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A simple and objective approach to identifying human round spermatids

Theofanis Angelopoulos[1], Lewis Krey[1], Andrew McCullough[2], Alexis Adler[1] and James A. Grifo[1,3]

[1]Department of Obstetrics and Gynecology and
[2]Department of Urology, New York University Medical Center, 317 East 34th Street, New York, NY 10016, USA

[3]To whom correspondence should be addressed

Although round spermatids have been studied extensively using staining techniques and electron microscopy, little information is available about their appearance in living conditions. We describe a method of collecting and identifying round spermatids from ejaculates and testicular biopsies. The validity of the selection procedure was confirmed by fluorescence in-situ hybridization. Based on cell size, morphological characteristics of nucleus and cytoplasm, and on the nucleus/cytoplasm ratio, we harvested a population of cells that was 84% haploid. This procedure can be applied to select spermatids for clinical or research purposes.

Key words: fluorescence in-situ hybridization/non-obstructive azoospermia/round spermatids/spermatogenic arrest/testicular biopsy

Introduction

Spermatogenesis, a complex mammalian cellular differentiation process, begins at puberty and continues throughout reproductive life. Meiosis results in haploid spermatids which undergo profound chemical and structural modifications in both the epididymis and testis to become functional spermatozoa. The entire process is tightly synchronized and integrated, so that pathological conditions which produce even very small deviations are likely to lead to infertility (Barratt, 1995).

Spermatogenic arrest, the inability of spermatogenetic cells to develop into male gametes within the gonads, has been reported in 4–30% of testicular biopsies of patients with severe oligospermia or azoospermia (Wong *et al.*, 1973; Levin, 1979; Colgan *et al.*, 1980; Soderstrom and Suominen, 1980; Nomen *et al.*, 1984). Spermatogenic arrest can occur at any stage of germ cell formation; primary spermatocyte arrest is most prominent, followed by spermatid arrest, and least commonly, spermatogonial arrest. Arrest at primary spermatocyte stage can be incomplete, so that a few secondary spermatocytes or spermatids are observed (Girgis *et al.*, 1969). Since the round spermatid contains a haploid set of chromosomes, the concept of whether these cells are able to participate in syngamy and produce offspring was investigated (Ng and Solter, 1992) and later demonstrated (Ogura and Yanagimachi, 1993). Indeed, animal studies have demonstrated that round spermatids have the potential to produce live, healthy and fertile offspring (Ogura *et al.*, 1994; Kimura and Yanagimachi, 1995; Sasagawa and Yanagimachi, 1997; Yanagimachi *et al.*, 1997).

In humans, spermatogenic arrest was considered a hopeless condition for couples desiring to conceive. However, the documented success of intracytoplasmic sperm injection (ICSI; Palermo *et al.*, 1992) has pointed to using this technique to inject spermatids into oocytes (Edwards *et al.* 1994; Ogura *et al.*, 1994). Recently, human elongated and round spermatids obtained from testicular biopsies have been reported to achieve fertilization (Vanderzwalmen *et al.*, 1995; Chen *et al.*, 1996; Fishel *et al.*, 1997; Yamanaka *et al.*, 1997) and pregnancies (Fishel *et al.*, 1995; Antinori *et al.*, 1997a). Moreover, Antinori and coworkers (1997b) announced an ongoing pregnancy with frozen-thawed round spermatids obtained from testicular biopsies.

Immature germ cells are present in ejaculates of subjects with a normal sperm count (Michael and Joel, 1937; Tomlinson *et al.*, 1992), oligozoospermia (Mac Leod, 1970;_Tomlinson *et al.*, 1993), or azoospermia (Kurilo *et al.*, 1993) and the presence of immature germ cells increases as the sperm count decreases (Sperling and Kaden, 1971). These immature cells appear to be viable; Tesarik and coworkers (1995; 1996) have reported two normal births following intracytoplasmic injection of ejaculated round spermatids from men with non-obstructive azoospermia.

Despite these achievements, fertilization rates with human round spermatid injection are low, between 20% and 30% (Chen *et al.*, 1996; Tesarik *et al.*, 1996; Fishel *et al.*, 1997), and even when higher fertilization rates are achieved, no pregnancies (Yamanaka *et al.*, 1997) or very low pregnancy rates are obtained (Antinori *et al.*, 1997a). Variations in centrosome function, oocyte activation, genomic imprinting, nuclear protein maturation and cell cycle asynchrony are possible causes (Kimura and Yanagimachi, 1995; Fishel *et al.*, 1996; Tesarik and Mendoza, 1996b); however, technical difficulties may also be responsible. Proper identification of round spermatids represents one technical difficulty. Although these cells have been thoroughly described by different staining techniques under light microscopy (Clermont, 1963; Papic *et al.*, 1988; Schenck and Schill, 1988; Adelman and Cahill, 1989; WHO, 1992) and electron microscopy (de Kretser, 1969; Holstein, 1976), not enough information has been available regarding the morphological features of live round spermatids to allow for their selection for injection into oocytes (Tesarik and Mendoza, 1996a). In this study, we present a simple and objective approach to collecting and identifying human round

spermatids that is based on the morphological features of these cells as observed under the inverted microscope.

Materials and methods

Source and preparation of spermatids

Ejaculated samples

A total of 115 ejaculates from 113 men attending the infertility clinic were evaluated according to World Health Organization guidelines (WHO, 1992). As a part of the routine semen analysis, we examined for round cells on 5 µl of semen in a Mackler counting chamber (Sefi Medical Instruments, Haifa, Israel). Normozoospermic samples ($n = 19$) and oligozoospermic samples ($n = 14$) containing $\geqslant 1\times10^6$ round cells/ml and all azoospermic samples ($n = 5$) were included in this study. To differentiate round spermatids from other round cells, 5 µl of each specimen were spread on prestained slides (Testsimplets®, Boehringer Mannheim, Mannheim, Germany).

In 16 samples with spermatozoa and $\geqslant 1\times10^6$ round cells/ml, cell separation was performed by Percoll (Pharmacia, Uppsala, Sweden) density gradient. Equal volumes of sperm and equilibrated sperm wash medium (SWM), consisting of human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA, USA) supplemented with 6% Plasmanate (National Hospital Specialties, Hackensack, NJ, USA) were mixed in a 15 ml tube (Falcon®, type 2095; Becton-Dickinson, Lincoln Park, NJ, USA). After centrifugation (300 *g* for 5 min), the supernatant was discarded and the pellets were resuspended in 1 ml of SWM and loaded on top of a discontinuous gradient column of 50% (1.5 ml), 70% (1 ml), 100% (1 ml) Percoll solutions. The samples were then centrifuged at 500 *g* for 20 min. In order to determine which layer contained the highest amount of spermatids, separated layers (50%; interphase of 50%; 70% fraction together with interphase of 50%; and 70% fraction) were allocated to different tubes, diluted 1:10 with SWM and centrifuged twice at 600 *g* for 10 min. The resulting cell pellet was resuspended to a concentration of approximately 500 000 cells/ml and assessed by Testsimplets® . This cell concentration facilitated the selection of cells with a micromanipulator (see below) since cells tend to aggregate at high concentrations (Meistrich, 1982).

Azoospermic specimens were diluted, after liquefaction, with equal volumes of SWM and centrifuged at 1800 *g* for 5 min. The pellets were resuspended at a concentration of approximately 500 000 cells/ml.

Testicular biopsies

Seven testicular biopsies were obtained from men undergoing a work-up for a clinical diagnosis of non-obstructive azoospermia. Testicular biopsies were performed through standard open surgical technique and the retrieved tissue was immersed in SWM in a sterile 500 µl microcentrifuge tube (Eppendorf, Westbury, NY, USA). The specimen was left for 1–2 h at 37°C in 5% CO_2 and then transferred to a Petri dish (Falcon®, type 1006) together with the SWM contained in the test tube. Tubular disruption was carefully carried out with two 22 gauge needles under a stereoscopic microscope. The tissue suspension

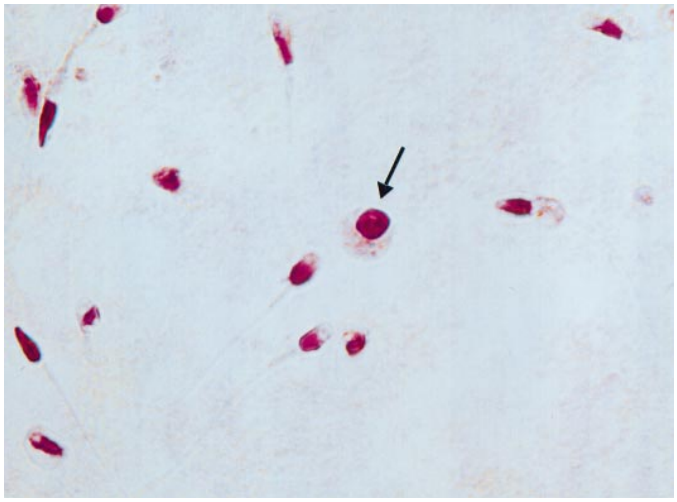

Figure 1. Ejaculated human round spermatid of 6 µm as visualized by Testsimplets® staining (bright field microscopy, original magnification ×1000). The acrosomal cap is clearly observed (arrow).

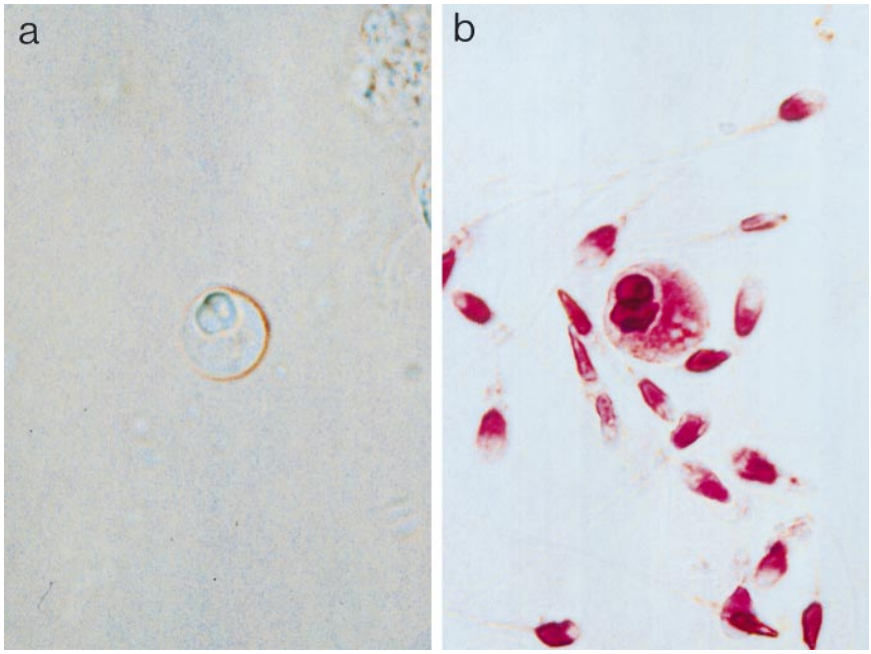

Figure 2. Multinucleated spermatids from an ejaculated sample observed under bright field microscopy at ×600 (original magnification) unstained (phase contrast; **a**) and stained by Testsimplets® (**b**).

was placed in a 15 ml tube (Falcon®) and mixed several times with a sterile Pasteur pipette. An equal volume of SWM was added and the suspension was centrifuged twice at 800 *g* for 15 min to detach the cells from each other. The pellet was resuspended at a final concentration of approximately 500 000 cells/ml, examined by Testsimplets® and then incubated for 1–2 h at 37°C in 5% CO_2 prior to further analysis.

Staining technique

The presence or absence of round spermatids, the morphology of round spermatids, as well as the distribution of different cell types (i.e., spermatids, leukocytes, other germ cells) was assessed by staining. Identification of round spermatids by different staining techniques has been reported in detail (Clermont, 1963; Papic *et al.*, 1988; Schenck and Schill, 1988; Adelman and Cahill, 1989; WHO, 1992). Staining with Testsimplets® is achieved with two dyes, N-methylene blue and cresyl violet acetate. The principle underlying the reaction of these two dyes with the cells can be visualized as being analogous to panchromatic staining. The different staining of the individual cell regions with the basic dyes is used for cell identification.

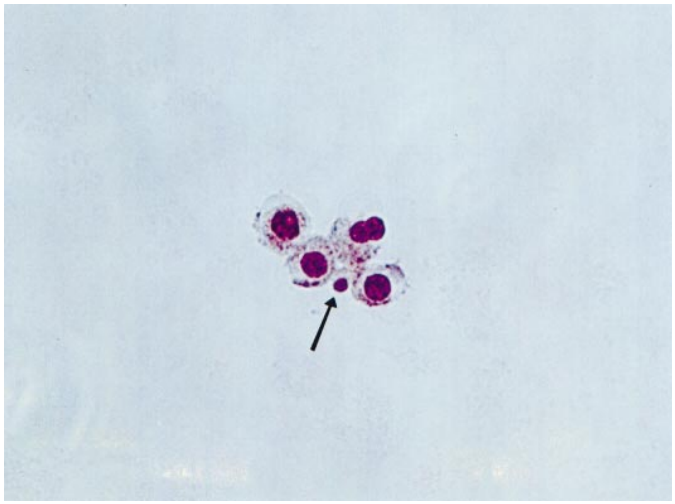

Figure 3. Testicular biopsy stained by Testsimplets® and observed under bright field microscopy at ×600 (original magnification). A round spermatid (arrow) together with four primary spermatocytes are observed.

The cell suspension (5 µl) was spread on the centre of a Testsimplets® slide. The slide was covered with a coverslip, allowed to stand for 15–30 min at room temperature and then examined by bright field microscopy at ×400, 600 and 1000. The microscope was equipped with an ocular micrometer to measure cell size (calibration accuracy of the grid: ±0.01 µm).

Illustrations and descriptions by Clermont (1963) were used to identify spermatids in stained smears. Round spermatids were visualized as spherical cells approximately 5–7.5 µm in diameter. In both Golgi and cap phases of spermiogenesis, the acrosomal vesicle or cap was identified as a magenta granule or a crescent shape protrusion on one side of the cell. The nucleus was generally round or, in the more mature stages, slightly oval when it made contact with the cytoplasmic membrane on the side of the cell. The nuclear chromatin was observed as a confluent network and stained purple in the immature forms. In later stages, the chromatin started to condense and the nucleus stained dark purple. The cytoplasm appeared as a pink halo that became more chromophilic as spermiogenesis advanced, sometimes showing tiny vacuoles. During initial stages, cytoplasm appeared abundant, due to rapid nuclear shrinkage. However, during elongation, cytoplasm became sparse, because part of it separated from the cell to become a residual body of amorphous structure (Figure 1; see also Figure 3). Spermatid aberrations were sometimes observed: multinucleation (Figure 2), abnormalities of nuclear condensation and acrosome vesicle, abundant cytoplasm in combination with small nuclei, and a large number of lipid droplets in the cytoplasm (Holstein, 1983).

It was difficult to differentiate very immature spermatids from secondary spermatocytes, the latter being distinguishable only by their larger size. Secondary spermatocytes usually possessed centrally located nuclei 7 µm in diameter and stained exactly as immature

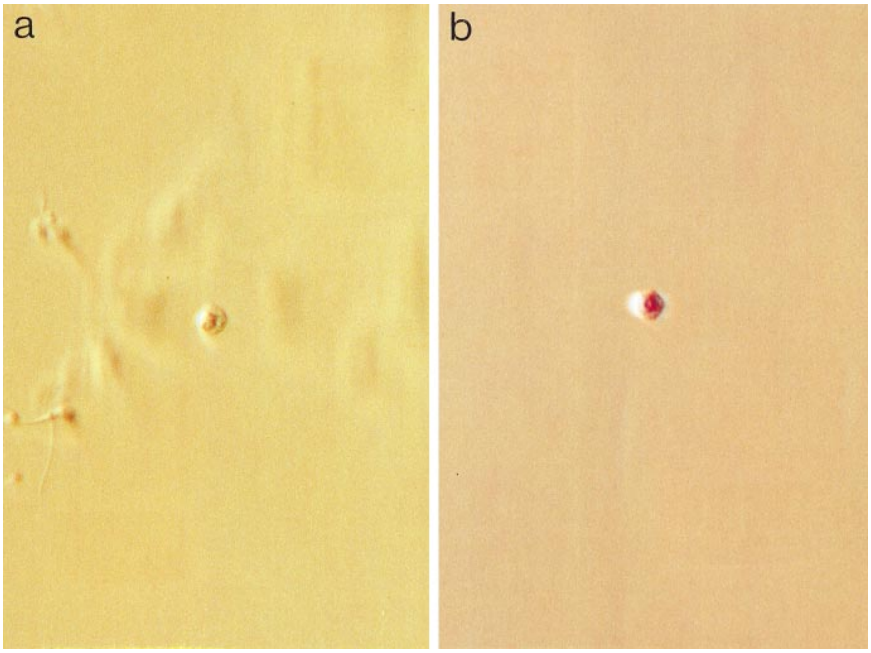

Figure 4. Round spermatid of 7.5 µm from an ejaculated sample observed under inverted microscopy **(a)** The same cell was collected with a microinjection pipette, delivered on a prestained slide and observed under inverted microscope **(b)** Original magnification ×600.

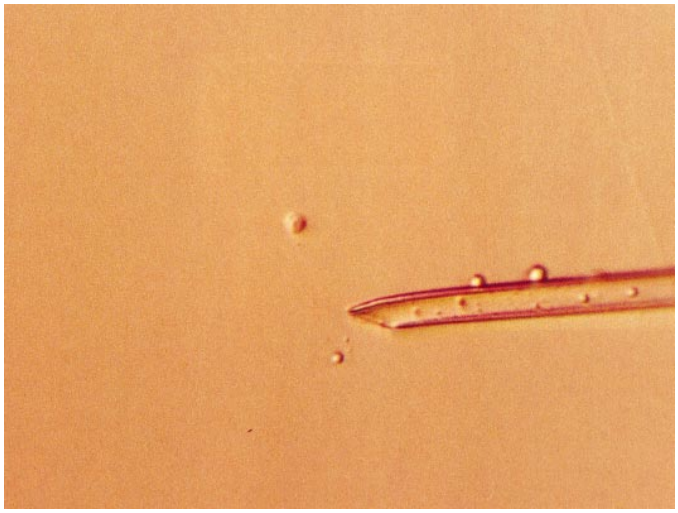

Figure 5. Round spermatid of 6 μm observed under inverted microscopy at ×400 (original magnification).

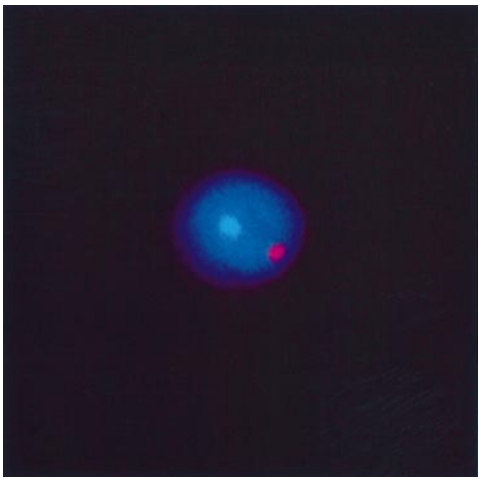

Figure 6. Fluorescence in-situ hybridization (FISH) performed on a single round spermatid selected with a microinjection pipette. By using a triple band pass filter, one signal for the Y chromosome (labelled red) and one signal for chromosome 18 (labelled green) are visualized.

spermatids with a scant light pink cytoplasm and rare, delicate, small cytoplasmic vacuoles. However, these cells were only seen occasionally because of their short life spans (Burger *et al.*, 1976; Barratt, 1995).

The remainder of the spermatogenic cells (i.e. primary spermatocytes, spermatogonia), Sertoli and Leydig cells could be easily identified because of their larger size relative to spermatids (Figure 3).

Special attention was paid to lymphocytes; these were roughly the same diameter as spermatids but distinguishable from them mainly by larger nuclear:cytoplasmic ratios. Their nuclei usually appeared slightly kidney-shaped or round and eccentric, and were dense, coarsely lumpy and possessed a marbled chromatin structure. They stained slightly darker than those of early spermatids, and lighter than the nuclei of late-stage spermatids. Lymphocytes had a very thin and discontinuous pink cytoplasmic zone, which occasionally appeared to be discretely vacuolized. Neutrophils were distinguished from multinucleated spermatids by the nuclei with interconnected lobes and by the granular cytoplasm observed in the former (Figure 2). Spermatids were distinguished from other white blood cell types by their smaller size and from the red blood cells, which lacked nuclei.

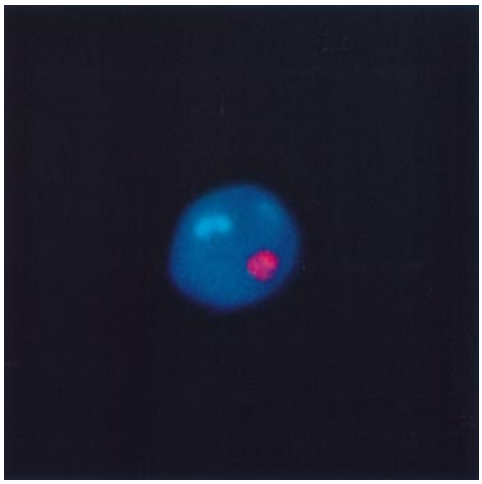

Figure 7. Fluorescence in-situ hybridization (FISH) analysis on a secondary spermatocyte collected with a microinjection pipette. By using a triple band pass filter, two double dotted signals, one for the Y chromosome and one for chromosome18, are seen (2n haploid cell).

Inverted microscopy

After processing of ejaculated samples and testicular biopsies, 1 μl of the cell suspensions was placed in a 5 μl droplet of HEPES-buffered HTF supplemented with 5% polyvinylpyrrolidone (PVP-K 90, molecular weight 360 000; ICN Biochemicals, Cleveland, OH, USA) in a plastic Petri dish (Falcon®, type 1006) under oil (BDH Limited, Poole, UK).

The selection of round spermatids was carried out under the inverted microscope (Olympus® IX 50) with 400 X Hoffman® Modulation Contrast Optics (Modulation Optics, Greenvale, NY, USA) and an ocular micrometer to measure the diameter of the cells. The microscope was also equipped with two motor-driven coarse control manipulators and two hydraulic micromanipulators (MM-188 and MO-109; Narishige Co. Ltd, Tokyo, Japan). The micropipettes were fitted to a tool holder controlled by two IM-6 microinjectors (Narishige). An injection pipette (Cook Veterinary Products, Bloomington, IN, USA) of 5.5–7 μm inner diameter and 7.5–9 μm outer diameter at the tip was used to collect the cells. The bevel of the injection needle was less sharp than that used for conventional ICSI (Tesarik and Mendoza., 1996a). Cells considered to be round spermatids were selected, photographed, and collected with the injection pipette.

In the first part of this study, Testsimplets® were used as a fast test to establish the validity of the criteria for spermatid selection under inverted microscope. For this purpose, a single cell was aspirated with the injection pipette. Then, the Petri dish was removed from the microscope stage and was replaced by a Testsimplets® slide containing a 3 μl droplet of HTF-Plasmanate. The cell was delivered into this droplet and observed at ×400 magnification. This technique allowed a rapid correlation of the morphological features of a single stained and unstained cell (Figure 4).

In the second part of this study, when the criteria were established with this procedure, we corroborated them by cytogenetic analysis.

Fluorescence in-situ hybridization on selected round cells

A total of 10 cells of the same diameter was aspirated with the injection pipette. As all cells had the same diameter, this selection

allowed for the establishment of a cut-off diameter to identify cells as spermatids (i.e., the larger diameter at which only haploid cells would be observed). Then the Petri dish was removed from the microscope stage and was replaced by another dish containing three 50 µl droplets of 5% PVP without oil. The injection pipette containing the cells was washed in these three droplets to remove the oil present on the surface of the pipette, since it might interfere with cell adhesion to the microslides. Next, a precleaned microslide (washed in ethanol for at least 48 h) containing a droplet of 5% PVP was placed on the microscope stage and the selected cells were delivered into this droplet. PVP allowed a better control of the injection needle and facilitated the positioning of the cells on the slides.

Once the cells were deposited on the microslides, the area was circled with a tungsten-carbide pencil. Slides were left at room temperature for 48 h, fixed with methanol:glacial acetic acid (3:1v/v) for 1 h and air dried. Slides were either analysed by fluorescence in-situ hybridization (FISH) directly or stored at -20°C until further processing. For FISH analysis, 7 µl of the hybridization solution consisting of 4.9 µl Spectrum® CEP hybridization buffer (Vysis, Downers Grove, IL, USA), 0.7 µl of Y Spectrum® Orange (Vysis), 0.7 µl of 18 Spectrum® Green (Vysis), 0.35 µl of X Spectrum® Orange (Vysis), 0.35 µl of X Spectrum® Green (Vysis) was placed on the slides. DNA probes were alpha-satellite repeat clusters in the centromeric region of X and 18 chromosomes, and the satellite-III DNA on the long arm of the Y chromosome. The area containing the cells was covered with a 11 mm circular coverslip, sealed with rubber cement, air dried for 5 min and placed on a dark slide warmer at 76°C for 10 min. Subsequently, slides were hybridized in a moist chamber at 37°C for 6–26 h. Slides were washed three times to remove excess probe, once in 50% formamide (Oncor, Gaithersburg, MD, USA)/standard saline citrate (SSC) (pH 7.0) at 42°C for 15 min and twice in phosphate-Nonidet buffer (PN buffer; 0.1 M sodium phosphate buffer; Sigma Chemical Co., St Louis, MO, USA), pH 8.0, 1% Nonidet-P40 (Sigma) for 15 min each at room temperature. Finally, they were counterstained with 10 µl of 4′,6-diamino-2-phenylindole (DAPI, Vysis) in antifade solution and analysed under a fluorescent microscope (Olympus® BX 60). A DAPI filter was first used to find the cell nuclei. The fluorescent signals were then observed with a double bandpass-filter: chromosome 18 appeared green, chromosome Y red, and chromosome X, a mixture of spectrums green and red, was visualized as yellow. A triple bandpass-filter allowed observation of both round cell nuclei and labelled chromosomes. The following scoring criteria were applied: a round cell was considered disomic when two fluorescent domains of the same colour were clearly positioned within the cell, they were comparable in brightness and size, and/or were at least one domain apart. One domain was considered to be the diameter of the signal (Hopman *et al.*, 1988).

FISH on smears of testicular biopsies

Spermatogenic arrest can occur at any stage of germ cell maturation, more frequently at the primary spermatocyte level (Girgis *et al.*, 1969). Collection of single cells was technically more complex and time-consuming than preparation of smears; thus, before collecting single cells in men lacking spermatozoa in their testicular biopsies, we smeared these biopsies to determine whether spermatids were present. If haploid cells were observed, the selection of individual round cells was performed as previously described.

Smears were processed using a method modified from Wyrobek and colleagues (1995). Samples were resuspended in 1% sodium citrate/6 mg/ml bovine serum albumin (BSA, Sigma) for 11 min at equal volumes. Cells suspensions were pelletted by centrifugation at 350 *g* for 5 min and fixed in 3:1 methanol/acetic acid solution. Following two washes and centrifugation with fixative at 350 *g* for 5 min, 10 µl of the cell suspension was smeared onto cold wet precleaned slides and then air dried and processed for FISH immediately. The hybridization procedure was performed as previously described for single cells, with the following modifications: 10 µl of hybridization solution was applied on the slides; 22×22 mm coverslips were used; 15 µl of DAPI in antifade (Vysis) was added to the slides.

Results

Staining technique

The 33 samples containing $\geqslant 1\times10^6$ round cells/ml had spermatids as assessed by Testsimplets®. Three out of five azoospermic samples had spermatids. The remaining two azoospermic samples contained only leukocytes, red blood cells and epithelial cells. These samples came from two men previously subjected to chemotherapy.

Clinical diagnosis of non-obstructive azoospermia was confirmed in seven men undergoing diagnostic testicular biopsy. Five biopsies showed spermatozoa and the complete germinal line was observed with Testsimplets® staining. Of the two biopsies without spermatozoa one had spermatids and the other had a spermatocytic arrest.

Efficiency of Percoll gradient to collect spermatids

The results of cell separation using Percoll gradient are shown in Table I. Although there was considerable variation in different cases, the 70% fraction consistently showed the highest selectivity for round spermatids. Spermatids with normal shape tended to group in this layer. However, when spermatid concentration was low in the samples, the 50% fraction was preferable because, although the number of other round cells was higher, the number of spermatids was also higher. The pooling of 70% fraction and the interphase of 50% fraction proved to be useful only in the few cases in which very few spermatids were present and the concentration of white blood cells was very low. In all other cases, the enrichment of spermatids by pooling these two fractions was hampered by high numbers of white blood cells and multinucleated spermatids that accumulated in the 50% interphase during centrifugation. The interphase of the 70% fraction had only red blood cells. Elongated spermatids were mostly found in the 70% fraction, while spermatocytes were in the 50% fraction.

Observation of spermatids under inverted microscope

The criteria for choosing spermatids were cell shape and size, nuclear characteristics, presence of an acrosome and cytoplasmic characteristics. Round spermatids had a diameter of 5–7.5 µm and the nucleus 4–6.5 µm, maintaining a stable nucleus/cytoplasmic ratio. The spherical, bright, homogeneous nucleus was centrally located in earlier stages, but slightly off-centre in late spermatids. The acrosome granule, occasionally observed, was located adjacent to the spermatid nucleus on one side of the cell and was observed as a small, light-grey spot, similar in colour to the acrosomal cap of the spermatozoa under inverted microscope. The cytoplasm surrounded the

Table I. Efficiency of Percoll density gradient to retrieve round spermatids

No. of patients	Percoll 50%	Interphase 50%	Percoll 70%+ Interphase 50%	Percoll 70%
5	++	++		+++
5	+	+		<+
3	++		+	
3	<+		+	

Concentration of round spermatids: <+ <1%; +1–5%, ++6–15%, +++16–30%. Percentages were calculated on total number of cells.

Table II. Ploidy of presumptive round spermatids as determined by fluorescence in-situ hybridization analysis

Cell diameter	No. of cells (%)[a]				
	Haploid	Aneuploid	2n haploid	Diploid	Total
5 μm	16 (94.1)	1 (5.9)	-	-	17
6 μm	12 (92.3)	1 (7.7)	-	-	13
7 μm	7 (77.8)	2 (22.2)	-	-	9
7.5 μm	6 (60.0)	-	1 (10.0)	3 (30.0)	10
Total	41 (83.7)	4 (8.2)	1 (2.0)	3 (6.1)	49

[a]Percentages were calculated on total number of cells of the same diameter

nucleus as a thin halo. The surface of the spermatid was smooth or showed some irregularities (Figure 5).

Round spermatids were distinguished from other spermatogenic cells by their size. The most difficult cell type to distinguish from spermatids was the secondary spermatocyte, since they shared the most morphological characteristics, but this cell type was usually larger. The secondary spermatocyte, however, was rarely observed in ejaculated and testicular biopsies, explained by the shorter life-span of this cell (24 h) compared with that of a primary spermatocyte (2 weeks or more) (Burger *et al.*, 1976).

Spermatids were easily distinguished from the larger blood cells. However, lymphocytes were of comparable diameter (6–12 μm) but they had a larger nucleus than spermatids. Moreover, lymphocyte nuclei were round or indented and the cytoplasm was very narrow, not surrounding the nucleus continuously, like a half moon. Vacuoles imitating the acrosomal granule of the spermatids were also sometimes present.

FISH analysis

Four ejaculates and seven testicular biopsies were processed by FISH. Spermatozoa were seen in all ejaculates. Of the testicular biopsies, only two had no spermatozoa and smears were performed. Analysis by FISH demonstrated that one smear did not have any haploid cells, whereas in others, several haploid cells were observed. These results were in agreement with the observations by the Testsimplets® technique.

Selection of individual cells with the micromanipulator was performed on the six biopsies that presented spermatids and on four ejaculated samples. A total of 100 cells were processed (10 slides containing 10 cells each); however, only 49 remained on slides after hybridization, all of which showed hybridization signals (Table II). Round spermatids had one signal for the 18 chromosome and one signal for the X or Y chromosome (Figure 6). The analysis revealed that 83.7% of presumed spermatids were haploid (Table II); of these, 18 cells were X/18 and 23 were Y/18. Three cells had a single signal for chromosome 18 but two sex chromosome signals (XY/18). One cell had one sex chromosome signal but two autosomal signals (Y/1818). Another had two double dotted signals, one for chromosome 18 and one for chromosome Y (Figure 7); following the nomenclature suggested by de Kretser and Kerr (1994) and used by Aitken and Irvine (1996), this was considered to be a 2n haploid cell, that is, a secondary spermatocyte. Of the diploid cells, all three were XY/1818.

Discussion

In this study, we used a simple approach to collect and identify round spermatids that can be utilized for patients with non-obstructive azoospermia. At present, routine semen analysis does not include the assessment of round spermatids. However, the establishment of this parameter might be of importance for the treatment of male infertility due to severe non-obstructive azoospermia. The presence of spermatids in the ejaculates of these men may be due to abnormalities in the cell junctions between Sertoli cells (Schleiermacher, 1980; Meyer *et al.*, 1992).

Two opposite approaches have been proposed to treat men suffering from non-obstructive azoospermia. One is to perform systematically an excisional testicular biopsy, since even in the severest cases of azoospermia foci of normal seminiferous tubules with spermatozoa may be found (Tournaye *et al.*, 1995). Although testicular biopsies are not exempted from risks (Schlegel, 1996), the injection of spermatozoa retrieved from the testis is very effective. The other approach is to inject ejaculated round spermatids without a prior testicular biopsy (Tesarik *et al.*, 1995, 1996). However, the injection of round spermatids has yielded low fertilization rates and a very limited number of pregnancies. We consider the testicular biopsy as

an essential diagnostic tool for patients with non-obstructive azoospermia. Since histopathology examines only a thin section of the sample, careful dissection and extensive examination of the tissue should be performed in order to find spermatozoa, particularly in cases of focal spermatogenesis. In addition, a meticulous study of the semen samples should be performed to ascertain the presence of spermatids. If spermatozoa are present, these should be utilized therapeutically. On the other hand, if patients have no spermatozoa and only spermatids both in ejaculates and testicular biopsies, ejaculated spermatids might be used to treat their infertility, avoiding the potential morbidity of a second surgical procedure.

Several methods have been employed for cell separation: elutriation (Grabske *et al.*, 1975), Statput technique (Bellvé *et al.*, 1977; Meistrich, 1977), and sedimentation velocity (Romrell *et al.*, 1976). The use of Percoll density gradient for separation of spermatogenetic cells had been already applied for human testicular biopsies (Blanchard *et al.*, 1991). Recently, Tesarik and Mendoza (1996a) reported the efficacy of the 70% fraction of Percoll to isolate human round spermatid from ejaculates of patients with non-obstructive azoospermia for intracytoplasmic injection. Our study shows that the 70% fraction is preferable when a high concentration of spermatids is observed in the sample. However, when few spermatids are found, 50% Percoll is preferred since, although less selective, it carries the highest concentration of spermatids. Due to the high cell loss observed with Percoll density gradient, azoospermic ejaculates and testicular biopsies should be processed by concentration and centrifugation and Percoll might only be considered when high concentrations of round cells are observed in the specimens. Preparation of testicular biopsies by mechanical means may provoke damage to the cells (Romrell *et al.*, 1976). However, fine mincing as performed in our study has been recently reported to be the most effective approach for processing testicular tissue (Verheyen *et al.*, 1995).

The differentiation between the various sperm precursors and leukocytes by light microscopy using Testsimplets® staining proved to be reasonably precise. However, the identification of round cells by staining techniques requires a highly trained eye for accurate diagnosis. Testsimplets® technique was preferred over permanent staining techniques (e.g., haematoxillin-eosin, Diff Quik, Schorr, Papanicolau, May-Grunwald-Giemsa) because it is faster and equally reliable. Furthermore, Testsimplets® allows the simultaneous comparison between fresh and coloured samples using inverted microscopy. This technique does not produce alterations of cell morphology that may occur with fixation for permanent stainings. Thus, cells maintain the same dimensions as those observed under inverted microscopy. Disadvantages of this technique are a short duration (no more than 24 h when refrigerated at 8°C) that impedes the storage of slides for future comparisons, and a faster fading of the colouration when combined with Percoll.

The velocity of sedimentation of a cell moving through an isotonic medium depends primarily on its size. However, density and shape of the cell and viscosity of the medium are also important factors (Loir and Lanneau, 1977). This led us to use a lower concentration of PVP than 10%. After about 10 min exposure to 5% PVP, cells tend to separate in different layers according to their size. Thus, all the cells of similar size are found at a certain level in the droplet of PVP, facilitating the detection of the cells and reducing the time of the procedure. Due to a higher viscosity, the cells remain floating in the 10% PVP droplet for a much longer time. In addition, 10% PVP induces the formation of cell aggregates in a short time period. Although cell aggregation also occurs with 5% PVP, it takes much longer. This is very useful when few spermatids are present in the samples because a longer time is required for their identification and collection. The properties of filtering debris and control of the injection pipette of 10% PVP remain unchanged with 5% PVP.

This study illustrates that using smears for FISH analysis is a simple and efficient method to assess the presence of round spermatids. Although the observations made by staining techniques are accurate, they require training in cytology. Furthermore, when only morphologically abnormal spermatids are found, the diagnosis by staining may become highly unreliable. In those cases, FISH may be used for to determine whether spermatids are present. This procedure might be proposed as an additional diagnostic tool in the work-up of the azoospermic patients. Another application of this technique would be to investigate the chromosomal status of round spermatids. In fact, a high incidence of cells with a XY/18 constitution was observed in one specimen (unpublished data). These cells may be chromosomally abnormal spermatids. If that is the case, the frequency of abnormal spermatids is higher than the expected incidence of chromosomal aneuploidies detected in sperm. This phenomenon would be consonant with the germinal cell loss occurring during spermatogenesis which may reduce the number of genetically abnormal gametes, not suitable for propagation (Roosen-Runge, 1973). Possible aetiologies of non-obstructive azoospermia are Klinefelter syndrome or 46XY/47XXY mosaicism (Persson *et al.*, 1996). Furthermore, non-obstructive azoospermic men with a normal peripheral karyotype can be mosaic with an aneuploid cell line confined to the germ tissue (Hendry *et al.*, 1976). Thus, these men may present a higher number of spermatids with chromosomal abnormalities. In these patients, FISH would be useful before considering the use of spermatids to fertilize oocytes. With this technique, however, diploid round spermatids would be overlooked. A method combining a morphological identification of the cell together with ploidy assessment would be ideal. Recently, the use of brightfield microscopical in-situ hybridization was reported (Martini *et al.*, 1996). This technique has the advantage of allowing simultaneous application of morphology and chromosome analysis. However, this procedure utilizes dithiothreitol (DTT) for DNA decondensation. The exposure of the cells to this compound would most likely deform them, preventing their proper identification. Furthermore, only chromosomes X and Y were assessed, preventing the differentiation between disomy and diploidy. In the present study, the labelling of an autosomal chromosome and both sex chromosomes allowed that distinction.

By conducting FISH on single cells, one can assess their ability to select round spermatids. The data obtained by direct morphometric and cytological evaluation shows a good

correlation with FISH. Haploid cells were detected in 84% of the cases. If the four abnormal cells are considered as aneuploid spermatids, this efficiency is even higher. The possibility that diploid cells may be abnormal spermatids should also be considered. Regardless of these considerations, our 84% correlation rate still compares favourably with that reported by Sofikitis and coworkers (1997) and Yamanaka and coworkers (1997). These reports using computer assisted image analysis for indirect morphometric cell selection have found that 76% of cells were round spermatids as confirmed by confocal scanning laser microscopy, and 84% with transmission electron microscopy. A technical matter still unresolved with the FISH technique is the high rate of cell loss during the washes performed after hybridization despite various meticulous manoeuvres to improve cell attachment to the microslides.

The cell with the double dotted signal was considered to be a secondary spermatocyte (Figure 7). The secondary spermatocyte is expected to show paired hybridization signals as it has a single univalent chromosome of each pair. This is homologous to what occurs in the oocyte (Munné *et al.*, 1995). Secondary spermatocytes can also display four different single dotted signals when the chromatids separate before completion of the second meiotic division leading to the generation of round spermatids. The cells visualized as XY/1818 may represent non-germinal cells, primary spermatocytes or spermatogonia, although these germ cells would hardly have been mistaken for spermatids due to their larger size. An interesting finding was that the 2n haploid cell and all the diploid cells were found among the population of 7.5 μm. Thus, cells smaller than 7.5 μm should be selected when they are available, since larger cells are likely to be secondary spermatocytes or white blood cells.

In summary, we report here a simple morphological method that allows us to collect a population of haploid spermatid cells from azoospermic men. Our study suggests that FISH is useful in the management of men with non-obstructive azoospermia for assessing the presence of round spermatids, as well as a 'quality control' for the technician's ability to select these cells. FISH can also be used as a screening test to determine the genetic constitution of round spermatids in diagnostic testicular biopsy.

At present, the use of round spermatids to treat severe male infertility is still in its early development. More basic research is needed fully to characterize these cells without compromising their viability. We have attempted to provide tools for isolating human round spermatids for both research and clinical purposes.

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