Journal of Andrology, Vol. 27, No. 2, March/April 2006

Relationship of Cytoplasmic Droplets to Motility, Migration in Mucus, and Volume Regulation of Human Spermatozoa

SABINA FETIC,* CHING-HEI YEUNG,* BARBARA SONNTAG,† EBERHARD NIESCHLAG,* AND TREVOR G. COOPER*

*From the *Institute of Reproductive Medicine and the †Department of Obstetrics and Gynaecology of the University of Münster, Germany.*

ABSTRACT: A significantly greater percentage of motile than immotile spermatozoa bore droplets at the osmolality of semen and cervical mucus. The percentage of spermatozoa with droplets was not significantly correlated with the osmolality of semen or the extent of cell swelling in response to quinine in hypotonic medium. Droplets appeared slightly more frequently (ie, were more obvious) in the presence of quinine, which blocks regulatory volume decrease, indicating that they are the major site of volume expansion. There was no selection for or against droplet-bearing spermatozoa migrating through viscous surrogate mucus at high or low osmolality. Sperm swelling in response to quinine at mucus osmolality was significantly greater in fathers than in patients whose partners had no fertility problem. Therefore, cytoplasmic droplets are not deleterious to sperm motility and may be related to physiological volume regulation, which may be predictive of some forms of human male infertility.

Key words: Fathers, mucus penetration, sperm swelling, semen analysis, diagnosis.

J Androl 2006;27:294–301

The majority of motile human ejaculated spermatozoa bear cytoplasmic droplets (CDs) (Cooper et al, 2004), indicating that they are not an abnormal feature of spermatozoa, although excess residual cytoplasm along the midpiece, often considered a CD by clinicians (Cooper, 2005), is associated with damaged cellular structures (Keating et al, 1997). The role of CDs in the assessment of human male fertility is largely unexplored, although retention of proximal and distal droplets on ejaculated spermatozoa is associated with infertility in domestic species (Waberski et al, 1994; Amann et al, 2000; Thundathil et al, 2001; Kuster et al, 2004).

In mice, CDs located at the end of the midpiece are the site of water entry, so in cases of defective volume regulation, the resultant swelling initiates flagellar angulation leading to hairpin-bend morphology (Yeung et al, 1999). This is a characteristic that can be mimicked by blocking the cation and anion channels involved in volume regulation (Yeung et al, 2002, 2005; Barfield et al, 2005) and which leads to failure of spermatozoa to reach the oviduct (Yeung et al, 2000). Osmotically sensitive ''mid-piece vesicles'' (MPVs) were described by Abraham-Peskir et al (2002) and Chantler and Abraham-Peskir (2004), our interpretation of which is that they are grossly swollen CDs (Cooper et al, 2004) categorized by the former authors as MPVs only when they extend the length of the midpiece.

Human ejaculated spermatozoa swell when presented with a physiologically relevant hypo-osmotic challenge (Yeung and Cooper, 2001) of cervical mucus osmolality (286 mmol/kg: Rossato et al, 1996), unless they are able to perform regulatory volume decrease (RVD). As quinine blocks the channels employed for osmolyte efflux during volume regulation (Yeung and Cooper, 2001; Yeung et al, 2003, 2005), a simple test of the volume-regulating status of spermatozoa is to monitor their size in the presence and absence of quinine. This drug would prevent RVD, leading to a larger volume only for cells capable of undergoing RVD. The smaller the difference in size in the presence and absence of quinine, the lower potential of the cell for regulating volume.

In this work, experiments were performed to examine the relationship, if any, among CDs, volume regulation, and mucus migration of spermatozoa from patients, volunteers, and fathers.

Supported by Deutsche Forschungsgemeinschaft grant YE37/6-1.

Correspondence to: Dr T. G. Cooper, Institute of Reproductive Medicine of the University of Münster, Domagkstrasse 11, D-48129 Münster, Germany.

Received for publication July 18, 2005; accepted for publication September 27, 2005.

DOI: 10.2164/jandrol.05122

Materials and Methods

Ejaculates

Ejaculates were obtained from 3 groups: 1) infertility patients attending the Institute of Reproductive Medicine; 2) healthy stu-

dent volunteers; and 3) recent fathers (recruited by, and partners of, women conceiving naturally and whose children were born within the last 12 months). Semen were analyzed according to the World Health Organization (WHO) manual (1999) for sperm concentration, ideal morphology, and motility and were analyzed within 60 minutes of production. The characteristics of the semen provided in this study are presented in Table 1. All patients gave informed consent for their semen to be used for research by signing a form approved by the local Ethics Committee.

Measurement of Osmotic Pressure and Composition of Media

The osmolality of 10 μL of fluids was measured with a vapor pressure osmometer (Wescor Vapro 5510, Kreienfeld Scientific Measuring Systems, Langenfeld, Germany) using no delay for media or a 2-minute delay to ensure chamber saturation and accurate results for liquefied semen. Various modifications of Biggers, Whittem, Whittingham (Biggers et al, 1971) media (BWW) were prepared with osmolality altered by addition or removal of NaCl and inclusion of 20 mM HEPES, pH 7.4, in addition to the bicarbonate buffer: BWW of 320 mmol/kg (BWW_{320}) was used to mimic the osmolality of freshly liquefied semen and to dissolve quinine in experiment 1; BWW_{230} was used in experiment 1 to reduce semen osmolality to that of mucus and in experiment 2 to achieve with BWW_{390} the desired osmolality of surrogate mucus; and BWW_{290} was used in experiment 3 to mimic the osmolality of human cervical mucus (286 mmol/kg: Rossato et al, 1996) in the preparation of Percoll. The osmolalities of surrogate mucus of hyaluronic acid (6 mg/mL: Tang et al, 1999; from rooster comb, Sigma H5388) and 4 mg/mL bovine serum albumin were 311 ± 3 mmol/kg and 267 ± 3 mmol/kg.

Experiment 1: Effect of Osmolality on Percentages of Cytoplasmic Droplets

These experiments were done in order to establish if CDs were more easily detected on sperm in semen of lower osmolality or when volume regulation was blocked by quinine.

Patients with sperm concentrations of more than 10×10^6/mL supplied semen for this study. For microscopic observations of spermatozoa in wet preparations, either quinine in BWW_{320} (10 μL stock) or BWW_{320} alone (10 μL as control) was added to 100 μL liquefied semen. After a 15-minute incubation at 37°C in 5% (vol/vol) CO_2, a 3-μL aliquot was placed under a 22 × 22–mm cover slip and examined by phase contrast microscopy (Olympus BH-20, Tokyo, Japan) with a 40× objective and 10× eyepiece on a heated stage at 37°C (Mini-Tüb, Tiefenbach, Germany). The percent motility (WHO grades a+b+c) was determined, and immotile and motile spermatozoa were separately assessed for the presence of I) CDs (regular distensions at the neck or along the midpiece), II) residual cytoplasm (large, irregular material along the midpiece), III) coiled or looped tails, or IV) none of the above categories (Cooper et al, 2004).

Similar preparations were examined in which semen was mixed with appropriate volumes of BWW_{230} to reach a final osmolality of 290 mmol/kg before the incubation with or without a final concentration 0.27 mM quinine. An equal volume of 7% (vol/vol) glutaraldehyde was added to 10 to 20 μL of these sam-

Table 1. *Characteristics of semen from patients, volunteers, and fathers used in this study**

	Experiment 1 Patients (27)	Experiment 1 Student Donors (12)	Experiment 2 All Subjects (31)	Experiment 3 Fathers (13)	Experiment 3 Patients (12)
Semen volume (mL)	4.2 ± 0.3 (1.6–7.2)	4.4 ± 0.4 (2.7–7.3)	4.2 ± 0.3 (1.5–9.4)	3.5 ± 0.4 (1.2–6.4)	4.7 ± 0.6 (2.6–10.5)
Sperm concentration (10^6/mL)	48.1 ± 7.8 (12–165)	50.6 ± 17.2 (12–201)	45.7 ± 7.3 (7–223)	86.2 ± 18.6 (3–223)	25.2 ± 5.9 (6–82)
Sperm count (10^6/ejaculate)	191.2 ± 29.2 (24–466)	185.6 ± 44.6 (60–534)	180.0 ± 29.6 (17–758)	265.8 ± 60.0 (9–758)	113.9 ± 26.3 (17–369)
Motility (%)					
Grade a	30.3 ± 2.5 (5–45)	33.1 ± 3.2 (12–47)	30.9 ± 2.0 (10–45)	38.0 ± 2.0 (23–48)	28.2 ± 3.3 (10–46)
Grade b	19.7 ± 1.7 (12–39)	18.1 ± 2.5 (8–32)	17.4 ± 2.6 (6–89)	13.0 ± 1.0 (6–19)	18.4 ± 2.3 (7–33)
Grade c	7.9 ± 0.8 (3–16)	8.3 ± 0.9 (5–14)	10.6 ± 1.1 (5–39)	8.5 ± 0.9 (5–13)	9.0 ± 1.3 (3–19)
Grade d	42.1 ± 1.8 (25–68)	40.5 ± 1.3 (36–51)	43.2 ± 9.2 (24–67)	40.4 ± 1.6 (33-54)	44.3 ± 2.3 (32–59)
Normal morphology (%)	12.1 ± 1.2 (2–25)	12.5 ± 2.3 (2–27)	11.08 ± 1.1 (2–30)	12.8 ± 1.3 (6–19)	10.0 ± 1.5 (3–21)

* Values are mean ± SEM (mimimum–maximum).

ples, and after 60 minutes at room temperature, the fixed cells were washed by addition of 1 mL phosphate-buffered saline and centrifuged at 500 × *g* for 5 minutes. The fixed pellet was examined as a wet preparation at 400× magnification for the presence of categories I through IV on the previous page. Centrifugation of fixed cells did not influence the percentages of CDs observed.

To assess if CDs were related to the regulation of cell volume, cell size (given in channel number) was measured by flow cytometry on other aliquots after dilution with the same medium.

Experiment 2: Sperm Migration Through Surrogate Mucus

These experiments were done to ascertain if the presence of CDs would hinder the migration of spermatozoa through fluid of cervical mucus viscosity and osmolality.

Twenty-one patients with sperm concentrations of more than 10×10^6/mL, 3 healthy volunteers, and 7 fathers provided semen for this study. The osmolality of the ejaculates after about 30 minutes' liquefaction was 321 plus or minus 4 mmol/kg.

Flat capillary tubes (10 cm long, 3 mm wide, and 0.3 mm deep; Camlab, Cambridge, United Kingdom) were filled with the mucus. One end was sealed with plasticine and the other end was inserted into an Eppendorf tube containing 75 μL liquefied ejaculate and incubated horizontally in a moist chamber at 37°C and 5% (vol/vol) CO_2 in air for 90 minutes. The number of sperm at 1 cm (representing penetrated sperm) and at 4 cm (representing migrated sperm) was counted, and the migration distance of vanguard sperm (the most distal field containing the most advanced 10 cells) in a low-power microscopic field (10× objective and 10× eyepiece) was measured.

In order to examine the presence of droplets on these spermatozoa, the capillary was scored with a diamond knife and snapped at 1 and 4 cm, a drop of the mucus was expressed by blowing the contents onto a slide, and 2 × 100 spermatozoa were evaluated with a 40× phase contrast objective. To test for the effect of mucus alone on the presence of CDs, 4-μL aliquots of the ejaculates were mixed with 20 μL mucus by stirring with the pipette tip and incubated in parallel with the capillary and analyzed at the same time point for the presence of CDs. For determination of percentages, 100 cells were counted in duplicate, except in cases in which fewer than 100 cells were seen, in which case all were counted.

Experiment 3: Volume Regulatory Capacity of Spermatozoa in Physiologically Hypotonic Medium

These experiments were done to determine if the regulatory response of spermatozoa from recent fathers to a physiologically relevant osmotic challenge differed from that of patients.

Aliquots of 200 μL freshly liquefied semen from patients or fathers were diluted in 200 μL BWW_{290} with or without a final concentration of 0.27 mM quinine and were incubated for 5 minutes at 37°C. To remove non-sperm particles, the semen was then washed through Percoll 30% (vol/vol)/60% (vol/vol) made up in modified BWW_{290} with or without 0.27 mM quinine; we gently layered the sperm sample over 1.4-mL Percoll in a 2-mL Eppendorf tube and centrifuged at 700 × *g* for 20 minutes. In this way, the osmotic challenge with and without quinine was already present during centrifugation.

Preliminary experiments revealed that this washing procedure did not select for quinine-responding or nonresponding spermatozoa. After removal of the supernatant the sperm pellet (about 50 μL) was then resuspended and the cell volume measured by flow cytometry after diluting a 5–10-μL aliquot in 200 μL of the same incubation medium. Measurements were also made on these washed samples after 5-minute demembranation by addition of 20-μL sample to 5 μL 10% (vol/vol) Triton X-100.

Flow Cytometry

Laser forward scatter by flow cytometry, as previously described and validated, was used as an index of cell volume (Yeung et al, 2003). The DNA stain propidium iodide (PI) was added to the sample (final concentration 6 μg/mL) before flow cytometric analysis to discriminate nonviable from viable sperm in the control and quinine-treated samples and to distinguish demembranated sperm from cell debris in the Triton-treated samples. The difference in cell volume (b − a) between nontreated viable sperm performing volume regulation (a) and quinine-treated sperm in which volume regulation was blocked (b) indicates the extent of volume regulation. This capacity was standardized against the cytoplasmic volume (a − c), in which c is the volume given by the demembranated sperm devoid of cytoplasm. The sperm volume regulation index was calculated as (b − a)/(a − c).

Statistics

Differences between populations were assessed by paired *t* test (% sperm with CDs with or without quinine), unpaired *t* tests (comparison of patients and fathers), or 3-way analysis of variance (% sperm with CDs for 3 sources of variation: patients or fathers; mucus of 267 or 311 mmol/kg; distance 0 (semen and mucus mixed in tube), 1, or 4 cm in capillaries) and relationships by linear regression. The statistical program SigmaStat (Erkrath, Germany) prompted the use of nonparametric tests when conditions of normality or equal variances were not met. Significance was accepted when $P < .05$.

Results

Experiment 1: Effect of Osmolality and Quinine on Motility and Percentages of Spermatozoa With Cytoplasmic Droplets

The percentage of motile spermatozoa with CDs was significantly higher than that of immotile cells, whether at the osmolality of semen or cervical mucus or in the absence or presence of quinine (Figure 1). The presence of quinine increased slightly but significantly the percentage of spermatozoa observed to have droplets in fixed preparations (41.2 ± 1.4 vs 37.6 ± 1.6) and in BWW_{290} (41.0 ± 1.7 vs 37.8 ± 1.7; n = 43).

There was a general trend toward lower sperm motility with semen of higher osmolality, but regression coeffi-

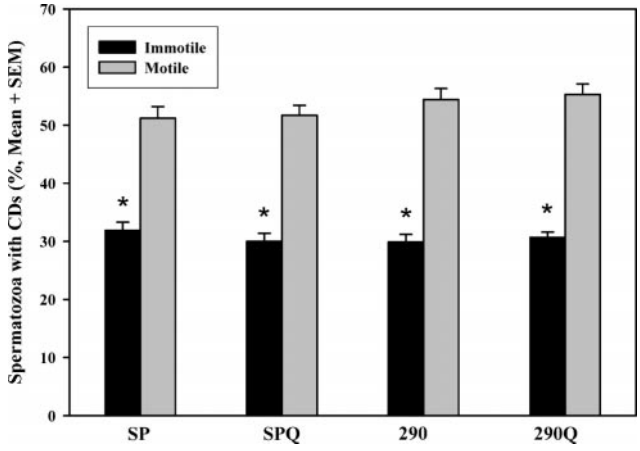

Figure 1. The percentage of motile (□) and immotile (■) human ejaculated spermatozoa bearing cytoplasmic droplets (ordinate, mean + SEM, n = 50) within 30 minutes of liquefaction, undiluted in seminal plasma (SP) or diluted in medium of 290 mmol/kg (290) in the absence (SP, 290) or presence (SPQ, 290Q) of 0.27 mM quinine. *, for each medium, significant difference between motile and immotile fractions ($P < .05$).

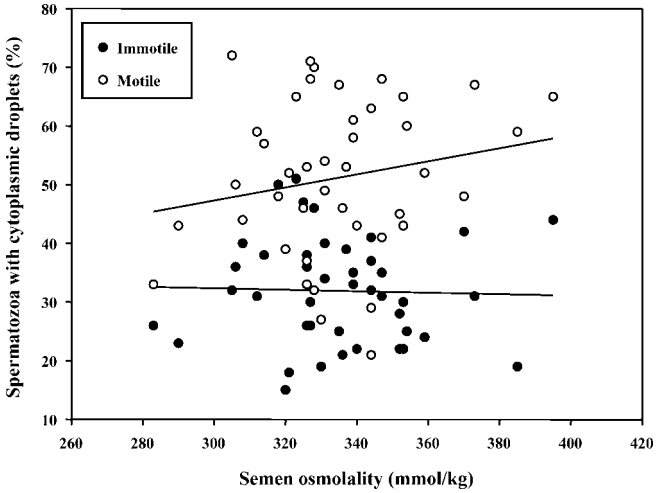

Figure 3. The percentage of spermatozoa bearing cytoplasmic droplets (ordinate) plotted against the osmolality of semen (abscissa) for motile (○) and immotile (●) cells.

cients were low (for seminal plasma, −0.229; for BWW_{290}, −0.203). The motility of spermatozoa in seminal plasma (mean ± SEM: 49.5% ± 2.7%) was not different from that in semen to which quinine had been added (49.1% ± 2.3%), whereas sperm motility in semen diluted in BWW_{290} (49.2% ± 2.7%) was significantly greater than that in BWW_{290} containing quinine (39.9% ± 2.6%; n = 43).

Flow cytometric determination of forward scatter revealed that there was no relationship between the size of sperm and the osmolality of seminal plasma (Figure 2). In the presence of quinine, live spermatozoa were significantly larger than in its absence both at the osmolality of native semen (mean forward scatter [FS, channel number] 569.7 ± 31.0 vs 543.6 ± 3.2) and of cervical mucus (290 mmol/kg) (FS: 560.5 ± 4.1 vs 536.4 ± 3.4), whereas nonviable cells permitting PI entry did not increase in size (FS: 531.1–537.1; differences between nontreated and quinine-treated were not significantly different from zero). As expected, Triton-demembranated sperm were smaller (FS 474.5 ± 3.4 ; n = 40), as membranes and their contained cytoplasm were lost.

The percentage of spermatozoa bearing CDs was related to neither semen osmolality (Figure 3) nor the RVD response (Figure 4).

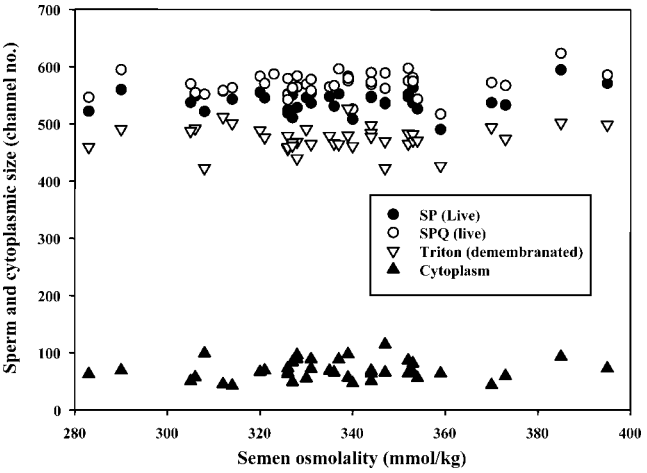

Figure 2. Relationship between the size of viable (propidium iodide–excluding) human ejaculated spermatozoa, reflected in laser light forward scatter (ordinate), to the osmolality of semen (abscissa). Data for intact spermatozoa in semen without (●) and with (○) quinine, in the presence of Triton X-100 (▽), and the calculated cytoplasmic volume (▲). Volume regulation within semen is indicated by the constant sperm size over the entire range of osmolality.

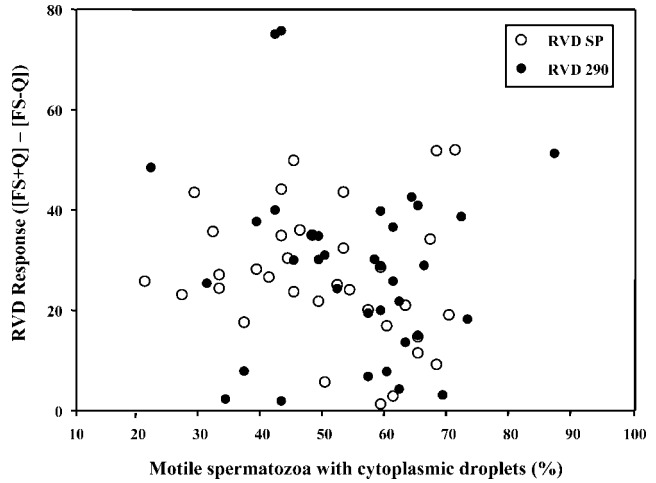

Figure 4. The response of spermatozoa to 0.27 mM quinine (ordinate) plotted against the percentage of motile spermatozoa bearing cytoplasmic droplets (abscissa) for spermatozoa in semen (○) and in physiologically hypotonic medium (●).

Table 2. *Percentages of motile spermatozoa bearing cytoplasmic droplets in native semen and in surrogate mucus with osmolality of 267 and 311 mmol/kg, respectively**

	Fathers (7)	Patients (21)	Volunteers (3)	All Subjects (31)
In semen	66.7 ± 5.1	60.6 ± 2.4	66.0 ± 5.1	63.4 ± 2.2
In mucus (311 mmol/kg)				
In tube	74.6 ± 5.3	67.9 ± 2.2†	65.0 ± 9.3	69.2 ± 2.0
In capillary				
At 1 cm	69.9 ± 4.7	63.4 ± 3.3†	69.0 ± 14.4	66.0 ± 2.5
At 4 cm	66.7 ± 6.8	58.5 ± 3.0†	75.0 ± 5.0	61.2 ± 2.6
In mucus (267 mmol/kg)				
In tube	74.6 ± 4.5	68.1 ± 2.8†	62.3 ± 9.9	69.7 ± 2.3
In capillary				
At 1 cm	77.0 ± 3.9	66.7 ± 2.5†	74.7 ± 6.0	69.8 ± 2.1
At 4 cm	69.6 ± 7.3	62.6 ± 3.0†	66.3 ± 10.6	65.0 ± 2.7

* Values are mean ± SEM.
† Significantly different from fathers.

Experiment 2: The Percentage of Spermatozoa With CDs After Migration Through Viscous Surrogate Mucus

The ejaculates used for this study came from 7 fathers, 3 student donors, and 21 patients. Since there was no difference between the groups in terms of how their spermatozoa behaved in mucus (Table 2), the results given are from all 31 subjects. As a group, the percentage of motile spermatozoa bearing CDs in the native semen (63% ± 3%, range: 40%–86%) was similar to the percentage of spermatozoa with CDs recovered from the 1-cm and 4-cm marks of capillaries containing mucus at high and low osmolalities (Table 2). The percentages of spermatozoa with CDs found within surrogate mucus at the 1-cm and 4-cm marks were similar at both osmolalities (Table 2), as were the vanguard distances (5.6 ± 0.3 cm [267 mmol/kg] and 5.7 ± 0.3 cm [311 mmol/kg]). The CDs on sperm in the mucus were of various sizes, ranging from small but distinguishable droplets at the neck region to a vesicle surrounding the whole of the midpiece (Figure 5); smaller ones were more frequently encountered. There was no correlation between the percentage of CDs and any values of the penetration score or the osmolality of the semen.

There was no significant difference between the osmolality of semen from fathers (317 ± 9 mmol/kg) and semen from patients (323 ± 4 mmol/kg). The percentage of spermatozoa with droplets within semen of fathers was not significantly higher than that of patients (Table 2). However, when mixed with surrogate mucus of either osmolality, or after penetrating 1 cm and 4 cm through mucus, there were consistently higher percentages of spermatozoa with droplets from fathers than with droplets from patients (Table 2). Three-way analysis of variance on these percentages revealed a significantly higher, albeit small, percentage of spermatozoa with droplets within the surrogate mucus when obtained from fathers rather than from patients (mean 7.5%).

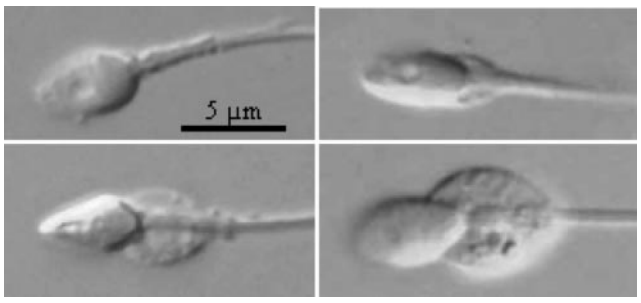

Figure 5. Differential interference micrographs of spermatozoa showing the absence (top left panel) and presence of cytoplasmic droplets of various sizes on the midpiece of the sperm tail after 80 minutes of surrogate mucus penetration. Smaller sized droplets were more frequently encountered. Scale bar = 5 μm.

Experiment 3: Volume Regulatory Capacity of Spermatozoa From Fathers and Patients to Physiologically Hypotonic Medium

There was no difference between fathers and patients in the uptake of the vital dye PI by spermatozoa from fathers (F: 74.3% ± 4.2% PI-negative, n = 13) or patients (P: 65.6% ± 4.5% PI-negative, n = 14) in the absence of quinine or in its presence (F: 74.1% ± 4.6%; P: 64.9% ± 4.1%), in their size (FS, channel number) in medium with the osmolality of human cervical mucus in the absence (F: 502.8 ± 7.7; P: 505.4 ± 5.9) or presence (F: 520.9 ± 7.5; P: 517.5 ± 6.3) of quinine, or in the size of demembranated spermatozoa (F: 441.2 ± 5.0; P: 442.0 ± 4.1) and, hence, their surmised cytoplasmic volume.

However, there was a significant difference between fathers and patients in the response of their spermatozoa to quinine at the osmolality of mucus, with a significantly greater quinine/control ratio for fathers (3.6% ± 0.5% increase in the presence of quinine) than for patients (2.4% ± 0.4% increase) (Table 3). When expressed as a percentage of the cytoplasmic volume, this value was significantly more marked in fathers (32.7% ± 4.7% increase) than in patients (18.7% ± 3.5%; Figure 6).

Discussion

Human spermatozoa are stored in the cauda epididymidis in a fluid with osmolality close to that of the vas deferens

Table 3. *Volume regulatory response to physiologically hypotonic medium in presence of quinine**

	Patients (n = 12)	Fathers (n = 13)	Significance (*P*)
Control samples at 290 mmol/kg			
Membrane intact (%)	63.8 ± 4.7	74.3 ± 4.2	.107
Size (channel number) of viable cells	506.2 ± 6.7	502.9 ± 7.7	.750
Samples in quinine (0.3 mM) at 290 mmol/kg			
Membrane intact (%)	63.3 ± 4.4	74.1 ± 4.6	.103
Size (channel number) of PI-negative cells	517.4 ± 7.0	520.9 ± 7.5	.732
Samples in Triton X-100			
Size (channel number) of demembranated samples	443.3 ± 4.5	441.2 ± 5.0	.763
Ratio of forward scatter (quinine/control)	1.022 ± 0.004	1.036 ± 0.005	.029

* Values are mean ± SEM; PI indicates propidium iodide.

(342 mmol/kg: Hinton et al, 1982) and form an ejaculate with an osmolality of around 294–304 mmol/kg within 5 minutes of production (Björndahl and Kvist, 2003; Cooper et al, 2005). Within the female tract, the sperm mass encounters similar osmolarities (mmol/kg) in cervical mucus (286.8 ± 30.7; Rossato et al, 1996), mid-cycle uterine fluid (284; 276–298; Casslén and Nilsson, 1984), tubal (hydrosalpinx) fluid (271–274; Granot et al, 1998; Ng et al, 2000), or follicular fluid (279.8 ± 7.0; Edwards, 1974). These hypo-osmotic media would promote swelling in the absence of volume regulation.

In mice, osmotic swelling occurs at the cytoplasmic droplet at the end of the midpiece and prevents passage through the uterotubal junction (Yeung et al, 2000). In man the droplet is proximal (Cooper et al, 2004), and its role in volume regulation and migration through cervical mucus is obscure. If such droplets are the major site of water influx in hypo-osmotic solutions and if volume regulation cannot occur, their size may physically hinder penetration through viscous media encountered in the female tract as mucus penetration is inhibited by quinine (Yeung and Cooper, 2001). Were this to be the case, assessing the presence of droplet-bearing spermatozoa may be of diagnostic importance for human infertility. This study examined whether the extent of swelling of human spermatozoa and their capacity for regulating volume was reflected in their CDs and whether the presence of CDs influenced sperm function (motility or penetration of a surrogate mucus).

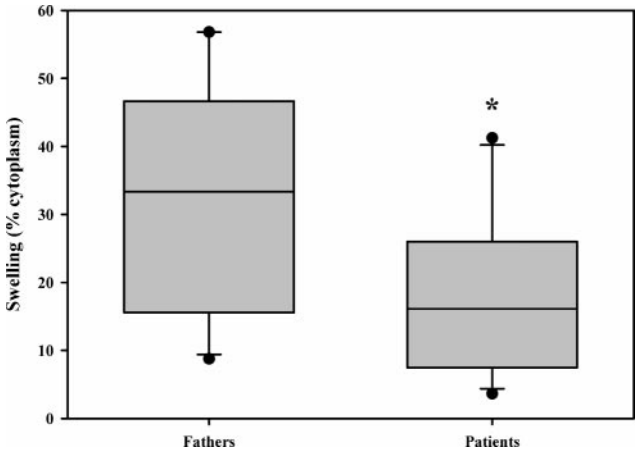

Figure 6. The extent of swelling of propidium iodide (PI)–excluding human ejaculated spermatozoa suspended in hypotonic medium (290 mmol/kg), expressed as the difference in size (forward scatter, channel number) in the presence and absence of 0.27 mM quinine as a percentage of the control cytosolic volume (size of intact sperm less that of Triton-demembranated sperm) in fathers and patients whose partners had no infertility problems.

The greater percentage of motile spermatozoa with droplets compared to immotile cells in semen has been noted before (Cooper et al, 2004) and extended here to semen diluted in medium of 290 mmol/kg. As judged from wet preparations, there was no clear relationship between the percentage of spermatozoa bearing droplets and the osmolality of seminal plasma. Although statistically significant, the increased percentage of spermatozoa bearing droplets in the presence of inhibitory concentrations of the channel blocker quinine, as recorded in fixed preparations, amounted only to 2.3%. As droplets were recorded regardless of their size (and not just those extending the length of the midpiece), the slight increase presumably reflects the slightly greater size of droplets in the presence of quinine that made them more obvious and thus caught the subjective attention of the observer. Objective measurements of sperm size by flow cytometry, indeed, indicated a significant increase in sperm size in the presence of quinine. These quinine-induced increases in size of spermatozoa at female tract osmolality reflect the extent of regulatory volume decrease that would otherwise occur in the absence of the drug. The observations demonstrate that with physiological challenges in the range of osmolalities found within liquefied semen and cervical mucus, the majority of spermatozoa were functioning to control their volume using osmolytes passing through quinine-sensitive channels. The response of spermatozoa to quinine was not related to the percentage bearing CDs, most likely because the majority of spermatozoa bore these structures. The geometry of the droplet at the

neck and not the annulus (Cooper et al, 2004) may prevent accumulation of cellular water in the droplet, as in mice, but may permit its incorporation along the entire flagellar length, which could account for the effect of quinine on human sperm kinematics (Yeung and Cooper, 2001).

As human mucus is difficult to obtain and variable in quality, use of a surrogate was deemed pertinent to answer questions relating to sperm migration through viscous media. In addition to the differences in rheological properties of hyaluronate (stemming from a polysaccharide) and those of human mucus (stemming from a range of mucin glycoproteins), soluble factors in human mucus may affect sperm progression and confound the interpretation of results. These components are lacking from the mucus substitute, which permits examination solely of viscosity effects at different osmolalities. In experiments with mucus of similar viscosity to that of mid-cycle cervical mucus, CDs neither hindered nor facilitated the penetration and migration of the spermatozoon into this matrix. These results are consistent with observations that spermatozoa within natural mucus (ie, selected for motility) are more likely to have droplets (midpiece vesicles) than (both motile and immotile) spermatozoa in semen (Abraham-Peskir et al, 2002), because mucus selects for motile sperm, which bear more droplets than immotile spermatozoa (data presented here; Cooper et al, 2004). The ability of droplet-bearing spermatozoa to penetrate mucus would also explain the presence of such spermatozoa in the Fallopian tube after artificial insemination (Mortimer et al, 1982).

The lack of differences in the migration of droplet-bearing spermatozoa within mucus implies efficient volume regulation by motile spermatozoa during exposure to a natural hypo-osmotic challenge of about 40 mmol/kg. Such efficient volume regulation may be required for mucus penetration, since quinine-treated spermatozoa were unable to penetrate surrogate mucus effectively (Yeung and Cooper, 2001), and thus, disturbances in volume regulation may be a cause of some cases of male infertility. That volume regulation has such an important role in fertility was indicated by the significantly greater swelling of spermatozoa from fathers compared to patients in response to quinine at female tract osmolality. This implies a greater RVD activity of spermatozoa from fathers and warrants a larger study to ascertain whether assessing volume regulation routinely in the Andrology laboratory would be a useful complement to the spermiogramme, especially for men with idiopathic infertility. The lack of relationship between RVD response and presence of CDs indicates that there is nothing to be gained by assessing the latter routinely for diagnostic purposes.

Acknowledgments

We thank Barbara Hellenkemper for performing the mucus penetration test and Ele Kürten, Sabine Rehr, Külli Nurmik, Margret Kloth, and Daniele Hanke for the routine semen analysis.

References

Abraham-Peskir JV, Chantler E, Uggerhøj R, Fedder J. Response of midpiece vesicles on human sperm to osmotic stress. *Hum Reprod.* 2002; 17:375–382.

Amann RP, Seidel GE, Mortimer RG. Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology.* 2000;54:1499–1515.

Barfield JP, Yeung CH, Cooper TG. The effects of putative K^+ channel blockers on volume regulation of murine spermatozoa. *Biol Reprod.* 2005;72:1275–1281.

Biggers JD, Whittem WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC Jr, ed. *Methods in Mammalian Embryology.* San Francisco, Calif: Freeman; 1971;86–116.

Björndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reprod Biomed Online.* 2003;7:440–448.

Casslén B, Nilsson B. Human uterine fluid, examined in undiluted samples for osmolarity and the concentrations of inorganic ions, albumin, glucose, and urea. *Am J Obstet Gynecol.* 1984;150:877–881.

Chantler E, Abraham-Peskir JV. Significance of midpiece vesicles and functional integrity of the membranes of human spermatozoa after osmotic stress. *Andrologia.* 2004;36:87–93.

Cooper TG. Cytoplasmic droplets: the good, the bad or just confusing? *Hum Reprod.* 2005;20:9–11.

Cooper TG, Barfield JP, Yeung CH. Changes in osmolality during liquefaction of human semen. *Int J Androl.* 2005;28:58–60.

Cooper TG, Yeung CH, Fetic S, Sobhani A, Nieschlag E. Cytoplasmic droplets are normal structures of human spermatozoa but are not well preserved by routine procedures for assessing sperm morphology. *Hum Reprod.* 2004;19:2283–2288.

Edwards RG. Follicular fluid. *J Reprod Fertil.* 1974;37:189–219.

Granot I, Dekel N, Segal I, Fieldust S, Shoham Z, Barash A. Is hydrosalpinx fluid cytotoxic? *Hum Reprod.* 1998;13:1620–1624.

Hinton BT, Pryor JP, Hirsch AV, Setchell BP. The concentration of some inorganic ions and organic compounds in the luminal fluid of the human ductus deferens. *Int J Androl.* 1982;4:457–461.

Keating J, Grundy CE, Fivey PS, Elliott M, Robinson J. Investigation of the association between the presence of cytoplasmic residues on the human sperm midpiece and defective sperm function. *J Reprod Fertil.* 1997;110:71–77.

Kuster CE, Hess RA, Althouse GC. Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. *J Androl.* 2004;25:340–347.

Mortimer D, Leslie EE, Kelly RW, Templeton AA. Morphological selection of human spermatozoa in vivo and in vitro. *J Reprod Fertil.* 1982;64:391–399.

Ng EH, Ajonuma LC, Lau EY, Yeung WS, Ho PC. Adverse effects of hydrosalpinx fluid on sperm motility and survival. *Hum Reprod.* 2000;15:772–777.

Rossato M, Di Virgillio F, Foresta C. Involvement of osmo-sensitive calcium influx in human sperm activation. *Mol Human Reprod.* 1996;2: 903–909.

Tang S, Garrett C, Baker HW. Comparison of human cervical mucus and artificial sperm penetration media. *Hum Reprod.* 1999;14:2812–2817.

Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine

sperm with proximal cytoplasmic droplets. *Anim Reprod Sci.* 2001; 65:181–192.

Waberski D, Meding S, Dirksen G, Weitze KF, Leiding C, Hahn R. Fertility of long-term–stored boar semen: influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. *Anim Reprod Sci.* 1994;36:145–151.

World Health Organization. *Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* 4th ed. Cambridge, United Kingdom: CUP; 1999.

Yeung C-H, Anapolski M, Cooper TG. Measurement of volume changes in mouse spermatozoa using an electronic sizing analyzer and a flow cytometer: validation and application to an infertile mouse model. *J Androl.* 2002;23:522–528.

Yeung C-H, Anapolski M, Depenbusch M, Zitzmann M, Cooper TG. Human sperm volume regulation. Response to physiological changes in osmolality, channel blockers and potential sperm osmolytes. *Hum Reprod.* 2003;18:1029–1036.

Yeung C-H, Barfield JP, Cooper TG. The role of anion channels and Ca^{2+} in addition to K^+ channels in the physiological volume regulation of murine spermatozoa. *Mol Reprod Dev.* 2005;71:368–379.

Yeung C-H, Cooper TG. Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. *Mol Hum Reprod.* 2001;7:819–828.

Yeung C-H, Sonnenberg-Riethmacher E, Cooper TG. Infertile spermatozoa of c-*ros* tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. *Biol Reprod.* 1999;61:1062–1069.

Yeung C-H, Wagenfeld A, Nieschlag E, Cooper TG. The cause of infertility of c-*ros* tyrosine kinase knockout male mice. *Biol Reprod.* 2000; 63:612–618.