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Influence of the male reproductive tract on the reproductive potential of round spermatids abnormally released from the seminiferous epithelium*

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Round spermatids can be collected from testicular biopsy material or occasionally from semen samples. We evaluated the influence of the passage of round spermatids through the male reproductive tract on their reproductive potential. A model of abnormal release of round spermatids from the seminiferous epithelium was created in mature male rats (group A). Additional sham-treated rats of the same age served as a control group (group B). Round spermatids were collected from the testicles of rats of both groups, the epididymides of rats of group A, and the vaginae of mature female rats mated with rats of group A. Isolated round spermatids were processed for ooplasmic injections. Injected oocytes were cultured. At 96 h post-injection, the blastocyst development rate was significantly higher in the groups of oocytes injected with testicular spermatids than the groups of oocytes injected with spermatids recovered from the vaginae, or the head, body, or tail of the epididymides. It appears that round spermatids recovered from testicular biopsy material have larger reproductive capacity than ejaculated round spermatids, due to mechanical or chemical detrimental influences of storage/passage through the male reproductive tract (outside the testicle) on the capacity of round spermatids to induce optimal early embryonic development.

Key words: infertility/rat/seminiferous epithelium/spermatid/testis

Introduction

Delivery of healthy human newborns has been reported after ooplasmic injections of secondary spermatocytes (Sofikitis *et al*., 1998a), round spermatids (Tesarik *et al*., 1995; Mansour *et al*., 1996; Antinori *et al*., 1997a,b; Vanderzwalmen *et al*., 1997), and elongating/elongated spermatids (Fishel *et al*., 1995; Araki *et al*., 1997; Amer *et al*., 1997; Bernabeu *et al*., 1998; Sofikitis *et al*., 1998b). Furthermore, ooplasmic injections of rabbit or mouse round spermatids and mouse secondary spermatocytes followed by embryo transfer techniques have resulted in delivery of healthy offspring (Ogura *et al*., 1994; Sofikitis *et al*., 1994a, 1996a; Kimura and Yanagimachi, 1995a,b). All the above studies indicate that in non-obstructed azoospermic men who are negative for testicular spermatozoa, but are positive for spermatids in their testicular biopsy material, ooplasmic injections of spermatids may serve as an alternative mode of treatment (Sofikitis *et al*., 1998c). Several studies indicate that some patients with spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome may have rare foci of spermatids somewhere in the testicle (Hannay, 1995; Tesarik *et al*., 1995; Mansour *et al*., 1996; Antinori *et al*., 1997a,b; Vanderzwalmen *et al*., 1997; Amer *et al*., 1997; Araki *et al*., 1997; Yamanaka *et al*., 1997; Bernabeu *et al*., 1998; Sofikitis *et al*., 1998b). Amer *et al*. (1997) used the term 'complete spermiogenesis failure' for men in whom the most advanced germ cell present in the testicular biopsy material is the round spermatid and the term 'incomplete spermiogenesis failure' for non-obstructed azoospermic men with a very limited number of elongated spermatids in their testicular biopsy material. It appears that in some men with spermatogenic arrest at the primary spermatocyte stage a number of germ cells can break the barrier of the premeiotic block and differentiate up to the stage of round or elongating spermatid (Amer *et al*., 1997; Yamanaka *et al*., 1997; Sofikitis *et al*., 1998c). Several biochemical mechanisms may be responsible for the inability of the round spermatid to undergo the elongation process. O'Donnell and co-workers have shown that suppression of intratesticular testosterone concentration may be one of the mechanisms (O'Donnell *et al*., 1996). Additional studies are necessary to clarify whether an intratesticular testosterone concentration below a specific threshold causes failure of elongation of round spermatids. If this hypothesis is correct, testicular pathophysiologies affecting optimal intratesticular testosterone concentration may result in complete spermiogenetic failure. It should be emphasized that varicocele, the most frequent cause of male infertility, known to cause azoospermia occasionally, is accompanied by a reduced intratesticular testosterone concentration (Rajfer *et al*., 1987).

Round spermatids are occasionally present in the seminal plasma of non-obstructed azoospermic men. These ejaculated round spermatids can be used for ooplasmic injections (Tesarik *et al*., 1995). It was reported that 69% of non-obstructed azoospermic men have round spermatids in their ejaculates (Mendoza and Tesarik, 1996). Tottori University International Research Group has reported that 23% of non-obstructed azoospermic men have ejaculates positive for round spermatids

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(Sofikitis *et al*., 1998c). It appears that ooplasmic injections of round spermatids/elongating spermatids recovered from the ejaculate represent an attractive alternative solution for the treatment of non-obstructed azoospermic men who do not wish to undergo a testicular biopsy. No significant difference was found in the fertilization rate after ooplasmic injection of human oocytes with spermatids recovered from the ejaculate versus the testicular biopsy material (Fishel *et al*., 1997). However, data on embryonic development rate/pregnancy rate were not revealed in that study. Our objective was to compare the fertilization rate, early embryonic development rate, and the overall live birth rate after ooplasmic injection of round spermatids recovered from the ejaculate versus the testicular tissue. For this purpose, we created an experimental model of abnormal release of round spermatids from the seminiferous epithelium in the rat (O'Donnell *et al*., 1996) and evaluated the influence of the passage of the round spermatid through the various regions of the male reproductive tract on the overall round spermatid fertilizing capacity and ability to induce optimal embryonic and fetal development. Several ethical considerations prohibit the performance of such a study in the human. Most importantly, the choice of human embryos for transfer depends on the embryonic quality and cannot be influenced by the source (testicle versus ejaculate) of the male gametes that fertilized the oocytes.

Materials and methods

Animals

Two groups (A and B) of 11-week-old male Wistar rats ($n = 8$ in each group) were housed under a 12 h light:12 h dark cycle with free access to food and water.

Chemicals

When the source of a chemical is not defined this chemical was purchased from Sigma Co (St Louis, MO, USA).

Temperature during procedures

When temperatures during experiments/procedures/techniques are not defined, procedures have been performed at 23–27°C. During germ cell or seminiferous tubule observation on an inverted microscope or stereo microscope, the temperature was maintained at 34°C using a heating stage (Konnishi Co, Yonago, Japan). During oocyte observation temperature was maintained at 37°C.

Micropipettes

For gamete micromanipulation micropipettes were prepared at our Reproductive Physiology and IVF Center using a pipette puller (P-97 IVF; Sutter Instruments Inc, Tokyo, Japan) and a microforge (MF-90, Narishige, Tokyo, Japan).

Creation of a model of abnormal release of spermatids

Rats of group A received intra-abdominal injections of testosterone daily (1 mg; Hayashi Co, Yonago, Japan) for 1 week to suppress luteinizing hormone (LH) while maintaining spermatogenesis (Robaire *et al*., 1979; O'Donnell *et al*., 1996). Then, animals of group A received s.c. administration of both testosterone (100 μg/day) and oestradiol-17β (1 μg/day; Hayashi Co, Yonago, Japan) daily for 7 weeks to suppress intratesticular testosterone and spermatogenesis (O'Donnell *et al*., 1996). During the 8 week experimental period animals of group B were treated by intra-abdominal (1 week) and s.c. administration (7 weeks) of normal saline (Hayashi Co) daily.

At the end of the experimental period each male rat from group A was placed in a cage with a mature female rat in oestrus as determined by vaginal smear examinations (Yamamoto *et al*., 1997). After mating, vaginal fluid was collected and observed via an inverted microscope (Olympus IX-70, Tokyo, Japan) computer-assisted system (Yamanaka *et al*., 1997) and a confocal laser scanning microscope computer-assisted system (Sofikitis *et al*., 1994a,b). Then, a part of the testicular tissue from each rat of both groups was processed for haematoxylin/eosin stain, a part was processed for evaluation of intratesticular testosterone concentration, and another part was processed for dispersion and extraction of spermatogenic cells. Furthermore, the head, body, and tail of epididymis of each rat of both groups were processed for recovery of cells from the lumens. Testicular and epididymal germ cells were observed via the inverted microscope-computer assisted system and the confocal laser scanning microscope computer-assisted system. Round spermatids isolated from the testicles, the vaginae, and the head, body, and tail of epididymides were processed for ooplasmic injection of oocytes that had completed the first meiotic division.

Intratesticular testosterone assay

Intratesticular testosterone was assayed by radioimmunoassay using kits from Nihon DPC Corporation (Tokyo, Japan), according to a previously published method (Coyotupa *et al*., 1972). Samples of testicular tissue were processed for intratesticular testosterone assay as previously described (Rajfer *et al*., 1987; Antypas *et al*., 1994).

Spermatogenic cell recovery from the testicle or the epididymis

Testicular fragments were placed in erythrocyte-lysing buffer (Sofikitis *et al*., 1996b). Seminiferous tubules were then washed in Dulbecco's phosphate-buffered saline (DPBS; Sofikitis *et al*., 1996a,b) and were cut into small pieces during observation under a stereo microscope (SZ-STS; Olympus, Tokyo, Japan). The cell suspension was flushed out through a 40 μm pore size metal mesh (Tomoda Co, Matsue, Japan). The filtrate was collected and centrifuged at 300 *g* for 15 min and the sedimented cells were resuspended in DPBS. Then the cells were observed using an inverted microscope computer-assisted system (Nomarski differential lens). Round spermatids could be easily identified by the presence of black spots (Figures 1 and 2). The latter spots represent the acrosomal granule(s) and become bright if there is excessive light during microscopical observation. In preliminary experiments, confocal laser scanning microscopy demonstrated that >96% of the cells characterized as rat round spermatids by inverted microscopy were indeed round spermatids. Confocal laser scanning microscopy has the capacity to recognize accurately the acrosomal granule(s)/cap and subsequently round spermatids (Sofikitis *et al*., 1996a,b; Yamanaka *et al*., 1997). It has also the ability to recognize the round spermatid stage (Sofikitis *et al*., 1997). Fixation/staining of cells is not necessary for observing cells with a confocal laser scanning microscope. Thus, undisturbed cells within a drop covered by oil were placed in an aluminium plate (Tomoda Co) and observed using a confocal laser scanning microscope in the above preliminary experiments.

The vast majority of round spermatids observed had a diameter larger than that of erythrocytes; these cells were processed for nuclei isolation (Figure 1). Occasionally, round spermatids with a diameter smaller than that of erythrocytes were observed; however, these were not processed further. The round spermatids observed which were larger than erythrocytes were collected via a 20 μm micropipette attached to a micromanipulator (MO-204, Narishige, Tokyo, Japan), transferred to SOF medium (Yamanaka *et al*., 1997; Sofikitis *et al*.,

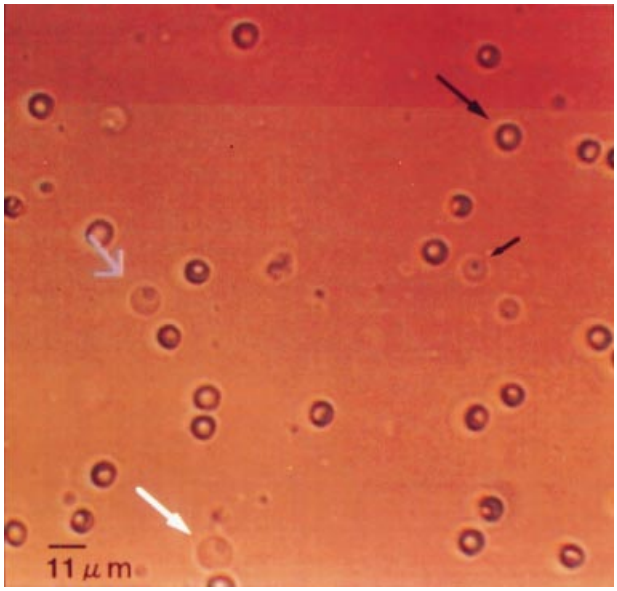

Figure 1. Observation via inverted microscope computer-assisted system. Erythrocytes are indicated by long black arrows. Cells with black spots and cellular size larger than erythrocytes (white arrows) are round spermatids and have been processed for nuclei isolation. Cells with black spots and cellular size smaller than erythrocytes (short black arrows) are also round spermatids, however, these cells have not been further processed.

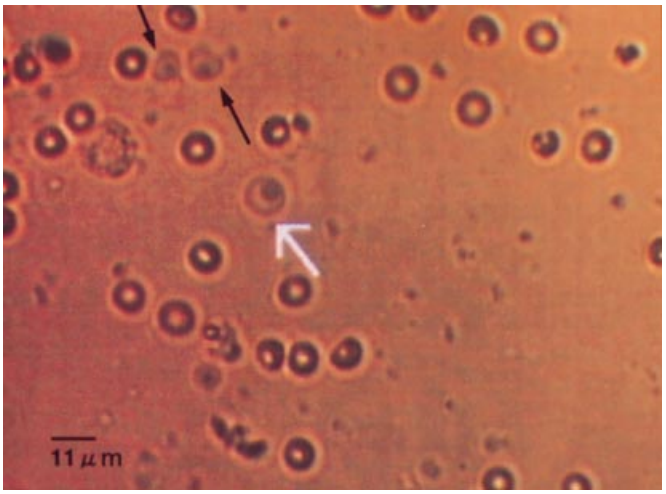

Figure 2. Degenerated round spermatids (black arrows) within minced epididymal tissue. A round spermatid with a normal shape is indicated by a white arrow. Observation via inverted microscope computer-assisted system.

1998b), and processed for cytoplasmic digestion. Similarly, the epididymal head, body, and tail were minced and filtered. Identified round spermatids (Figure 2) were transferred to SOF medium and processed for cytoplasmic digestion. In addition, round spermatids recovered from the vaginal fluid were transferred to SOF medium and processed for cytoplasmic digestion. Prior to nuclei isolation, the percentage of viable round spermatids was calculated in each spermatid fraction by Trypan Blue staining (Ogura and Yanagimachi, 1993). To calculate the percentage of live spermatids, >500 cells were checked per animal sample.

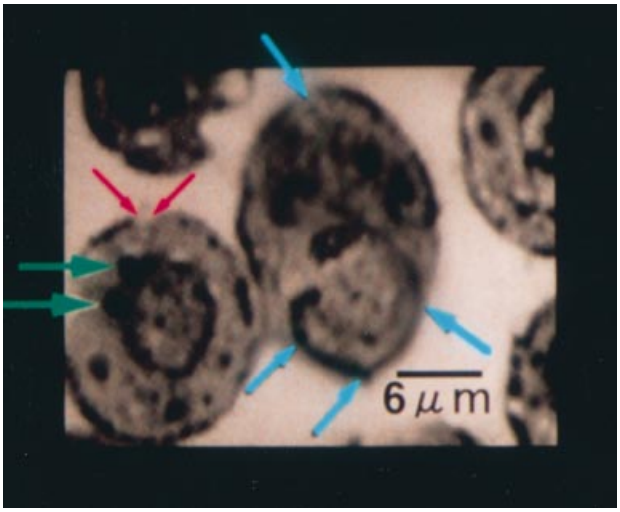

Figure 3. The cell surrounded by blue arrows is a round spermatid of step 8. The cell indicated by pink arrows is a round spermatid of step 2. Within the latter cell, two proacrosomal granules (black spots) are indicated by green arrows. Observation using a confocal laser scanning microscope computer-assisted system.

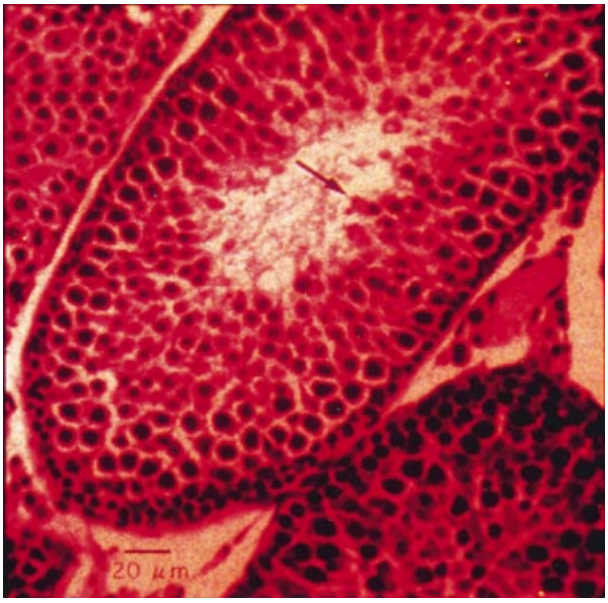

Figure 4. Haematoxylin/eosin stain of rat testicular tissue after treatment with testosterone plus oestradiol for 7 weeks following 1 week of testosterone administration demonstrates spermatogenic arrest at the round spermatid stage. The arrow indicates a round spermatid with morphology consistent with step 8.

Nuclei isolation

Within each group of selected spermatids, undamaged round spermatids that did not demonstrate even a minor degree of elongation (steps 2–7; step 1 round spermatids are negative for black/dark spots, due to the lack of acrosomal granules and were neglected; step 8 round spermatids show a small degree of elongation and were not processed further; Figure 3 shows observations using a confocal laser scanning microscope) were transferred from droplets of SOF medium to droplets of a hypertonic solution (prepared in our facilities as previously described; Sofikitis *et al.*, 1996a) using micropipettes (internal diameter: 15 μm) and resuspended in droplets of the same solution containing Triton X-100 (0.025%; v/v). After gentle pipetting

via micropipettes for 6 min, the nuclei (Sofikitis *et al*., 1996a,b; Yamanaka *et al*., 1997) surrounded by a thin cytoplasmic layer were washed repeatedly in SOF medium and finally resuspended in the same medium supplemented with 1 mM $MgCl_2$ and 10% polyvinylpyrrolidone (molecular weight 360 000, PVP K 90; ICN Biochemicals, Costa Mesa, CA, USA) and kept at 34°C (normal testicular temperature). Previous studies have shown that when round spermatids are exposed to a higher temperature, the cell physiology and fertilizing capacity are detrimentally influenced (Nakamura *et al*., 1978; Sofikitis *et al*., 1996a,b).

Recovery and preparation of oocytes

Ovulation was stimulated in immature female Wistar rats as was previously described (Toyoda and Chang, 1974; Yamamoto *et al*., 1997). Cumulus masses were dispersed by a brief incubation with hyaluronidase (type VIII; 320 IU/mg solid; Sigma Chemical Co) (Sofikitis *et al*., 1994a). Mature oocytes (the first polar body had been extruded into the perivitelline space) were collected and transferred to Toyoda and Chang (1974) fertilization medium without Phenol Red (TC medium). Oocytes were preincubated at 10°C in an atmosphere of 5% CO_2 in air for 2 h (Sofikitis *et al*., 1997). Previous studies have shown that incubation of oocytes for 2–3 h at 10°C prior to electrical stimulation and round spermatid nuclei injection (ROSNI) has beneficial effects on oocyte activation and fertilization (Sofikitis *et al*., 1996a,b). Oocytes then underwent an electrical stimulation with a single electric pulse (1000 V/cm; 100 μs) as previously described (Ogura and Yanagimachi, 1993; Kimura and Yanagimachi, 1995a; Sofikitis *et al*., 1996a). One nucleus was injected into an oocyte 20 min post-electrical stimulation.

Round spermatid nuclei injection (ROSNI) into oocytes

Within each group of injected oocytes, the nuclei that were processed for ooplasmic injections had been obtained from all animals of the group A or B. Injecting micropipettes with a 9 μm diameter were used. Each nucleus was aggressively compressed by the tip of the injecting micropipette several times. These manoeuvres resulted in removal of an additional amount of cytoplasm from the spermatid nucleus–cytoplasmic layer complex (Sofikitis *et al*., 1998b,c). The nuclei were finally aspirated easily into another injecting micropipette without exerting a significant force during the aspiration technique. One round spermatid was placed at the tip of the injecting micropipette. This position ensured the injection of a minimal amount of medium within the oocyte.

Placing the round spermatid at the tip of the injecting micropipette required skill at injection to avoid losing the spermatid during penetration of the sticky rat oocyte coverings. A Piezo Micromanipulator Model (PMAS-CT140; Prima Meat Packers, Tsuchiura, Japan) was used to direct forward the injecting micropipette. This unit has the capacity to advance the pipette holder a very short distance at a time at a very high speed. Each oocyte was placed with the first polar body at the 12 o'clock position. The tip of the injecting micropipette was brought into contact with the zona at the three o'clock position and Piezo pulses (intensity: 1–2, speed: 1–2) were applied to move the micropipette through the zona. Finally the injecting micropipette entered the ooplasm by applying additional Piezo pulses (intensity: 1–2, speed 1–2). A vigorous ooplasmic aspiration/expelling manipulation was performed and the round spermatid nucleus was expelled within the ooplasm.

To determine the influence of the capacity of the rat oocytes to undergo parthenogenetic activation on post-ROSNI embryonic development, an additional group (C) of 20 rat mature oocytes were sham-injected with medium only using the above described technique for ooplasmic injections. The latter oocytes prior to ooplasmic injections had been exposed to a temperature of 10°C and subsequently underwent electrical stimulation in the same fashion as oocytes of groups A and B. Injected oocytes were kept in TC medium for 15 min on the cooled stage (17–19°C) of the microscope (Kimura and Yanagimachi, 1995c). It is known that the latter temperatures help the ooplasm to heal post-injection.

Embryo culture

Following injections all oocytes were transferred to TC medium supplemented with taurine (1 mmol/l; Sofikitis *et al*., 1997) and cultured at 37°C under 5% CO_2 in air. Taurine is an antioxidant and is known to have beneficial effects on the in-vitro development of embryos generated from the fertilization of oocytes with round spermatids (Sofikitis *et al*., 1998c). Oocytes/embryos were carefully observed at 9, 24, and 96 h post-injection (Sofikitis *et al*., 1997). An activated oocyte was defined as one with a first and second polar body extruded into the perivitelline space and at least one (female) pronucleus (Ogura and Yanagimachi, 1993; Kimura and Yanagimachi, 1995a,b). A normally fertilized oocyte was defined as one with a first and second polar body extruded into the perivitelline space and two pronuclei (male and female). At 9 and 24 h post-injection oocytes were placed in modified R1ECM medium (Miyoshi *et al*., 1997) supplemented with taurine (5 mmol/l). At the end of the experimental period embryos were carefully observed using an inverted microscope. Within group A, blastocysts generated from the fertilization of oocytes by testicular round spermatids, epididymal head-round spermatids, epididymal body-round spermatids, and epididymal caudal-round spermatids (see Results) were transferred to the uterine horns of three, two, one, and one pseudopregnant recipients respectively. Of the blastocysts generated from group B animals, 20 were transferred to two pseudopregnant recipients. Five blastocysts derived from fertilization of oocytes with ejaculated round spermatids were transferred to one pseudopregnant recipient. Post-transfer the vaginal smears of the recipients were examined daily. They were killed if pro-oestrus/oestrus was demonstrated and their uterine horns and uterine bodies were examined for implantation sites. The number of resorption sites/implantation sites was recorded.

Pregnant female recipients were allowed to litter. The remaining 17 blastocysts generated from fertilization of oocytes with spermatids of group B and the remaining one blastocyst generated from the fertilization of one oocyte with one spermatid collected from a vaginal sample of group A were not transferred.

Treatment of pseudopregnant recipients

To induce pseudopregnancy, mature female Wistar rats who had demonstrated at least three consecutive regular 4 day oestrus cycles were mated with vasectomized mature male Wistar rats that had been proven to be azoospermic. Mating was arranged between 19:30 and 20:30 h on the day of pro-oestrus. The day of mating was determined to be the day 0 of pseudopregnancy. Blastocysts were transferred on day 4 (between 12:00 and 14:00 h).

Statistical analysis

The recovery/processing of round spermatids of stages 2–7 for ooplasmic injections and observations of the testicular tissue histology (Figure 4), spermatid fractions, oocytes (Figure 5), and embryos (Figures 6 and 7) were performed in a blinded fashion. Statistical analysis was performed using the χ^2 test (qualitative parameters) or Wilcoxon's test (comparison of a quantitative parameter between two groups), or analysis of variance plus Duncan's test (comparison of a quantitative parameter among three or more groups). $P < 0.05$ was considered to be statistically significant. Values were expressed as mean ± SD.

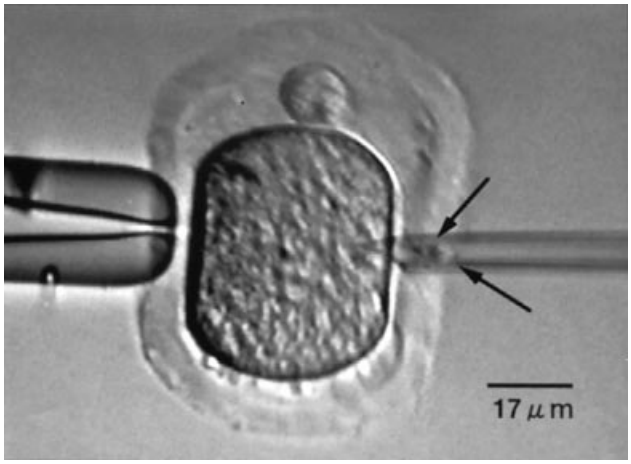

Figure 5. Performance of round spermatid nuclei injection (ROSNI) in the rat. The black arrows indicate the round spermatid nucleus.

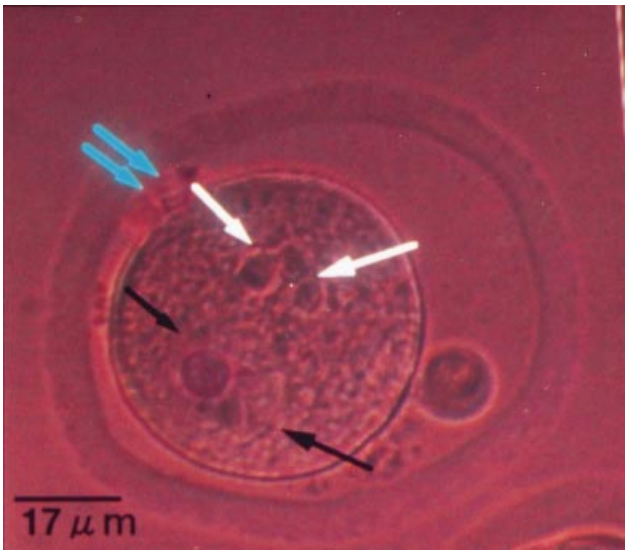

Figure 6. Normally fertilized oocyte at 9 h post-round spermatid nuclei injection (ROSNI). One pronucleus is indicated by black arrows and another one by white arrows (see nucleolus-like structures within each pronucleus). One polar body is clearly visible while the second polar body is out of focus (blue arrows).

Results

Intratesticular testosterone concentration

The intratesticular testosterone concentration was significantly smaller in group A than in group B (Table I).

ROSNI outcome

There were no significant differences in the proportion of activated oocytes to injected oocytes, the ratio of normally fertilized oocytes to injected oocytes, and the proportion of normally fertilized oocytes that subsequently cleaved to injected oocytes among testicular, vaginal, and epididymal samples within group A. Furthermore, differences in the latter parameters of testicular samples between groups A and B were not significant (Table II). In contrast, the ratio of blastocysts to injected oocytes at 96 h was significantly larger in the testicular samples of groups A and B than in the epididymal samples of group A and the vaginal samples of group A (Table II). Ten of the 20 oocytes of group C were activated (parthenogenetic activation; observation at 9 h post-injection) and seven of the latter oocytes completed the first cleavage (observation at 24 h post-injection). Within group C no oocyte developed up to the blastocyst stage (observation at 96 h post-injection).

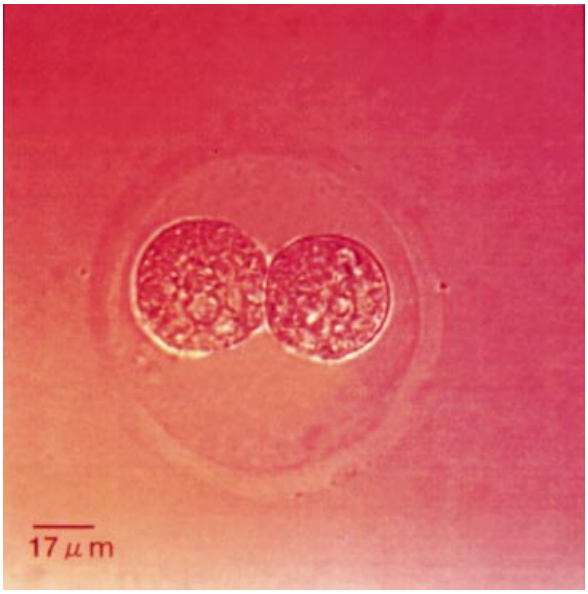

Figure 7. A 2-cell embryo at 24 h post-round spermatid nuclei injections (ROSNI).

Table I. Intratesticular testosterone concentration in rats treated with testosterone plus oestradiol for 7 weeks following 1 week of testosterone administration

Group	Treatment	Testosterone (ng/g testis)
A	Testosterone plus oestradiol	5.0 ± 1.4[a]
B	Sham-treatment	109.8 ± 24.9[a]

[a]Significant difference between the groups ($P < 0.05$).

Table II. Outcome of round spermatid nuclei injection (ROSNI) using round spermatids collected from the male reproductive tract or the ejaculate of rats treated with testosterone plus oestradiol for 7 weeks following one week of testosterone administration

Source of round spermatids	IO	AO	FO	CO	BLS-96
Testicle (group B)	80	60	53	46	37[a]
Testicle (group A)	80	57	51	44	33[a]
Epididymal head (group A)	80	61	52	43	16[b]
Epididymal body (group A)	80	56	47	41	12[b]
Epididymal tail (group A)	80	55	49	42	13[b]
Ejaculate (group A)	40	28	24[a]	21	6[b]

IO = no. of injected oocytes; AO = no. of activated oocytes at 9 h post-injection; FO = no. of normally fertilized oocytes at 9 h post-injection; CO = no. of normally fertilized oocytes that subsequently cleaved (observation at 24 h); BLS-96 = no. of blastocysts at 96 h.
[a,b]Values within each column with same superscripts are significantly different ($P < 0.05$).

Table III. Viability of round spermatids collected from the male reproductive tract or the ejaculate of rats treated with testosterone plus oestradiol for 7 weeks following one week of testosterone administration

Source of round spermatids	Viability of round spermatids (%)
Testicle (group B)	93 ± 3[a]
Testicle (group A)	91 ± 3[a]
Epididymal head (group A)	80 ± 5[b]
Epididymal body (group A)	76 ± 7[b]
Epididymal tail (group A)	77 ± 8[b]
Ejaculate (group A)	59 ± 7[c]

[a,b,c]Values with different superscripts are significantly different ($P < 0.05$).

Round spermatid viability

Round spermatid viability was significantly larger in the testicular samples of groups A and B than in the epididymal samples of group A and the vaginal samples of group A. Within group A, ejaculated round spermatids demonstrated significantly smaller viability than round spermatid fractions isolated from the lumen of the head, body, or tail of epididymis (Table III).

Histology of testicular tissue evaluated by haematoxylin/eosin stain

Rats of group A demonstrated spermatogenic arrest at the round spermatid stage (Figure 4) in the majority of seminiferous tubules. Elongating/elongated spermatids were rarely seen.

Inverted microscopy of minced/filtered testicular/epididymal specimens and vaginal samples

Inverted microscopy of dispersed cells from testicular, epididymal, and vaginal samples of group A revealed several round spermatids. Deformities were observable in several round spermatids collected from epididymides and vaginae (Figure 2). There were no round spermatids in the epididymal head, body, and tail of rats of group B.

Confocal laser scanning microscopy of minced/filtered testicular/epididymal specimens and vaginal samples

Confocal laser scanning microscopy of minced testicular tissue revealed that the vast majority of the testicular spermatids of group A were of step 8 or below. Step 8 round spermatids and round spermatids at steps 9–10 were occasionally observed within the minced testicular samples. Spermatids of step 10 or below were rarely seen within the minced testicular tissue. Within the group A, a large number of round spermatids in the cellular fractions recovered from the epididymal head, body, and tail, and the vaginae were of step 8 (Figure 3). Within the epididymal and vaginal samples, several round spermatids of steps 2–7 were also found. The latter spermatids were considered for ooplasmic injection.

In-vivo embryonic development

One of the two recipients who underwent transfer of blastocysts generated from the fertilization of oocytes with testicular spermatids of group B delivered five healthy offspring. The second recipient who underwent transfer of group B blastocysts died suddenly. All the three recipients who underwent transfer of blastocysts generated from the fertilization of oocytes with testicular spermatids of group A delivered healthy offspring (seven, three, and three offspring/recipient).

No pregnancy was achieved post-transfer of blastocysts generated from the fertilization of oocytes with ejaculated spermatids or spermatids recovered from the epididymal head, body, or tail. Within group A, the ratio of live offspring/transferred blastocysts was significantly higher in testicular samples (13 out of 33) than in the three epididymal samples taken together (none out of 41).

Within group A, among the recipients that showed pro-oestrus/oestrus vaginal smears 12–20 days post-transfer, implantation scars were observed due to the implantation of blastocysts generated from the fertilization of oocytes with epididymal head spermatids (two recipients: two and one scars, respectively), epididymal body spermatids (one recipient: one scar), and epididymal tail spermatids (one recipient: three scars). Within group A, no implantation scars were observed in the recipient who underwent transfer of blastocysts derived from fertilization of oocytes with ejaculated spermatids.

Discussion

A significant percentage of non-obstructed azoospermic men have a limited number of spermatozoa within their testicular biopsy material (see for review Sofikitis *et al*., 1998c). Ooplasmic injection of testicular spermatozoa [intracytoplasmic sperm injection (ICSI) techniques] is an attractive mode of treatment for these men. Among the non-obstructed azoospermic men who are negative for spermatozoa in their testicular tissue a subpopulation demonstrates testicular foci of spermatids (Hannay *et al*., 1995; Amer *et al*., 1997; Antinori *et al*., 1997a,b; Vanderzwalmen *et al*., 1997; Bernabeu *et al*., 1998; Sofikitis *et al*., 1998b). Ooplasmic injection of spermatids is the only hope for the latter men to father their own children. The first pregnancies after ooplasmic injections of round spermatids were achieved by the Tottori University International Research Group (Hannay, 1995; Sofikitis *et al*., 1995). Additional pregnancies after ooplasmic injection of spermatids have been reported by other workers (Fishel *et al*., 1995; Tesarik *et al*., 1995; Mansour *et al*., 1996; Vanderzwalmen *et al*., 1997; Amer *et al*., 1997; Antinori *et al*., 1997a,b; Barak *et al*., 1998; Kahraman *et al*., 1998; Sofikitis *et al*., 1998b).

Two studies (Mendoza and Tesarik, 1996; Tesarik *et al*., 1998) showed that the great majority of non-obstructed azoospermic men have ejaculates positive for spermatids. A previous study (Tesarik *et al*., 1995) isolated human round spermatids from ejaculates of non-obstructed azoospermic men, injected them into the ooplasm, and achieved full-term pregnancies. A perplexing problem in the management of non-obstructed azoospermic men with ejaculates positive for round spermatids is the choice between ROSNI techniques using round spermatids recovered from the ejaculate, and ICSI or ROSNI procedures using spermatozoa or round spermatids respectively, isolated from therapeutic testicular biopsy material. A therapeutic testicular biopsy is the most promising approach because it offers the possibility to identify spermatozoa, if they exist, in the testicular tissue. However, a significant

percentage of non-obstructed azoospermic men are not willing to undergo a therapeutic testicular biopsy when spermatids are present in their ejaculates. Attempting to evaluate further the above therapeutic dilemma we compared the ROSNI outcome using testicular round spermatids versus abnormally-released round spermatids from the seminiferous epithelium. We chose the rat as an experimental animal because a model of abnormal release of round spermatids from the rat seminiferous epithelium has been well established (O'Donnell *et al*., 1996). Furthermore, after the successful performance of ROSNI techniques in the rabbit (Sofikitis *et al*., 1994a) and mouse (Ogura *et al*., 1994), it was a challenge to evaluate the reproductive capacity of the early haploid male gamete in an additional species.

The results of the current study show clearly that the overall outcome of ROSNI techniques using testicular round spermatids is superior to ROSNI results using abnormally released (from the seminiferous epithelium) epididymal round spermatids or ejaculated round spermatids. Although abnormally-released round spermatids have a non-significantly altered capacity to: (i) contribute to the oocyte activation (assuming that the oocyte activating factor of the round spermatid acts synergetically with the electrical pulse); (ii) fertilize oocytes; and (iii) induce/contribute to the first mitotic division of the zygote, early embryonic development after the first mitotic division of the zygote is significantly impaired when the latter round spermatids are used. The smaller potential of the abnormally released round spermatids to trigger optimal early embryonic development might not be attributed to the reduced average round spermatid viability in the fractions of epididymal or vaginal spermatids, because (i) although the average round spermatid viability was significantly smaller in ejaculated samples than in epididymal samples there were no significant differences in the blastocyst development rate (Table II); (ii) deformed, non-round, degenerated, or irregular round spermatids were not processed for ROSNI techniques; and (iii) there was no significant difference in the capacity of the nucleus of the injected round spermatid to transform into a male pronucleus with nucleolus-like structure (indicating live genetic material and active DNA synthesis) among testicular, epididymal, and ejaculated samples. Chemical and/or mechanical factors may contribute to the diminished capacity of abnormally released round spermatids to induce optimal early embryonic development.

Epididymal secretions or seminal vesicular secretions may influence detrimentally the reproductive potential of round spermatids during the epididymal storage of round spermatids or at the process of ejaculation respectively. Alternatively, contractions of the vas deferens and the ejaculatory duct during the non-physiological passage of round spermatids through the ducts of the male reproductive tract may influence mechanically their integrity and/or the overall reproductive potential of the round spermatid. In fact, deformities were commonly found in the epididymal and ejaculated round spermatids. The impaired reproductive potential of abnormally released round spermatids is additionally supported by the lack of offspring after transfer of blastocysts generated from the fertilization of oocytes with epididymal or ejaculated round spermatids. Furthermore, the total number of implantation sites was small (i.e. seven implantation sites after the transfer of 41 blastocysts) in the four recipients who received blastocysts developed from the fertilization of oocytes with epididymal spermatids. We suggest that round spermatids within non-physiological regions of the male reproductive tract (i.e. outside the testis) have, not only an impaired capacity to induce optimal early embryonic development post-fertilization, but also an impaired ability to contribute (together with female factors) to the generation of embryos with adequate potential for the implantation process and the post-implantation development. Several recent studies suggest that there is a paternal contribution to the early embryonic development and capacity for implantation (Janny and Ménézo, 1994; Ono *et al*., 1997; Sofikitis *et al*., 1998b). It appears that the passage of the round spermatid through the ducts of the male reproductive tract has an adverse effect on the factors of the male gamete that are responsible for the paternal influence on early embryonic development and potential for implantation.

Previous studies have demonstrated delivery of healthy offspring after ooplasmic injections of round spermatids and embryo transfer techniques in the rabbit (Sofikitis *et al*., 1994a), mouse (Ogura *et al*., 1994), and human (Tesarik *et al*., 1995). The present study is the first report in the literature showing that ooplasmic injection of the early haploid male gamete can induce efficient in-vitro and in-vivo embryonic development in the rat. The failure of the sham-injected oocytes (group C) to develop up to the blastocyst stage suggests that the advanced in-vitro embryonic development of rat oocytes injected with round spermatid nuclei cannot be attributed to parthenogenetic activation of the oocytes.

O'Donnell *et al*. (1996) have shown that when exogenous testosterone is administered to adult rats, producing slightly supraphysiological serum testosterone concentrations, pituitary LH is suppressed, resulting in a suppression of intratesticular testosterone concentration and almost undetectable levels of elongated spermatids (O'Donnell *et al*., 1996). Low doses of oestradiol in combination with testosterone have a synergistic effect in suppressing testicular function presumably due to the more profound suppression of LH (Robaire *et al*., 1979; McLachlan *et al*., 1994). Testosterone is critical for the differentiation of round to elongated spermatids (McLachlan *et al*., 1994). This animal model is considered to be primarily one of LH and testicular testosterone deficiency since follicle stimulating hormone (FSH) concentrations are either unaffected or partially affected (Robaire *et al*., 1979; Zirkin *et al*., 1989). We created the same model as that studied by O'Donnell *et al*. (1996) with the difference that in the present study testosterone and oestradiol were administered exogenously via daily injections, instead of being released by silastic implants (Robaire *et al*., 1979). We administered exogenously (daily) similar doses of testosterone and oestradiol to those that were released daily by the silastic implants in the model of O'Donnell *et al*. (1996) (Robaire *et al*., 1979). We chose hormonal administration via injections because preliminary experiments showed that in ~20 and 10% of silastic implants similar to those previously described (Robaire *et al*., 1979; O'Donnell *et al*., 1994, 1996), the release of testosterone was significantly

faster and significantly slower respectively, than that reported by Robaire *et al*. (1979). In the current study, we confirmed the adverse effect of exogenous testosterone administration under doses that do not alter significantly the serum testosterone concentrations (data not shown; see Robaire *et al*., 1979; O'Donnell *et al*., 1994, 1996) on (i) intratesticular testosterone concentration (Table I); and (ii) differentiation of round spermatids to elongating spermatids (see arrest at the round spermatid stage in Figure 4). Furthermore, the release of round spermatids from the seminiferous epithelium was confirmed in the current animal model by identifying round spermatids within the epididymis or the ejaculate. It has been suggested that following intratesticular testosterone suppression, round spermatids are unable to proceed through the transition between steps 7 and 8 and are, therefore, unable to complete the elongation process (O'Donnell *et al*., 1994, 1996). The failure of round spermatids to complete spermiogenesis following intratesticular testosterone suppression may be due to detachment of round spermatids from the seminiferous epithelium. Although O'Donnell *et al*. (1996) proposed a stage-specific (between steps 7 and 8) detachment of round spermatids from the rat seminiferous epithelium, confocal laser scanning microscopy in the current study recognized several round spermatids of steps 2–7 in addition to the released step 8 round spermatids within the epididymal lumen (Figure 3). The former round spermatids were processed for nuclei isolation and ooplasmic injections. In the study by O'Donnell *et al*., the probability that few round spermatids of steps 2–7 were also released could not be ruled out since the authors (O'Donnell *et al*., 1996) used periodic acid–Schiff staining for identification of round spermatids within the epididymis and not the gold standards, transmission electron microscopy or confocal laser scanning microscopy. Further studies are necessary to clarify the stages in the early spermiogenetic process during which abnormal release of round spermatids occurs as a response to suppression of intratesticular testosterone concentration. The present study demonstrates that the rat early haploid male gamete has reproductive potential. Testicular round spermatids have the ability to induce better early embryonic development and subsequently larger pregnancy rates than round spermatids abnormally released from the seminiferous epithelium.

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