ANDROLOGY

Ooplasmic Round Spermatid Nuclear Injection Procedures as an Experimental Treatment for Nonobstructive Azoospermia

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Purpose: Our objective was to apply ooplasmic round spermatid nuclear injections for the treatment of nonobstructive azoospermia.

Materials: Participants were nine azoospermic men who had previously undergone diagnostic testicular biopsy. Spermatogenetic arrest was diagnosed at the round spermatid stage ($n = 6$) or primary spermatocyte stage ($n = 3$). A second (therapeutic) testicular biopsy was performed and round spermatid nuclei were recovered from all the participants.

Results: Forty-nine mature oocytes were successfully injected with nuclei and then cultured for 72 hr. Twenty-four embryos were transferred to nine women. No pregnancy was achieved.

Conclusions: Round spermatids can be recovered from therapeutic testicular biopsy material of men negative for round spermatids in previous routine diagnostic testicular biopsy specimens. Round spermatid nuclear injections may play a role in the treatment of nonobstructive azoospermia.

KEY WORDS: azoospermia; infertility; oocyte; round spermatid.

INTRODUCTION

The male gamete that has just completed the second meiotic division in the testis is the round spermatid, and therefore, the nucleus of the round spermatid contains a complete haploid set of chromosomes. Previous studies have shown fertilization and delivery of healthy offspring after transferring round spermatid nuclei into mouse or rabbit oocytes via electrofusion or microsurgical methods, respectively (1–3). Recently, two human pregnancies have been achieved by ooplasmic injections of intact round spermatid cells (4). One healthy male infant was delivered (4). Round spermatid nuclear injections into oocytes have also led to pregnancies (5). However, the latter pregnancies have resulted in abortions due to maternal factors (5).

Ooplasmic injection of the intact round spermatid cell ensures the transfer of all the cytoplasmic components of the male gamete into the maternal gamete and is less time-consuming than round spermatid nucleus injection. Furthermore, manipulations of the nuclear matrix and envelop are avoided via intact spermatid cell injections. In contrast, integral round spermatid cell injection has two disadvantages: (a) injecting micropipettes of larger diameters are necessary, and consequently the probability of injuring oocytes during injections is higher; and (b) persistence of a large amount of cytoplasm around the round spermatid nucleus may impede its transformation into male pronucleus (3,6). In the mouse, transferring the round spermatid nucleus into oocytes via electrofusion or microsurgical techniques is a far more efficient proce-

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dure in achieving fertilization and pregnancy than transferring the intact round spermatid cell (3,6).

In the current study we report the results of ooplasmic injections of round spermatid nuclei for the treatment of male infertility due to nonobstructive azoospermia.

MATERIALS AND METHODS

Participants

The participants in the present study were nine non-obstructed azoospermic men with karyotypes 46-XY aged 29 to 43 years who attended our infertility clinics. Their wives were 33 to 44 years old. Peripheral serum follicle stimulating hormone levels (FSH) in the male patients were 31 to 47 IU/L. Normal levels of FSH in our facilities are considered to be between 3 and 10 IU/L. All nine men had previously undergone routine diagnostic testicular biopsy. Evaluation of the fixed/stained slides suggested that spermatogenesis was arrested at the round spermatid stage ($n = 6$) or primary spermatocyte stage ($n = 3$). All the couples were aware that ooplasmic injections of round spermatid nuclei had not previously resulted in a live birth. The three couples with spermatogenetic arrest at the primary spermatocyte stage were additionally informed that round spermatids might not be identified in the second (therapeutic) testicular biopsy material and, in that case, the cycles would be canceled. Informed consent was obtained from all the couples.

Oocyte Preparation

Details of ovarian stimulation and oocyte recovery in our facilities have been published previously (7). After treatment with 0.1% bovine testicular hyaluronidase (type VIII; Sigma Co., St. Louis, MO) for 2 min, the oocytes were freed from cumulus or coronal cells and transferred into Human Tubal Fluid medium (Irvine Scientific, Santa Ana, CA) supplemented with 15% patients serum (modified HTF medium) and incubated at 37°C under 5% CO₂ for 3 to 5 hr. Oocytes which had already completed the first meiotic division were injected.

Therapeutic Testicular Biopsy/Isolation of Round Spermatids

A small amount of tissue (therapeutic testicular biopsy; 203 to 288 mg) was resected from the contra-

lateral testis to the side of the previous diagnostic biopsy. The testicular tissue was washed three times with normal saline. The seminiferous tubules were then washed in Dulbecco's phosphate-buffered saline (Sigma Co.) containing 5.6 mM glucose and 5.8 mM sodium lactate (modified DPBS) and subsequently minced into small pieces. Conventional microsurgical instruments were used to cut the tissue. Samples were kept constantly at 5°C. Preliminary studies in the rabbit showed that a low temperature (4–7°C) during testicular tissue process for isolation of round spermatids and extraction of nuclei prolongs the round spermatid viability and nucleus fertilizing capacity. A dissecting microscope (Olympus SZ-STS, Olympus, Tokyo) facilitated cutting the tissue into smaller fragments with minimal blood contamination, since pieces of tissue having blood vessels in their surfaces were not cut further. The overall mincing process lasted 1 hr. The samples were then centrifuged at 500g for 20 min and the pellet was suspended in modified DPBS. The samples were subsequently filtered via a filter paper of 20- to 30- μ m pore size (Whatman Co., New York; Grade No. 4, Catalog No. 1004-042). The filtrate was collected, then centrifuged at 750g for 30 min, and the pellet was resuspended in the latter medium. Then the cells were observed via an inverted microscope (X600; IX-70, Olympus, Tokyo)—computer-assisted system (Apple Computers Inc., Cupertino, CA). The television monitor was precalibrated, allowing instantaneous measurements of distances and angles. Images were occasionally transferred from the television monitor to the computer and further magnified on the computer screen. Computer-assisted image analysis allowed us to appreciate the maximal diameter of a given cell. The middle point of the maximal diameter was then defined. The diameter passing through the middle point of the maximal diameter at a 90° angle was defined as the minimal diameter of the cell. The oblique diameter was defined as that passing through the middle point of the maximal diameter at a 45° angle.

We calculated three morphometric parameters instead of one, attempting to appreciate better the cellular size and increase the accuracy of our method of round spermatid identification. "Round cells" other than red blood cells, having a maximal diameter between 7 and 15 μ m, a minimal diameter between 6 and 10 μ m, and an oblique diameter of less than 13 μ m were recovered via the micromanipulator pipette. The internal diameter of the micropipette was 25 μ m. Most of the cells with the above quantitative characteristics were collected. Irregularly shaped cells were neglected since our preliminary studies have shown

that most of these cells are pachytene spermatocytes. In preliminary experiments human cadaveric testicular cells with morphometric parameters satisfying the above quantitative criteria were recovered and processed for transmission electron microscopy. It was found that 63 and 16% of the cells isolated were round spermatids of stages 2 to 5 and stages 6 to 8 of spermiogenesis, respectively. The remaining cells were elongating spermatids, spermatocytes/spermatogonia, and leukocytes. To confirm further that our methods result in the recovery of a high percentage of round spermatids, a fraction of cells recovered from four participants was processed for transmission electron microscopy (1,2) and another fraction of cells from five participants was processed for confocal scanning laser microscopy (7). Microscopic techniques using standard procedures were applied (1,2,7). Recovered round spermatids were transferred to a medium (SOF medium) containing CaCl_2 (0.9 mM), KCl (5.4 mM), MgSO_4 (0.6 mM), Na-lactate (5.8 mM), $\text{Fe}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$ (1.0×10^{-4} mM), NaHCO_3 (34 mM), glucose (8.4 mM), Arginine (7.5×10^{-1} mM), cystine (88.3×10^{-2} mM), glutamine (3.0 mM), glycine (2.6×10^{-1} mM), histidine (1.5×10^{-1} mM), hydroxyproline (8.0×10^{-2} mM), lysine (5.1×10^{-1} mM), methionine (1.5×10^{-1} mM), phenylalanine (2.3×10^{-1} mM), proline (9.0×10^{-2} mM), serine (3.4×10^{-1} mM), tyrosine (2.5×10^{-1} mM), valine (4.9×10^{-1} mM), biotin (4.0×10^{-4} mM), folic acid (5.0×10^{-3} mM), inositol (1.2×10^{-1} mM), thiamine (7.0×10^{-3} mM), riboflavin (8×10^{-4} mM), phenol red (2.0×10^{-2} mM), nicotinamide (2.0×10^{-2} mM), and cholesterol (5.2×10^{-4} mM). Before use, 2.85 μg of bicarbonate was added/ml of SOF medium.

Isolation of Round Spermatid Nuclei

Recovered round spermatids were transferred from the SOF medium in a sucrose solution consisting of 50 mM Tris (Sigma Co.), 10 mM MgCl_2 , and 550 mM sucrose (pH 7.4). Then they were centrifuged at 750g for 10 min and resuspended in the same solution containing Triton X-100 (0.015%, v/v). After gentle pipetting for 30 sec and further centrifugation at 1000g for 7 min, the pellet (nuclei) was collected and transferred into modified DPBS. The samples were then centrifuged at 750g for 10 min. The pellet was collected and washed twice in SOF medium, and finally, nuclei were suspended in SOF medium without cholesterol supplemented with 1 mM MgCl_2 and 4% polyvinylpyrrolidone (K90; MW, 360,000; ICN Biochemicals, Costa Mesa, CA; Catalog No. 102787).

Nuclei were kept at 34°C. Some of the nuclei were fixed and observed via a transmission electron microscopy-computer-assisted system as we have described previously (1, 2).

Round Spermatid Nuclei Injection into Oocytes

For manipulations of oocytes Hepes-buffered modified HTF medium was used.

Round spermatid nuclei were injected into oocytes within 60 min of isolation. To inject the nucleus into an oocyte the injecting micropipette penetrated the zona pellucida and vitelline membrane, a minimal amount of cytoplasm was aspirated into the micropipette to confirm the rupture of the vitelline membrane, and then the cytoplasm together with the nucleus and a small amount of medium was expelled into the oocyte. Additional cytoplasmic aspiration/expelling manipulations were not performed during nuclei injections. In only a few nonsuccessfully injected oocytes, the nuclei either failed to enter the oocytes or were extruded. The internal diameter of the injecting micropipette was 7 to 8 μm . After injections all the oocytes were transferred to modified HTF medium and cultured at 37°C under 5% CO_2 for 72 hr. Medium was changed 48 hr after injections. Oocytes were carefully observed 9 hr after injections. In a previous study, the peak of the two-pronuclei appearance curve in a group of human oocytes injected with round spermatid nuclei was 9 hr after injections (8). At that time all the normally fertilized oocytes revealed two pronuclei, whereas 2 hr later both pronuclei disappeared in 20% of the oocytes (8).

An oocyte was considered activated when the first and second polar bodies and a well-developed female pronucleus were discernible (3, 6). Fertilization was assessed by looking for the presence of two pronuclei and polar bodies (Table 1).

Embryo Transfer

At the end of the culture period two- to eight-cell-stage embryos which had been at the two pronuclei stage 9 hr after injections were transferred to the wives of the participants by standard techniques (7). Two-cell-stage embryos after 72 hr of culture are generally considered as slowly developing or blocked. In the present study two-cell-stage embryos were transferred considering that their potential for implantation cannot be ruled out. Round spermatid nuclear injection procedures are currently offered at a limited number of IVF centers. The couples treated in the present study may not have the opportunity to participate in an additional

Table I. Outcome of Human Ooplasmic Round Spermatid Nuclear Injections^a

Parameter assessed	Hours of culture	
No. of couples		9
Injected oocytes		53
Successfully injected oocytes		49
Activated oocytes	9	47
One-cell-stage oocytes without pronuclei	9	2
One-pronucleus stage	9	13
Two-pronuclei stage	9	34
One-cell-stage oocytes without pronuclei	72	2
One-pronucleus stage	72	13
Oocytes arrested at the process of the first cleavage	72	1
Two-pronuclei stage	72	3
Cleaved oocytes	72	30
Transferred embryos	72	24
No. of women undergoing embryo transfer		9

^aFor additional information, see the Results.

cycle in the future and the highest probability for pregnancy was offered them by transferring all the oocytes which had developed two pronuclei and a second polar body and subsequently cleaved.

Embryo Culture

In one case 12 oocytes were fertilized. In that cycle, six embryos were transferred, three embryos were cryopreserved (four-cell stage) (9), and the remaining three embryos (four-cell stage) were cultured at 37°C under 5% CO₂ for an additional 36 hr (cultured for a total 108 hr). Medium was changed 96 hr after injections. At the end of the culture period two embryos reached the expanding blastocyst stage. The above two blastocysts were analyzed for chromosomal synthesis. Chromosome spreads were made by a direct preparation (2). Slides were stained with Giemsa (2). Chromosomes were analyzed in a minimum of 35 cells for each blastocyst. Six karyotypes were constructed from each blastocyst.

RESULTS

Round Spermatid Nuclear Injection Outcome

Data on activated, cleaved, and transferred oocytes after ooplasmic nuclear injections are presented in Table I.

At least one embryo was transferred to every woman. Eight of the transferred embryos were at the

two- to four-cell-stage and the remaining 16 transferred embryos had reached the five- to eight-cell stage. At least one embryo in each cycle developed to the four-cell stage or further. No pregnancy was achieved. Two of the three four-cell-stage embryos cultured for 108 hr developed to the expanding blastocyst stage. The development of the remaining one embryo was arrested at the eight-cell stage. All six karyotypes prepared from each blastocyst were diploid. In both blastocysts 46-XY karyotypes were found.

In three couples round spermatids had not been identified in the diagnostic testicular biopsy specimen. However, cells satisfying our quantitative criteria for identification of round spermatids were isolated from the therapeutic testicular biopsy material. These cells were considered to be round spermatids and processed for nuclei extraction and ooplasmic injections.

Transmission Electron Microscopy of Selected Cells and Nuclei

Transmission electron microscopy identified 84% of the cells isolated as round spermatids of stages 2 to 7 of spermiogenesis (Fig. 1). Spermatocytes/spermatogonia, leukocytes (neutrophils and eosinophils), and elongating spermatids were also observed.

A layer of cytoplasm around the nuclear envelope was found in all nuclei. No defects in the nuclear membrane were demonstrated (Figs. 2a and b).

Confocal Scanning Laser Microscopy of Spermatids

Confocal scanning laser microscopy recognized 76% of the cells collected as round spermatids (Fig. 3). Remaining cells were spermatogonia/spermatocytes, leukocytes, and elongating spermatids.

Since it is difficult to differentiate accurately between round spermatids of stage 1 of spermiogenesis and secondary spermatocytes via confocal scanning laser microscopy or transmission electron microscopy, all cells with characteristics of the round spermatid of stage 1 were recorded as secondary spermatocytes. Thus, the actual number of round spermatids isolated might be larger than the number recorded.

DISCUSSION

The results of the present study are consistent with a previous report (4) showing that ooplasmic injections of human round spermatids can result in fertilization

Fig. 1. Observation of a human round spermatid cell via the transmission electron microscope-computer-assisted system. Original magnification, $\times 9600$.

and subsequent embryonic development *in vivo*. In addition, human oocytes fertilized by round spermatid nuclei could further develop *in vitro* up to the expanding blastocyst stage. Normal diploid karyotypes were found in the latter blastocysts. Therefore, we suggest that the chromosomes of the human round spermatid can pair with the chromosomes of the oocyte to participate in syngamy and further embryonic development. Furthermore, we support the thesis that post-meiotic modifications of the human round spermatid are not required for the final events of the fertilization process (i.e., after the entrance of the male gamete within the ooplasm).

The transfer of the early haploid male gamete nucleus within oocytes which had received no electrical or mechanical ooplasmic prestimulation resulted in activation of 95% of the successfully injected oocytes. It has been suggested that activation of oocytes depends on a male gamete nucleus-associated oocyte-activating factor present in both mature (i.e., epididymal) and immature (i.e., testicular) spermatozoa (6). The above factor has been considered to appear/to be activated in the mouse male gamete after the round spermatid stage (6). In contrast, our findings

show that transferring human round spermatid nuclei into the oocyte results in oocyte activation in a high percentage of the cases. In preliminary experiments nine human mature oocytes were collected from super-ovulated women. A minimal amount of ooplasm was aspirated into an injecting micropipette as described above (see Materials and Methods) and the oocytes were injected with an amount of medium equivalent to that used for injections in the present study. No oocyte was activated. Since the injection of medium or the technique applied for injections alone did not activate human oocytes, it must be the nuclei that did this in the present study. It appears that in the human, the oocyte-activating factor has already appeared or been activated at or before the first stages of spermiogenesis. It should be emphasized also that the technique applied for human male gamete/male gamete nucleus injections into oocytes may influence the oocyte activation process. It has been reported recently that vigorous aspiration of oocyte cytoplasm during injections may facilitate oocyte activation by increasing the Ca^{2+} load of the oocytes (10). This increased Ca^{2+} load may support the action of the male gamete nucleus-

Fig. 2. (a,b) Observation of human round spermatid nuclei via the transmission electron microscope-computer-assisted system. A thin cytoplasmic layer (white) surrounds the nucleus (black). Original magnification, $\times 12,000$.

associated oocyte-activating factor to trigger oocyte activation (10).

There is indirect evidence that mammalian mature oocytes (with the exception of mouse oocytes) have lost their centrosomic material and that paternally inherited centrosome participates in the mitotic divi-

sions of the zygote (11). In contrast, cleavage of oocytes fertilized by nude nuclear material (i.e., without any cytoplasmic content) extracted from rabbit early spermatids has been demonstrated in three previous studies (1, 2, 12). In the present study, transmission electron microscopy revealed that the nuclei isolated

Fig. 3. Observation of a round spermatid cell via the confocal scanning laser microscope-computer-assisted system. A large proacrosomic granule (large black spot) is observable between the cytoplasmic membrane and the nuclear envelop. Original magnification, $\times 7200$.

by our methods were surrounded by a layer of cytoplasm. Therefore, we cannot comment on whether centrosomic material has been injected into the oocyte together with the nucleus.

"Round cells" satisfying the quantitative criteria for round spermatids were found in the therapeutic testicular biopsy material of all the participants. However, transmission electron microscopy and confocal scanning laser microscopy revealed that a small but significant percentage of the "round cells" processed for injections were not round spermatids. Thus, the fertilization rate after ooplasmic nuclear injections might have been higher if all the nuclei injected were round spermatid nuclei. In animal experiments when a confocal scanning laser microscope-micromanipulator system was used for identification of round spermatids, 97% of the recovered cells were proven to be round spermatids (1, 2). It is clear that the method chosen for round spermatid isolation is a major factor affecting the outcome of nuclear injection procedures.

In the current study 13 oocytes were identified as one-pronucleus plus second polar body (1PN-2SPB) stage after 9 hr of culture. These embryos did not further develop. Kimura and Yanagimachi (6) demonstrated that 1PN-2SPB-stage mouse zygotes generated after ooplasmic round spermatid nuclear injections seldom developed into blastocysts and live offspring. In

fact, it was suggested that some of the zygotes which were identified as 1PN-2SPB must have had a minute male pronucleus (6). More experimental and clinical studies are necessary to evaluate the developmental potential of 1PN-2SPB zygotes after ooplasmic nuclear injections.

Although three of the participants had been found to be negative for round spermatids after diagnostic testicular biopsy, at least one embryo ≥ 4 -cell stage was developed in each of these cycles. It appears that even in patients whose routine diagnostic testicular biopsy failed to demonstrate any round spermatids, a more thorough search of therapeutic testicular biopsy material revealed a few round spermatids, which were then processed for nuclei extraction and nuclear ooplasmic injections. Our findings suggest that some of the patients with the diagnosis of spermatogenic arrest at the primary spermatocyte stage, in truth, may have rare foci of round spermatids somewhere in the testes. Silbert and co-workers have suggested that even spermatozoa can be found in the therapeutic testicular biopsy in some men with nonobstructive azoospermia (13). Thus, we emphasize the need to collect an adequate amount of testicular tissue (>200 mg) for accurate demonstration of round spermatids by therapeutic testicular biopsy procedures in men who are negative for round spermatids in the routine diagnostic testicular

biopsy specimen. In the present study the therapeutic biopsy weight was $>5\times$ diagnostic biopsy weight).

We may suggest that ooplasmic round spermatid nuclear injections have a role in the treatment of male infertility due to nonobstructive azoospermia since (a) at least one fertilized oocyte developed to ≥ 4 -cell stage embryo in each cycle, (b) some fertilized oocytes developed up to the 8-cell stage, and (c) diploid XY karyotypes were found in cells from embryos cultured up to the expanding blastocyst stage. More research efforts are necessary to understand better the physiology of round spermatids and subsequently improve the outcome of round spermatid nuclear injection procedures.

APPENDIX

The present paper does not refer to the outcome of transfers of frozen/thawed embryos derived from ROSNI procedures during 1995. Results of the above transfers will be published when the permission of our Ethical Committees is granted. All of the authors agree with the official thesis of the Japanese Association of Obstetrics and Gynecologists that ROSNI procedures are experimental.

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