

Enhanced Binding of Sperm With Superior Volume Regulation to Oviductal Epithelium

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ABSTRACT: The plasma membrane is a key organelle with respect to sperm fertilizing ability. A sensitive way of testing plasma membrane functionality is to examine the sperm ability to moderate its swelling in response to hypo-osmotic stress (volume regulatory ability) using an electronic cell counter to assess cell volume changes. In this study of frozen-thawed bull sperm, we examined the relationship among sperm-oviductal epithelium binding capacity, osmotically induced swelling response, volume regulatory ability, and standard spermatologic parameters. Sperm cell volume distributions were measured under iso-osmotic conditions and after hypo-osmotic stress. The relative volume shift was calculated by comparing modal values of the cell volume distributions during transition from iso-osmotic to hypo-osmotic conditions. Significant correlations were found between volumetric parameters and sperm-oviduct binding capacity. Both the relative volume shift and regulative volume decrease correlated positively and significantly with the sperm-

oviduct binding capacity. No significant correlations were found between sperm volumetric parameters and any of the standard sperm parameters with the exception of forward motility of Percoll-washed sperm. However, the use of multiple regression models improved the prediction level for binding capacity when motility parameters were combined with membrane integrity and volumetric parameters ($R^2 = .84$). Spermatozoa of bulls with high nonreturn rates responded to hypotonicity as "perfect osmometers." Subfertile bulls had lower binding indices and deficiencies in volume recovery after hypotonic challenge, indicating that intact volume regulatory ability is a necessary prerequisite for binding to oviductal epithelium and is related to fertility. Volumetric parameters therefore could be used as tools in semen evaluation programs.

Key words: Sperm function, oviduct, plasma membrane, cell volume, fertility.

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Introduction

Numerous studies have reported the predictive value of semen parameters to aid in the determination of in vitro fertilization and pregnancy outcomes. Such parameters include concentration of motile spermatozoa (Fetterolf and Rogers, 1990; Liu et al, 1991), quantitative and computerized measurements of spermatozoa motility (Krause, 1995; Macleod and Irvine, 1995), morphology (Menkveld and Kruger, 1995; Eggert-Kruse et al, 1996), zona pellucida binding (Coddington et al, 1994), and occurrence of the acrosome reaction (Cummins et al,

1991; Aitken et al, 1994). The standard procedure for evaluating the fertility of semen from sires is to determine pregnancy data following artificial insemination. This procedure is time consuming and expensive because of the large number of young bulls entering the breeding program (Zhang et al, 1999). Several studies have already been conducted to find a simple and reliable test for fertility (Larsson and Rodriguez-Martinez, 2000). Because fertilization requires several sperm activities, it would be better to combine different sperm traits to achieve a better correlation between in vitro tests and in vivo fertility (Amann and Hammerstedt, 1993; Farrell et al, 1998; Zhang et al, 1999).

The integrity of the plasma membrane of the sperm cell is of crucial importance for sperm function—only an intact cell is able to undergo the series of complex changes in the female leading to capacitation, acrosome reaction, and finally acquisition of the ability to fertilize an oocyte (Yanagimachi, 1994). A range of spermatologic assays has been used to test the membrane integrity of spermatozoa, based either on the permeability of the membrane to different dyes or on the ability of the cells to respond to stress. The best-known procedure in the second group is the hypo-osmotic swelling test (HOST). The percentage of swollen cells within a sperm popula-

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tion has been suggested to be indicative for membrane integrity and fertility of human spermatozoa (Jeyendran et al, 1984). Avery and colleagues (1990) reported that there were positive correlations between the percentage of swollen spermatozoa in HOST and in vitro fertilization. A number of other investigations, however, revealed that there is no clear correlation between the outcome of in vitro fertilization and the results of HOST. Okada and coworkers (1990) reported positive correlations between the results of HOST and hamster egg penetration for specimens with high or low fertility rates (ie, ejaculate with a low swelling rate demonstrated low fertility), but correlations were not always found in the middle range of rates. In contrast, McClure and Tom (1991) found no significant correlations for spermatozoa from infertile patients but only for spermatozoa from fertile donors. Nevertheless, it was shown that a combination of HOST with other spermatologic parameters could improve its predictive potential (Chan et al, 1991). A modified version of HOST, using electronic volume measurements (Petzoldt and Engel, 1994), evaluated the percentage of swollen cells and the swelling level. For spermatozoa of many species, such as bulls, boars, and humans, which act as osmometers (Drevious, 1972; Gilmore et al, 1995; Willoughby et al, 1996; Petrunkina and Töpfer-Petersen, 2000) and show a visible response to changing osmotic conditions, the electronic evaluation of cell volume has many advantages. It allows the assessment of large cell populations and the evaluation of different subpopulation responses. The practical approach for the volumetric evaluation of boar and bull sperm populations within an ejaculate using an electronic cell counter has been described in our previous reports (Petrunkina et al, 2001a,b). Different clinical studies indicate that HOST as a single assay is not sufficient to predict the fertilizing capacity of an ejaculate (Jeyendran et al, 1992). Combining HOST results with other spermatologic parameters (eg, motility and vitality) is useful to improve correlations with the fertility rate (Van der Ven et al, 1986; McClure and Tom, 1991; Jeyendran et al, 1992; Ramirez et al, 1992).

The purpose of this study was to evaluate the osmotically induced response of frozen-thawed spermatozoa and to investigate its relationship to oviductal epithelium binding and routine sperm parameters in a bovine model. For the studies on sperm-oviduct interaction, this model has been shown to be a good alternative to humans, for which the collection of oviductal tissues represents ethical and technical problems (Ellington et al, 1999). Besides, the volume regulation in bull and human sperm appears to involve similar mechanisms like quinine-sensitive potassium ion channels (Petrunkina et al, 2001a; Yeung and Cooper, 2001), justifying the importance of the findings about the

functional relationship between sperm volume regulation and sperm-oviduct binding for human andrology.

Materials and Methods

Chemicals

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma Chemical Company (Steinheim, Germany). All were of suitably high purity.

Media

Media used for this study were TALP (Parrish et al, 1988), Percoll-saline medium (Parrish et al, 1995), and phosphate-buffered saline (PBS; as used by Petrunkina et al, 2001c).

Two variants of a standard HEPES-buffered saline medium (HBS; Petrunkina et al, 2004a) were used as the vehicles for experimentation. Isotonic HBSM (300 mOsm/kg) consisted of 137 mM NaCl, 10 mM glucose, 2.5 mM KOH, and 20 mM HEPES buffered with NaOH to pH 7.4 at 39°C. Hypotonic HBSM (180 mOsm/kg) was prepared by adjusting the NaCl content to the correct osmolality. To minimize detection of particulate "noise" during cell volume measurements, the media were passed through a 0.2- μ m filter before use (Minisart; Sartorius AG, Göttingen, Germany).

Semen Source and Routine Analysis

For the first experimental series, frozen semen straws from 30 bulls (3 ejaculates from each bull) of a commercial source obtained from the artificial insemination stations were used (generously provided by NORDRIND Rinderproduktion Niedersachsen, GmbH, Bremen-Hannover, Germany). In the first experimental series, the relationship between sperm-oviduct binding capacity and volume regulatory ability was investigated. For the second experimental series, frozen semen from 6 bulls with known nonreturn rates (NRR) (3 straws from 3 different ejaculates per bull) was obtained from Besamungsverein Neustadt a.d. Aisch (Neustadt a.d. Aisch, Germany). In this experimental series, it was investigated if there were differences between fertile and subfertile bulls with respect to volume regulatory ability and sperm-oviduct binding. Unless otherwise stated, individual straws were thawed rapidly in a warm water bath at 38°C for 30 seconds.

Analysis of thawed sperm routinely includes morphologic evaluation, computer-assisted motility analysis, and subjective evaluations of sperm motility and sperm viability. The assessment of morphologically abnormal sperm was performed after fixation in formol citrate (Hancock, 1957) according to the classification described by Krause (1965). Viability of thawed spermatozoa for the first experimental series was evaluated using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Göttingen, Germany) and according to the method described by Garner and Johnson (1995). In the second experimental series, the membrane state was evaluated by flow cytometry using combined propidium iodide/fluorescein isothiocyanate-conjugated peanut agglutinin staining.

The methodology and instrument settings for membrane state assessment were essentially as previously described (Petrunina et al, 2005a,b,c).

For the oviduct explant assay and assessment of volume regulation, sperm were processed by centrifugation through discontinuous Percoll density gradient as described by Parrish and colleagues (1995). These assessments were performed on different straws within the same ejaculate to provide a sufficient amount of spermatozoa for the examination. Prior to further experiments, Percoll-washed spermatozoa were resuspended in TALP medium (Parrish et al, 1988). Motility of Percoll-washed spermatozoa was assessed by microscopic evaluation.

Volumetric Measurements

The method described by Petrunina et al (2001a) was used as a guide. The original volume distributions were obtained by means of a CASY1 cell counter (Schärfe Systems GmbH, Reutlingen, Germany). Because the 2 test solutions (iso- and hypo-osmotic) had different electrical conductivities, it was necessary to use a correction factor obtained by comparison of volume measurements of standard latex beads (3.4 μm diameter; Sigma) in 3 solutions of different osmolality (150 mOsm/kg, 300 mOsm/kg, and 450 mOsm/kg) at 38°C. The volume of latex beads measured at different osmolalities was fitted to a linear regression model, and the resulting coefficient was used to calculate the cell volume under hypo-osmotic conditions as described previously (Petrunina and Töpfer-Petersen, 2000). The relative volume shift (V_r) and regulatory volume decrease (RVD) are given in relative units according to the definition; real cell volumes are presented for the modal values of hypo-osmotic volume distribution (V_{iso}) and iso-osmotic volume distribution (V_{hypo}) in femtoliters (1 fL = 10^{-15} L).

HBS was used as sperm incubation medium. Samples (2–10 μL) were taken from the Percoll-washed semen suspension and added to 6 mL of iso-osmotic (300 mOsm/kg) and hypo-osmotic (180 mOsm/kg) HBS solution contained in snap-cap glass vials preincubated at 38°C (5-mL incubation volumes were used in the second set of experiments). After incubation periods of 5 and 20 minutes, the samples were passed through a CASY1 cell counter.

Analysis of Sperm Volumetric Data

Much of the volumetric methodology used was based on earlier studies of sperm volumetric behavior (Petrunina and Töpfer-Petersen, 2000; Petrunina et al, 2004a,b). Briefly, the modal value of the volume distribution curve was taken into consideration, as it was found to be a more sensitive parameter of volume change than the mean value (Petrunina and Töpfer-Petersen, 2000). The V_r was used as a measure of volume regulation in response to hypo-osmotic conditions (Petzoldt and Engel, 1994; Petrunina et al, 2001a). It was defined as $V_r = V_{hypo}/V_{iso}$. A cell subpopulation was considered as osmotically active if its V_r was greater than 1 (Petrunina et al, 2001a). RVD, also used as an evaluation parameter, was defined as the difference between the relative volume shifts after 5 and 20 minutes of exposure to hypotonic

conditions: $\text{RVD} = V_{r,5} - V_{r,20}$. RVD represents the measure of the relative cell volume recovery (Petrunina et al, 2001a,2004a).

At each sampling time point, volume distributions were collected from a single iso-osmotic dilution and a single hypo-osmotic dilution using a CASY1 sample volume setting of 200 μL and a size scale of 10 μm . Particles of effective size less than 2.3 μm and larger than 6 μm were eliminated from the study by setting cursors to appropriate scale to standardize the experiment. In each sampling the data were obtained from more than 20 000 sperm cells.

Oviduct Explant Assay

The following assay was based on the methodology described by Lefebvre and coworkers (1996).

Collection of Oviducts and Preparation of Explants—Oviducts (including isthmus, ampulla, infundibulum, fimbria, and a small part of the uterotubal junction and mesosalpinx) were collected from both cows and mature heifers at the local slaughterhouse. The uterus and ovaries were examined for anomalies and pathologic lesions as well as for pregnancy before collection of the oviducts. Each oviduct was washed with sterile PBS and then transported on ice to the laboratory. Upon arrival, the oviducts were thoroughly washed with PBS and then dissected free of the surrounding tissues (mesosalpinx). The ampullary and isthmic segments were cut into 2 to 3 pieces and placed in a large Petri dish containing PBS. Each piece was taken with a watchmaker's forceps and gently squeezed along the outside toward the wide end with another watchmaker's forceps to expel epithelium. For every experiment, both oviducts (right and left) from 3 to 4 animals were pooled to avoid individual cow effects as well as any local hormonal effects. The expelled epithelial tissue sheets were disaggregated into small pieces by passage through a 25-gauge needle attached to a 1-mL syringe, transferred to a test tube containing 5 mL of sperm-TALP, and then allowed to stand for 10 minutes. After the cellular material had sedimented, the supernatant was removed and the pellet was resuspended in 5 mL of fresh sperm-TALP. The supernatant volume was removed after a further 10 minutes (second sedimentation). The resulting oviduct epithelial cell sheets were resuspended in 0.5 mL of TALP and incubated at 39°C in a humidified atmosphere containing 5% CO_2 . Within about 30 minutes, the epithelial cell sheets formed clumps or everted vesicles with beating cilia and the apical surfaces of the epithelial cells on the external surface. These everted vesicles are referred to as explants.

Co-incubation of Sperm and Oviductal Epithelial Explants and Evaluation of Sperm-Oviduct Binding—Both explants and semen samples were equilibrated for 10 minutes at 39°C in a humidified atmosphere containing 5% CO_2 . Then a 10- μL aliquot was taken from the dense layer of explants and transferred to a 50- μL droplet of TALP in a small Petri dish. Next, 20 μL of the semen suspension was added to the droplet and gently mixed so that the final droplet volume was 80 μL and the final sperm cell concentration was 1.25×10^6 sperm/mL. After 15 minutes of co-incubation in a CO_2 incubator, the explants were washed free of unbound, loosely attached sperm

by drawing them up into a 100- μ L micropipette and transferring them into fresh 80- μ L TALP droplets. The washing process was repeated 3 times to assure that all unbound sperm were removed. The explants with bound sperm were then transferred to prewarmed slides and covered with prewarmed cover slips supported by silicon grease. Videomicroscopy and image analysis were performed essentially as described by Petrunkina and collaborators (2001c). Briefly, explants on each slide were viewed under 256 \times magnification in each slide; the images of 12 regions (referred as to “fragments” here and elsewhere) of about 6 explants (2 fragments/explant) were recorded on videotape. For each experimental replicate, beside the bound sperm cells, the image of a scale was recorded at the same magnification as the explants. Recording was completed within 15 minutes for each slide. For analysis, the videotapes were reviewed to count the number of spermatozoa bound to the side of the oviduct explants facing the camera. The surface areas of the videotaped explant and its fragments were estimated with the help of an image-analysis, computer-assisted, surface area-measuring program “Aida” (version 2.0; Mika Medical GmbH Image Analysis; Rosenheim, Germany) as described by Petrunkina and coworkers (2001c). A transparent film was placed over the image plane of the monitor, the bound spermatozoa were then marked with a water-soluble marker, and these markings were counted. The parameter of sperm-oviduct binding capability was termed the binding index (BI), defined as the number of spermatozoa bound to 0.01 mm² explant surface. The method of calculating BI was adapted from Petrunkina and colleagues (2001c). The surface areas of 36 fragments per bull (12 fragments/ejaculate and 3 ejaculates/bull) and their bound sperm numbers were used to estimate the BI for each ejaculate according to the formula:

$$BI = \frac{\sum N_1 + N_2 + \dots + N_{12}}{\sum S_1 + S_2 + \dots + S_{12}}$$

N_{1-12} = the number of bound spermatozoa/fragment; S_{1-12} = the surface areas of the explant's fragments. The BI for each bull was calculated as the mean value of the binding indices of the 3 ejaculates.

Computer-Assisted Motility Analysis

For the first experimental series, computer-assisted motility analysis was performed using a Stromberg Mika Cell Motion Analyzer (SM-CMA, Strömberg-Mika; Bad Feilnbach, Germany). The general system settings, sample preparation, and parameters used were as previously described (Petrunkina et al, 2003b). The particular settings for bull spermatozoa were as suggested by the manufacturer: number of frames per analysis, 32; time between 2 video pictures to detect immotile objects, 20 ms; cell size range, 35 to 300 pixels; threshold velocity for immotile objects, 10 μ m/s; threshold velocity for nonprogressively motile object, 25 μ m/s, minimum number of frames, 15; and maximum radius for circular motile spermatozoa, 25 μ m.

For the second experimental series, sperm motion analysis was performed using Sperm Vision—CASA System (Minitüb, Tiefenbach, Germany). Aliquots (2.3 μ L) of frozen-thawed

semen were placed in one of the chambers of the prewarmed 38°C measuring slide (Leja, Nieuw-Vennep, The Netherlands), and the chambers were filled by capillary force. The depth of the chamber was 20 μ m. Seven fields per chamber and at least 1500 spermatozoa were analyzed. The particular settings for bull spermatozoa were as suggested by the manufacturer: the system was set to detect the cells in the real head surface range from 22 μ m² to 60 μ m². The kinetic characteristics of spermatozoa such as average path velocity (VAP), straight path velocity (VSL), curvilinear velocity, amplitude of lateral head displacement, and flagellar beat-cross frequency (BCF) were analyzed.

Statistical Analysis

Unless otherwise specified, standard spermatologic and volumetric parameters and binding indices are presented as arithmetic mean values and standard deviations (mean \pm SD). The differences between individual bulls or groups were verified by means of analysis of variance (parameter-free, Proc NPAR1WAY; SAS Institute, 1989). The relationships between individual parameters were examined by Pearson's correlation analysis (Proc CORR; SAS Institute, 1989). To investigate if the binding ability to oviductal epithelium can be predicted by other spermatologic parameters, the multiple regression procedure (Proc STEPWISE; SAS Institute, 1989) was used.

Results

Motility and Morphology

The descriptive statistics of motility and morphology sperm parameters are given in Table 1. Both motility parameters and the percentage of morphologic alterations were in the normal range for frozen-thawed sperm. Computer-assisted motility analysis revealed high levels of sperm path velocities, confirming that sperm met the general requirements with respect to conventional parameters (Table 1).

Oviduct Explant Assay

Bull sperm attached rapidly to the oviductal explants. Despite gentle swirling after sperm addition and before videotaping, attached spermatozoa were not evenly distributed over the surfaces of the oviductal explants. They were spaced closely in some areas, sparsely in others, and absent in a few areas. Sperm appeared to adhere to the oviductal explants by rostral surface of the head, and most of them remained motile (98%). Viability of the oviductal explants was judged by the ciliary activity of the epithelial cells. The ciliary beats were strong and were apparent during the entire experiment. The overall mean of the BI was 15.1 \pm 2.9 sperm/0.01 mm² (Table 2).

Table 1. Main motility and morphology parameters for 30 bulls (3 ejaculates each)

Parameter*	Mean ± SD
CMA-forward (%)	59.2 ± 8.4
CMA-local (%)	8.1 ± 2.9
Subj-forward (%)	60.4 ± 8.2
Subj-local (%)	8.9 ± 2.5
VAP (μm/s)	62.8 ± 3.9
VCL (μm/s)	111.9 ± 8.1
VSL (μm/s)	55.9 ± 4.7
Percoll-forward (%)	74.5 ± 7.5
Percoll-local (%)	9.4 ± 2.3
MAS (%)	37.3 ± 7.6
Head (%)	30.4 ± 8.6
Head primary (%)	0.8 ± 0.7
Others (%)	6.6 ± 4.1

* CMA-forward indicates progressive motility (determined by computer-assisted motility analysis); CMA-local, nonprogressive motility (determined by computer-assisted motility analysis); subj-forward, progressive motility (determined by subjective microscopic evaluation); subj-local, nonprogressive motility (determined by subjective microscopic evaluation); VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; Percoll-forward, progressive motility (determined after Percoll washing procedure by subjective microscopic evaluation); Percoll-local, nonprogressive motility (determined after Percoll washing procedure by subjective microscopic evaluation); MAS, morphologically altered spermatozoa; head, sperm head abnormalities including acrosomal abnormalities; head primary, primary head abnormalities; and others, other sperm abnormalities.

Statistical analysis of the data revealed a significant ($P \leq .01$) effect of individual bulls on the ability of sperm to bind to the oviduct epithelial explants (Figure 1).

Volumetric Parameters

Two typical plots of volume distributions of frozen-thawed bull spermatozoa are shown in Figure 2. The volume distributions were either uni- or bimodal. In the case of bimodal distributions, the values of the first osmotically active peak were used for calculations because these cells correspond to the functionally stable sperm subpopulation within an ejaculate (Petrunikina et al, 2005a).

The overall mean of the modal values of sperm cell volume distribution under isotonic conditions remained nearly constant during the 20-minute incubation period (15.3 fL at 5 minutes vs. 15.8 fL at 20 minutes). After exposure to hypotonic conditions, the cell volume was greater (24.5 fL) after 5 minutes of incubation at 38°C; a subsequent reduction was observed after 20 minutes of incubation (Table 2).

The overall mean value of the relative sperm volume increase (V_r) was 1.65 ± 0.41 . Most of the bulls recorded values between 1.30 and 2.0. However, 7 bulls showed values outside this range. Within this group, 3 bulls had

Table 2. Mean advanced spermatologic parameters for 30 bulls (3 ejaculates each)

Parameter*	Mean	SD
$V_{iso,5}$ (fL)	15.3	4.1
$V_{iso,20}$ (fL)	15.8	4.0
$V_{hypo,5}$ (fL)	24.5	5.5
$V_{hypo,20}$ (fL)	21.5	5.6
V_r	1.65	0.41
RVD	0.25	0.55
BI	15.1	2.9
Alive (%)	65.5	10.7

* $V_{iso,5}$ indicates modal value of sperm cell volume after 5 minutes under isotonic conditions; $V_{iso,20}$, modal value of sperm cell volume after 20 minutes under isotonic conditions; $V_{hypo,5}$, modal value of sperm cell volume after 5 minutes under hypotonic conditions; $V_{hypo,20}$, modal value of sperm cell volume after 20 minutes under hypotonic conditions; V_r , relative volume shift after 5 minutes of exposure to hypotonic conditions; RVD, regulatory volume decrease after exposure to hypotonic conditions; BI, binding index (number of sperm bound to 0.01 mm² of explant); and alive, percentage of live spermatozoa determined by LIVE/DEAD Sperm Viability Kit.

very high initial osmotic responses ($V_{r,5} = 2.14, 2.22,$ and 2.29) and 4 bulls had very low initial responses ($V_{r,5} = 1.24, 1.16, 1.25,$ and 1.22). The differences were significant between individual bulls ($P < .05$, Figure 3).

After 20 minutes the overall relative volume shift decreased significantly (RVD = 0.25 ± 0.55), demonstrating the ability of frozen-thawed bull sperm to recover their cell volume after exposure to hypotonic conditions. This subsequent shrinking was observed in 18 bulls (RVD > 0); spermatozoa of another 5 bulls were able to maintain a nearly constant volume (RVD of approximately 0). In the remaining 7 bulls, the prolonged incubation under hypotonic conditions resulted in further swelling rather than shrinking (RVD < 0). There were significant differences between tested bulls with respect to this ability ($P < .05$; Figure 3). Particularly, 2 bulls demonstrated a high level of RVD (0.85 and 1.03) while 2 other bulls demonstrated enhanced progressive swelling (RVD = -0.46 and -0.37); these values were outside of the range of the mean ± SD. Recovery of the relative cell volume in the first osmotically active subpopulation was not due to cell death because, for all 18 bulls with positive RVD, V_r remained greater than 1 after 20 minutes of incubation under hypotonic conditions.

Correlation Between Different Spermatologic Parameters

The correlation matrix between conventional and advanced sperm parameters is given in Table 3. There were significant positive correlations between the ability of sperm to swell in response to hypotonic stress (V_r) and between the ability of sperm to recover after initial

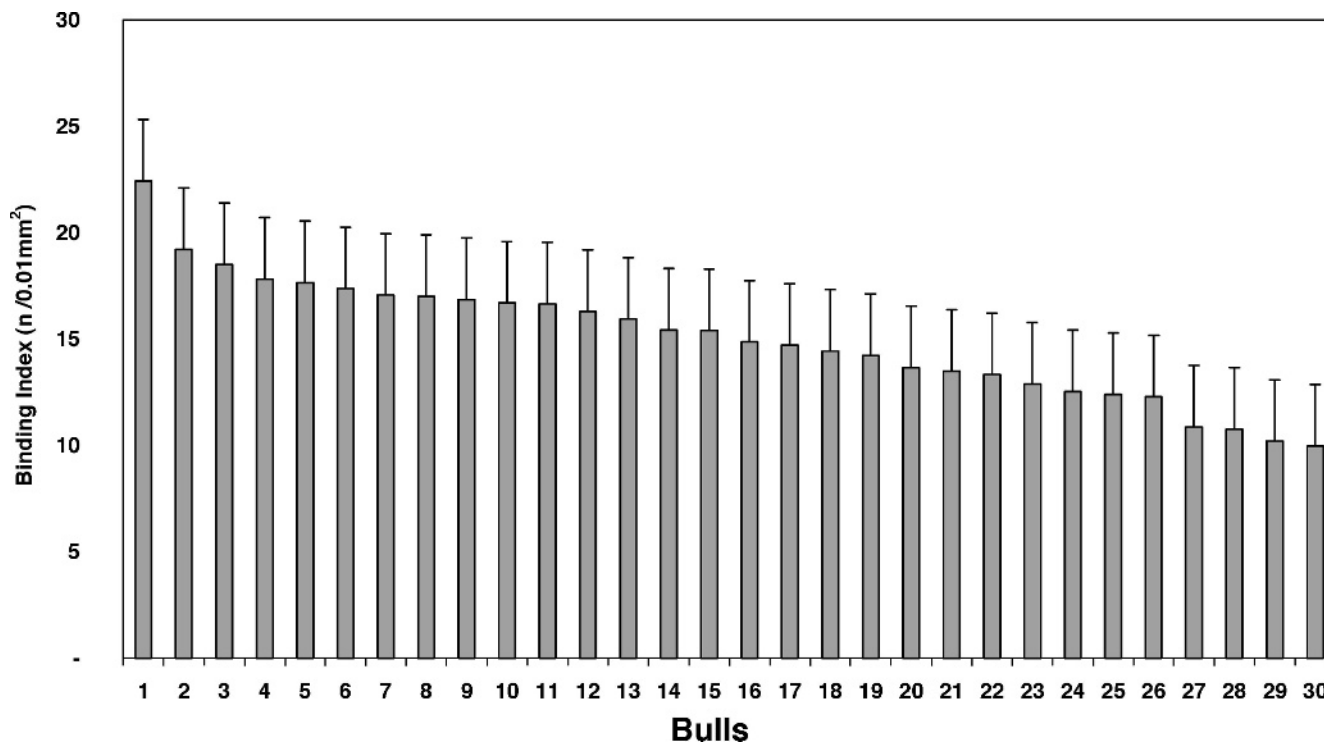


Figure 1. Binding index (sperm bound to 0.01 mm² surface area of an oviductal explant) (n = 90 ejaculates from 30 bulls; 3 ejaculates per bull). There were significant differences in the ability of sperm in the ejaculates of individual bulls to bind to the oviductal epithelial explants ($P < .05$).

swelling (RVD) and the BI ($r = .50$ and $r = .42$, respectively). This correlation is presented in Figure 3. There were no significant correlations between sperm volumetric parameters and any of the conventional spermatologic parameters. However, there was a significant correlation between the progressive motility percentage of Percoll-washed spermatozoa and the BI ($P \leq .0001$).

Relationship Between Binding Index and Other Spermatologic Parameters

As the partial correlations have been found only between BI, RVD, and forward motility of Percoll-washed sperm, we tested the hypothesis that the level of prediction for sperm binding ability to oviductal epithelium can be improved by using multiple predictors. Parameters routinely assessed in frozen-thawed sperm (computer-assisted cell motility, viability, and morphology) and the volumetric parameters and forward motility after the Percoll-washing procedure were used for optimized multiple regression analysis. Multiple forward regression analysis improved the prediction level for the BI if parameters assessed after the Percoll-washing procedure were included in the model. A 7-predictor, optimized model was obtained which consisted of volume regulatory ability, VAP, percentage of sperm showing circle motion, isotonic cell volume,

percentage of live sperm, and both local and forward motilities after the Percoll-washing procedure ($R^2 = .84$, $P < .0001$ vs best partial model $R^2 = .49$). This model showed a negative regression of BI with the isotonic volume, VAP, and circle-moving sperm but positive regression with the ability to regulate the cell volume, percentage of live sperm, and motility after Percoll washing (Table 4).

Differences Between Fertile and Subfertile Bulls With Respect to Spermatologic Characteristics

Bulls investigated in the second experimental series were divided into 2 groups according to NRR, fertile and subfertile: NRR = $72.3 \pm 3.0\%$ and $58.9 \pm 6.6\%$, respectively (mean NRR of each ejaculate based on 26 to 399 inseminations). There were significant differences between BIs with respect to group classification (Table 5). Bulls from the fertile group demonstrated higher BIs than the bulls from the subfertile group (BI = $23.5 \pm 2.6\%$ vs $19.3 \pm 3.6\%$, $P < .05$).

Also, primary hypotonic swelling in the subfertile group was much higher than in the fertile group, demonstrating a higher degree of instability and/or diminished volume regulatory function (Table 5). Indeed, bull spermatozoa respond to anisotonicity initially as “perfect osmometers” with an osmotically inactive cell volume of 61% (Guthrie et al, 2002). According to

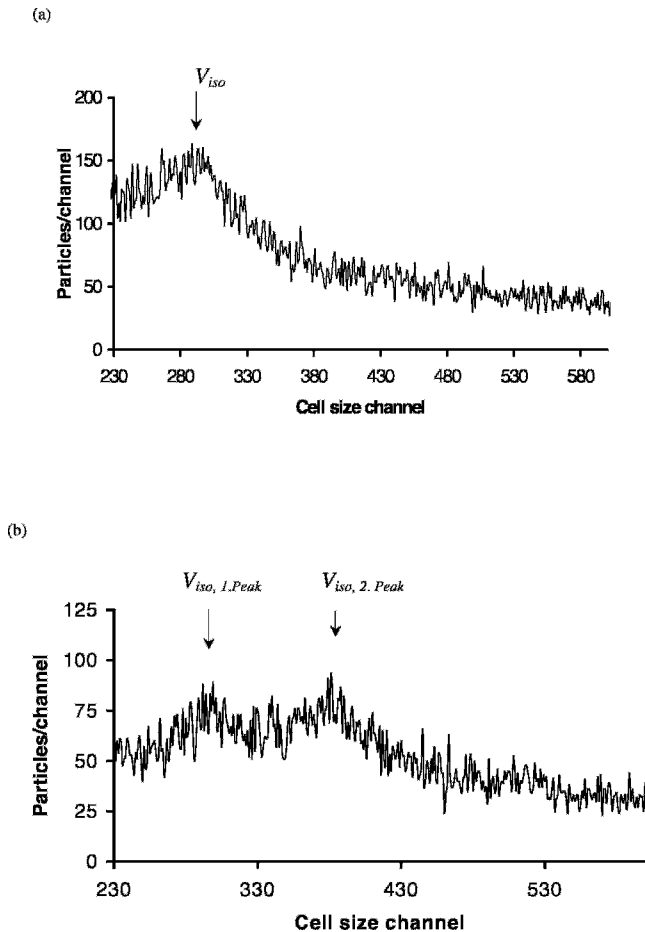


Figure 2. Plots of cell volume distributions. **(a)** Unimodal cell volume distribution curve of bovine spermatozoa. **(b)** Bimodal cell volume distribution curve of bovine spermatozoa. Volumetric measurements were made after 5 minutes of incubation under isotonic conditions. Modal values are indicated with arrows. In some samples, 2 peaks in the size range corresponding to spermatozoa were observed. The presence of such subpopulations provides additional information about heterogeneity within an ejaculate, and the osmotic response of particular subpopulations can be used for advanced analysis.

the Boyle Van't Hoff relationship, the expected cell volume under hypotonic conditions should be $V_{\text{hypo}} = 1.26 \cdot V_{\text{iso}}$ (Petrunkina and Töpfer-Petersen, 2000). In the present experiments, the spermatozoa of fertile bulls swelled to $V_{\text{hypo}} = 15.0$ fL (ie, $1.28 \cdot V_{\text{iso}}$), which corresponded to the expected values. However, not only was the swelling in the subfertile group higher than expected (18.9 fL), but the final recovery of cell volume after a 20-minute exposure to hypotonic conditions was less efficient than in the group of fertile bulls: $V_{r,20} = 1.42 \pm 0.24$ in the subfertile group vs $V_{r,20} = 1.13 \pm 0.16$ in the fertile group ($P < .05$).

Both VSL and BCF were significantly higher in the subfertile group (Table 5). No differences in other spermatologic parameters were detected.

Discussion

The mammalian oviduct has been shown to act as a functional sperm reservoir (Hunter, 1981), responsible for selection of a fertilization-competent sperm population, modulation of sperm capacitation, and regulation of sperm transport to minimize polyspermic fertilization (Hunter, 1996; Hunter et al, 1999; Petrunkina et al, 2001a,2003b,2004c; Gualtieri and Talevi, 2003; Hunter and Rodriguez-Martinez, 2004). It has been shown in numerous studies that the binding of sperm to the oviduct presents a mechanism for selecting a competent sperm population characterized by morphology, motility, membrane integrity, cytosolic calcium levels, sperm protein tyrosine phosphorylation, capacitation, and a high fertilizing competence (Thomas et al, 1994; Thomas and Ball, 1996; Ellington et al, 1999; Fazeli et al, 1999; Petrunkina et al, 2001c,d; Gualtieri and Talevi, 2003). However, this is the first study to date that links RVD with oviductal binding.

The ability to bind to the oviductal epithelium correlated both with the sperm's initial ability to swell in response to hypotonic stress and with the recovery of cell volume after exposure to hypotonic conditions. The ability of cells to swell in response to hypotonicity is clearly associated with the integrity of the sperm plasma membrane (Jeyendran et al, 1984), thus deviations from such behavior reflect defects either in the latter's functional state or in associated signaling mechanisms (Petrunkina and Töpfer-Petersen, 2000; Petrunkina et al, 2005a). Thus, a correlation between the V_r and BI may simply reflect the requirement that cells be viable to bind. However, the relationship between the ability to recover from swelling (RVD) and oviductal binding ability represents a new independent requirement.

Cell volume regulation is a very important physiologic property which has been linked with natural fertility. Human spermatozoa with compromised volume regulatory ability were found to be unable to migrate through mucus (Yeung and Cooper, 2001). In a comparison of caput, corpus, and cauda spermatozoa from fertile and infertile transgenic mice, it was found that sperm from the infertile strain showed abnormal motility characteristics commensurate with poor volume regulation (Yeung et al, 1999). Moreover, it has been demonstrated that boars with good volume regulatory ability have higher farrowing rates than those with poor or moderate volume regulation (Petrunkina et al, 2004a). In the present study, although we found highly significant correlations between the motility of spermatozoa in Percoll-washed suspensions and sperm-oviduct binding, interestingly there was no correlation between motility and RVD (Table 3). Therefore, it would appear

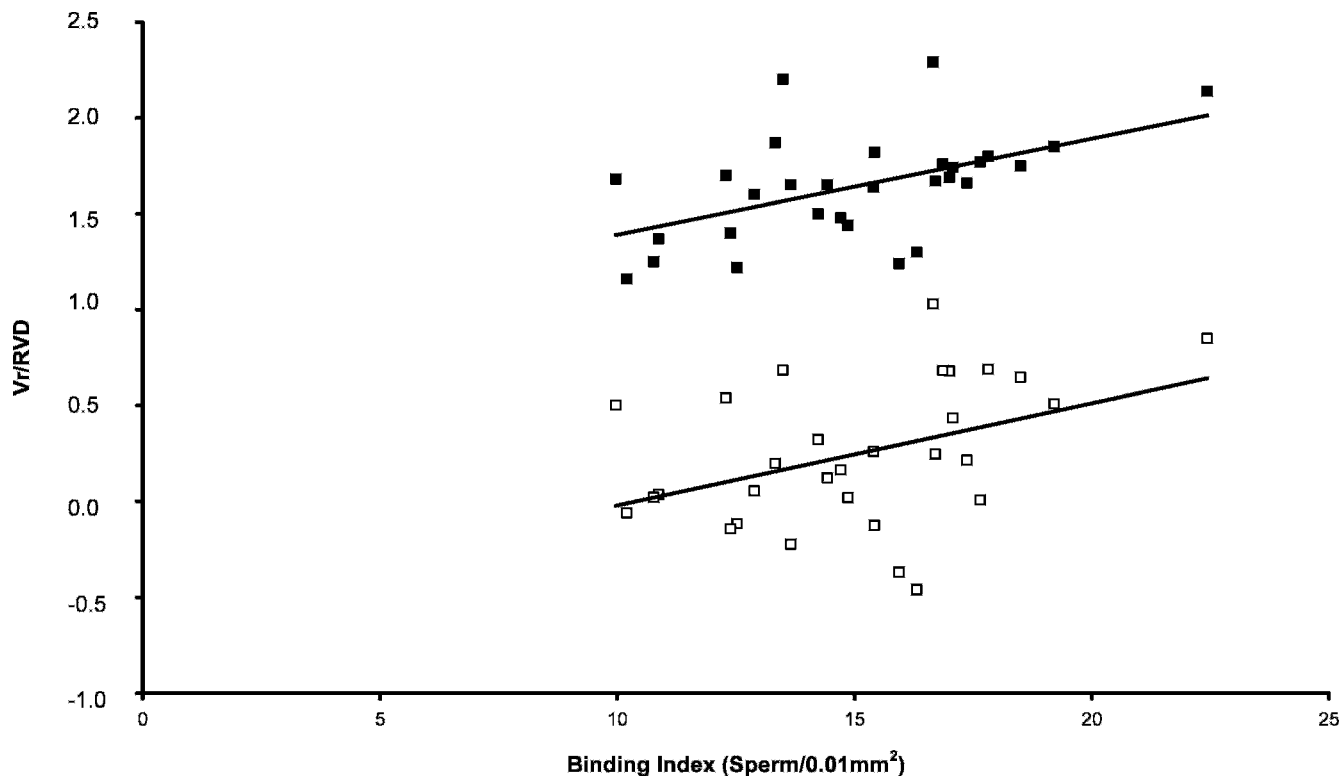


Figure 3. Relationship between binding index (BI) and volumetric parameters ($n = 90$ ejaculates from 30 bulls; 3 ejaculates per bull). Filled squares: primary osmotic response (relative volume shift $V_{r,5} = V_{\text{hypo},5}/V_{\text{iso},5}$). There were significant differences in the primary osmotic response (5 minutes) to hypo-osmotic conditions (V_r of modal cell volume) between individual bulls ($P < .05$). There was a significant linear regression between V_r and BI: $\text{BI} = 0.050 \cdot V_r + 0.89$. Open squares: regulatory volume decrease (RVD). There were significant differences in RVD ($\text{RVD} = V_{r,5} - V_{r,20}$) of sperm populations from different bull ejaculates ($P < .05$). There was a significant linear regression between RVD and BI: $\text{BI} = 0.053 \cdot \text{RVD} - 0.55$.

that the failure of sperm with poor volume regulation to bind to the oviductal epithelium cannot be ascribed directly to poor motility. It is more likely that motility and volume regulatory activity play independent roles in oviduct binding (ie, such binding comes about through the action of multifunctional processes). In support of this supposition, in a multiple regression analysis, the BI correlated with motility parameters, volumetric parameters, and percentage of viable sperm with a high goodness of fit ($R^2 = .84$). There was a negative correlation between isotonic sperm volume and BI. This latter result is in agreement with our previous findings for bull spermatozoa, underlining the negative correlations between isotonic sperm volume and fertility in terms of NRR in bull sperm (Petrunkina et al, 2001b). Sperm with elevated isotonic volumes likely have dysfunctional signaling pathways or transport mechanisms leading to impaired physiologic volume regulation, a pathophysiologic response which may account for decreased fertility in these samples (Petrunkina et al, 2001a,b,2005a; Yeung et al, 2005).

The oviduct is known to bind noncapacitated sperm selectively (Lefebvre and Suarez, 1996; Fazeli et al,

1999). Moreover, frozen-thawed sperm have been reported to be in a so-called “cryocapacitated” state (Cormier and Bailey, 2003). Thus, the differences observed between our bull samples in relation to their BIs may reflect differences in the degree of “cryocapacitation” within these samples. However, defects in volume regulation cannot be solely attributed to cryopreservation. The ability to regulate volume in the face of osmotic challenge appears to develop during maturation: when spermatozoa from wild-type mice were exposed to hypotonic conditions, corpus and cauda cells returned to a smaller volume after initial slight swelling whereas cells from the caput swelled but did not subsequently shrink (Yeung et al, 2002). It has been hypothesized by Cooper and Yeung (2003) that the acquisition of osmolytes during epididymal passage of spermatozoa may be driven by the regulatory volume increase (RVI) invoked by the hypertonicity of the epididymal fluid, and the first experimental evidence for the ability of mammalian spermatozoa to express RVI has been recently provided (Petrunkina et al, 2005a). The functional importance of RVI and RVD becomes apparent when one considers the sudden exposure to

Table 3. Correlation matrix of conventional and advanced sperm parameters

Parameter*		BI	V_r	RVD	SubjF	PercF	Alive	Head	MAS
V_r	<i>r</i>	.51							
	<i>P</i>	.004							
RVD	<i>r</i>	.42	.78						
	<i>P</i>	.02	.0001						
SubjF	<i>r</i>	.20	.05	-.01					
	<i>P</i>	.30	.78	.97					
PercF	<i>r</i>	.70	.23	.13	.69				
	<i>P</i>	.0001	.22	.48	.0001				
Alive	<i>r</i>	.23	.25	.01	.27	.18			
	<i>P</i>	.21	.18	.94	.15	.34			
Head	<i>r</i>	-.03	.14	.04	-.76	-.44	-.03		
	<i>P</i>	.87	.46	.82	.0001	.02	.87		
MAS	<i>r</i>	-.06	.05	-.02	-.76	-.45	-.13	.93	
	<i>P</i>	.73	.80	.93	.0001	.01	.49	.0001	

* BI indicates binding index (number of sperm bound to 0.01 mm² of explant); V_r , relative volume shift after 5 minutes of exposure to hypotonic conditions; RVD, regulatory volume decrease after exposure to hypotonic conditions; SubjF, progressive motility (determined by subjective microscopic evaluation); PercF, progressive motility (determined after Percoll-washing procedure by subjective microscopic evaluation); alive, percentage of live spermatozoa determined by LIVE/DEAD Sperm Viability Kit; head, sperm head abnormalities including acrosomal abnormalities; and MAS, morphologically altered spermatozoa.

a hypotonic environment that the sperm experience during ejaculation. One may deduce that any shortcomings in the development of volume regulatory ability during epididymal maturation would result in a significant disturbance of sperm transport in the female tract: the swelling induced by the osmotic shock would likely disrupt sperm intracellular organization, with serious consequences for membrane and motility functions. Epididymal sperm show reduced ability to bind to the oviduct (Petrunina et al, 2001a; Gwathmey et al, 2003), although whether this is due to their maturational state or to the lack of contact with seminal plasma remains to be seen. Nevertheless, our findings indicate that maturational defects in the development of cell volume regulation may be one of the underlying reasons for a low BI. Thus, maturational state is a key aspect to consider in subsequent investigations.

The nature of the functional relationship between volume regulation and sperm-oviduct binding remains unclear. At this stage one can only speculate about any mutual association of underlying mechanisms. As the properties of the plasma membrane are obviously crucial for both volume regulation and oviduct binding, faults in the organization of the sperm surface could be a common factor. Not only could such faults lead to impaired ion transport across the plasma membrane, with resultant disruption of the processes for maintenance of osmotic equilibrium (Petrunina et al, 2004b), but they could also lead to a failure of interaction between the protein/carbohydrate binding sites on the sperm surface and glycosylated ligands on the oviductal epithelium.

In this study, we were able to detect significant differences between individual bulls with respect to their

Table 4. Multiple regressions between binding index and other spermatologic parameters

Parameter*	Estimate	Partial Pr > F	Partial R ²	Model Pr > F	Model R ²
PercF	0.28	.0001	.486	.0001	.486
RVD	2.00	.027	.111	.009	.597
PercLoc	0.41	.01	.084	.001	.681
Circular	-0.50	.05	.044	.001	.726
VAP	-0.21	.02	.055	.01	.781
$V_{iso,20}$	-0.24	.08	.027	.025	.808
Alive	0.05	.04	.034	.04	.842

* PercF indicates progressive motility (determined after Percoll-washing procedure by subjective microscopic evaluation); RVD, regulatory volume decrease after 20 minutes of exposure to hypotonic conditions; PercLoc, nonprogressive motility (determined after Percoll-washing procedure by subjective microscopic evaluation); circular, percentage of spermatozoa swimming in circles in frozen-thawed sample; VAP, average path velocity in frozen-thawed sample; $V_{iso,20}$, isotonic cell volume after 20 minutes of incubation; alive, percentage of live spermatozoa determined by LIVE/DEAD Sperm Viability Kit; estimate, coefficient of an entered predictor in the model; partial Pr > F, partial level of probability for regression between each entered predictor and model parameter; partial R², improvement of model's goodness of fit by each entered predictor; model Pr > F, level of probability of a predictor in final improved model; and model R², goodness of fit of final improved model.

Table 5. Difference between groups of bull ejaculates with different nonreturn rates (6 bulls; n = 11–18 ejaculates)

Parameter§	Group I†	Group II†
NRR (%)	72.3 ± 3*	58.9 ± 6.6†
BI	23.5 ± 2.6*	19.3 ± 3.6†
V _{iso,5} (fL)	11.7 ± 1.7	12.9 ± 3.2
V _{iso,20} (fL)	13.0 ± 1.9	12.8 ± 2.4
V _{hypo,5} (fL)	15.0 ± 1.3*	18.9 ± 4.9†
V _{hypo,20} (fL)	14.6 ± 3.1	18.2 ± 4.4
MAS (%)	35.3 ± 8.5	39.5 ± 7.6
MOT (%)	65 ± 9.3	57.8 ± 10
PNA/PI ⁻ (%)	86.2 ± 2.5	83.5 ± 5.9
VAP (μm/s)	58.7 ± 4.1	60.9 ± 3.2
VSL (μm/s)	47.0 ± 3.5*	50.3 ± 2.8†
BCF (1/s)	24.2 ± 1.5*	27.0 ± 2.5†

*† Values with different symbols within one row differed significantly ($P < .05$); † Group I, fertile; group II, subfertile; § NRR indicates nonreturn rates; BI, binding index (number of sperm bound to 0.01mm² of explant); V_{iso,5}, cell volume after 5 minutes of incubation under isotonic conditions; V_{iso,20}, cell volume after 20 minutes of incubation under isotonic conditions; V_{hypo,5}, cell volume after 5 minutes of incubation under hypotonic conditions; V_{hypo,20}, cell volume after 20 minutes of incubation under hypotonic conditions; MAS, proportion of morphologically altered spermatozoa; MOT, total motility; PNA/PI⁻, percentage of propidium iodide/fluorescein isothiocyanate-conjugated peanut agglutinin negative cells (membrane- and acrosome-intact sperm); VAP, average path velocity; VSL, straight-line velocity; and BCF, beat-cross frequency.

BIs. The ability of sperm to bind to the oviductal epithelium appears to be a highly individualized property which could be used for diagnostic purposes (Petrunkina et al, 2001a; Waberski et al, 2005): a direct association between poor sperm-oviduct binding and low fertility has already been found in pilot studies in a small group of boars and bulls (De Pauw et al, 2002; Waberski et al, 2006). Similarly, the tested bull individuals differed significantly in sperm volume regulatory properties. Volume-regulatory tests have been already established for diluted boar semen and both fresh and frozen-thawed dog semen (Petrunkina et al, 2004a,b,d,2005b), and the present study extends these to frozen-thawed bull semen. We found differences in both volumetric response and sperm-oviduct binding capacity between bull ejaculates that differed in their NRR. It appears that spermatozoa of bulls with higher NRR have more efficient volume recovery after exposure to hypotonic stress as well as enhanced capacity for sperm-oviduct binding. Obviously, these findings must be confirmed on a larger number of bulls in a more focused field study. However, it is interesting that although both groups demonstrated initial progressive swelling in response to hypotonic conditions, subfertile bulls showed higher hypotonic sperm volumes. This preliminary finding supports the hypothesis that a suboptimal rather than a minimal response to challenge is associated with subfertility (Petrunkina et al,

2005c). Whereas the bulls in the fertile group responded to hypotonicity according to the Boyle Van't Hoff relationship, the bulls in the subfertile group showed a significantly higher initial response than expected according to the "perfect osmometer model" and were not able to regulate their sperm volumes. Furthermore, these results show that the assessment of sperm initial osmotic response (ie, HOST) needs to be completed by a volume-regulation test to ensure more information and correct interpretation of data. The increased sperm velocity and beat frequency parameters in the subfertile group indicated higher levels of hyperactivation, perhaps due to cryopreservation-triggered activation of capacitation signaling pathways (Chamberland et al, 2000, Cormier and Bailey, 2003).

Assessments of sperm volumetric behavior and/or sperm binding capacity would seem to represent a good strategy for sperm quality evaluation since our findings suggest that they are sensitive parameters associated with NRR. Volumetric behavior tests in particular may be very useful for selecting so-called "good freezers." During cryopreservation, spermatozoa shrink due to the formation of extracellular ice; as thawing occurs, under relatively hypo-osmotic conditions, the spermatozoa must be able to restore their volume. Thus the insights gained here could be useful both to improve cryopreservation protocols and for routine sperm laboratory diagnostics. Volumetric tests are especially easy to perform; they allow the recording of precise quantitative data and do not involve high costs or tissue preparation. As a sperm parameter reflecting different levels of functional regulation such as sperm ability to withstand cryopreservation, bind to the oviductal epithelium, and undergo capacitation, volume regulatory ability offers an exciting new test to be applied to the field of human andrology.

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