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ESR-DETERMINED OSMOTIC BEHAVIOR OF BULL SPERMATOZOA

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Introduction

Our laboratories are pursuing a fundamental approach to the problems of semen cryopreservation. For many cell types (human red cells, yeast, HeLa) it has been demonstrated that there is an optimum cooling rate for cryopreservation. Faster rates allow insufficient time for cell dehydration and result in intracellular ice formation and cell death (Mazur, 1984). It is possible to predict this optimal rate provided that the cell acts as an ideal osmometer and several other cell parameters are known such as the membrane hydraulic conductivity. It is the purpose of this work to examine the osmotic response of bull sperm to sucrose (0.122 to 0.524 Osm) and NaCl (0.25 to 1.5 Osm) utilizing electron spin resonance (ESR) to measure cell volume.

The determination of cell osmotic behavior requires the measurement of cell volume in solutions of differing osmotic strengths. Because of the highly non-spherical shape and relatively fragile nature of sperm, conventional methods for cell volume measurements such as direct photo-micrography, light scattering, Coulter counter, and spermatocrit all suffer various disadvantages. Indeed many earlier reports using some of these methods were unable to detect osmotic volume changes. ESR methods are cell shape independent, and thus particularly well suited for these measurements. Additionally, Colvin et al. (1988) have demonstrated the relatively non-toxic nature of tempone and CrOx to sperm. The high osmolalities tested in this study are especially appropriate to the hypertonic solutions experienced by sperm during osmotic dehydration and freezing. Previous studies of bovine sperm osmotic behavior include those of Drevius (1972b) and Hammerstedt et. al. (1978), but these were either restricted to the hypotonic range or covered a limited range of osmolalities.

For calibration purposes we also measured the ESR response of human red cells (RBC), the osmotic response of which is well documented with other methods.

Materials and Methods

Materials: The RBC were handled in phosphate buffered saline (PBS) and the sperm in a modified Tyrode's medium supplemented with 4 mg/ml of bovine serum albumin (BSA), lactate, and 33 μl/ml pyruvate (TL Hepes W/BSA) (Bavister, 1983). Tempone was obtained from Molecular Probes (Eugene, OR) and CrOx ($K_3[Cr(C_2O_4)_3]\cdot 3H_2O$) was synthesized according to the procedure of Bailer and Jones (1939). RBC were obtained by venepuncture from three normal adults using acid citrate dextrose anticoagulant, washed in PBS, and used within 10 hours.

Spermatozoa Isolation: Bovine semen from seven bulls (four for the sucrose and three for the NaCl studies) was obtained by electroejaculation from the Purdue University Animal Breeding Unit, diluted 1:3 with buffer, transported at 37°C to Indianapolis, analyzed via computer assisted semen analysis (CellSoft, Cryo Resources, Ltd, NY), and held in an incubator at 37°C until used (within 10 hrs). There were some minor variations in the handling of the cells up to the point of use;

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e.g. a higher dilution ratio than 1:3 or resuspension in Mann's Ringer. No apparent effect of these variations was seen in the ESR data. In all cases sperm motility exceeded 80% and typically was 95%.

ESR sample preparation: The NaCl experiment samples were prepared using 2 μl of 50 mM tempone (final concentration 2 mM), 10 μl of 250 mM CrOx (final concentration 50 mM), 28 μl of NaCl solution (83, 153, 345, 694, 1217, and 2270 mOsm) and 10 μl loosely pelleted cells (400 x g) yielding 50 μl samples of final concentration 250, 290, 400, 600, 900, and 1500 mOsm.

The sucrose experiment samples contained 4 μl 25 mM tempone plus: (i) 14 μl 115 mM CrOx, 5 μl cells and 27 μl sucrose (0, 27, 77, 216 mOsm), or (ii) 25 μl 115 mM CrOx and 21 μl cells or (iii) 25 μl 115 mM CrOx, 5 μl cells and 16 μl of 1261 mOsm sucrose. The net osmolalites of the resulting samples were 122, 137, 164, 239, 284, and 524 mOsm.

For both experiments 10 μl of the sample was removed for trypan blue staining and counting of membrane intact cells. The remaining sample was drawn by capillary action into a 50 μl disposable micropipet for ESR measurement. All the final osmotic concentrations were determined by interpolation of the tables by Scatchard et al. (1938) and linear addition assumed for the differing solutes. With a given ejaculate, an entire NaCl or sucrose experiment was run in one day. For each sample, an ESR background control sample was prepared by omitting the cells and using pure buffer in place of the cell pellet.

ESR Data Acquisition and Analysis: ESR spectra were obtained on a Varian E109 X-Band spectrometer using 20 mW power, 25 gauss sweep, 0.5 gauss modulation, 20 $\pm$ 0.2°C temperature, 0.128 sec time constant, and variable rate sweep with digital averaging (Kleinhans, 1985) yielding a total sweep time of 350 sec. Tempone was used to label the aqueous regions of the sample and the broadening agent CrOx was used to broaden the extracellular signal to near extinction (Yager et al., 1979). Residual extracellular signal was eliminated by digital subtraction of the signals from samples with and without cells. The intensity (W^2h) of the mid-field tempone line relative to a 2 mM tempone standard yields the fractional volume, F_v, of the cell suspension. The average volume per cell, V_c, is obtained as $V_c = F_v/C_c$ where C_c is the concentration of membrane intact cells measured on a hemocytometer. For each day's experiment, the cell volumes were normalized by fitting the data with a least squares linear fit, computing the volume at 290 mOsm, and dividing all the data by that value. The reported isotonic volumes are averages of the fitted volumes at 290 mOsm. Data are reported as means $\pm$ standard deviations.

The osmotic data were analyzed by generating Boyle van't Hoff plots in which the sperm volume is plotted as a function of reciprocal osmolality. A linear relationship indicates an ideal osmotic response (Dick, 1966).

Results and Discussion

Calibration using RBC: Human RBC volumes were measured at 290 mOsm as a test of the ESR methodology. An intracellular aqueous volume of 58 $\pm$ 5 μm^3 was obtained, in good agreement with the recent ESR measurements (59.2 μm^3) of Moronne et al.(1990). The assumption that the aqueous volume of RBC's is 71.5 % of the total (Mazur and Miller, 1976) yields a net RBC volume of 81 $\pm$ 7 μm^3 in good agreement with typical literature values, e.g. 80 to 100 μm^3 (Miale, 1982).

Aqueous volume of isotonic sperm: For the bovine sperm, nine replicates of the sucrose series were completed and four replicates of the NaCl series have been completed to date. Isotonic intracellular aqueous sperm volumes of 20.1 ± 2.2, 24.1 ± 1.7, and 21.3 ± 2.8 μm^3 were obtained for the sucrose, NaCl, and combined data, respectively. In comparison Hammerstedt (1978) reported an ESR determined aqueous volume of 20 ± 1 μm^3 and Drevius (1972b) a total cell volume of 36 μm^3 which yields an aqueous volume of 21 μm^3 assuming water is 58.6% (Drevius, 1972a) of the total.

Osmotic linearity: Both the sucrose and the NaCl data gave linear Boyle van't Hoff plots with a coefficient of determination, r^2, of 0.96 and 0.998, respectively. The combined data are shown in Fig. 1 and yield an r^2 of 0.98 reflecting good osmotic linearity of bovine spermatozoa over the entire range of 122 to 1500 mOsm.

Previously Drevius (1972b), using spermatocrit measurements at 2000 x g, obtained a linear osmotic response for epididymal bull sperm but his data only cover the hypotonic range (51 to 353 mOsm). As a means of measuring cell volumes, spermatocrit data suffer from the presence of trapped extracellular medium. Hammerstedt et al. have made extensive studies of mammalian sperm utilizing ESR and for bull sperm reported (1978) a linear response between 300 to 700 mOsm but with some deviations from linearity below 300 mOsm. At the time of their studies the broadening agent CrOx was not in use. Instead, they used $NiCl_2$ which is known to be more damaging to cells and may account for the observed non-ideal osmotic behavior observed in hypotonic media.

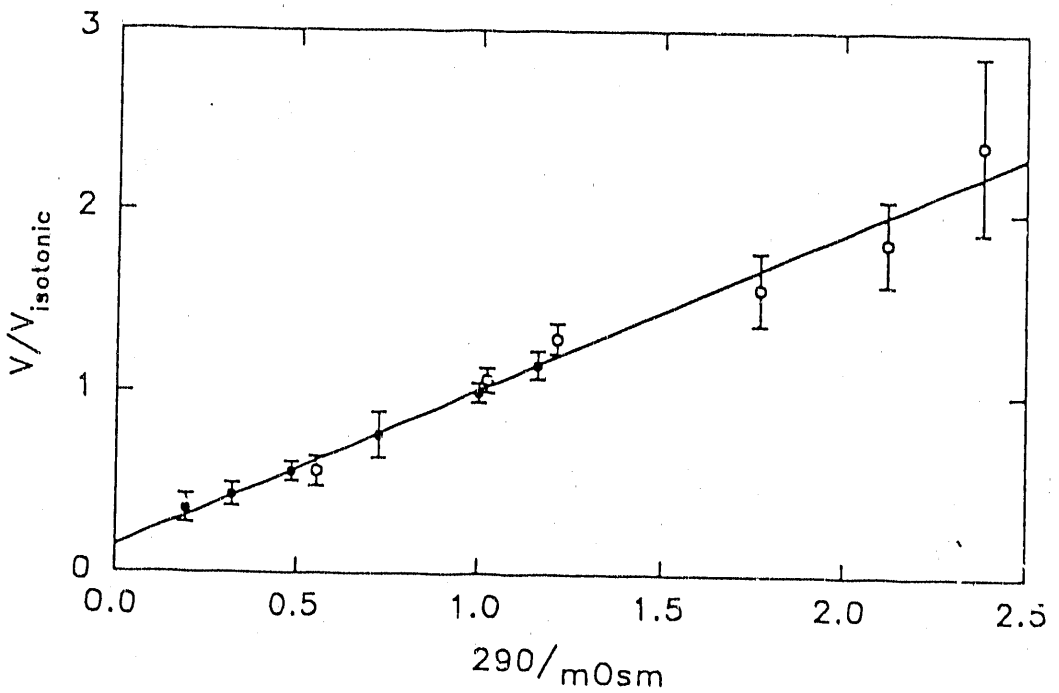

Figure 1. Osmotic response of bovine sperm using sucrose (o) and NaCl (•) to vary osmolality. The intracellular water volume, normalized to one at 290 mOsm, is plotted on the vertical axis. Means ± S.D. plotted with n = 9 for sucrose and n = 4 for NaCl.

Osmotically inactive water: In the usual Boyle van't Hoff plots of total cell volume versus reciprocal osmolality, the Y intercept is generally interpreted as the cell solids volume. Osmotically inactive water can not be determined from such a plot. In the ESR measurements, however, intracellular water volume is directly measured and in the simple case would be expected to fall to zero (Y intercept = 0) at infinite osmolality. The non-zero intercept (0.144 → V = 3 μm^3) actually obtained, Fig. 1, reflects a tempone accessible, osmotically inactive component of intracellular water. Expressed as a Ponder's R value, the intercept of 0.144 yields R = 0.86.

There are many hypotheses (Dick, 1966; Mazur and Schneider, 1986) to explain the existence of osmotically inactive water but the matter is not adequately resolved. The Ponder's R value of 0.86 is similar to the values found in RBC (Dick, 1966) and in bull sperm (Drevius, 1972b). The Boyle van't Hoff bull sperm plot by Hammerstedt et al. (1978) yields an unrealistic, negative intercept and sheds no light on this issue.

Summary: ESR spin label methods were used to directly measure cell volumes and it was found that bull sperm have an isotonic, intracellular aqueous volume of 21 μm^3, exhibit ideal osmotic behavior over the range 122 to 1500 mOsm, and exhibit a tempone accessible, osmotically inactive volume of 3 μm^3.

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