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Development of a hyperosmotic shrinkage test to measure the functional integrity of the membrane of human round spermatids retrieved from testes for use in ICSI*

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ABSTRACT

Objective: To develop methods for selection of viable round spermatids for use in ICSI.

Design: A prospective study.

Patients: Round spermatids (RS) were recovered from testes of eight azoospermic (non-obstructive) males via testicular biopsies.

Interventions: The quality of the round spermatids harvested from testes, was determined via a standardized hyperosmotic shrinkage test (HYOS-test) and a supra-vital stain to assess their membrane integrity.

Results: The results from the applied measurements pointed out that spermatids recovered via the traditional methods of testicular biopsy, exhibited significant deficiencies in their cell membrane profiles, as measured via the supra-vital stain and the HYOS-test. Only 13% showed intact membranes (live-dead) and even more importantly, only 7% showed any response to the HYOS- test. Exposure of RS to the HYOS-test resulted in volume reduction and subsequent crenation. The occurrence of this cellular membrane behavior, suggests that fluid transport from the cell was allowed in an attempt to reach osmotic equilibrium with the surrounding hyperosmotic medium.

Conclusions: The findings generated in this study suggest that spermatids (RS) when recovered via the traditional manner, undergo irreversible membrane damage which could have detrimental effects in the ability of those immature male gametes to be actively involved in complete fertilization and furthermore, in the proper development of the embryos during embryonic and fetal development. It is possible that those deficiencies noted in the current study could be due to the tearing of the cells during separation of the cells from the parenchymal testes and could offer an explanation of the extremely low rate of pregnancies established during ICSI using round spermatids.

Key words: Round spermatids, non-obstructive azoospermia, Hyperosmotic shrinkage test.

With the advent of Assisted Reproductive Technologies (ART's) and specifically Intracytoplasmic Sperm Injection (ICSI), many male patients with obstructive or non-obstructive azoospermia now have a better opportunity to conceive. More specifically, in humans, spermatids can be used as substitutes for mature spermatozoa if men are unable to produce spermatozoa in their testes (1, 2).

Round spermatids (RS) are male gametes that have just completed the second meiotic division in the testis and therefore their nuclei contain a complete haploid set of chromosomes. It was demonstrated recently that whole RS injected

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(ROSI) into human and animal oocytes formed pronuclei and also participated in a syngamy yielding full pregnancies (1-3). Generated results suggested that 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 to activate oocytes during ROSNI. Results generated by Ogura and Yanagimachi (4) indicated that, without electrical pre-stimulation, approximately 25% of mechanically stimulated oocytes remained inactivated after injection of RS nuclei. Because of the low pregnancy rates achieved from such efforts, other concerns have been raised such as the ability of RS to deliver (i) the proper genetic material, (ii) the centrosome and (iii) the putative cytosolic factor to initiate oocyte activation. No doubt, one must also be concerned about the physiological and microanatomical intactness of spermatids used in ICSI, especially those of testicular origin. 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 can help successfully 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 intact, physiologically active RS, which is, in these authors opinion, one of the key elements for a successful ROSI program and a prerequisite for widespread use of ROSI as a new treatment for the nonobstructive and obstructive azoospermic patient.

The objective of this study was to develop proper methodologies for selection of viable RS for use in ICSI. It is thought that the low pregnancy results from ICSI using RS could be associated with the low quality of the RS employed in such efforts.

MATERIALS AND METHODS

Spermatid Collection and Evaluation

Round spermatids (RS) were recovered via testicular biopsies, from testes of eight azoospermic (non-obstructive) males, undergoing male infertility evaluation at the Andrology Institute of America. The experimental protocol of the current study was reviewed and approved by the Institutional Review Board of the Andrology Institute of America. Testicular biopsies were performed under local infiltration anesthesia. A fragment of testicular tissue (3-4 mm in diameter) was excised, washed with HEPES-buffered Ham's F-10 medium and the seminiferous tubules were identified and dissected. Each biopsy specimen was morselized in a petri dish and the spermatids were isolated among other morselized tissues, as previously described (7), transferred to culture tubes, washed and then tested for membrane integrity via the use of a standard eosin-nigrosin supra-vital stain (live-dead ratio) and also for the ability to selectively permit the transport of fluids and certain molecules across their membrane. For the selective permeability measurements, the spermatids were exposed to hyperosmotic conditions using a fructose-sodium citrate mixture adjusted at 450 mOsm/L. The results of the various tests and cell morphological characteristics were compiled and compared.

Protocol for Spermatid Isolation and Purification

The isolated spermatids were transferred into a test tube and washed in sperm preparation media twice and reconstituted in 0.5 ml aliquot. 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 5 minutes. The final pellet was reconstituted in 0.5 ml Ham's F-10 for microscopic evaluation or 0.5 ml of hyperosmotic solution for the performance of the Hyperosmotic Shrinkage (HYOS) test.

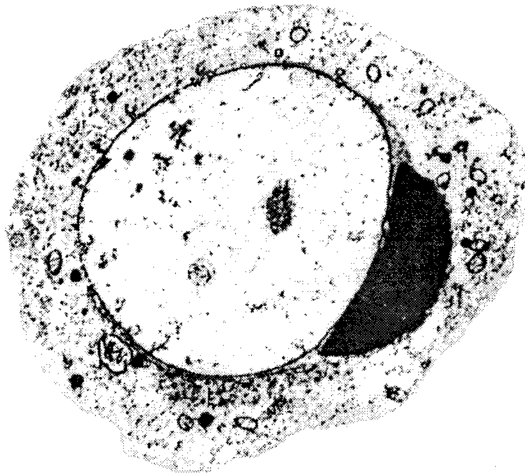

Figure 1. Electron micrograph of a cap phase spermatid. Distinctive characteristics are the size of the cells (approximately 8 μm), size of the nucleus (4 to 5 μm), relationship of the cytoplasmic to the nuclear area (ratio = 0.5 to 0.6) and most important the presence of a large acrosomal vesicle (cap) with perinuclear orientation. Those features are visible under light microscope (430x).

Microscopic Evaluation of Spermatids

Spermatids were identified in the recovered fraction (430x), aspirated into a 12 μm micro-injection needle and transferred onto a slide with cover slip for further close microscopic examination and characterization, as previously described (7). Round spermatid containing samples were prepared for morphological examinations under light microscope (Papanicolaou-stained smears) and transmission electron microscope (Figure 1).

Table 1. Percentage of Round Spermatids (RS) responding to the quality tests applied (mean ± SD).

Tests Performed	Percentage Reacted RS (%)*
Supra-vital stain	12.8±3.6
HYOS-test**	7.2±2.8

*One hundred cells tested per patient (RS).
**HYOS-test: Hyperosmotic Shrinkage test.

Spermatids and other round cells (RC) were characterized before and after the HYOS test (Figure 2). The RS were distinguished from other RC on the basis of their size (approximately 8 μm), size of their nucleus (4 to 5 μm in diameter) and most importantly, the presence of a large acrosomal vesicle with perinuclear orientation (Figure 1).

RESULTS

The results from the applied measurements pointed out that spermatids recovered via the traditional methods of testicular biopsy exhibited significant deficiencies in their cell membrane profiles, as measured via the supra-vital stain and the HYOS-test. Only 13% showed intact membranes (live-dead) and even more importantly, only 7% showed any response to the HYOS test (Table 1). After exposure of the RS to the hyperosmotic solution (0.5 hr), the membrane of intact spermatids shrank and ridges along with a "notching pattern", began to appear along the cell surface along with subsequent crenation, suggesting that fluid could exit the cell in an attempt to reach osmotic equilibrium with the surrounding hyperosmotic medium. Both modes of morphological evaluation revealed the presence of high numbers and purity of RS.

DISCUSSION

The development and employment of microsurgical techniques for testicular sperm and spermatid aspiration in the treatment of male infertility gave new hopes to a group of infertile males that were considered "untreatable". The employment of RS in the treatment of severe male infertility is considered to be a significant breakthrough. However, the success obtained via ICSI and the use of RS retrieved either surgically or post-voided, may be considered too low compared to ICSI with mature spermatozoa. Additional efforts should be made to develop better techniques to further assist this previously "hopeless" group of patients. A number of attempts

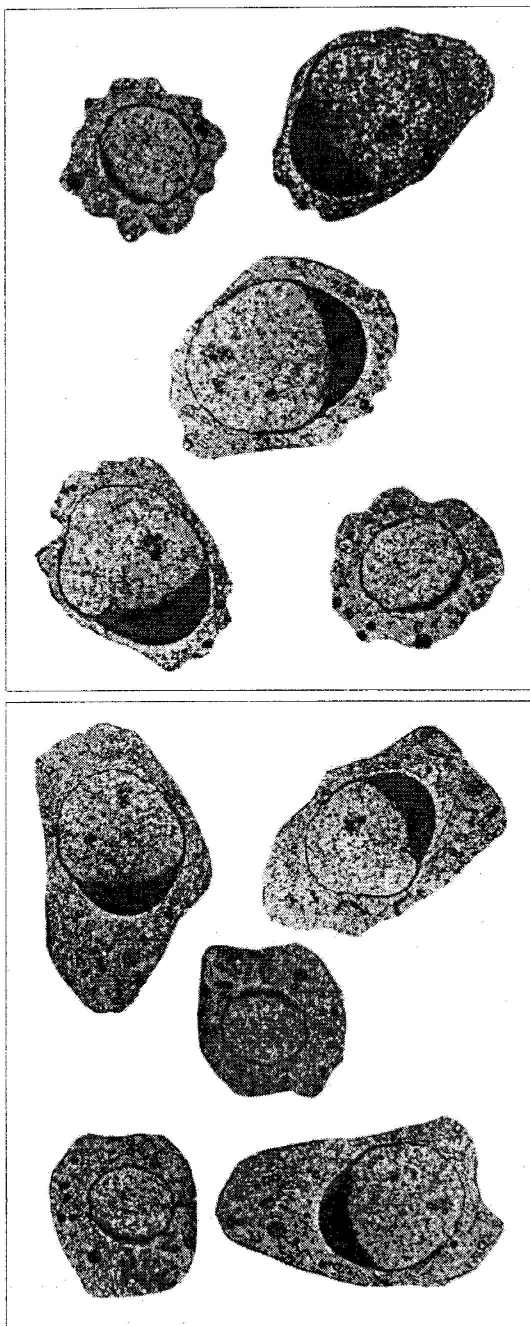

Figure 2. Round spermatids that either responded (upper plate) or did not respond (lower plate) to the HYOS-test (430x). The size and ratio of the sub-cellular compartments (cytosol:nucleus) were altered after the response to the HYOS-test. Also the "notching pattern" was evident to the HYOS-test reacted spermatids.

have been made at our facilities to develop such techniques in order to bring about improvements in the diagnosis and treatment of male infertility.

In the current study, we have attempted to address the importance of distinguishing and selecting high quality, viable RS from other RC, by employing techniques that have been previously described (4, 7). Also, as noted previously, the presence of debris was 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 oocytes (7). 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 also in previous publications (2,4,7). RS 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 the tissue preparations. However, the applied purification techniques (7) enabled the proper selection of the round spermatids for proper testing and evaluation to take place.

Achievement of fertilization, embryonic development and pregnancies after ROSI and ROSNI have been reported in humans and other animal models (1-3,5,6,8,9). However, using surgically retrieved spermatids, although the technique offers a valuable therapeutic option with a reasonable success rate, it is also quite limiting when compared to the use of mature spermatozoa in ICSI, as far as pregnancy outcome is concerned (10). It is these authors opinion that after the proper isolation, identification, purification (6,7) and quality assessment of the RS as recommended and established in the current study via the HYOS-test, the injection of those cells into human oocytes 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 properly isolated, quality assessed via the HYOS-test and microinjected RS can be relatively successful, as a therapeutic option, in establishing pregnancies and most importantly, be extremely beneficial for patients with impaired spermatogenesis.

Further studies are underway at our facilities to better understand the possible interactions between

the HYOS-tested RS and their involvement in the proper syngamy of the male and female gametes. Such understanding will be necessary in the employment and adequate interpretation of the in-vitro developments in RS and their implications in the overall in-vivo behavior and participation in the fertilization process.

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