The early haploid male gamete develops a capacity for fertilization after the coalescence of the proacrosomal granules

Nikolaos Sofikitis, Yasuhisa Yamamoto, Tadahiro Isoyama and Ikuo Miyagawa
Reproductive Physiology and IVF Center, Department of Urology, Tottori University School of Medicine, 36 Nishimachi, Yonago 683, Japan

Introduction

Male gamete alterations during spermiogenesis can be grouped into formation of the acrosome, nuclear changes, development of the flagellum and reorganization of the cytoplasm. Alterations in the shape/size of the acrosomal vesicles/granules can be used as a marker to define different stages in the early spermiogenisis. Stage –1 begins with the formation of a new group of spermatids as a result of the second meiotic division (Oakberg, 1956). These spermatids lack specific characteristics. They may be most commonly confused with secondary spermatocytes but they are 30–40% smaller than secondary spermatocytes (Russel et al., 1990). The appearance of two or three proacrosomal granules marks stage –2. Fusion of the proacrosomal granules into a single granule adjacent to the nucleus defines stage –3 (Oakberg, 1956). The acrosomal granule subsequently becomes larger, flattens onto and extends over the nucleus.

Round spermatid nuclei injections (ROSNI) into the ooplasm have resulted in fertilization and pregnancies in the rabbit (Sofikitis et al., 1994a), mouse (Kimura and Yanagimachi, 1995a, b), and human (Hannay, 1995; Sofikitis et al., 1995a). Blastocyst formation has been demonstrated after ooplasmic injections of round spermatids into bovine oocytes (Goto et al., 1996). In addition, intact round spermatid injections (ROSI) into human oocytes have been followed by delivery of two healthy children (Tesarik et al., 1995, 1996; Tesarik and Mendoza, 1996). Additional pregnancies achieved with ROSI techniques or ooplasmic injections of elongating spermatids have been reported by Antinori (1997a, b), Fishel and co-workers (1995), and Araki and co-workers (1997). However, in the above studies, the exact stage of the round spermatids injected into oocytes was not defined. It appears that human ROSNI/ROSI procedures can be used as experimental techniques for the treatment of non-obstructive azoospermia with maturation arrest at the round spermatid stage (Edwards et al., 1994; Fishel et al., 1996; Sofikitis et al., 1997). Furthermore, Yamanaka and co-workers (1997) have shown that ROSNI procedures can be occasionally applied in the treatment of men with arrest in spermatogenesis at the primary spermatocyte stage. It appears that in patients whose routine diagnostic testicular biopsy has demonstrated primary spermatocytes only, a more thorough search of therapeutic testicular biopsy material may reveal few round spermatids (Yamanaka et al., 1997).

Our objective was to compare the reproductive capacity of the round spermatid prior to appearance of the proacrosomal granules (stage –1), prior to coalescence of two or three proacrosomal granules (stage –2), and after fusion of the proacrosomal granules and before the spreading acrosome reaches an angle of 90° (stages –3 to –5) (Russel et al., 1990). Such a study may be clinically important because it could define the round spermatid stage with the maximal probability for fertilization after ooplasmic injections.

Materials and methods

Recovery and preparation of oocytes

Mature New Zealand white female rabbits were superovulated as previously described (Sofikitis et al., 1996a, b). Mature oocytes were collected, washed three times in modified RD medium (Sofikitis et al., 1996a), and freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (Type VIII; 320 units/mg solid; Sigma Co., St Louis, MO, USA) in modified RD medium. Cumulus free oocytes were rinsed and kept in modified RD medium at 37°C under 5% carbon dioxide in air.
Isolation of round spermatids

Spermatogenic cells were recovered from male New Zealand white rabbits (8 months old) and resuspended in modified Dulbecco’s phosphate buffered saline (DPBS) as previously described (Sofikitis et al., 1994a, 1996a,b). Then the cells were observed by confocal scanning laser microscopy (1LM06; Lasertec, Yokohama, Japan) with a computer assisted system (Apple Computers Inc., Cupertino, CA, USA) attached to a micromanipulator (MO-204 Narishige, Tokyo, Japan). The confocal scanning laser microscope has the capacity to provide three-dimensional images of live cells under high magnification without requiring the staining of materials or damaging the cells (Hyodo et al., 1991; Sofikitis et al., 1994b). Thus, live cells could be used for a variety of further in vitro procedures after observation. Furthermore, the confocal scanning laser microscope allows selective intracellular observation at various depths. Round spermatids of stages –3 to –5 could be identified by the presence of a single acrosomal granule adjacent to the nucleus (Sofikitis et al., 1996b). Careful observation also allowed the identification of round spermatids of stage –2 (i.e., two or three proacrosomal granules adjacent to the nucleus; Figure 2). Round spermatids of stage –1 could be identified by the absence of proacrosomal/acrosomal granules and their size (10 µm; Figure 1). Cells of similar shape and intracellular characteristics (i.e., absence of proacrosomal granules) with a diameter of 11–12 µm were considered to be secondary spermatocytes. Round spermatids of stage –1 (group A), stage –2 (group B), and stages –3 to –5 (group C) were selected via the micromanipulator pipette, after confocal imaging.

When the angle subtended by the single acrosomal granule in group C cells extended to a maximum of 90°C (≈90°; Figure 3)(Russel et al., 1990) the spermatids were considered to be stages –3 to –5 (Russel et al., 1990). The internal diameter of the pipette was 20 µm. Selected round spermatids were transferred to SOF medium (Yamanaka et al., 1997). Before use, 2.85 µg of bicarbonate was added/ml of SOF medium. Our methodology has been proven to be accurate enough to select round spermatids of the first stages of spermiogenesis (Sofikitis et al., 1996a, b). However, taking into consideration that the identification of round spermatids of stage –1 was based mainly on their size, the probability that a small number of secondary spermatocytes was included in group A cells could not be ruled out.

Transmission electron microscopy (TEM) of selected cells

To confirm that the confocal scanning laser microscopy methodology applied was sufficient to distinguish different stages of round spermatids, a large number of round spermatids of stages –1 to –5 were selected using the micromanipulator pipette. These fractions of round spermatids were observed via confocal scanning laser microscopy and subsequently were processed for TEM as previously described (Figure 4)(Sofikitis et al., 1996a). Percentages of round spermatids of stage –1, stage –2, and stages –3 to –5 were recorded during confocal scanning laser microscopy and TEM. The mean percentage of round spermatids of stage –1, stage –2, or stages –3 to –5 recorded
in the same medium supplemented with 1 mM MgCl2 and 10% w/v polyvinylpyrrolidone (molecular weight 360 000, PVP K 90; ICN Biochemicals, Costa Mesa, CA, USA) and kept at 34°C.

ROSNI procedures
Cumulus free mature oocytes were preincubated at 10°C in an atmosphere of 5% carbon dioxide in air for 2 h. 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. The oocytes were subsequently transferred to modified RD medium for injections. A single round spermatid nucleus was injected into each oocyte. The time interval between treatment of oocytes with A23187 and ooplasmic injections was 30–45 min. The technique for ooplasmic injections was different from the one previously applied (Sofikitis et al., 1996a) because of the smaller size of the round spermatid nucleus and subsequently the smaller volume it occupies within the injecting micropipette. The injected oocytes were transferred to modified RD medium without antioxidants and incubated at 37°C under 5% carbon dioxide in air for 72 h. Oocytes were observed at 5, 9 and 24 h post-injection. Previous studies have shown that the optimal time for observation of two pronuclei after ROSNI procedures is 9 h post-injection (Sofikitis et al., 1995b; Yamanaka et al., 1997). Medium was changed at 24 h post-injection. An activated oocyte was defined as one with first and second polar bodies and at least one female pronucleus present (Ogura and Yanagimachi, 1993; Sofikitis et al., 1996a).

Observations of the oocytes and embryos were performed in a blinded fashion. Statistical analysis was performed using chi-square test. A probability <0.05 was considered to be statistically significant.

Results
Oocyte activation
There were no significant differences in the proportion of activated oocytes to injected oocytes among groups A, B and C.

Fertilization
There was no significant difference in the proportion of fertilized oocytes to injected oocytes between groups B and C. However, the proportion of fertilized oocytes to injected oocytes was significantly lower in group A (Table I).

Zygote cleavage
The proportion of cleaved oocytes to injected oocytes was significantly lower in groups A and B than in group C. In contrast, the difference in the proportion of cleaved oocytes to injected oocytes between groups A and B was not significant.

Embryonic development
The proportion of morulae/blastocysts to injected oocytes was significantly lower in groups A and B than in group C. However, the proportion of morulae/blastocysts to injected oocytes was not significantly different between groups A and B.

Parthenogenic activation
Approximately one third of the oocytes injected with medium were activated. One and three of them showed two and three female pronuclei, respectively. The parthenogenetically activated oocytes did not complete the first cleavage.

Figure 4. Round spermatid of stage ≥3. Observation via transmission electron microscopy (×2000) with a computer assisted system (×3).
the group of round spermatids of stages −3 to −5 the percentage of activated oocytes was higher than that we previously reported when applying a minor mechanical stimulation only (Sofikitis et al., 1996a). Furthermore, the combination of mechanical and chemical stimulation applied was sufficient to support/induce activation in a high percentage of oocytes injected with less mature cells (i.e., round spermatids of stages −1 or −2). Preliminary experiments have indicated that the combination of chemical plus multiple mechanical stimulation during ROSNI procedures results in a larger activation rate (90% in the present study; 92% in preliminary experiments) than a mild/minor mechanical stimulation alone (85%; Sofikitis et al., 1996a), a single, vigorous mechanical stimulation alone (76%), or a multiple, vigorous mechanical stimulation alone (87%). Oocytes are incubated in the presence of ionophore after intracytoplasmic sperm injections in several assisted reproduction programmes (Hoshi et al., 1995; Tesarik and Sousa, 1995a).

We applied the chemical stimulus prior to ROSNI techniques in the current study since previous studies and preliminary experiments showed better activation rates for rabbit oocytes when the electrical (Sofikitis et al., 1996a) or chemical stimulation was applied before ROSNI procedures than after these techniques. Furthermore, Kimura and Yanagimachi (1995b) reported that mouse ROSNI procedures were more effective when oocytes were activated first and injection was performed at the telophase of the second meiotic division of the oocyte. Oocytes had been incubated at 10°C before the activating stimulus was applied because we previously showed that incubation of rabbit oocytes at 5–10°C prior to electrical stimulation plus ROSNI techniques had beneficial effects on oocyte activation and fertilization process (Sofikitis et al., 1996a). Mouse round spermatids lack the oocyte-activating factor (OAF) (Kimura and Yanagimachi, 1995a, b). However, there is much controversy over whether the rabbit OAF is expressed at the round spermatid stage. The authors believe that rabbit OAF is expressed at/before the round spermatid stage since (i) healthy offspring were born after ROSNI into oocytes treated with a minor mechanical stimulation (Sofikitis et al., 1994, 1996a), (ii) the percentage of activated oocytes after minor mechanical ooplasmic stimulation plus ROSNI techniques was relatively high in a previous study (Sofikitis et al., 1996a) or chemical stimulation of the oocyte prior to ROSNI techniques may support the functionality/action of the OAF and subsequently have beneficial effects on oocyte activation and fertilization. This thesis is consistent with the suggestion of Tesarik and Sousa (1995b) that a vigorous mechanical stimulation during intracytoplasmic sperm injection making the oocyte undergo a substantially high Ca$^{2+}$ load may support the action of the human OAF supposed to trigger oocyte activation.

Recently, Yamanaka and co-workers (1997) and Sousa and

**Discussion**

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 zygotes obtained was not evaluated in that study. In another study, intact round spermatids were injected into the perivitelline space of mature hamster or mouse oocytes and a fusion pulse was applied to fuse the spermatids with the oocytes (Ogura et al., 1993). It was found that the nuclei commonly failed to develop into large pronuclei. In one additional study it was found that when mouse intact round spermatids were successfully fused with the oocytes, some of the zygotes developed into normal offspring (Ogura et al., 1994). The overall success rate of the electrofusion of intact spermatids with oocytes was low due to the difficulty of fusing large cells like oocytes with small cells like spermatids without lysis of the larger cells (Ogura et al., 1994). To avoid oocyte damage due to the fusion process we chose a microsurgical approach to transfer round spermatid nuclei into oocytes and achieved the first pregnancies in mammalian species via microsurgical ROSNI techniques (Sofikitis et al., 1994a). In that study the proportion of implanted embryos to the number of injected oocytes and the ratio of offspring to the number of injected oocytes were low. The low values of these parameters may be attributable to low developmental potential of the injected oocytes due to inadequate mechanical stimulation applied to activate oocytes prior to ROSNI procedures. For this reason we designed another study, the objective of which was to evaluate the effects of electrical stimulation of oocytes before ROSNI procedures on oocyte activation and subsequent embryonic development (Sofikitis et al., 1996a).

That study provided information on the optimal stimulation required for oocyte activation, fertilization, and normal embryonic development when rabbit ROSNI-embryo transfer procedures are scheduled. It was found that electrical stimulation of oocytes prior to ROSNI techniques had beneficial effects on oocyte activation, fertilization, and subsequent embryonic development. In the present study we applied a combination of chemical and multiple, vigorous mechanical stimulation to activate the rabbit oocytes. Within

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**Table I. Injections of round spermatids at different stages into rabbit oocytes**

<table>
<thead>
<tr>
<th>Spermatid groups</th>
<th>Control group D (medium)</th>
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</thead>
<tbody>
<tr>
<td>A (stage–1)</td>
<td>49</td>
</tr>
<tr>
<td>B (stage–2)</td>
<td>41 (83)a</td>
</tr>
<tr>
<td>C (stage –3 to –5)</td>
<td>18 (36)a</td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>49</td>
</tr>
<tr>
<td>Activated oocytes (%)</td>
<td>8 (16)a</td>
</tr>
<tr>
<td>Cleaved oocytes (%)</td>
<td>2 (4)a</td>
</tr>
</tbody>
</table>

Within each line, values not sharing the same superscript a, b or c are significantly different ($P < 0.05$).
co-workers (1996) suggested that the OAF is present at the human round spermatid.

After the encouraging messages from the above experimental studies an attractive challenge was to apply ROSNI procedures for the treatment of non-obstructed azoospermic men. The first human pregnancies with ROSNI techniques were achieved in 1994 and reported in April 1995 (Hannay, 1995; Sofikitis et al., 1995a). However, these pregnancies resulted in abortions. A few months later, Tesarik et al. (1995) reported two human pregnancies. The latter pregnancies resulted in deliveries of healthy children (Tesarik and Mendoza, 1996; Tesarik et al., 1996). Fishel and co-workers (1995) reported a pregnancy after an elongated spermatid injection into an oocyte. Vanderzwalm and co-workers (1995), Chen et al. (1996), and Yamanaka and co-workers (1997) fertilized human oocytes with late-stage spermatids, intact round spermatids, and round spermatid nuclei, respectively. Additional ROSI pregnancies have been reported by Antinori and co-workers (1997a, b). However, in all of the above studies the exact stage of the round spermatids injected into oocytes was not defined. We used the rabbit as an experimental model to evaluate whether there are alterations in the reproductive capacity of the early haploid male gamete during the first stages of spermiogenesis.

We compared the fertilizing capacity of round spermatids without proacrosomal/acrosomal granules (stage –1), round spermatids with two or three proacrosomal granules (stage –2), and round spermatids with a single acrosomal granule rounded on the nuclear surface (stage –3) (Russel et al., 1990) or a single acrosomal vesicle flattened/extended on the nuclear membrane to an angle smaller than 90° (stages –1990; the mouse classification of stages –1990) or a single acrosomal vesicle flattened/extended on the nuclear membrane and subsequently without cytoplasmic contents of the round spermatid nuclei, respectively. Additional ROSI pregnancies have been reported by Antinori and co-workers (1997a, b). However, in all of the above studies the exact stage of the round spermatids injected into oocytes was not defined. We used the rabbit as an experimental model to evaluate whether there are alterations in the reproductive capacity of the early haploid male gamete during the first stages of spermiogenesis.

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More studies are necessary to clarify whether paternal factors contributing to the initiation of the early embryonic development are expressed/activated at stage –1 or –2 of spermiogenesis.

The results of the present study are consistent with a previous report (Sofikitis et al., 1996a) showing that rabbit oocytes treated with an activation stimulus plus medium injection have low potential for cleavage and further development. In contrast, the proportion of morulae/blastocysts to the oocytes injected with stages –3 to –5 round spermatids was smaller in the current study than that previously reported (Sofikitis et al., 1996a). This difference may be attributed to the different stimulus applied for support/induction of oocyte activation in the present study or to the absence of antioxidants (i.e., taurine) in the embryo culture medium. Li and co-workers (1993) have demonstrated that addition of antioxidants to rabbit embryo culture medium results in faster embryonic development.

Centrosomic components (i.e., the reproducing element of the centrosome, the microtubule organizer and a γ-tubulin-binding protein) of the rabbit zygote are considered to be paternally inherited (Longo, 1976; Schatten, 1994). In three previous studies (Sofikitis et al., 1994a, 1996a, b) and the current study normal embryonic development was achieved both in vitro and in vivo by introducing an early haploid nucleus without any cytoplasmic coverings and subsequently without centrosomal material within a rabbit oocyte. The above observations, the artificial parthenogenesis in several mammalian species, and the observations of Ozil (1990) that rabbit oocytes electrically stimulated repeatedly could implant and further develop up to the day –10 of pregnancy, could be interpreted as a challenge to the classical theory of centrosomes (Schatten, 1994) and tend to support the speculation of Mazia (1984) that when paternal centrosomal material is absent, novel maternal spindle organizing centres can develop or previously denatured or non-functional female centrosomal material can undergo resuscitation after oocyte activation. Alternatively, the idea that centrosomal material is firmly attached to the male gamete nucleus and subsequently introduced into the oocyte via nuclear injections (Navara et al., 1994) could explain the development of oocytes injected with round spermatid nuclei to embryos, fetuses.
and offspring (Sofikitis et al., 1996a). The present study reveals that the rabbit round spermatid of stages 3 to 5 has larger capacity to fertilize oocytes and initiate embryonic development than round spermatids of earlier stages. If the results of the present study could be transferred to the human, recovery of round spermatids of stages ≥3 would be recommended in assisted reproduction programmes applying ROSNI procedures for the treatment of non-obstructive azoosperma. Although human pregnancies have been achieved by ROSNI or ROHI techniques (Sofikitis et al., 1995a; Tesarik et al., 1995; Antinori et al., 1997a, b) genetic implications of ROSNI procedures should be carefully considered. To evaluate the genetic risks of assisted reproductive technologies, one has to consider the genetic risk inherent in the procedure performed and the genetic risk inherent to treatment population. Genetic risks inherent to ROSNI/ROSI techniques may involve (i) centrosomomic abnormalities resulting in an aberrant spindle formation and subsequently in an increased risk of mosaicism, (ii) injection of diploid or disomic genetic material which could give rise at fertilization to a triploid or trisomic embryo/fetus (the presence of diploid spermatids/spermatooza is well documented in the mammalian testis with severe spermatogenic impairment) (Levy and Burgoyne, 1986) and (iii) genomic imprinting abnormalities. A genetic risk of ROSNI/ROSSI procedures inherent to a population of non-obstructed azoospermic men is transferring an azoospermia-related causing gene to the next generation. An additional problem concerning the application of human ROSNI/ROSI techniques in centres without a confocal scanning laser microscope is the identification of the human round spermatid. To identify human round spermatids quantitative (Yamanaka et al., 1997) or qualitative criteria (Mendoza and Tesarik, 1996) can be used. However, qualitative criteria are highly subjective. More experimental studies are necessary to overcome the above drawbacks to human ROSNI procedures.

References


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