Ooplastic injections of rabbit round spermatid nuclei or intact round spermatids from fresh, cryopreserved and cryostored samples*

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We compared the outcome of ooplastic round spermatid nuclear injections (ROSNI) versus intact round spermatid injections (ROSI). Rabbit round spermatid nuclei and intact round spermatids were recovered and injected into rabbit oocytes (groups A and B, respectively). Fertilization, cleavage and embryonic development rates were compared. In additional studies, five protocols for cryopreservation of round spermatids and two protocols for cryostorage of round spermatids were applied. The outcome of ROSNI techniques using frozen–thawed or cryostored~warmed round spermatids was evaluated. The cleavage rate and the overall morula plus blastocyst development rate were significantly larger in group A than group B. ROSNI procedures are superior to ROSI techniques in the rabbit. The largest fertilization, cleavage and embryonic development rates after ROSNI techniques using cryopreserved or cryostored round spermatids were demonstrated in groups of round spermatids in which a mixture of seminal plasma plus test yolk buffer was employed as an extender, and dimethyl sulphoxide plus a high concentration of glycerol served as cryoprotectants. It appears that the seminal plasma contains factors protecting round spermatids during cryopreservation or cryostorage, and/or the employment of two cryoprotectants has a beneficial role in the maintenance of round spermatid reproductive capacity.

Key words: cryopreservation/cryostorage/cryptorchidism/rabbits/spermatids

Introduction

The male gamete that has just completed the second meiotic division in the testis is the round spermatid. Therefore, the nucleus of the round spermatid contains a complete haploid set of chromosomes. It has been shown that round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy (Ogura and Yanagimachi, 1993). However, the developmental potential of the obtained zygotes was not evaluated in that study. Recent studies have demonstrated that ooplastic injections of round spermatid nuclei (ROSNI) or intact round spermatids (ROSI) in the rabbit (Sofikitis et al., 1994a, 1996a,b, 1997a), mouse (Ogura et al., 1993, 1994; Sasagawa and Yanagimachi, 1997) and bovine (Goto et al., 1996) species can result in fertilization and pregnancies.

The above studies raised the possibility of treating non-obstructive azoospermia with ROSNI or ROSI techniques (Edwards et al., 1994; Sofikitis et al., 1994a). It is known that patients with a diagnosis of spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome, occasionally may have rare foci of round spermatids somewhere in the testicles (for review, see Sofikitis et al., 1998a). The term ‘complete spermiogenesis failure’ was used for men in whom the most advanced germ cell present in the therapeutic testicular biopsy material is the round spermatid (Amer et al., 1997).

For the latter men, ROSNI and ROSI techniques represent the only hope to father their own children. The first human ROSNI pregnancies were achieved in 1994 and reported in 1995 (Hannay, 1995; Sofikitis et al., 1995a). However, these pregnancies resulted in abortions. A few months later, deliveries of two healthy children after ROSI techniques were reported (Tesarik et al., 1995). A high activation and fertilization rate of human oocytes following ROSNI procedures was reported (Yamanaka et al., 1997). Full-term pregnancies after ROSNI/ROSI techniques were achieved by a number of authors (Antinori et al., 1997a,b; Vanderzwalmen et al., 1997; Sofikitis et al., 1997b and Barak et al., 1998).

In the mouse, transferring the intact round spermatid into oocytes results in a lower fertilization rate than transferring the round spermatid nucleus (Ogura et al., 1993; Kimura and Yanagimachi, 1995a). This may be due to the following two reasons: first, the use of injecting micropipettes of larger diameter is necessary for ROSI techniques and subsequently the probability of injuring oocytes is higher during ROSI procedures; and second, the persistence of a significant amount of cytoplasm around the round spermatid nucleus may impede its transformation into a male pronucleus within the ooplasm (Kimura and Yanagimachi, 1995a,b). In contrast, ROSI techniques have the following theoretical advantages over ROSNI procedures: (i) ooplastic injection of the intact round spermatid cell ensures the transfer of all the cytoplasmic components of the male gamete into the female gamete; (ii) ROSI techniques are less time-consuming than ROSNI techniques; and (iii) mechanical or chemical manipulations of the nuclear matrix and envelope of the male gamete are avoided when ROSI procedures are applied.

The first objective of the present study was to compare the


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capacity of the intact round spermatid versus the round spermatid nucleus to activate oocytes, fertilize oocytes, and initiate embryonic development. Furthermore, the speed of embryonic development following ROSNI or ROSI techniques was compared. Such a study is clinically important to appreciate whether the early haploid male gamete should be ooplasmically injected intact or after rupture of its cytoplasm.

A second objective was to develop an optimal protocol for cryopreservation of round spermatids. There are only two reports in the international literature which deal with cryopreservation of round spermatids (Ogura et al., 1996; Antinori et al., 1997b). However, the latter studies did not attempt to develop new media specific for the cryopreservation of round spermatids, but rather applied standard protocols originally developed for spermatozoal cryopreservation. Considering that there are cytostructural differences between round spermatids and spermatoozoa, and that the cryopreservation process is a cell-specific technique (Mazur, 1970), our efforts were directed to the development of media ‘specific’ for cryopreservation of round spermatids. The overall purpose was to compare the fertilizing capacity of groups of round spermatids frozen in media containing different extenders and cryoprotectants. Such a study is clinically important since successful cryopreservation of round spermatids offers the possibility to non-obstructed azoospermic men who have failed in a ROSNI/ROSI trial to participate in an additional cycle using frozen–thawed spermatids instead of undergoing another testicular biopsy (Antinori et al., 1997b).

A third objective of the current study was to develop a simple method for short-term cryostorage of round spermatids that would not require specific equipment and could be readily available in most laboratories. A significant percentage of non-obstructed azoospermic men have ejaculates positive for round spermatids (Mendoza and Tesarik, 1996; Sofikitis et al., 1998a). However, preliminary studies in our facilities have demonstrated that there is a great degree of variation regarding the presence, stage and number of round germ cells present in different ejaculates from one individual (Sofikitis et al., 1998a). Isolation of round spermatids from ejaculates of non-obstructed azoospermic men a few days prior to the oocyte retrieval day and successful short-term cryostorage offers them the possibility to avoid a therapeutic testicular biopsy if the semen sample(s) collected at the day of oocyte recovery is/are negative for round spermatids.

**Materials and methods**

**Comparison of ROSI versus ROSNI**

Recovery and preparation of oocytes: New Zealand White female rabbits were superovulated as previously described (Sofikitis et al., 1994a, 1996a,b). Oocytes were collected and washed three times in modified RD medium (standard mixture of RPMI medium and Dulbecco’s medium; Sofikitis et al., 1994a, 1996a,b). At 3–5 h post-recovery, oocytes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (type VIII; 320 units/mg solid; Sigma Chemical Co., St Louis, MO, USA). Cumulus-free oocytes were rinsed and mature oocytes processed for ooplasmic injections. Induction of left cryptorchidism: Six-month-old male New Zealand White rabbits ($n = 8$) were anaesthetized with intravenous injections of sodium pentobarbital (30 mg/kg; Nembutal, Abbott Laboratories, Chicago, IL, USA) as previously described (Sofikitis et al., 1994b). The abdominal cavity was opened longitudinally and the left testis placed into the abdominal cavity and fixed to the posterior abdominal wall muscles by placing a nylon suture into the tunica albuginea of the testis. At 6 weeks after surgery, a small piece from each left testis was processed for haematoxylin and eosin staining. The remaining fragment of the left testis was processed for the isolation of round spermatids.

Isolation of round spermatids: At 6 weeks post-cryptorchidism induction, spermatogenic cells were dispersed from the seminiferous tubules of the left testicles of the cryptorchid rabbits and resuspended in modified Dulbecco’s phosphate-buffered saline (DPBS) as described previously (Sofikitis et al., 1994a, 1996a,b). The spermatogenic cells were then observed via a confocal laser scanning microscope, computer-assisted system (Apple Computer Inc., Cupertino, CA, USA) attached to a micromanipulator (MO-204 Narishige, Tokyo, Japan) as described previously (Sofikitis et al., 1994c). The confocal laser scanning microscope had the capacity to provide three-dimensional images of live cells under high magnification without staining or damaging the cells (Hyodo et al., 1991). Thus, live cells could be used further for a variety of in-vitro procedures after observation. Furthermore, the confocal laser scanning microscope allows selective intracellular observation at various depths. Rabbit round spermatids of stages 3 to 5 were recognized as described previously (Sofikitis et al., 1997a), aspirated via a micropipette, and transferred to SOF medium (Yamanaka et al., 1997; Sofikitis et al., 1998b). The internal diameter of the micropipette was 20 µm. The above methodology has been proven to be accurate to select round spermatids of a specific stage (Sofikitis et al., 1997a, 1998a).

Nuclei isolation: A subpopulation of selected round spermatids was transferred from droplets of SOF medium to droplets of a hypertonic sucrose solution (Sofikitis et al., 1994a, 1996a,b) and subsequently resuspended in the same hypertonic solution containing Triton X-100 (0.035%, v/v). The round spermatids remained within droplets of this hypertonic solution for 8–10 min. During the latter period, gentle pipettings were applied. Finally, the nuclei of the round spermatids were collected from the hypertonic solution via a micropipette and transferred to droplets of SOF medium.

**ROSNI/ROSI procedures**: Isolated nuclei and intact round spermatids were washed five more times in SOF medium, resuspended in the same medium without cholesterol, supplemented with 1 mM MgCl$_2$ and 10% polyvinylpyrrolidone (molecular weight 360 000 Da; PVP K90, ICN Biochemicals, Costa Mesa, CA, USA), and kept at 34°C.

Oocytes had been preincubated at 10°C under 5% carbon dioxide in air for 2 h (Sofikitis et al., 1996a). They were then incubated in the presence of A23187 (10 µM; C7522, Sigma) at 37°C under 5% carbon dioxide in air for 8 min (Sofikitis et al., 1997a). The oocytes were subsequently transferred to modified RD medium (Sofikitis et al., 1997a) for injections. A single round spermatid nucleus or an intact round spermatid was injected into each oocyte. The time interval between treatment of oocytes with A23187 and ooplasmic injections was 30–40 min. Each nucleus or intact round spermatid had been aggressively compressed by the tip of the injecting micro-pipette eight to ten times. Preliminary experiments demonstrated that the above action on the nuclear surface or intact round spermatid surface prior to ooplasmic injections has beneficial effects on the oocyte fertilization process (Sofikitis et al., 1997a). The internal diameter of the injecting micropipettes for both ROSNI and ROSI techniques was 9–10 µm. Each oocyte was positioned with the first polar body at 12 o’clock and punctured directly at 3 o’clock by the
micropipette. During ooplasmic injections a great deal of care was taken to introduce as little medium as possible into the oocyte by placing the nucleus or the intact round spermatid close to the tip of the micropipette prior to entry into the oocytes. After the tip of the injecting micropipette had penetrated the vitelline membrane and approached the centre of the oocyte, ooplasmic content and the nucleus or the intact round spermatid were drawn into the micropipette to a distance equal to approximately twice the oocyte diameter, the aspirated ooplasm was expelled into the cytoplasm of the oocyte, and the nucleus or the intact round spermatid was placed again at the tip of the micropipette. The above ooplasmic aspirating/expelling manoeuvres were repeated five times (Sofikitis et al., 1997a) and finally the aspirated cytoplasm of the oocyte, together with the spermatid nucleus or the intact round spermatid and a small amount of medium were expelled into the oocyte. Forty-one (group A) mature oocytes were successfully injected with round spermatid nuclei, whereas 43 oocytes were successfully injected in a similar fashion with intact round spermatids (group B). Five to six round spermatid nuclei or intact round spermatids from each cryptorchid rabbit were processed for ooplasmic injections of five to six oocytes of groups A or B, respectively. The injections were performed under ×400 magnification provided by an inverted microscope (Olympus IX-70, Tokyo, Japan). The injected oocytes were transferred to modified RD medium supplemented with antioxidants (taurine, 5 mmol/l; Sigma) (Li et al., 1993) and incubated at 37°C under 5% carbon dioxide for 72 h. Oocytes were observed at 5, 9, 18, 24 and 72 h post-injection in an attempt to better appreciate the speed of embryonic development after ROSNI or ROSI techniques. Previous studies have shown that the optimal time for observation of two pronuclei after ROSNI procedures is 9 h post-injection (Sofikitis et al., 1995a, 1998a).

Medium was changed at 24 h post injection. An activated oocyte was defined as one with a female pronucleus and a second polar body (Kimura and Yanagimachi, 1995a).

Statistical analysis on activation, fertilization, cleavage and embryonic development rates in all the experiments of the current study (A, B and C; see below) was performed using the chi-square test. A probability \( P < 0.05 \) was considered to be statistically significant.

Studies on cryopreservation of round spermatids

Round spermatids of stages 3 to 5 were isolated from the left testicles of the cryptorchid rabbits and their viability was evaluated using Trypan blue (Sofikitis et al., 1996a). They were then suspended in droplets of extenders C, D, E, F and G (groups of round spermatids C, D, E, F and G respectively). More than 300 round spermatids of each group were suspended in each droplet (volume equal to 400 µl; four droplets/group) of the respective extender. Extender C contained Tris and TES buffers (Sigma) (TEST-buffer), each of them having an osmolarity of 330 mOsm and a pH equal to 7.2. Fresh egg yolk and glycerol were added to the TEST buffer (Zwitterion extender) at 20% and 10% respectively. Penicillin G (0.060 g/l) was also added to the TEST buffer. Extender D was prepared as extender C with the difference that glycerol had been added at 30%.

Extender E was prepared as the extender D. However, it additionally contained citrate (7.2 mg/ml), cholesterol (10.3 mg/ml), and fructose (8.4 mg/ml). To prepare the extender F (Table I), a volume of extender D was mixed with an equal volume of seminal plasma from semen samples produced by a mature healthy rabbit. Semen samples were collected using an artificial vagina, as described previously (Sofikitis et al., 1994b), centrifuged at 7000 g for 30 min, and the supernatant (seminal plasma) was collected. Microscopic observation of the seminal plasma samples post-centrifugation revealed absence of either spermatozoa or round cells. Extender F also contained dimethyl sulphoxide (0.5 mmol/l). Milk containing 3.6% of fatty acids diluted with an equal volume of extender D served as extender G. Before suspension of spermatids to the extender G, milk was heated at 95°C for 10 min to inactivate lactein, an anti-streptococcal substance which is toxic for the male gamete. The final concentration of glycerol in extenders F and G was 30%. After transferring the round spermatids of each group to the droplets of the respective extender, all droplets were transferred to 500 µl microtubes and refrigerated at 2–4°C to allow a slow cooling. After a 60-min period, each sample was allowed to freeze slowly in liquid nitrogen. Microtubes were placed at –2°C for 25 min, –40°C for 10 min, and –90°C for 10 min. The sample was taken to –196°C finally by plunging it directly into liquid nitrogen.

Samples were removed from liquid nitrogen 5–6 months later. They were then incubated within a water bath at 34°C and diluted with an equal volume of SOF medium. The viability of fractions of frozen–thawed round spermatids was evaluated. Round spermatids were then processed for nuclear isolation. Recovered nuclei from each group were processed for ROSNI techniques. Injected oocytes (50, 48, 52, 48 and 51 oocytes of groups C, D, E, F and G respectively) were cultured in modified RD medium supplemented with antioxidants (see above) for 72 h. Medium was changed at 24 h of culture. Oocytes were observed at 9, 24 and 72 h post-injection. Proportions of fertilized oocytes (oocytes with two pronuclei plus second polar body) to injected oocytes, cleaved oocytes to injected oocytes, and morulae plus blastocysts to injected oocytes were compared among groups C, D, E, F and G. Values of the percentage of live spermatids were expressed as mean ± SD. Differences among mean values of the latter parameter were analysed statistically using analysis of variance plus Duncan’s multiple range test.

Studies on short-term cryostorage of round spermatids

Round spermatids of stages 3 to 5 were isolated from the left testicles of healthy mature New Zealand White rabbits as described above for cryptorchid rabbits. The percentage of live round spermatids was measured and fractions of round spermatids were suspended in droplets (400 µl) of extender F (round spermatids of group D-1) or droplets of equal volume of extender D (round spermatids of group D-1). More than 400 round spermatids were suspended in each droplet of the respective extender (four droplets per group). Droplets of groups F-1 and D-1 were gradually cooled to 5°C for 52 to 63 h in a manner similar to that described by Kofinas and Zavos (1992) for short-term cryopreservation of spermatozoa (Kofinas and Zavos, 1992). At the end of the cryostorage period samples were incubated at 34°C under 5% carbon dioxide in air for 1 h and subsequently diluted with an equal volume of SOF medium. Viability of fractions of round spermatids was assessed and samples were then processed for nuclear isolation and nuclear ooplasmic injections. Forty-seven and 50 rabbit mature oocytes were successfully injected with round spermatid nuclei of groups F-1 and D-1 respectively. Oocytes were cultured for 72 h post-injection. Fertilization, cleavage and embryonic development rates were recorded.

Results

ROSNI versus ROSI

The peak of oocyte activation after ROSNI or ROSI techniques was demonstrated at 5 or 9 h post-injection respectively. The peak of the oocytes with two pronuclei plus second polar body (normally fertilized oocytes) was demonstrated 9 h following the former procedure, whereas it was shown 18 h following the latter technique. Within each group A or B, the largest number of cleaved oocytes was
Immature germ cell ooplasmic injections

Table I. Media for cryopreservation of round spermatids

<table>
<thead>
<tr>
<th>Group</th>
<th>Extender</th>
<th>Cryoprotectant</th>
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<tbody>
<tr>
<td>C</td>
<td>TEST buffer–egg yolk</td>
<td>Glycerol (10%)</td>
</tr>
<tr>
<td>D</td>
<td>TEST buffer–egg yolk</td>
<td>Glycerol (30%)</td>
</tr>
<tr>
<td>E</td>
<td>TEST buffer–egg yolk</td>
<td>Glycerol (30%)</td>
</tr>
<tr>
<td>F</td>
<td>TEST buffer–egg yolk–seminal plasma</td>
<td>Glycerol (30%) and DMSO (0.5 M)</td>
</tr>
<tr>
<td>G</td>
<td>TEST buffer–egg yolk–milk</td>
<td>Glycerol (30%)</td>
</tr>
</tbody>
</table>

DMSO = dimethyl sulphoxide.

*See also Materials and methods.

Table II. Comparison of round spermatid nuclear injection (ROSNI, group A) versus round spermatid injection (ROSI, group B) outcome: dynamics of embryonic development

<table>
<thead>
<tr>
<th>Post-injection period</th>
<th>Injected oocytes</th>
<th>Activated oocytes (ooocytes that demonstrate or have demonstrated 2nd PB plus at least one female PN)</th>
<th>Oocytes demonstrating 2PN (male and female) plus 2nd PB</th>
<th>Normally fertilized oocytes at the process of cleavage or with at least one mitotic division completed</th>
<th>Normally fertilized oocytes developed up to the stage of morula or blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 h</td>
<td>9 h</td>
<td>18 h</td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Injected oocytes</td>
<td>41</td>
<td>43</td>
<td>41</td>
<td>43</td>
<td>41</td>
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<tr>
<td>Activated oocytes</td>
<td>36</td>
<td>21</td>
<td>36</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Activated oocytes</td>
<td>36</td>
<td>21</td>
<td>36</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocytes demonstrating 2PN (male and female) plus 2nd PB</td>
<td>9</td>
<td>2</td>
<td>33</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normally fertilized oocytes at the process of cleavage or with at least one mitotic division completed</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td></td>
<td></td>
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<tr>
<td>Normally fertilized oocytes developed up to the stage of morula or blastocyst</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

9 h: Activated oocytes/injected oocytes, ROSNI versus ROSI (statistically non-significant).
18 h: Fertilized oocytes/injected oocytes, ROSNI versus ROSI, P < 0.05 (statistically significant).
24 h: Cleaved oocytes/injected oocytes, ROSNI versus ROSI, P < 0.05 (statistically significant).
72 h: (Morulae plus blastocysts)/injected oocytes, ROSNI versus ROSI, P < 0.05 (statistically significant).

PB = polar body; PN = pronucleus.

At 24 h post-injection, the number of morulae/blastocysts was also significantly larger in group A than in group B (Table II).

Cryopreservation of round spermatids recovered from cryptorchid rabbits

The percentage of live round spermatids was significantly larger (P < 0.05) in fractions of round spermatids prior to cryopreservation (89 ± 6%) than post-cryopreservation in group C (67 ± 7%). In addition, the percentage of live round spermatids post-cryopreservation was significantly smaller in group C than in groups D (77 ± 7%), E (80 ± 11%), F (84 ± 8%) and G (79 ± 9%).

The proportion of fertilized oocytes to injected oocytes (fertilization rate) at 9 h post-injection, the proportion of cleaved oocytes to injected oocytes (cleavage rate) at 24 h post-injection, and the proportion of morulae plus blastocysts to injected oocytes (embryonic development rate) were significantly larger (P < 0.05) in groups E, F and G than in groups C and D (Table III). The values of the latter three parameters were significantly smaller in groups C and D than in group A (fresh round spermatids from cryptorchid rabbits; Table II). In contrast, differences in the fertilization rate, cleavage rate and embryonic development rate between group A and groups E, F and G were not significant. Among the protocols for
cryopreservation, the highest fertilization rate, cleavage rate and embryonic development rate were observed in group F. However, values of the latter three parameters were not significantly different among groups E, F and G (Table III).

**Short-term cryostorage of round spermatids recovered from healthy rabbits**

Differences in the percentage of live round spermatids in fractions of round spermatids before cryostorage (93 ± 6%) and post-cryostorage in groups F-1 (88 ± 8%) and D-1 (86 ± 9%) were not significant. Differences in the fertilization rate, cleavage rate and embryonic development rate between groups F-1 and D-1 were significant (P < 0.05; Table IV).

Haematoxylin and eosin staining of left testicular cryptorchid tissue revealed spermatogenic arrest at the primary spermatocyte stage in the great majority of the seminiferous tubules (Figure 1). However, in a small number of tubules round spermatids were observed.

**Discussion**

The first human pregnancies using ooplasmic injections of round spermatid nuclei were reported in April 1995 (Hannay, 1995; Sofikitis et al., 1995a,b). However, these pregnancies resulted in abortions. During the following four years, additional human pregnancies and the delivery of healthy children after ROSI/ROSNI techniques were reported (Tesarik et al., 1995; Mansour et al., 1996a; Antinori et al., 1997a,b; Vanderzwalmen et al., 1997; Barak et al., 1998). Ooplasmic injections of round spermatids or round spermatid nuclei recovered from men with complete spermatogenic arrest resulted in the delivery of healthy offspring. Candidates for ROSNI/ROSI techniques may be men with complete spermiogenesis failure (Amer et al., 1997), spermatogenic arrest at the round spermatid stage, or men with spermatogenic arrest at the primary spermatogenic stage or Sertoli cell-only syndrome in whom a number of germ cells in a limited number of seminiferous tubules can break the pre-meiotic spermatogenic block and differentiate up to the round spermatid stage (Sofikitis et al., 1998a). Objections to the ROSNI/ROSI techniques were raised (Sailboat and Johnson, 1998) since it was contended that when spermatozoa are absent in the testicular biopsy specimen, round spermatids are also absent. However, the thesis of Sailboat and Johnson (1998) may not be adopted since: (a) their observations refer to a limited number of participants; (b) they attempted to identify human...
Immature germ cell ooplasmic injections

Figure 1. Absence of spermatozoa and spermatids within the lumen of a seminiferous tubule of a cryptorchid rabbit (original magnification ×600). The most advanced spermatogenic cells are primary spermatocytes (arrows). Scale bar = 30 µm.

Figure 2. Observation of a rabbit round spermatid via a confocal laser scanning microscope (original magnification ×2500); computer-assisted system (×1).

Figure 3. Ooplasmic injection of a rabbit round spermatid nucleus (arrow) into a rabbit oocyte. Original magnification ×400.

Figure 4. Observation of a rabbit round spermatid (black arrow) via Nomarski lens (original magnification ×600). Within the round spermatid a black spot (white arrow) representing the acrosomal granule/cap is observable.

round spermatids via Nomarski or Hoffman lenses, although they admit that it was very difficult for them to do so using this technology; (c) the most reliable methodology for round spermatid identification (i.e. transmission electron microscopy or confocal laser scanning microscopy) were not applied; (d) their results refer mainly to histological observations of diagnostic testicular biopsy images and not to detailed observations under high magnification of dispersed cells of therapeutic testicular biopsy material (Silbert et al., 1997; Silbert and Johnson, 1998); (e) several studies by independent groups clearly indicate that complete arrest in spermiation is not rare in non-obstructed azoospermic men (Sofikitis et al., 1998a); and (f) specific testicular pathophysiology (i.e. reduction in intratesticular testosterone content) are known to be accompanied by inability of round spermatids to undergo the elongation process (O'Donnell et al., 1996; Tesarik et al., 1998b).

The present study shows clearly that the fertilization rate, cleavage rate and embryonic development rate in vitro are significantly lower when the whole (intact) round spermatid (Figures 2–4) is injected into the oocyte. The lower outcome of ooplasmic injections of integral round spermatids may not result from injury of the oocytes due to the larger size of micropipettes used for injections since micropipettes of the same diameter were used for both techniques. To explain the superior outcome of ROSNI techniques versus ROSI procedures in the rabbit and the faster speed of oocyte activation, fertilization, cleavage and embryonic development after the former techniques, we may adopt the thesis that the presence of a significant amount of cytoplasm around the round spermatid nucleus may impede or delay its transformation into the male pronucleus (Ogura et al., 1993; Kimura and
et al, and subsequently for male pronucleus formation (Sofikitis, prerequisite for the decondensation of the male gamete nucleus and nuclear decondensation. It is known that rupture of the male gamete nuclear membrane within the ooplasm is a prerequisite for the decondensation of the male gamete nucleus and subsequently for male pronucleus formation (Sofikitis et al., 1998a). Furthermore, a large amount of cytoplasm around the round spermatid nucleus may impede or delay the liberation of the nucleus-associated oocyte-activating substance present in spermatids/spERMatozoa (OASIS; Kimura and Yanagimachi, 1995a; Sofikitis et al., 1998a) and may affect subsequently the process of oocyte activation which is a prerequisite for the development of male and female pronuclei. The above mechanism(s) may offer additionally an explanation for the delayed peak of the number of oocytes with two pronuclei plus second polar body after ROSI techniques compared with ROSNI techniques (18 h versus 9 h respectively). Furthermore, the above hypotheses may explain the early beginning of cleavage after ROSNI techniques (at 18 h). At that time, no ROSI-embryo had begun or completed the first mitotic division. Another interesting finding is that 19 out of 29 (65%) cleaved ROSNI-embryos developed further up to the stage of morulae or blastocysts, whereas only one out of nine (11%) cleaved ROSI-embryos developed up to the morula or blastocyst stage. It appears that rabbit ROSI-embryos have less potential to develop further.

To evaluate further whether the presence of a large amount of cytoplasm around the round spermatid nucleus is responsible or contributes to the lower outcome of ROSI techniques than ROSNI procedures, an additional experiment was performed. Round spermatids recovered from cryptorchid rabbits were aspirated and expelled (with difficulty) several times via relatively narrow injecting micropipettes of internal diameter equal to 8–9 µm. These manoeuvres resulted in the removal of a small amount of cytoplasm from the round spermatid. In fact, confocal laser scanning microscopy revealed that a small amount of cytoplasm had been mechanically removed and a significant amount of cytoplasm remained around the nucleus of the round spermatid. The latter nuclei were processed for injections of 43 oocytes using micropipettes of diameter equal to 9–10 µm. An injecting micropipette aspirated each nucleus and the respective removed cytoplasmic fragment together and transferred both of them into one oocyte. Within the injecting micropipette, the distance between the nucleus and the cytoplasmic fragment was equal to 10–15 µm approximately.

Fertilization (14 out of 43 injected oocytes) and cleavage (12 out of 43 injected oocytes) rates were slightly larger (but non-significantly) than those achieved after ooplasmic injections of intact round spermatids (Table II); however, they were significantly smaller than the fertilization and cleavage rates achieved after ROSNI techniques (Table II). Although small, statistically non-significant improvements in fertilization and cleavage rates were demonstrated after ooplasmic injections of round spermatids that had undergone removal of a small amount of cytoplasm compared with ooplasmic injections of intact round spermatids, the importance of this additional experiment may be limited by the fact that the injected nuclei were still surrounded by an amount of cytoplasm. It should be emphasized that it is not possible to remove mechanically all the cytoplasm surrounding the rabbit round spermatid nucleus. Complete removal of the rabbit round spermatid cytoplasm can be achieved only by chemical detergents; however, when chemical treatment is applied, the removed cytoplasm is destroyed and subsequently cannot be processed for ooplasmic injection.

The present study indicates that transfer of all the cytoplasmic components of the early haploid male gamete into the female gamete is not accompanied by an enhanced fertilization rate. Main cytoplasmic epigenetic contributions of the male gamete to zygote are the OASIS and the reproducing element of the centrosome (Schatten, 1994). Ooplasmic injections of rabbit intact round spermatids secure the transfer of the above cytoplasmic factors into the female gamete, but do not result in enhanced fertilization, cleavage and embryonic development rates. The delivery of healthy rabbits after injections of bare round spermatid nuclei into non-prestimulated oocytes (Sofikitis et al., 1994a) allowed us to suggest that: (a) the rabbit OASIS has been expressed at the rabbit round spermatid stage (Sofikitis et al., 1997a); and (b) the rabbit OASIS is associated with the nuclear membrane (for review, see Sofikitis et al., 1998a). A close association of the rabbit OASIS with the round spermatid nuclear membrane is consistent with the results of several studies (Sofikitis et al., 1994a, 1996a, 1997a), proving that the absence of the cytoplasm of the rabbit round spermatid has no detrimental effects on the efficiency of the OASIS. It should be emphasized that application of exogenous electrical (Sofikitis et al., 1996a) or chemical (Sofikitis et al., 1997a) stimuli may support the functionality of round spermatid OASIS or may act synergistically with OASIS. The potential of the rabbit zygote for cleavage additionally depends on the presence and/or functionality of the reproducing element of the centrosome that is paternally inherited in the rabbit (Longo, 1976; Schatten, 1994). The large embryonic development rate (46%) up to morula or blastocyst stages after ooplasmic injections of bare round spermatid nuclei in the current study and several previous studies in our laboratory may counter the argument that assisted reproduction techniques should ensure transfer of all the cytoplasmic components of the male gamete into the ooplasm, and challenges the classical theory of the centrosomes (Schatten, 1994; Sofikitis et al., 1998a).

To explain the mitotic divisions after rabbit ROSNI techniques, we raised the probability that when paternally inherited centrosomic material is absent during the transfer of male gamete genetic material within the ooplasm, previously denatured or inactivated female centrosomic material and/or spindle organizing centres may be reactivated, resuscitated or undergo renaturation after oocyte activation (Sofikitis et al., 1998a). The latter speculation is also supported by observations (Mazia, 1984). However, the probability that the paternally inherited components of the centrosome are firmly attached to the male gamete nuclear membrane and are subsequently transferred into the ooplasm during ROSNI techniques cannot be excluded (Navara et al., 1994).

The number of human pregnancies achieved via ROSI

Y.Yamamoto, N.Sofikitis and I.Miyagawa
techniques (Tesarik et al., 1995; Mansour et al., 1996; Antinori et al., 1997a,b; Vanderzwalmen et al., 1997) is larger than the number of ROSNI-pregnancies (Sofikitis et al., 1995a, 1997b). This difference is in the ROSI technique’s favour, but is false for the following reason: ROSI techniques are relatively simple and applied by a large number of centres internationally. In contrast, ROSNI procedures are time consuming and relatively complicated techniques and applied by Japanese centres only. It should be emphasized that the results of the current study cannot be transferred unequivocally to the human. In fact, it appears that in the human, both ROSNI and ROSI procedures can be applied efficiently. The inconsistency between the poor outcome of rabbit ROSI techniques and the encouraging and relatively satisfactory results of human ROSI procedures may be due to anatomical or functional differences between the human and rabbit male and female gametes. The rabbit oocyte may have a limited capacity to handle, dilute or eliminate the cytoplasmic content of the early haploid male gamete. In contrast, the human oocyte is a relatively large oocyte (Kimura and Yanagimachi, 1995c) and may have an adequate capacity to eliminate or digest the cytoplasmic layer of the round spermatid. Furthermore, differences in the size of the acrosomal granule between the rabbit and human round spermatid may contribute to the inconsistency between the results of human and rabbit ROSI techniques. The rabbit acrosomal cap and granule are relatively large (Kimura and Yanagimachi, 1995c). If the acrosomal granule of the rabbit round spermatid cannot be digested or eliminated within the ooplasm, the overall result may be the presence or liberation of several active enzymes within the rabbit ooplasm that probably exert a detrimental effect on the fertilization process. In contrast, the human acrosomal cap and granule are relatively small (Kimura and Yanagimachi, 1995c) and can probably be easily digested within the human ooplasm.

In most of the experimental studies in the international literature dealing with the reproductive potential of the early haploid male gamete, round spermatids were recovered from healthy animals (Ogura and Yanagimachi, 1993; Ogura et al., 1993; Sofikitis et al., 1994a; Kimura and Yanagimachi, 1995a). In contrast, candidates for human ROSNI or ROSI techniques are men with primary testicular damage. This was the rationale on which we evaluated the fertilizing capacity of round spermatids and round spermatid nuclei from rabbit testicles with impaired function in most of the experiments (studies A and B) of the current study.

Human spermatozoa were among the first living cells studied for the effects of freezing and thawing (Sherman, 1977). Although there are several studies in the international literature dealing with cryopreservation of spermatozoa, there are only two reports assessing the reproductive potential of frozen–thawed round spermatids (Ogura et al., 1996; Antinori et al., 1997b). To cryopreserve round spermatids in the latter two studies, protocols previously developed for cryopreservation of mature spermatozoa were employed. We attempted to develop new protocols for round spermatid cryopreservation taking into consideration that cryopreservation is a cell-specific process. The round spermatid and the mature spermatozoon differ in the cytoplasmic content and subsequently in the amount of intracellular water. Differences in the permeability of the cellular membrane may also exist. Glycerol is the most popular cryoprotectant for freezing spermatozoa. We compared freezing protocols of standard (10%) and high (30%) concentrations of glycerol, considering the large amount of the cytoplasm of the male gamete at the round spermatid stage. Since round spermatid viability assay outcome, fertilization rate and cleavage rate in group D were significantly larger than in group C, we may suggest that high glycerol concentrations are preferable. The protective effects of glycerol may be due to its action to depress the freezing point and reduce the electrolyte concentration to which the cells are exposed during freezing procedures (Lovelock and Polge, 1954). It may also moderate the effect of slow-cooling cell injury and may have a beneficial role in maintaining the pH and changing the temperature at which the liquid-to-solid phase is reached. Citrate was added to extender E to bind free hydrogen and hydroxyl ions in the surrounding medium, aiding the dehydration process. Cholesterol was also employed in protocol E to stabilize further the plasma membrane of the round spermatid. To prevent the round spermatid’s undesirable use of its own intracellular phospholipids, fructose was chosen as an additional component of the extender E. Comparing the fertilization, cleavage and embryonic development rates between groups D and E it is obvious that the addition of citrate, cholesterol and fructose had beneficial effects on the maintenance of the reproductive capacity of the round spermatid during freezing (Table III). To evaluate the role of seminal plasma as an extender for freezing round spermatids, extender F was devised and tested. Protocol F contained an additional (second) cryoprotectant, dimethyl sulphoxide. The latter protocol was proven to be the best for freezing round spermatids, as the highest fertilization, cleavage and embryonic development rates were achieved (Table III). It appears that seminal plasma contains factors protecting the early haploid male gamete during freezing, and/or that the addition of a second cryoprotectant has a role in the cryopreservation of round spermatids. Polymamines, citrate, cholesterol and fructose may be among the components of the seminal plasma responsible for its beneficial role in the maintenance of the fertilizing capacity of round spermatids during cryopreservation.

Milk is readily available, inexpensive, and is a natural fluid. It has been used for dilution or cryostorage of spermatozoa (Barisic et al., 1994, 1995). Satisfactory fertilization, cleavage and embryonic development rates were demonstrated using extender D plus milk as a medium to cryopreserve round spermatids. The latter parameters were significantly larger in the group G than in the classical protocols, C and D.

The current study shows that the viability and/or reproductive potential of the round spermatid can be maintained during freezing–thawing procedures. This is of clinical importance: cryopreservation of round spermatids may be performed in all non-obstructed azoospermic men negative for spermatozoa but positive for round spermatids in therapeutic testicular biopsy material. If the first ROSNI/ROSI trial fails to result in pregnancy, frozen–thawed round spermatids may be used in a second cycle. Thus, an additional testicular biopsy may be
avoided. The latter thesis is supported by the absence of significant differences in fertilization, cleavage and embryonic development rates between ooplasmic injections of nuclei extracted from fresh round spermatids (group A) and frozen–thawed round spermatids (groups E, F and G). When complete spermiogenesis arrest is diagnosed during a ROSI/ROSNI cycle, candidates for cryopreservation of round spermatids are those men: (a) who are about to undergo surgery of the reproductive system, as well as radiation or chemotherapy (the latter treatments may degrade testicular function, and spermatogenic arrest at the round spermatid stage may be replaced by spermatogenic arrest at the primary spermatocyte stage); (b) whose general health does not allow an additional testicular biopsy; and (c) who will be outside the country for a long time.

The present study is the first report in the international literature dealing with cryostorage of round spermatids, and indicates that short-term cryostorage of early haploid male gametes using seminal plasma plus TEST–yolk buffer as an extender and two major cryoprotectants results in maintenance of round spermatid capacity to fertilize oocytes and initiate early embryonic development in vitro. It has been suggested that the vast majority of non-obstructed azoospermic men have ejaculates positive for round spermatids (Mendoza and Tesarik, 1996). A perplexing problem in centres applying ROSNI/ROSI techniques is that a non-obstructed azoospermic individual may produce within a short period ejaculates both positive and negative for round spermatids. Therefore, ROSNI/ROSI candidates who do not wish to undergo a therapeutic testicular biopsy may be advised to produce ejaculates at 60 and 24 h before oocyte recovery day, and if round spermatids are identified they may be cryostored and serve as an alternative solution for an ejaculate negative for round spermatids at the oocyte recovery day.

The present study showed that the outcome of rabbit ROSNI techniques is superior to that of ROSI procedures. We also provided evidence that media containing seminal plasma plus TEST–yolk buffer as an extender, and glycerol and dimethyl sulphoxide as cryoprotectants, offer satisfactory maintenance of the round spermatid reproductive capacity during cryopreservation or cryostorage.

References


Immature germ cell ooplasmic injections


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