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Measurement of Volume Changes in Mouse Spermatozoa Using an Electronic Sizing Analyzer and a Flow Cytometer: Validation and Application to an Infertile Mouse Model

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ABSTRACT: The importance of sperm volume has recently been highlighted in a knockout mouse model in which infertility was caused by defects in volume regulation, which led to sperm transport failure in the female tract. Inhibition of volume regulation by human sperm, resulting in failure of penetration of cervical mucus in vitro, has also been reported. The present work aims to establish a sensitive and convenient method for monitoring changes in sperm volume for functional studies. Mature murine sperm obtained from the cauda epididymidis were analyzed by flow cytometry for their forward and side (90°C) scatter of a 488-nm excitation wavelength laser, and the data were compared with volumes measured by electronic sizing using a Coulter counter. Changes in cell volume were induced by releasing or diluting sperm into culture media of various osmolalities (208–520 mmol/kg). Forward scatter signal (FSS) intensity correlated well with volume measurement obtained by a Coulter counter ($R = .83$; $P < .001$), confirming that FSS reflects Coulter counter findings as for somatic cells. Sperm swelling was also induced by the presence of quinine, a wide-spectrum channel blocker, in a medium of 330 mmol/kg, which is similar to the osmolality of uterine fluid. The effect of quinine on sperm volume was more obvious when analyzed by flow cytometry than by electronic sizing. This effect was even more marked after dead sperm identified by fluorescent dye were eliminated from analysis using flow cytometry. Swelling was characterized by an increase in forward scatter and side scatter, generating a subpopulation of sperm that correlated well ($R = .79$; $P < .0001$) with the population of sperm exhibiting an angulation of the tail, which is a morphological manifestation of swollen murine sperm. Flow cytometric analysis revealed that infertile sperm released from the cauda epididymidis of c-*ros* knockout mice were significantly larger than those of fertile sperm from heterozygous mice. This finding directly substantiates the suggestion that infertile sperm are defective in their volume regulation. Laser scatter analysis of viable murine sperm by flow cytometry offers a convenient and sensitive method for the study of sperm volume regulation.

Key words: Coulter counter, c-*ros* knockout mice, hypo-osmotic swelling, regulatory volume decrease, sperm function.

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The volume of a mature spermatozoon has always been regarded as an inherent species-specific property. Spermatozoa have been described as perfect osmometers over wide ranges of osmolality, and changes in sperm volume have often been considered as a manifestation of biophysical characteristics resulting from osmotically driven water fluxes (Drevius, 1972a; Du et al, 1994; Willoughby et al, 1996). These studies examine sperm cell volume from 44 to 2000 mmol/kg at large osmotic intervals. Hypo-osmotic swelling of human sperm has been incorporated as a clinical test for the physical integrity of the plasma membrane (Jeyendran et al, 1984; World Health Organization, 1999). Despite many studies of somatic cell volume regulation, changes in sperm volume over the physiological range of osmolalities as well as the importance of sperm volume regulation and its mechanisms have been largely neglected. Recently, a defect in sperm volume regulation has been identified as the cause of sterility in the protein tyrosine kinase receptor c-*ros* knockout mouse (Yeung et al, 2000). These defective sperm exhibit tail angulation at the cytoplasmic droplet due to swelling when diluted from cauda epididymidal fluid of around 420 mmol/kg into culture medium of 310 mmol/kg, or the uterine environment of 330 mmol/kg, preventing their migration through the uterotubal junction into the oviduct. Such swelling-induced tail angulation can be generated in normal mouse sperm by blockers of ion channels involved in cell volume regulation, including quinine, or with hypo-osmotic solutions (Yeung et al, 1999). In humans, quinine treatment of sperm in the ejaculate or after washing increases cell size and alters their swimming pattern such that their ability to penetrate a surrogate cervical mucus is impaired (Yeung and Cooper, 2001). To understand volume regulation in spermatozoa and its role in their function, a sensitive, robust, and convenient method is required to monitor small changes in sperm volume under physiological conditions.

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Measurement of sperm volume is complicated by the small size and peculiar shape of spermatozoa (a tiny, asymmetrical head and an extremely narrow and long flagellum with little cytoplasm). The most frequent method used is electronic sizing by a Coulter counter (eg, Bredderman and Foote, 1971; Brotherton, 1975; Willoughby et al, 1996). Other methods reported in the literature include calculation from spermatocrit (Drevius 1972a), stereology by electron microscopy (eg, Curry et al, 1996), volume exclusion methods using differential radioisotope labeling of intercellular and intracellular spaces (Ford and Harrison, 1983; Curry et al, 1996), and estimation from the concentration of entrapped fluorophore (Curry et al, 2000).

Detection of laser light scattering by flow cytometry has long been employed in clinical hematology for distinguishing between cell types based on their size (see Shapiro 1995). Although flow cytometry has also been used successfully to monitor volume changes in other somatic cells (eg, to study swelling of glial cells; Staub et al, 1994), it has not been applied to the study of sperm cell volume. The present work describes such an application in mice with validation of the method using an established electronic sizing (Coulter counter) technique and demonstration of volume changes under osmotic challenge. Differences in light scatter, which reflect differences in cell volume between sperm from sterile knockout mice and fertile, heterozygous c-*ros* transgenic mice, were detectable by this method and are documented in this work.

Materials and Methods

Animals and Media

Maintenance and use of mice for the present experiments were conducted according to German federal laws on the care and use of laboratory animals (license 41/98). Adult heterozygous male mice from a colony of c-*ros* transgenic animals derived from cross-breeding C57BL6 and Ola129 strains were used (Sonnenberg-Riethmacher et al, 1996). Because these mice have identical phenotypes and similar fertility as wild type mice, they were used as normal controls for infertile c-*ros* knockout males. Mature sperm were collected from the cauda epididymidis by gently expressing the contents through slits cut in a lobule of tubule into the incubation medium (see below). Sperm from the same collection were simultaneously analyzed for cell volume using both a Coulter counter size analyzer and a flow cytometer (see below) immediately after dispersion for 1 minute at 37°C in 5% (v/v) CO_2 in air, or at various time points during further incubation as indicated in the ''Results'' section. One measurement was obtained from each mouse. Incubation media were similar to Biggers Whitten Whittingham medium (BWW; Biggers et al, 1971), except for the osmolality, which was adjusted to 208–520 mmol/kg by altering the concentrations of NaCl. Media contained bovine serum albumin at 4 mg/mL when used for incubation, but not when they were used for dilution for volume measurements. All media were filtered (0.45 μm pore size, Millipore, Molsheim, France) before use. In some experiments, the medium at 330 mmol/kg (BWW330; the osmolality of uterine fluid) with or without the ion channel–blocker quinine (1 mM; Sigma, Taufkirchen, Germany) was used to produce swollen spermatozoa without decreasing extracellular osmolality. To obtain the minimal sperm volume for measurement in some experiments, aliquots of incubated sperm were demembranated by the addition of 1% (v/v) Triton X-100 for 2 minutes.

Volume Analysis Based on Electrical Conductivity Using the Coulter Counter (Electronic Sizing)

The Coulter counter (model Z2, Beckman Coulter, Krefeld, Germany) calculates particle size from the change in electrical resistance to a constant current detected as the particle displaces its volume of the conducting solution (sperm incubation medium) when it passes a small aperture (50 μm) between 2 electrodes. Volume measurement was calibrated using 5-μm-diameter standard beads (Coulter Electronics Ltd, Luton, England) suspended in BWW330. When there was a change in medium that differed in osmolality between readings, blank medium was run through the counter 3 times before the sperm sample was analyzed in order to establish stable baseline conductivity.

For each measurement, an aliquot (30 μL) of the sperm suspension was diluted into 2 mL of the same medium without albumin in the sampling cuvette and read for 10 seconds at a flow rate of 10 μL/s, with 8000–15 000 sperm cells being measured. The size distribution profile of the measured particles was analyzed using Accucomp software provided by the manufacturer, and mean volume of the sperm population was calculated after cell debris and aggregates were eliminated.

Volume Analysis Based on Laser Light Scattering Using Flow Cytometry

In flow cytometry, forward scatter of the laser by the cell is proportional to its size, and side (90°) scatter reflects its surface or structural complexity. An aliquot (50 μL) of sperm suspension was diluted with 250 μL of the same medium without albumin, but it contained the vital stain propidium iodide (PI) at 5 μg/mL. Forward scatter signal (FSS) and PI fluorescence intensities (channel number) were analyzed using the flow cytometer (Epics XL, version 3.0, Coulter, Krefeld, Germany) with laser excitation set at 488 nm. Viable (PI negative) and dead (PI positive) spermatozoa were analyzed separately or together as 1 population (total 10 000) for their mean intensity (ie, channel number) of forward scatter and side scatter signals.

Assessment of Sperm Tail Morphology

In 6 experiments, aliquots of incubated sperm taken at the same time points as sperm volume measurements, were fixed in 3% glutaraldehyde. For each sample, the shape of the sperm tail (as an indication of cell swelling) was examined in 100 sperm with phase contrast microscopy and classified into 4 different categories based on the extent of angulation of the flagellum: hairpin form, acute angulation, slight angulation, and straight (no angulation; see micrographs in Yeung et al, 1999). Percentages of

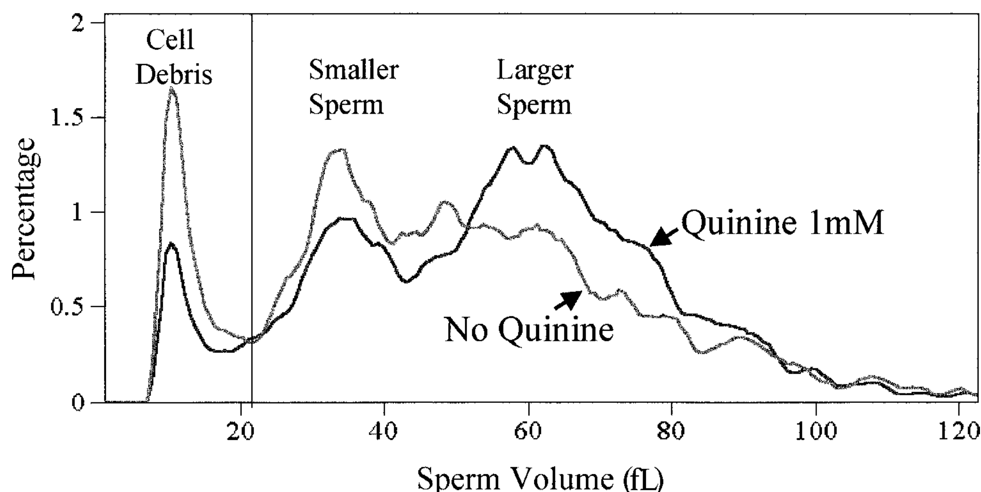

Figure 1. Cell volume distribution curves of murine sperm in an incubation medium of 330 mmol/kg analyzed with the Coulter counter. The peak on the left represents particles smaller than spermatozoa, presumably cell debris, which were eliminated from analysis of sperm volume. The rugged profile indicates the heterogeneity of sperm size. Swelling in the presence of 1 mM quinine is reflected by a shift of the distribution to the right.

all hairpin and angulated forms were added together to represent swollen sperm.

Statistical Analysis

Linear and nonlinear regressions were analyzed using analysis of variance and differences between genotypes were tested by the Student-Newman-Keuls method using SigmaStat statistical software (version 2.03, SPSS Inc, Chicago, Ill). Differences at $P < .05$ were considered statistically significant.

Results

Profiles of Sperm Volume as Electrical Resistance Properties

The profile of particle size distribution in a sperm sample measured as electrical resistance by the Coulter counter is given in Figure 1. Excluding from analysis the first peak on the left, which represents cellular debris, the profile appeared first as a high peak, which was followed by a few merged peaks of increasing cell volume, indicating sperm size heterogeneity. Heterogeneity within and among samples was such that no large or small sperm subpopulations could be systematically and objectively identified. In preliminary experiments, measuring sample aliquots of increasing dilutions did not change the range of values or the means, suggesting that high values were not produced by agglutinated cells, although this could not be entirely excluded. For each sample, the mean volume of the whole population was taken to represent average sperm size, which varied between 45 and 65 fL, depending on the osmolality of the medium used (see the next section). When swelling was induced by inclusion of 1 mM quinine in the incubation medium (also below), the major peak shifted to the right, representing a predominance of larger sperm (Figure 1). The initial volume measurement made after cauda epididymidal sperm had been dispersed in basal medium of osmolality between 410 (similar to cauda luminal fluid) and 330 (similar to estrous uterine fluid) mmol/kg was 53.5 ± 3.5 fL (mean ± SEM; n = 10). Volume after demembranation with Triton X-100 was 21.6 ± 0.8 fL (n = 7).

Profiles of Sperm Volume as Laser Scatter Signal Intensities

In flow cytometry, spermatozoa in the basal medium also appeared heterogeneous in their forward and side scatter properties, with hints of a minor subpopulation displaying signals with large side scatter (LSS). In the presence of quinine, the large signals were displayed by the majority of cells that also showed increases in FSS intensities (Figure 2). For each sample, the mean FSS of all the sperm, which indicates average cell size, as well as the percentage of sperm in the LSS subpopulation, were analyzed. The same parameters were also obtained for viable sperm in the sample after gating out the PI-positive cells.

Effects of Osmolality of Medium on Measurements of Cell Size

To validate cell volume measurements by both methods described above, mature mouse sperm were released into medium of 410 mmol/kg (similar to the osmolality of cauda epididymidal fluid), which was further diluted and analyzed within 2 minutes in media of increasing or decreasing osmolalities to induce cell shrinkage and swelling, respectively. In 2 experiments, both the Coulter counter and the flow cytometer registered similar profiles of increasing cell volume and laser forward scatter, respectively, with decreasing osmolality (Figure 3).

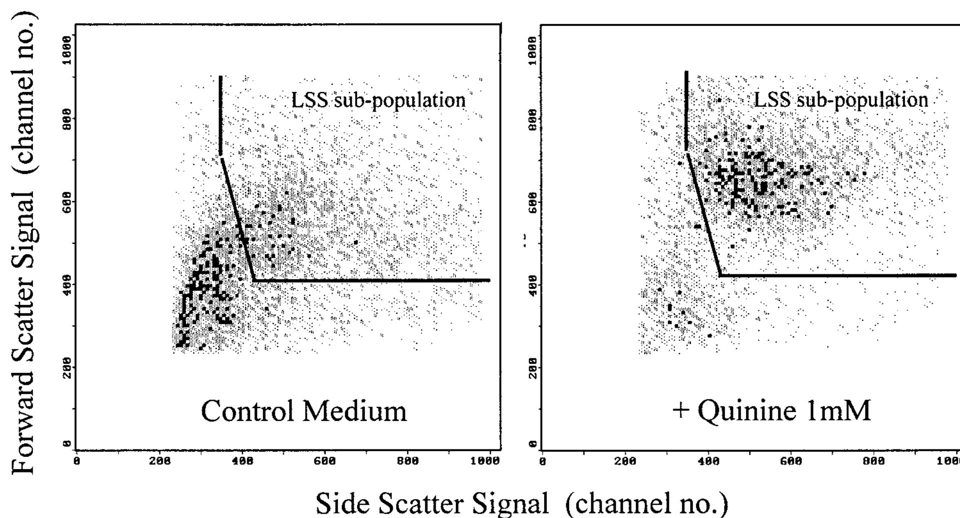

Figure 2. A dual parameter dot plot showing the distribution of murine sperm according to their laser forward and side (90°) scatter signals analyzed by flow cytometry. Note the enlargement of the LSS subpopulation in the sample treated with 1 mM quinine.

Effect of Quinine on Measurements of Cell Size

In the presence of 1 mM quinine, sperm volume obtained by electronic sizing and laser FSS increased within 5 minutes, and the effect was maintained throughout the 50-minute incubation. There was also a similar, sustainable increase in the population of sperm displaying LSS (see Figure 2). In flow cytometry, in which viable sperm were discernible by their lack of PI staining, the effects of quinine were more markedly demonstrated in viable sperm (as shown in Figure 4) than in the whole sperm population. The overall average increase was 9% in volume measured by the Coulter counter, 17% in forward scatter of whole sperm population, and 32% in forward scatter of viable sperm. In comparison to the consistent and stable increases in light scatter measurements, the Coulter counter measurements were more variable and fluctuational (Figure 4).

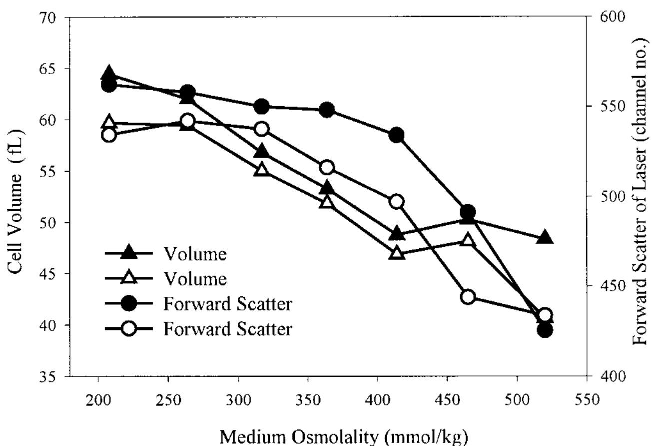

Figure 3. Data from 2 separate experiments showing the size of murine sperm suspended in media of various osmolalities for 1 minute, measured by electronic sizing (Coulter counter data measured as mean volume, left-hand axis, triangles) and as reflected by the intensity of laser forward scatter signal (right-hand axis, circles).

Correlation of Sperm Volume Measurements by Different Methods and Sperm Tail Morphology

The mean volume of sperm measured in fL by the Coulter counter obtained for each sample correlated well with the mean laser forward scatter of the whole sperm population (Figure 5). The percentages of sperm with tail angulation showed a significant ($P < .001$) albeit weak ($R = .33$) correlation with the mean sperm volume of the sample. On the other hand, the extent of tail angulation was much more strongly correlated with the mean forward scatter (Figure 6) as well as the size of the subpopulation displaying LSS (Figure 7), as shown by nonlinear regression.

Comparison of Sperm Size Between c-ros Heterozygous and Homozygous Mice

Using the flow cytometric method, in which nonviable sperm could be eliminated from analysis, the size of mature sperm freshly released from the cauda epididymidis into medium with uterine fluid osmolality (330 mmol/kg) was measured as FSS and compared. This was found to be significantly larger in the infertile knockouts (n = 8) than in fertile heterozygous mice (n = 10), whether taking into account all sperm (471.3 ± 13.1 vs. 411.0 ± 7.8 channel number, mean ± SEM, respectively) or only viable sperm (491.4 ± 14.8 vs 447.0 ± 5.6). The percentage of sperm appearing as the LSS subpopulation was also significantly higher in knockout mice (47.0% ± 2.6% vs. 29.5% ± 1.7% of all sperm, and 55.2% ± 2.8% vs. 40.4% ± 1.8% of viable sperm).

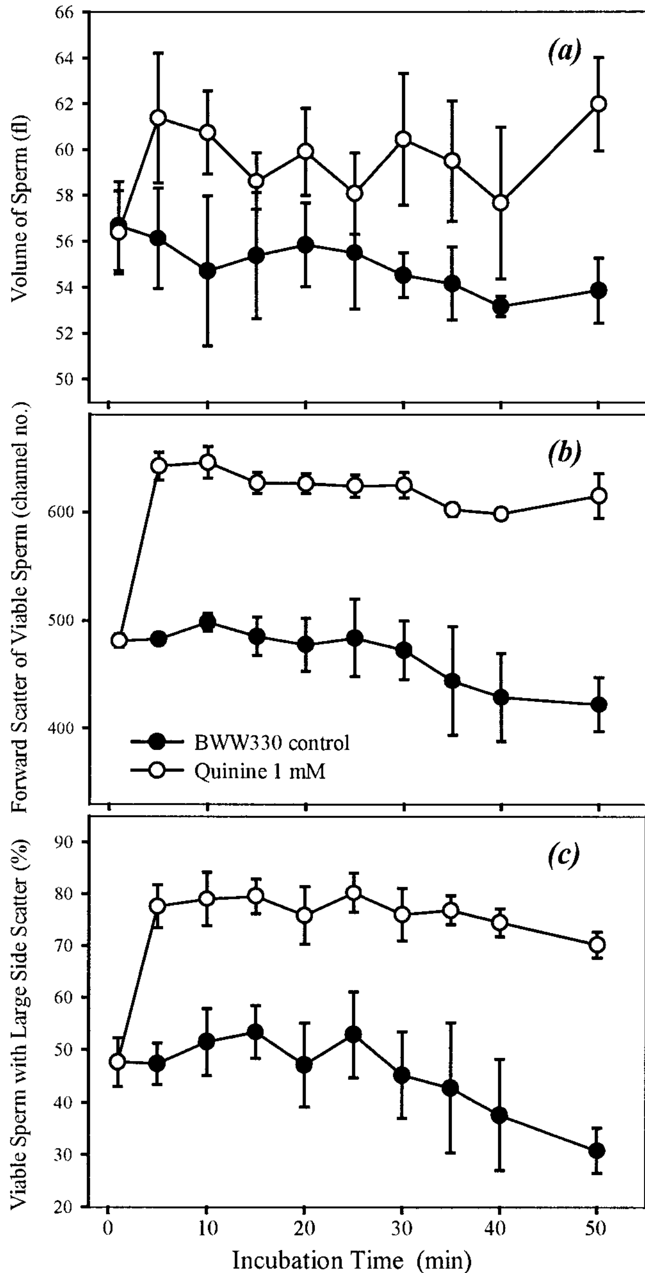

Figure 4. Induction of swelling of mature murine sperm by 1 mM quinine over 50 minutes of incubation at an osmolality of 330 mmol/kg, indicated by volume measurement using the Coulter counter **(a)** and by the forward scatter of laser light analyzed by flow cytometry **(b)**. Effect of quinine on the percentage of sperm in the subpopulation showing large size scatter **(c)** is also shown. Values are mean ± SEM (n = 4).

Discussion

Compared to the multitude of reports on various sperm properties and function, cell volume and its regulation are rarely documented in the literature. In the present work, murine sperm volume suspended in physiological osmolalities (between cauda epididymidal fluid of 410 mmol/kg and uterine fluid of 330 mmol/kg; see Yeung et al, 1999, 2000) was estimated at an average of 53.5 ± 3.5 fL for intact sperm and 21.6 ± 0.8 fL for Triton-demembranated sperm. This agrees well with the values of 53–56 fL measured for intact sperm at 290 mmol/kg with the Coulter counter (Willoughby et al, 1996). The volume of intracellular water of murine sperm suspended in a 290-mmol/kg solution measured by electron paramagnetic resonance is 43.3 fL (Du et al, 1994). Using the Coulter counter and the manufacturer's medium (Isoton, about 310 mmol/kg), the volume of intact mouse sperm was reported to be 61 and 70 fL, and after treatment with the detergent zaponin, it was 39 and 33 fL (Brotherton, 1975).

Despite the routine use of flow cytometry for cell size

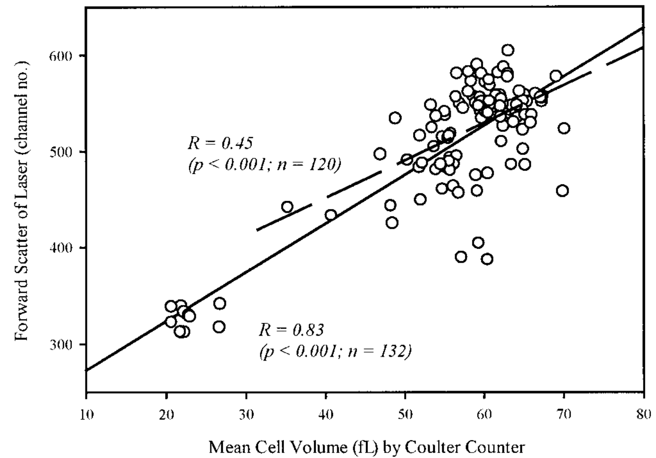

Figure 5. Linear correlation of laser forward scatter (analyzed by flow cytometry) and cell volume (measured by electronic sizing) of murine sperm. Each data point represents the 2 mean values of all sperm analyzed in each sample. The solid line indicates regression of data from all samples; the dashed line indicates regression of data excluding the group in the lower left of the graph that represents samples treated with Triton X-100 for demembranation.

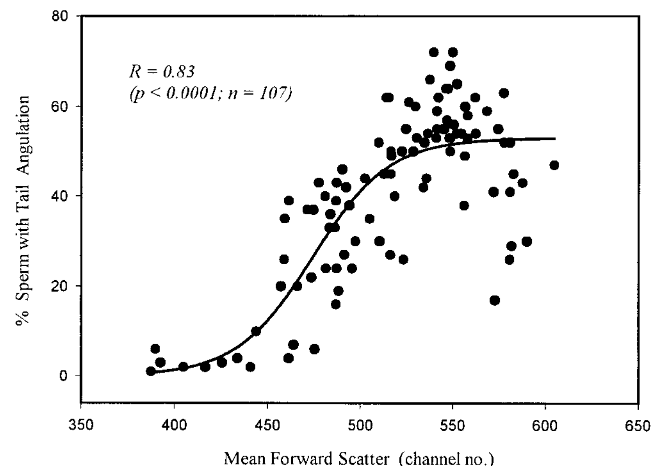

Figure 6. Nonlinear correlation of mean laser forward scatter in flow cytometry and percentages of sperm showing angulation of the tail, showing a sigmoidal regression.

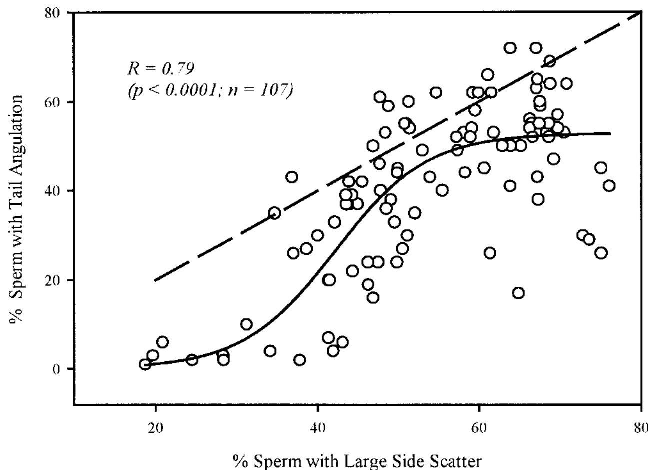

Figure 7. Nonlinear correlation of the percentage of sperm found in the LSS subpopulation (x axis) and the percentage of sperm showing angulation of the tail (y axis), showing a sigmoidal regression. Dashed line marks the hypothetical line for identical values of both parameters, indicating $x > y$ for most samples.

measurements in recognition of the various types of leukocytes, this is the first report of its application to quantify sperm volume, in particular to monitor volume change. The latter is specially useful in functional studies, which was our intention in establishing the method. For this purpose, sensitivity and accuracy are more important than the absolute value. Laser scatter in flow cytometry is detected and quantified in channel numbers that are not absolute values, but are highly dependent on the voltage set for the photomultiplier. For sensitive detection of sperm volume changes, the size range detectable within the optimized voltage setting employed in the present method was not wide enough to cover the sizes of standard beads available commercially. Therefore, it was not possible to establish a standard curve to calibrate absolute values. To validate that measurement of FSS intensity reflects cell volume, regression was tested on the data obtained by the Coulter counter, which is a more conventional method of enabling direct assessment of cell volume. A good linear correlation was obtained from the mean values of the same samples measured by the 2 methods, and heterogeneity within samples was apparent with both. One important advantage of flow cytometry over the Coulter counter is the feasibility of eliminating nonviable sperm from analysis with the use of fluorescent vital stain, providing more physiological results.

Both methods of volume measurement detected immediate changes in the expected direction when sperm were exposed to different osmolalities, and to a physiological osmolality (that of uterine fluid, 330 mmol/kg) when incubated in the presence of the channel blocker, quinine. Mouse sperm are readily permeable to water and their volume has been shown to be inversely proportional to the external osmolality, which varies in a steep gradient from 75 to 1200 mmol/kg (Du et al, 1994; Willoughby et al, 1996). In the present study, with a smaller gradient within a much narrower and more physiological range, the inverse correlation was not exactly linear. This was probably due to an immediate onset of volume regulation upon swelling. Regulatory volume decrease under hypo-osmotic conditions, as described for somatic cells, has been documented in bovine sperm and can be inhibited by quinine (Kulkarni et al, 1997). The present findings with both methods demonstrated volume increases in mouse sperm induced by quinine. The effect detected by the Coulter counter was not as marked as that detected by the flow cytometer, because the latter analysis excluded the nonviable and presumably nonfunctional sperm that failed to respond.

It is well known that when mammalian sperm swell in hypo-osmotic solutions, the shape of the tail changes as a means to withstand an increase in cell volume without a similar increase in surface area, which would overstretch the plasma membrane. This is exhibited as coiling of the tail, as in bovine and human sperm (Drevius and Eriksson, 1966; Drevius, 1972b; Jeyendran et al, 1984), or angulation of the tail at the cytoplasmic droplet, as in rodents (Serres and Kann, 1984; Cooper, 1985; Willoughby et al, 1996; Yeung et al, 1999). This morphological manifestation of swelling was indeed reflected by the mean cell volume monitored by both methods. Correlation of the percentage of sperm showing tail angulation with measurements by the Coulter counter was weak, albeit statistically significant. On the other hand, correlation with laser forward scatter was much stronger, and showed a sigmoidal regression. This suggests that the sperm tail can tolerate moderate increases in volume while maintaining the straight form. Kinking of the tail occurs only when the spermatozoon swells beyond a threshold, which may depend on the physical-chemical status of the plasma membrane, and was heterogeneous among the sperm population as reflected by the slope of the sigmoidal regression curve. Once the tail forms an angle, any further increase in cell volume can no longer be detected by light microscopy, accounting for the plateau of the regression.

In conditions in which considerable swelling was induced, which occurred, for example, with treatment by 1 mM quinine, a subpopulation of sperm was clearly discernible in the dot plot of the flow cytometric data when both forward and side scatter signals were considered, regardless of inclusion or exclusion of nonviable cells. The so-called LSS group showed a good correlation in population size with angulated sperm as classified by light microscopy. However, the 2 populations were not identical, because in most samples, there were more sperm in the LSS group than sperm with angulated tails. In addition to cell size, small angle forward scatter is also affected by other cell properties such as cytoplasmic gran-

ulation, whereas side (90°) scatter signals are known to be influenced by factors such as ruffling of the cell membrane, and intracellular vacuoles or inclusions (see Shapiro, 1995). The present findings suggest that deformation of the sperm tail is preceded by such changes that are detectable by light scatter, which may also be volume-related.

Flow cytometry is a sensitive, efficient, and easy method for measuring a large sample size, which is important for statistical consideration in view of the heterogeneous nature of spermatozoa. As shown by the good correlation with the conventional electron sizing method, analysis of sperm volume using flow cytometry would also enable functional studies with the feasibility of exclusion of non-viable sperm and give an indirect, objective indication of changes in tail form. The latter is a critical factor in the failure of sperm transport in the female tract of infertile c-*ros* knockout mice (Yeung et al, 2000). Comparison of sperm from these infertile knockout mice with those from the phenotypically normal, fertile, heterozygous mice indeed demonstrated slight but significant differences in FSS and LSS group sizes, substantiating the suggestion of a larger sperm volume due to failure in volume regulation, and confirming the increase in the extent of tail angulation assessed previously by subjective light microscopy evaluation (Yeung et al, 1999). Therefore, flow cytometry offers a useful method for the study of sperm volume regulation, which is an important sperm function that has also been demonstrated in men (Yeung and Cooper, 2001).

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