes are available for syngamy, the spermatic chromosomes will already have developed PCC resulting in failed fertilization. Under these circumstances, nuclei of round spermatids are more vulnerable than those of elongated spermatids because advanced histone to protamine transition in elongated spermatids will protect them from ooplasmic MPF. This might explain why in this study, the incidence of fertilization was significantly better with elongated spermatids as compared to round spermatids for each category.

When the effect of in-vitro culture on the motility of fresh and frozen-thawed human testicular spermatozoa obtained from both obstructive azoospermic and non-obstructive azoo- spermic patients was evaluated, the results showed the significant improvement of sperm motility (Liu et al., 1997; Zhu et al., 1997). During the present study, a positive but not significant effect of in-vitro culture on the incidence of fertilization was obtained. This might be explained by the duration of histone to protamine transition during spermatid maturation (Grimes, 1986). Radioautograph studies have deter- mined that during human spermatogenesis one cycle of the seminiferous epithelium is completed in 16 days (i.e. spermio- genesis in 17–18 days) and the whole of spermatogenesis in approximately 64 days (Heller and Clermont, 1963). Spermatid maturation, which takes about 16 days to complete in vivo, cannot be accelerated in vitro, especially within 48 h. However, the growth of flagella provided an excellent means of identi- fication of viable round spermatids. This approach may be useful if identification is a problem; and a lower incidence of fertilization than occurred in this study could be attributed to erroneous identification.

Recently, a pregnancy with a cryopreserved spermatid was reported with the normal karyotype at 16 weeks of gestation (Antinori et al., 1997). Results of the present study also confirmed that cryopreserved spermatids can be used to procure fertilization. Although cryopreservation significantly reduces the incidence of 2 PN formation, cryopreservation of spermatids could save azoospermic patients repeating the surgical proced-
ure each time a treatment cycle is planned. Enough spermatids can be isolated from a single testicular biopsy which can be cryopreserved for the subsequent use.

The results of the present study confirmed that human spermatids from azoospermic patients can be microinjected into oocytes to procure fertilization when spermaotroa are not available for clinical use, that the more mature the spermatid, the higher the incidence of fertilization, and that cryopreserved spermatids are viable. However, the results of this study also confirm that the incidence of fertilization with spermatids remains significantly lower than with spermaotroa. Further studies are necessary to find a safe mechanism to optimize the incidence of fertilization, and to determine molecular aspects, including the onset of genomic imprinting, during spermatid maturation.

References


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