

DETERMINATION OF BOAR SPERMATOZOA WATER VOLUME
AND OSMOTIC RESPONSE

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Received for publication: February 4, 1994

Accepted: September 30, 1994

ABSTRACT

Boar spermatozoa water volume and osmotic response were determined by a shape-independent method for measuring cellular volume, electron paramagnetic resonance (EPR), employing the spin label, tempone, and the broadening agent, potassium chromium oxalate (CrOx). A water volume of $18.4 \pm 1.6 \mu\text{m}^3$ ($\bar{X} \pm \text{SD}$) was obtained for individual boar spermatozoa at 290 milliosmolal (mOsm) which, after correction for the presence of cytoplasmic droplets, yields a boar sperm water volume of 13.0 to 15.0 μm^3 . Assuming 59% of the total cell volume is water, the total cell volume of boar spermatozoa is 22.0 to 25.4 μm^3 . In addition, the experiment indicated that the relative water volume versus the reciprocal of the external osmolality (Boyle van't Hoff plot) was linear over the range of 210 to 1500 mOsm of sodium chloride ($r^2 = 0.996$), supporting the hypothesis that boar spermatozoa act as ideal osmometers. A non-zero y axis intercept of 0.23 from the Boyle van't Hoff plot indicated a 23% spin label accessible, but osmotically inactive water component.

Key words: boar spermatozoa, water volume, Boyle van't Hoff, electron paramagnetic resonance, osmotic water

Acknowledgments

This research was supported by a grant from the USDA/NRI Competitive Grants Program (93-37203-9272), by a Career Development Award from the NIH (KO4-HD00980 to JKC) and a NATO Collaborative Research Grant (CRG 920170). The authors gratefully acknowledge Mrs. Katherine Vernon for secretarial assistance.

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INTRODUCTION

Although boar spermatozoa were successfully frozen more than 20 yr ago, the fundamental cryobiological factors that affect the ability of spermatozoa to participate in fertilization after cooling and warming remain largely unknown. Current methods used to cryopreserve boar semen result in only about 30 to 40% cryosurvival. Additionally, the fertility of deep-frozen boar spermatozoa is lower than that of fresh or liquid-stored spermatozoa (1,16). To improve the efficiency for artificial insemination using cryopreserved boar spermatozoa, a more complete understanding of the underlying biophysical properties affecting cryosurvival needs to be developed. Knowledge of the permeability of a cell to water and to cryoprotectant solutes can be powerful tools in predicting the likely optimal values for the major steps involved in cryosurvival. From the value of the permeability coefficient for the cryoprotectant one can compute the optimum procedure for adding and removing the cryoprotectant without osmotic damage. Arnaud and Pegg illustrate an application of this concept to human platelets in which a multi-step dilution sequence is used to remove glycerol or propylene glycol while limiting osmotic excursions (3). Knowledge of the permeability of the cell to water, and its temperature coefficient, allows one to predict the cooling rate likely to be low enough to preclude lethal intracellular freezing. Noiles et al. and Mazur illustrate the application of these ideas to human sperm and mammalian embryos, respectively (21,20). Basically, a cell must be cooled slowly enough to allow it to substantially dehydrate before intracellular freezing occurs. If appreciable intracellular water is present at the time of intracellular freezing, cell death generally results. The standard modeling theories used to determine the permeability of a cell to water and to cryoprotectant requires information about the cell water volume and the osmotic behavior of the cell. The cell volume of boar sperm has previously been measured by Hammerstedt et al. and O'Donnell while the osmotic response has not been (14,22). The cell volume and osmotic response of boar spermatozoa are the subject of this paper.

Many types of cells behave as ideal osmometers which means that the cell volume changes linearly as the reciprocal of the osmolality of the external solution (Boyle van't Hoff relationship). It has been demonstrated that bovine, human, and mouse spermatozoa act as ideal osmometers (10-12). The current experiment was designed to determine the isotonic water volume of boar spermatozoa and whether boar spermatozoa act as ideal osmometers to provide the basis for the calculation of water and cryoprotectant permeabilities.

In this experiment, a shape-independent method of measuring cellular volume, electron paramagnetic resonance (EPR), employing the spin label, tempone, and the broadening agent, potassium chromium oxalate (CrOx) was used to study the osmotic response of the boar spermatozoa in the range of 210 to 1500 mOsm.

MATERIALS AND METHODS

Media Components

The spin label, ^{15}N -tempone (4-oxo-2,2,6,6-tetramethyl-piperidine-1- ^{15}N -1-oxyl) was purchased from MSD Isotopes, Montreal, Canada. Potassium chromium oxalate ($\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$; [CrOx]) was synthesized according to the procedure of Bailar and Jones (4). Hoechst 33258 was obtained from Sigma Co. (St. Louis, MO). The medium used for boar

spermatozoa was a modified Tyrode's medium, TL-HEPES, supplemented with pyruvic acid (36 $\mu\text{g}/\text{ml}$, Sigma) and bovine serum albumin (BSA, 4 mg/ml , Sigma) and was referred to as TL-HEPES/BSA (5).

Animals

Five mature Yorkshire boars (18 mo to 3 yr old, weighing 450 to 600 lbs) and one mature Landrace boar (16 mo old, weighing approximately 400 lbs) were used in this study between September 24 and October 7, 1993. The boars had free access to move inside a barn with straw or to go outside on a concrete slab. They were fed once a day with a mixed corn-soybean ration according to each boar's body condition and allowed access to water ad libitum.

Boar Spermatozoa Preparation

One semen sample from each of six boars was collected by manual manipulation using a dummy and gloved hand. Semen from the sperm rich portion was diluted 1:3 or 1:2 times in TL-HEPES/BSA medium in 15 ml conical tubes (Sarstedt, Newton, NC) and transported in a 37°C water bath (thermos) during the 1 h transport time. A computer assisted semen analysis (CASA) was performed (CellSoft®, CryoResources, Ltd., New York) to determine cell concentration and percent motility (19). Then the samples were set on the bench top at room temperature (20-22°C) until being used within 10 h.

EPR Sample Preparation

The EPR osmotic samples were prepared by mixing 2.4 μl of 50 mM ^{15}N -tempone, 12 μl of 250 mM CrOx, 33.6 μl of NaCl solutions (16, 85, 155, 348, 698, 1220, 1745, or 2272 mOsm), and 12 μl packed cells in TL-HEPES/BSA (centrifuged at 200g for 5 min). The mixed EPR solutions had a total volume of 60 μl with final concentrations of 2 mM ^{15}N -tempone, 50 mM CrOx, and total osmotic concentrations of 210, 250, 290, 400, 600, 900, 1200, and 1500 mOsm excluding ^{15}N -tempone (11). Ten microliters of each mixed solution were transferred into another tube and 10 μl of 1500 mOsm NaCl solution were added to stop the cell motility. Then the mixed solution was diluted by a precise amount of medium for cell counting under a light microscope using a hemocytometer. Another 10 μl of the EPR sample was used for Hoechst 33258 staining to assess membrane integrity. The remaining sample was drawn into a 50- μl disposable micropipet (Clay Adams #4622, Parsippany, NJ) by capillary action, and sealed with Critoseal® (Sherwood Medical, St. Louis, MO) for EPR measurement. The final osmotic concentrations were determined by linear addition of the individual osmolalities of the TL-HEPES/BSA, NaCl, and CrOx (non-permeating solutes) and were confirmed on a freezing point depression osmometer (Advanced DigiMatic Osmometer, Model 3D2), which yielded agreement within 3%.

A calibration sample was prepared using 2.4 μl of 50 mM ^{15}N -tempone and 57.6 μl TL-HEPES/BSA. When extracellular tempone is broadened by CrOx, a broad residual signal remains. The residual signal is eliminated using digital subtraction of a "background" reference signal at each osmotic strength (11). The "background" reference samples were prepared in parallel with the regular samples, except that the cells were replaced by buffer.

Cell Membrane Integrity

For each EPR osmotic sample, 10 μ l of the solution were transferred into 1 ml NaCl solution of the same osmolality as the sample and ten μ l of Hoechst 33258 (0.01 mg/ml stock solution) were added to the cell suspension. Hoechst 33258 is nearly impermeant to the plasma membrane, binds to the DNA in the head of spermatozoa with disrupted membranes, and fluoresces. After Hoechst 33258 was equilibrated in the solution for 5 min, 30 μ l of salmon testis DNA solution (5 mg/ml) was added to remove excess external Hoechst 33258. The percentage of stained cells was determined using a FACStar Plus Flow Cytometer (Becton Dickinson, San Jose, CA) with 3 watt Krypton laser operated at 200 mW power, a UV wavelength of 350 nm, and a barrier filter, 450DF65. The Hoechst 33258 negative cells were counted as membrane-intact cells.

EPR Data Acquisition and Analysis

A Varian X-Band E109 spectrometer with a rectangular cavity and an HP9825 data system with custom software for biological spin label work was used for the EPR measurements (17). The temperature was controlled to $20 \pm 0.5^\circ\text{C}$.

The spectrometer parameters used for these experiments were a) power 15 mW, b) magnetic field 3269 gauss, c) field sweep 32.5 gauss, d) modulation amplitude 0.5 gauss, e) amplifier time constant 0.064 sec, and f) variable rate sweep yielding a total sweep time of 275 sec. For background spectra, the time constant was increased to 0.128 sec and the total sweep time increased to 550 sec. The variable rate sweep was used to improve the signal to noise ratio by spending additional time in the peak regions of the spectrum, with digital signal averaging (17).

EPR Water Volume Measurement and BVH Relationship

The details of the EPR cell water volume measurements have been previously described (11, 18). Very briefly, the spin label tempone labels all aqueous regions of the sample, and CrOx (which is membrane impermeable) broadens the extracellular tempone signal nearly to extinction. The remaining tempone signal is proportional to the intracellular volume of the sample, and after amplitude calibration and adjustment for the cell concentration, yields the intracellular volume of tempone accessible water. The method measures the intracellular water volume of all the membrane-bound structures in the sample. Thus, if cytoplasmic droplets are present, a correction must be made for them. A corrected water volume, V_c , may be computed from the raw EPR water volume, V , using

$$V_c = V - r_d \cdot V_d \cdot f \quad 1)$$

where r_d is the ratio of cytoplasmic droplets to sperm in the sample, V_d is the droplet volume, and f is fraction of the droplet which is water.

The Boyle van't Hoff (BVH) relationship is

$$V_w = V_i(M_i/M) + V_a \quad 2)$$

where V_w is the total volume of cell water at osmolality M , V_i is the volume of osmotically

active water at the isotonic osmolality, M_i , and V_a is the volume of osmotically inactive (but temporarily accessible) water. Normalizing this equation by dividing by the total cell isotonic water and expressing it in terms of Ponder's R value, the BVH relationship can be expressed as (2,11)

$$y = R(M_i/M) + (1 - R) \quad 3)$$

To analyze the data, cell volumes were normalized to 290 mOsm. The BVH relation is generated by plotting the normalized spermatozoa volume versus 290/mOsm (11). In replicate experiments, the sequence of exposure to high tonicity and low tonicity solutions was reversed to avoid any systematic effects of time.

Morphometric Volume Determination

The boar sperm was assumed to consist of a flat head and a cylindrical midpiece, principal piece and tail-end piece for the purpose of estimating the volume from microscopic dimensional data. The volume of the head is just its area x thickness, and the cylindrical volumes are given by the length of each cylinder by its cross sectional area. Thus

$$V = A \cdot t + \pi \cdot [ml \cdot (md/2)^2 + pl \cdot (pd/2)^2 + tl \cdot (td/2)^2] \quad 4)$$

where A is the cross sectional head area, t the head thickness, ml and md the midpiece length and diameter, respectively, pl and pd the principal piece length and diameter, respectively, and tl and td are the tail-end length and diameter, respectively.

RESULTS

Boar Spermatozoa Water Volume and Osmotic Response

The uncorrected boar spermatozoa intracellular water volume in 290 mOsm NaCl was determined to be $18.4 \pm 1.6 \mu\text{m}^3$ ($\bar{X} \pm \text{SD}$, $n = 6$). However, the sperm samples contained a large proportion of cytoplasmic droplets for which a correction must be applied. The ratio of droplets to sperm (r_d) is estimated to be 50 to 80%, the droplet volume is taken to be $9 \mu\text{m}^3$ (22), and the droplets are assumed to be 75% water by volume. Using these data, a corrected boar sperm intracellular water volume of 13.0 to $15.0 \mu\text{m}^3$ is obtained (equation 1). To compute the total cell volume, boar sperm are assumed to have the same percentage, 59% by volume, of water as do bovine and human spermatozoa (9,18), yielding a total cell volume of 22.0 to $25.4 \mu\text{m}^3$.

The normalized boar spermatozoa water volume versus the reciprocal of the external osmolality (Boyle van't Hoff plot, equation 3) is shown in Figure 1. A linear fit ($y = 0.765x + 0.234$; $r^2 = 0.996$) was obtained using a least squares fit.

Cell Fragility

The results of the Hoechst 33258 staining from flow cytometry are listed in Table 1. The percentage of plasma membrane-intact cells was $> 90\%$ over the 250 to 1200 mOsm range. The percentage of plasma membrane intact cells in 210 and 1500 mOsm were $89.2 \pm 2.5\%$ and $88.8 \pm 3.9\%$, respectively.

Table 1. The percentage of membrane-intact boar spermatozoa at different NaCl osmolalities:

mOsm	% membrane-intact cells ($\bar{X} \pm SD$)
210	89.2 \pm 2.5
250	92.6 \pm 2.3
290	92.2 \pm 3.0
400	92.8 \pm 2.1
600	92.4 \pm 2.1
900	91.5 \pm 2.8
1200	90.4 \pm 2.4
1500	88.8 \pm 3.9

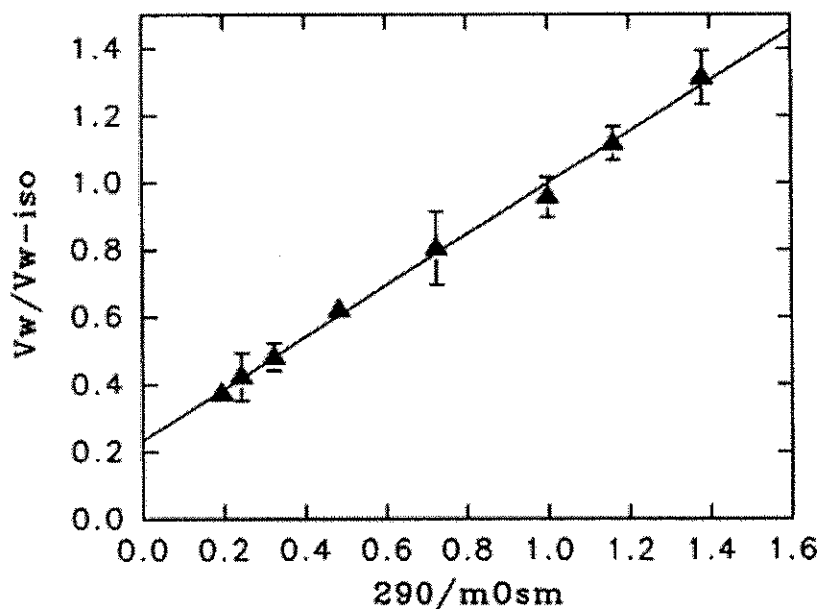


Figure 1. Osmotic (Boyle van't Hoff, equation 3) response of boar spermatozoa. Relative EPR determined water volume of boar spermatozoa versus reciprocal buffer osmolality in the range of 210 to 1500 mOsm is shown (mean \pm SD; n = 6). Least squares fit yields: $y = 0.765x + 0.234$; $r^2 = 0.996$.

DISCUSSION

The uncorrected boar spermatozoa water volume of $18.4 \mu\text{m}^3$ in 290 mOsm NaCl is essentially equivalent to the $20 \mu\text{m}^3$ at 300 mOsm obtained by Hammerstedt et al., (14) using an EPR technique employing the spin label, ^2H -tempone, and the broadening agent, NiCl_2 . Our estimated total volume of 22.0 to $25.4 \mu\text{m}^3$ for boar spermatozoa is comparable to, but somewhat larger, than the electronic counter value of $21 \mu\text{m}^3$ reported by O'Donnell (22).

The boar sperm volume may also be determined using microscopically determined dimensional data and geometric formula (equation 4). We calculated a volume of $23.5 \mu\text{m}^3$, in good agreement with the EPR and electronic counter volumes, using the following dimensional data: The head cross sectional area is $37.6 \mu\text{m}^2$ based on the average of areas reported in van Duijn (13) and the head thickness is $0.35 \mu\text{m}$ according to Bonet and Briz (6). The midpiece length, ml, and diameter, md, are $10.6 \mu\text{m}$ and $0.75 \mu\text{m}$, respectively, based on an average of data in Cummins and Woodall (8), Bonet and Briz (6), and Hancock (15) for the length and Bonet and Briz (6) for the diameter. The principal piece length (pl) and diameter (pd) are $28.6 \mu\text{m}$ and $0.5 \mu\text{m}$, respectively, based on data from the same sources used for the mid-piece. The tail-end length (tl) and diameter (td) are $2.5 \mu\text{m}$ and $\approx 0.2 \mu\text{m}$, respectively, based on data in Bonet and Briz (6) and Bustos-Obregon and Flechon (7), respectively. Other dimensional data in Bustos-Obregon and Flechon (7) are consistent with the above numbers.

The dimensionally determined volume of $23.5 \mu\text{m}^3$ is quite sensitive to the assumed head thickness. A head thickness of $0.35 \mu\text{m}$ was used here based on SEM measurements of glutaraldehyde fixed sperm (6). This represents a lower limit for head thickness due to the likelihood of specimen shrinkage on preparation and fixing. Increasing the head thickness from 0.35 to $0.45 \mu\text{m}$ increases the computed sperm volume to $27.3 \mu\text{m}^3$. Thus the microscopic data do not yield a firm value for the boar sperm volume.

The BVH plot is linear in the range of 210 to 1500 mOsm which indicates that the boar spermatozoa act as ideal osmometers. The non-zero y axis intercept of 0.234 indicates that 23% of the isotonic water is tempone accessible but osmotically inactive (equation 3). The non-zero y axis intercept also can be translated (equation 3) to a Ponder's R value of 0.77 which is much smaller than that of the human (0.87), bovine (0.86), and mouse (0.87) spermatozoa (10-12).

The linear BVH behavior observed implies that the cells maintain membrane integrity over the range 210 to 1500 mOsm, which we presume to be a prerequisite for cell survival. During cryopreservation cells are generally exposed to an even wider range of osmolalities, but these are beyond the range of our current EPR methodology. The large BVH intercept of 23% is interesting as it is nearly twice the value we have found for human, bovine, or mouse spermatozoa. Cryopreservation protocols, which do not involve vitrification, are generally designed to osmotically remove water from cells during the cooling process in order to prevent appreciable intracellular ice formation. This is generally considered to be lethal. The large percentage of osmotically inactive water implied by our BVH data suggests that it is more difficult to osmotically dehydrate boar semen during cryopreservation and thus they may be more sensitive to freezing injury. It is not obvious that the large fraction of osmotically inactive water has any relation to the cold shock sensitivity of boar sperm.

The Hoechst 33258 staining results for boar spermatozoa indicate that osmotic stress, by itself, is not a major factor in causing cell membrane damage over the range of 210 to 1500 mOsm.

The EPR technique is an effective method for determining the volume characteristics of irregular shaped cells, especially sperm cells. The EPR technique also can give information about the osmotic behavior and osmotically active water components. These data together with several other fundamental biophysical parameters are useful for the development of an optimal cryopreservation protocol for boar spermatozoa. Ongoing experiments to determine boar semen membrane water permeability (L_p) and cryoprotectant permeability (P_c), will allow the modeling of optimal cooling and warming protocol for the cryopreservation of boar spermatozoa.

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