Round spermatid separation and in-vitro maturation

Hassan S. Jamal, *MD, CABOG,* ***Zouhair O. Amarin,*** *MD, FRCOG.*

ABSTRACT

Objective: To combine 2 methods; spermatid isolation and in-vitro maturation to improve their quality and enhance their ability of fertilization.

Methods: A discontinuous Percoll gradient was used to separate immature germ cells. Co-culture with Vero cells was attempted to convert round spermatids into more mature forms. In total 87 spermatids were studied.

Results: Of a final number of 77 round spermatids only 12 (15.5%) showed a certain degree of maturation in 3 out of 7 patients (42%). Of those 12 maturing spermatids, only 4 developed to an early elongated spermatid stage Sc1, but without flagella.

Conclusion: Considering the limited in-vitro round spermatid maturation achieved in this study, the low fertilization and pregnancy rate with spermatids in general and round spermatids in particular, further refinements of this technology have to be achieved before its regular implementation in routine clinical practice is justifiable.

Keywords: Round spermatid, percoll gradient, in-vitro, maturation.

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Spermatozoa may not be available in some azoospermic men with various pathological conditions such as Sertoli cell only syndrome, maturation arrest, post-cryptorchidism tubular atrophy, post-chemotherapy testicular atrophy, post mumps orchitis and Kleinefelter syndrome. In such cases attention needs to turn to less mature cells. Spermatids are the first haploid cells in the complex spermatogenic cell series with the relevant functional capability to affect successful fertilization after microinjection. Recently, the injection of spermatids into human oocytes has became a novel approach to the treatment of male factor infertility. Such clinical studies were prompted by initial animal experiments showing fertilization of hampster and mouse oocytes with round spermatids.[1] In 1995 came the first reports of live human births resulting from the transfer of oocytes fertilized with round[2] and elongated spermatids.[3] To date 35 cases have been reported in the medical literature. Twenty seven were obtained by Elongated Spermatid Injection (ELSI). Eight by Round Spermatid Injection (ROSI). Five of which were round spermatids obtained by testicular biopsy and 3 were ejaculate round spermatids. With this type of technology, we are faced with 2 predominant problems that concern us. The first one is the difficulty in identifying immature germ cells in unstained fresh samples. This has led most laboratories to use the term “round cells” which encompasses leukocytes. The 2nd concern is the low incidence of fertilization with round spermatid microinjection.

This paper had two aims, one, to test the use of a discontinuous Percoll gradient method to enable good separation of immature germ cells. This technique demonstrated a high immature germ cell concentration in gradient fractions with 30% to 45% Percoll with a small concentration of leukocytes.[4] The other aim was to report an attempt to convert round spermatids into more mature forms, by using

From the Department of Obstetrics and Gynecology, King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. H. Jamal, Department of Obstetrics & Gynecology, King Abdulaziz Hospital, PO Box 6615, Jeddah 21452, Kingdom of Saudi Arabia.

in-vitro co-culture with Vero cells. The incentive being an effort to overcome the maturation block and improve the fertilization rate. Aslam and Fishel demonstrated that about 22% of round spermatids can grow flagella under in-vitro conditions.[5] Cremades et al, demonstrated that 30% developed flagella, 46% matured to elongating and 19% to elongated spermatids.[6] To our knowledge, this is the first time those 2 techniques had been combined.

Methods. To test the efficiency of the method, round and elongated spermatids were selected for experimental purposes with informed consent from subjects with various andrological pathologies. Either ejaculates, after 3 days of abstinence, or open testicular biopsies were studied. Four patients with azoospermia, 2 of which were obstructive, one with severe oligoasthenozoospermia, one with asthenozoospermia and one with borderline normozoospermia were included. All gave consent to experimentally co-culture their gametes on Vero cells monolayers to promote maturation for research purposes. In no patient was clinical treatment implemented with in-vitro matured cells. No karyotype tests were performed. Of those 7 patients 2 underwent testicular biopsy, one underwent both testicular and ejaculate sampling co-culture. The remaining 4 were subjected to ejaculate testing only. Testicular samples were collected in pre-incubated sperm preparation medium (SPM), teased in a Petri dish under the microscope with the use of tuberculin syringes, then washed with 2ml SPM. Prepared testicular samples or 2ml of ejaculates were gently layered on top of a gradient Percoll column prepared by obtaining Isotonic 100% Percoll (Sigma Chemical Co, USA) through the addition of 9 parts of Percoll to one part of Earle's balanced salt solution 10 X (Imperial, UK). The 100% Percoll was diluted again to obtain dilution increments of 5%, from 30 to 100% in a 10ml test tube washed with 100% SPM. One ml of each concentration was gently stratified starting with 100% using a Pasteur pipette followed by centrifugation for 25 minutes at 800g at room temperature. The single Percoll fractions were separated and analyzed to select the best ones. These fractions were mixed with Earle's medium (1:2) and centrifuged at 150g for 10 minutes The pellet was resuspended in 1ml of Earle's medium and the cell concentration was examined. The various spermatids were developmentally classified into 4 stages according to Krester's and Tezarik's criteria (Figure l). Sa spermatids show acrosomal vesicle. Sa1 have no flagella. Sa2 have short ones <8um. In Sb spermatids, the acrosome takes caplike form. Sb1 flagellum is >8um with a central position of the nucleus. Sb2 nuclei start taking on an oval shape and move peripherally with the beginning of the spermatid elongation process. Sc spermatids have a more elongated shape with a smaller nucleus that begins to protrude in Sc1 and up to one half does so in Sc2. This culminates in Sd1 where more than half the nucleus protrudes followed by whole nucleus protrusion in Sd2 mature spermatozoa.[7,8] Cells were selected with a micropipette with 7-8um inner diameter and transferred to 20ul microdrops containing Vero Cells microlayers on a plastic Petri dish covered by paraffin oil at 37°C with 5% CO_2 in air. Vero cells (American Type Culture Collection) frozen in liquid nitrogen were thawed at 37°C and placed in a 15ml sterile tube. Ten ml of Minimum Essential Medium Eagle 10 X (MEM) (Sigma Aldrich Co. Ltd) containing 10% Foetal Bovine

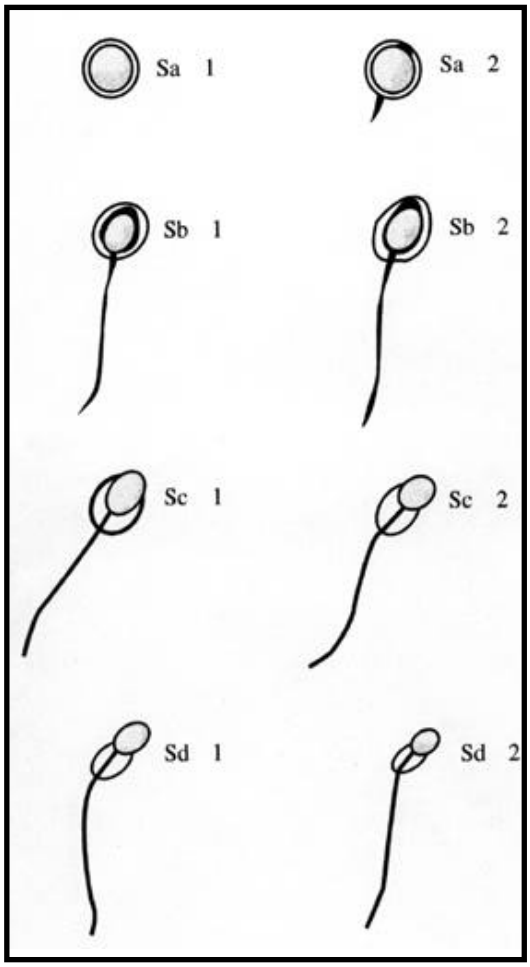

Figure 1 - Schematic presentation of spermatid developmental stages.
Sa - show acrosome vesicle
Sa1 - no flagellum
Sa2 - has a short flagellum < 8 um
Sb - acrosome takes caplike form
Sb1 - flagellum is > 8 um with central nucleous
Sb2 - nucleous is oval and periferal
Sc - more elongated with small nucleous
Sc1 - slight nucleous protrusion
Sc2 - up to one half nucleous protrusion
Sd1 - more than half nucleous protrusion
Sd2 - whole nucleous protrusion

Serum (Gibco BRL, Life Technologies, 10108/165) was added then centrifuged for 10 minutes at 800g. Pellets were resuspended at concentration of $5x10^6$ cells/10ml in a 25ml tissue culture flask and incubated at 37°C in 5% CO_2 (Forma incubator). Media was changed every 24 hours to maintain healthy cells. When the cells became confluent (complete monolayer), trypsinization was performed. The medium was aspirated from the flask. Ten ml of Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich CHEMIE Gmbh) was used to wash the monolayer. Two ml of Trypsin-Ethylenediamineteraacetic acid (EDTA) solution (1X) (Sigma Chemical Co, T - 3924) was added to the monolayer and was observed using inverted microscope for about 5 minutes. On disperation 2 ml of 10% FBS in MEM was added followed by transferring the contents into another tube with 4 ml MEM solution containing 10% FBS. After 10 minutes of centrifugation at 800g, the supernatant was discarded. The pellet was suspended in 3ml of MEM supplemented with 10% FBS, adjusted to 10^5 cells/ml then labelled. Three microdrops of 20ul were transferred on each Petri dish then covered with paraffin oil.

Results. ***Patient 1.*** This patient with obstructive azoospermia underwent testicular biopsy. Only round and elongated spermatids were recovered. Fourteen round spermatids were co-cultured. After observation for 5 days, no conclusive changes were observed.

Patient 2. This patient with non obstructive azoospermia also underwent testicular biopsy. Only round spermatids were recovered. Co-culture of 10 spermatids for 5 days did not show any conclusive signs of a maturing process. An ejaculate sample from the same patient demonstrated similar round spermatids only. Five days of co-culture produced no development in 12 round spermatids.

Patient 3. An ejaculate sample with less than $1x10^6$/ml spermatozoa and 0-1 round cells/high power field (HPF). Thirteen Sa1 and Sa2 type spermatids were co-cultured. At 3 days 2 out of 3 Sa2 spermatids lost their flagella. On day 5, one of the remaining Sa2 spermatids matured to Sb2 type.

Patient 4. Another ejaculate from an azoospermic patient. Ten round and 10 elongated spermatids were co-cultured. Unfortunately the process had to be abandoned on day 2 in view of a fungal growth.

Patient 5. An ejaculate sample with $25x10^6$/ml density and 30% motility and Grade 1 (sluggish, wavering with minimal forward progression) and Grade 2 (meandering, poor to fair activity) progression and 1-2 round cells/HPF. Fifteen round spermatids were co-cultured. After 4 days, one Sa1 developed into Sa2 and 2 out of 3 Sa2 matured to Sb1 .

Patient 6. A borderline nomospermic patient with $20x10^6$/ml density. Out of 15 Sa1, 4 developed into Sc1 but without flagella, 2 changed to Sa2 and 2 to Sb1 after 3 days.

Patient 7. This patient with obstructive azoospermia underwent testicular biopsy. Zero to 1 immotile/cover slip spermatozoa were observed. Ten round spermatids were co-cultured. Over 5 days of study, no change was observed.

In summary, if we exclude the yeast contaminated sample of 10 round spermatids, of 77 round

Table 1 - Summary of results.

Case No.	Sperm count	Spermatid Source	Spermatid type found	Spermatid type and number cultured	Days of co-culture	Outcome	%
1	Azoospermia	Biopsy	Round and elongated	14 Round	5	No change	0.0
2	Azoospermia	Biopsy	Round only	10 Round	5	No change	0.0
3	< 1 x 10^6	Ejaculate	Round and elongated	13 Round	5	one Sa_2 -> Sb_2	8.0
4	Azoospermia	Ejaculate	Round and elongated	10 Round 10 Elongated	2	Abandoned yeast contamination	20.0
5	25 x 10^6	Ejaculate	Round and elongated	15 Round	4	one Sa_1 -> Sa_2 two Sa_2 -> Sb_1	53.0
6	20 x 10^6	Ejaculate	Round and elongated	15 Round	3	four Sa_1 -> Sc_1 two Sa_1 -> Sa_2 two Sa_1 -> Sb_1	
7	Azoospermia	Biopsy	Round and elongated	10 Round	5	No change	0.0
Total				**87 Round *77**		**12**	**15.5**
*77 - 10 spermatids have been excluded due to contamination							

spermatids only 12 (15.5%) showed certain degrees of maturation in 3 out of 7 patients (42%). Of those 12 spermatids that showed a certain degree of maturation, only 4 developed to an early elongated spermatid stage Sc1 and without flagella (Table l).

Discussion. Recent progress in the field of advanced reproductive technology has dramatically modified the concept and management of patients with azoospermia. Spermatids could easily be obtained from ejaculates and testicular biopsies. Results of the search for round spermatids performed in the ejaculate of 124 men with non-obstructive azoospermia gave positive results in 69% of these patients.[9] The distinction of round spermatids from other round cells is mainly based on the size and shape of both cell and nucleus. The spermatogonia, spermatocytes and polymorphonuclear leukocytes are larger cells with different forms of nucleus. Lymphocytes have similar diameters, but have a higher nuclear/cytoplasm ratio. Round spermatids posses a clear cytoplasmic zone around the nucleus. A developing acrosome can be seen as a small vesicle adjacent to the nucleus. This feature is quite distinctive. Identification of round cells is simple when cytologic staining procedures are used. Those tests, apart from not being supravital, would only give an indication as to the percentage of spermatids available in a given sample, but it would not eliminate the chance of an error in choosing which round cell to use for microinjection.

The discontinuous Percoll gradient method has confirmed a good separation of immature germ cells in gradient fraction with 30% to 45% Percoll with a small number of leukocytes. With this technique the identification problem is solved to a major extent on one hand. On the other hand, round cells do not produce a high percentage of fertilization when microinjected into oocytes.[10,11] Poor round spermatid fertilization in round spermatid injection (ROSI) and round spermatid nuclear injection (ROSNI) may be explained biochemically by the incomplete transition of nuclear proteins. Past research has shown that the nuclei of mammalian spermatozoa undergo condensation during which histones bound to DNA are replaced by protamines, which then become bridged by tightly packed di-sulphate cross links. These morphological changes take place at intervals within spermiogenesis. The premature exposure of the spermatid nucleus to the maturation promoting factors of the oocyte may result in premature chromosome condensation leading to fertilization failure. Another problem using spermatids with intracytoplasmic sperm injection, is the possible absence of sperm-associated oocyte activating factor (SAOAF) responsible for Ca++ oscillations. In this research project, the co-culture of Percoll gradient separated spermatids had 2 objectives in mind, to verify the competence of the separation technique by observing objective morphological changes in spermatids and the possible utilization of the more mature forms for microinjection so that better fertilization results are obtained.

In our study about 15% of the round spermatids tested showed a minor degree of maturation in 3 out of 7 patients. Considering the limited success of this particular technique in conjunction with the low fertilization in round spermatid intracytoplasmic injections, further research is required before it is clinically implemented with confidence.

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