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Methods for isolation and purification of post-ejaculate human round spermatids for possible use in intracytoplasmic round spermatid injection*

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ABSTRACT

Objective: To develop methods for the isolation and purification of round spermatids (RS) from ejaculated semen specimens recovered from patients suffering from nonobstructive azoospermia for the purpose of performing intracytoplasmic round spermatid injections (ROSI) into human oocytes.

Design: Prospective study.

Setting: Andrology Institute of Lexington, Lexington, Kentucky.

Patients: Six men with nonobstructive azoospermia.

Interventions: Semen specimens were washed via centrifugation prior to selection and purification of RS. The RS were isolated via a multi-layer Percoll (90, 80, 70, 60 and 40% Percoll) centrifugation procedure. The isolated RS were purified via a second multi-layer Percoll (70, 40, 30 and 20% Percoll) centrifugation procedure. Purified RS were washed twice, resuspended in polyvinylpyrrolidone (PVP) and identified via microscopy.

Main Outcome Measures: Isolation and purification of RS from other round cells (RC) and seminal debris.

Results: High recovery of RS (66 to 76% of the original RS population) with minimal background (round cells and debris) after Percoll isolation and the various purification steps.

Conclusions: Large quantities of RS were detected in the original ejaculates and subsequently isolated and purified. Intracytoplasmic injection techniques with ejaculated and purified spermatids can be beneficial for patients with defective spermatogenesis such as nonobstructive azoospermia, without any required urologic intervention.

Key words: Round spermatids, nonobstructive azoospermia, spermatid isolation and purification

The male gamete that has just completed the second meiotic division in the testis is the round spermatid (RS) and therefore the nucleus of the RS contains a complete haploid set of chromosomes. It was demonstrated recently that whole RS injected (ROSI) into human oocytes formed pronuclei and also participated in a syngamy yielding full pregnancies (1,2). We previously reported achievement of pregnancies after RS nuclear injections (ROSNI) into rabbit oocytes followed with embryo transfer (3). However, low developmental ratios in embryos from such injections may be attributable to low developmental potential of the injected oocytes due to inadequate mechanical stimulation applied

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to activate oocytes during ROSNI. Ogura and Yanagimachi (4) reported that, without electrical prestimulation, approximately 25% of mechanically stimulated oocytes remained inactivated after injection of RS nuclei. Meanwhile, intracytoplasmic sperm injection (ICSI) techniques have been proven to be highly effective in treating those patients with severe oligoasthenoteratozoospermia and fertilization failure in vitro (5,6). Furthermore, it successfully can help obstructive azoospermic patients who have undergone microsurgical epididymal sperm aspiration or testicular biopsy (3). Unfortunately, in some infertile men, spermatozoa may not be available because of arrested maturation at the spermatid stage. It is believed that ROSI may solve this problem but it can also assist the nonobstructive azoospermic patient (1). A major and extremely important aspect in the performance of ROSI today is the development of proper techniques for isolation of RS, which is the key element for a successful ROSI program and a prerequisite for widespread use of ROSI as a new treatment for the nonobstructive azoospermic patient. The objective of this study was to develop proper methodologies for isolation and purification of RS from post-ejaculated seminal specimens for possible use in a ROSI program.

MATERIALS AND METHODS

Ejaculate Collection and Evaluation

Ejaculates (N=10) were collected from 6 patients undergoing male infertility evaluation at the Andrology Institute of Lexington. The experimental protocol of the current study was reviewed and approved by the Institutional Review Board of the Andrology Institute of Lexington. All six patients had undergone complete physical examinations and were diagnosed as suffering from nonobstructive azoospermia. All patients produced their seminal specimens with 3 to 4 days of sexual abstinence each time. Also, all patients collected their own ejaculates using a condom-shaped seminal collection device (Male Factor Pak ; ZDL, Inc., Lexington, KY, USA) at intercourse.

Semen Evaluation

After semen samples were produced and completely liquefied (within 15 to 30 minutes), each specimen was evaluated according to standard procedures recommended by the World Health Organization (WHO) with a phase contrast microscope. Semen measures included volume, pH, numbers of spermatozoa and characterization of the round cells if present. All seminal parameters were evaluated by two technicians. Cases in which spermatozoa were found in the ejaculate were excluded from this study.

Protocol for Round Spermatid Isolation and Purification

1. Wash ejaculate in sperm preparation media twice and reconstitute in 0.5 mL aliquot (Step 1; sperm wash). This procedure was accomplished via dilution (1:1) with modified Ham's F-10 medium containing 3% (w/v) bovine serum albumin (SpermPrep media; ZDL, Inc.), followed by centrifugation at 350 x g for 7 minutes. Reconstitute the final pellet in 0.7 mL Ham's F-10.
2. Prepare a multi-gradient Percoll column using isotonic solutions containing 90, 80, 70, 60 and 40% Percoll. Overlayer the Percoll solutions (1.0 mL per layer) on top of each other, beginning with the 90% solution at the bottom of a 15 mL conical centrifuge tube.
3. Overlayer the washed seminal specimen (0.7 mL) onto the 40% Percoll layer and centrifuge at 330 x g for 20 minutes (Step 2; Percoll RS isolation).
4. Remove the 70% Percoll layer, dilute 1:2 and wash with Ham's F-10 medium (350 x g for 7 minutes) and reconstitute again in 0.5 mL Ham's F-10.
5. Prepare a second multi-gradient Percoll column using the following gradients: 70, 40, 30 and 20 (1.0 mL volume per layer).
6. Overlayer the 0.5 mL aliquot recovered after the RS isolation step onto second multi-gradient Percoll column and centrifuge at 330 x g for 15 minutes (Step 3; Percoll RS purification).

7. Remove the 70% gradient portion, dilute 1:2 and wash twice in Ham's F-10 medium, to remove silica gel particles, and resuspend the cell pellet in a 10% polyvinylpyrrolidone (PVP) fraction (MW 360,000, Sigma, St. Louis, MO, USA).
8. A drop of the PVP suspension is placed in a culture dish and covered with mineral oil. Spermatids are identified in this fraction (430x), aspirated into a 12 μm micro-injection needle and transferred onto a slide with coverslip for further microscopic examination and characterization.

Round spermatid samples were prepared separately for morphological examinations under light microscope (Papanicolaou-stained smears) and transmission electron microscope (Fig. 1). Round spermatids and other round cells (RC) were characterized before and after ejaculate wash and Percoll isolation and purification steps.

RESULTS

The ejaculate characteristics before Percoll purification and selection were: 3.2±0.7 mL (volume), 1.8±0.3 x10^6/mL (concentration of RC), 2.9±0.4 x10^6/mL (concentration of RS), pH of 7.6±0.3 and 4.0±0.0 presence or absence of debris (range of 0 to 4). A significant improvement in the ratio of RS:RC and removal of debris was obtained following the two-step Percoll centrifugation. Also, a high proportion of the original number of RS was recovered following the first (75.5%) and second (65.9%) Percoll wash. The Percoll purification step proved to be quite essential in improving the concentration of RS and significantly reducing the amount of debris present (Table 1).

Both modes of morphological evaluation revealed the presence of high numbers and purity of RS. The RS were distinguished from other RC on the basis of their size (approximately 8μm), size of their nucleus (4 to 5 μm in diameter) and most importantly, the presence of a large acrosomal vesicle with perinuclear orientation (Fig. 1).

DISCUSSION

The importance of distinguishing RS from other RC has been recognized in the current study, as well as previously (4). The presence of debris was also an important aspect which could interfere with the performance of the ROSI micromanipulation steps and especially during the identification, pick-up and microinjection of the RS into the oocyte. Round spermatids can be recognized by their size, form of nucleus, a defined cytoplasm surrounding the nucleus and the developing acrosome vesicle as noted in the current study and previous publications (2,4). Round spermatids can also be differentiated from other cells by staining and labeling techniques (4). All patients considered in this study had a rather large number of RS, RC and debris in their ejaculates. Following the first and second Percoll centrifugation steps, a significant number of RS were recovered. Of significant importance, approximately 88% of the debris in the ejaculate and RS preparations was removed following the Percoll isolation and purification steps.

Table 1 Numbers of Round Spermatids (RS) and Ratio of RS:RC (round cells) identified during the various isolation and purification steps (means±SD).

	Seminal Specimen Characteristics		
Treatments/Steps	RS (x10^6)	RS:RC ratio	Debris (0 to 4)
Semen wash (Step 1)	9.4±0.7	5.8:4.2	4.0±0.0
Percoll isolation (Step 2)	7.1±0.5	7.7:2.3	2.5±0.4
Percoll purification (Step 3)	6.2±0.4	8.1:1.9	0.5±0.0

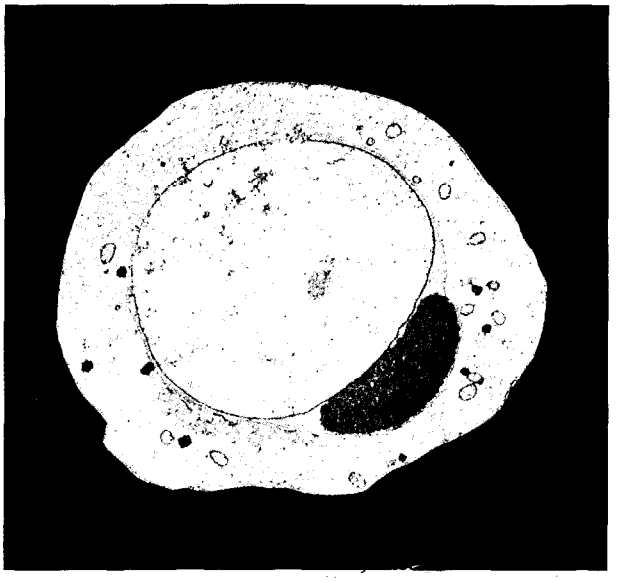

Figure 1 Electron micrograph of a cap phase spermatid. Distinctive characteristics are the size of the cells (approximately 8 m), size of the nucleus (4 to 5 m), relationship of the cytoplasmic to the nuclear area (ratio=0.5 to 0.6) and most important the presence of a large acrosomal vesicle (cap) with perinuclear orientation. Those features are visible under light microscope.

Achievement of fertilization, embryonic development and pregnancies after ROSI and ROSNI have been reported in humans and rabbits, respectively (1-3,5,6). It is these authors opinion that after the proper isolation, identification and purification of the RS, the injection of RS into the human oocyte should be relatively simple, since the technology and instrumentation for ROSI is quite similar to those methods currently employed in ICSI requiring only minute modifications. Furthermore, the ROSI technique with ejaculated RS can be extremely beneficial for patients with defective spermatogenesis , and more specifically the process of spermiogenesis, without any required urologic intervention.

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