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Osmotic Tolerance of Equine Spermatozoa and the Effects of Soluble Cryoprotectants on Equine Sperm Motility, Viability, and Mitochondrial Membrane Potential

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ABSTRACT: Osmotic stress attributed to differences in the relative permeability of cryoprotectants, such as glycerol and water, appears to be an important factor in cryodamage. The objective of this study was to characterize the osmotic tolerance of equine spermatozoa, and to evaluate the effects of addition and removal of cryoprotectants from equine spermatozoa on their motility, and membrane and acrosomal integrity, as well as their mitochondrial membrane potential. Equine spermatozoa had a limited osmotic tolerance to anisosmotic conditions. Although the addition of increasing concentrations of glycerol decreased the motility and viability of equine spermatozoa, the rapid removal of glycerol by dilution in isosmotic media resulted in an even greater decline in motility and viability compared with spermatozoa maintained under anisosmotic conditions. Likewise, the addition and rapid removal of 1.0 M glycerol, ethylene glycol, dimethylsulfoxide, or propylene glycol resulted in a significant decline in sperm motility and viability. Among these cryoprotectants, ethylene glycol had the least detrimental effect on either viability or motility of spermatozoa following the rapid addition and removal of these cryoprotectants. These data demonstrate that equine spermatozoa have a limited osmotic tolerance compared with published reports for mouse or human spermatozoa, and appear to be more similar to boar spermatozoa in their osmotic tolerance. Of the 4 cryoprotectants evaluated in equine spermatozoa, the addition and removal of glycerol resulted in a more marked osmotic stress as indicated by alterations in motility, viability, and acrosomal integrity. These data suggest that alternative cryoprotectants should be considered for cryopreservation of equine spermatozoa in order to reduce osmotic stress associated with the addition of these agents during semen freezing.

Key words: Sperm, osmotic stress, horse, cryopreservation, glycerol, ethylene glycol, sperm function.

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Although the first foal born after insemination of cryopreserved equine semen was reported in 1957 (Barker and Gandier, 1957), many aspects of semen cryopreservation in the horse remain empirical with relatively little information available on the basic cryobiologic and biophysical stresses imposed during freezing and thawing. The success of cryopreservation of equine semen varies widely between stallions, and our understanding of the factors associated with this large intermale variation is poor (Amann and Pickett 1987; Vidament et al, 1997). Improvements in the success of semen cryopreservation in the horse require an understanding of the cryobiology of spermatozoa from this species. Because there are marked differences in the ability of spermatozoa from different species to survive cryopreservation, it is difficult to extrapolate across species relative to specific aspects of cryodamage. Therefore, parameters such as osmotic tolerance must be determined for the species of concern.

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Damage to spermatozoa during freezing and thawing can result in a reduction in the proportion of surviving spermatozoa, as well as an altered functional capacity of those spermatozoa that do survive (Watson, 2000). Damage during cryopreservation has been attributed to changes in temperature, ice crystal formation, oxidative damage, alterations in sperm membrane, DNA damage, toxicity of cryoprotectants, and osmotic stress (Watson 1995, 2000; Gao et al, 1997). Spermatozoa appear to be sensitive to osmotic stress associated with the addition and removal of molar amounts of cryoprotectants as well as alterations in solute concentrations during freezing (Gao et al, 1997; Watson 2000).

Osmotic stress attributed to differences in the relative permeabilities of cryoprotectants and water as well as the temperature dependence of these permeabilities has been the focus of considerable research efforts in spermatozoa of human (Gao et al, 1992; Gilmore et al, 1995, 1997) boar (Caiza de La Cueva et al, 1997c; Gilmore et al, 1998) bull (Watson et al, 1992; Liu and Foote 1998), ram (Curry and Watson, 1994; Curry et al, 2000), mouse (Willoughby et al, 1996; Phelps et al, 1999), and rabbit (Curry et al, 2000). Unfortunately, there are few published studies concerning the osmotic tolerance of equine sperma-

tozoa or the effects of cryoprotectants such as glycerol on sperm function. Noiles et al (1992) estimated a relatively high hydraulic conductivity (Lp) of equine spermatozoa (26 μM min^{-1} atm^{-1}) with a critical osmolality (osmolality at which 50% of spermatozoa lyse) of 47 mOsm. Other investigators have examined the relative increase in equine sperm volume as determined by spermatocrit over the range of 300 to 50 mOsm (Lagares et al, 2000) with concomitant reduction in sperm viability as determined by eosin exclusion. In two studies, Caiza de la Cueva et al (1997a,b) characterized the effects of osmotic stress on equine sperm viability and motility as well as ultrastructural changes associated with anisosmotic conditions. These studies point to osmotic stress as an additional factor to consider in cryodamage to equine spermatozoa.

Biophysical changes in the sperm plasma membrane during solute addition and removal appear to account for a least a portion of the damage incurred during cryopreservation. When glycerol-loaded cells are abruptly transferred to an isotonic, glycerol-free medium (such as skim milk extenders or to the female reproductive tract secretions) the intracellular environment is hyperosmotic relative to the extracellular environment. Because the permeability of most cryoprotectants is much lower than that of water, there is a net influx of water with a resulting increase in cell volume. Mammalian spermatozoa appear to behave as linear osmometers, and cell death occurs if the spermatozoa swells or shrinks beyond species-specific osmotic tolerances (Gilmore et al, 1995, 1996, 1998; Willoughby et al, 1996). In addition to cell lysis, membrane damage may occur secondary to rapid water movement across the membrane (Muldrew and McGann 1990; Curry and Watson, 1994).

The objectives of the current study were to characterize the osmotic tolerance of equine spermatozoa and to determine the effects of rapid addition and removal of permeable cryoprotectants on sperm motility, viability, acrosomal integrity, and mitochondrial membrane potential.

Materials and Methods

Sample Preparation

Semen samples were collected with an artificial vagina from light-horse stallions and filtered to remove gel. Raw semen was layered on a discontinuous Percoll gradient (40%/80%), and centrifuged (300 × *g* for 20 minutes) to isolate motile spermatozoa. Spermatozoa were then washed in isosmotic Tyrodes albumin lactate pyruvate (TALP; 325 mOsm) and resuspended to a final concentration of 600 × 10^6 cells/mL.

Media Preparation

A modified isosmotic Tyrodes medium (Bavister, 1989) was used in all experiments. Hypotonic TALP was prepared by reducing the amount of NaCl. Hypertonic TALP was made by adding NaCl, sucrose, or cryoprotectants to isosmotic TALP. In each experiment, osmolality was confirmed by measurement with a freezing point depression osmometer (Model 5004, Precision System Inc, Natick, Mass).

Experimental Design

Validation of Propidium Iodide Staining for Viability—The use of propidium iodide (PI) for determination of sperm viability via a microplate assay was validated by the use of fixed ratios of viable:dead spermatozoa (100:0, 75:25, 50:50, 25:75, and 0:100) with 4 ejaculates from 4 stallions (Papaioannou et al, 1997). After separation of spermatozoa from seminal plasma via Percoll gradient centrifugation, aliquots of spermatozoa were killed by plunging them into liquid nitrogen and rewarming to 37°C (3 cycles). Membrane integrity was determined by PI staining (12 μM final concentration; Molecular Probes, Eugene, Ore). After 5 minutes of incubation with PI at 22°C, the samples were aliquoted in duplicate wells (200 μL each) and read by a fluorescence microplate reader (Model HTS 7000; Perkin Elmer, Norwalk, Conn). The $\lambda_{excitation}$ and $\lambda_{emission}$ for PI were 550 nm and 635 nm, respectively. The uptake and fluorescence of PI was also determined in frozen-thawed spermatozoa after permeabilization with digitonin (10 μg/mL) in order to control for the effects of osmolality. After blank correction, PI fluorescence was evaluated by regression analysis.

Experiment 1: Effect of Anisosmotic Conditions on Motility and Membrane Integrity of Equine Spermatozoa—The effect of osmolality on sperm motility and membrane integrity was evaluated by incubation of equine spermatozoa in TALP with osmolality ranging between 90–600 mOsm. For this study, spermatozoa (325 mOsm, 600 × 10^6 cells/mL) were diluted (1:15) in each anisosmotic TALP (90, 150, 200, 325, 400, 500, or 600 mOsm final osmolality) and incubated for 10 minutes at 22°C. Sperm motility was determined in each sample by computer-assisted semen analysis (CASA; CEROS Analyzer; Hamilton Thorne Research, Beverly, Mass). Membrane integrity was determined by exclusion of PI as described above. In a subset of samples, spermatozoa were returned to near isosmotic conditions by dilution (1:6) in isosmotic TALP, and motility was determined by CASA at 1- and 5-minute intervals after dilution.

Experiment 2: Effect of Glycerol Addition and Removal on Motility, Mitochondrial Membrane Potential, Viability, and Acrosomal Integrity of Equine Spermatozoa—The effect of addition and removal of the membrane-permeable cryoprotectant, glycerol, on sperm motility and viability was determined in a series of experiments. Spermatozoa were isolated on Percoll density gradients, washed, and resuspended at a final concentration of 600 × 10^6 cells/mL in isosmotic TALP. This stock sperm suspension was subsequently diluted (1:2.14) by the rapid addition of TALP with glycerol to yield final osmolalities of 325, 600, 900, 1200, 1500, and 1800 mOsm. Spermatozoa were incubated for 10 minutes, and motility was determined by CASA. Each treatment was subsequently diluted (1:7; final sperm concentration of 40 × 10^6 cells/mL) in either the corresponding anisosmotic TALP or in isosmotic TALP, and motility was again determined by CASA at 5 and 10 minutes after dilution. Samples were loaded with PI (as in experiment 1), and fluorescence was

determined in duplicate wells for determination of membrane integrity.

Effects on Mitochondrial Membrane Potential—For this experiment, spermatozoa were prepared as above (experiment 2) to yield a final concentration of 40 × 10^6 cells/mL. A 5.56-μL volume of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; 250 μM; Molecular Probes) was added to each 700-μL sample, and samples were incubated for 20 minutes at 22°C. Samples were loaded in 96-well microplates as triplicate wells and orange ($\lambda_{excitation}$ = 485; $\lambda_{emission}$ = 595) and green ($\lambda_{excitation}$ = 485; $\lambda_{emission}$ = 535) fluorescence was determined. Mitochondrial membrane potential was expressed as the ratio of orange:green fluorescence normalized to control values (325 mOsm; Gravance et al, 2000).

Effects on Viability and Acrosomal Integrity—Samples were prepared as above, except final cell concentration was adjusted to 25 × 10^6 cells/mL and viability and acrosomal integrity were evaluated based upon a modification of the method of Wilhelm et al (1996). To each aliquot of 100-μL sample, 4 μL of fluorescein isothiocyanate–*Pisum sativum* agglutinin (FITC-PSA; 5 mg/mL) was added and incubated for 10 minutes prior to the addition of 2 μL of PI (2 mg/mL). After 5 minutes of incubation, samples were further diluted to 5 × 10^6 cells/mL before assessment of individual samples for PI and FITC-PSA staining. A total of approximately 10 000 gated events were analyzed per sample with the flow cytometer (FACScan; Becton-Dickinson, Franklin Lakes, NJ). The green fluorescence 1 (FL1-H: FITC-PSA) was collected through a 525-nm bandpass filter and the red fluorescence 2 (FL2-H: PI) was collected through a 635-nm bandpass filter. The relative distribution of green- and red-stained sperm populations were collected as dot plot histograms for FL1-H and FL2-H, and expressed as the percentage of spermatozoa in each histogram. Populations of spermatozoa were classified as live-acrosome–intact, live-acrosome–damaged, dead-acrosome–intact, or dead-acrosome–damaged based on analysis of scatter plots.

Experiment 3: Effects of Addition and Removal of 1.0 M Concentrations of Permeable Cryoprotectants on Equine Sperm Motility, Viability, and Acrosomal Integrity—Spermatozoa were separated from seminal plasma as described in experiment 2 and resuspended in TALP at a final concentration of 600 × 10^6 cells/mL in isosmotic TALP. This stock sperm suspension was subsequently diluted (1:1) by the rapid addition of TALP containing either 2.0 M glycerol, dimethylsulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), or control (isosmotic TALP). Samples were incubated for 5 minutes at 22°C prior to dilution (1:6) in a 1.0 M concentration of the corresponding TALP (1.0 M group) or dilution in isosmotic TALP (return to isosmolar group). Samples were subsequently incubated for 10 minutes prior to determination of total and progressive motility via CASA. Additional aliquots from each treatment were labeled with PI and evaluated via a fluorescence microplate reader (see experiment 1 for methods). The uptake and fluorescence of PI was also determined in frozen-thawed spermatozoa after permeabilization with digitonin (10 μg/mL) in order to control for the effects of cryoprotectants. In a separate experiment, spermatozoa were treated as above and viability and acrosomal integrity were determined via flow cytometry (see experiment 2 for methods).

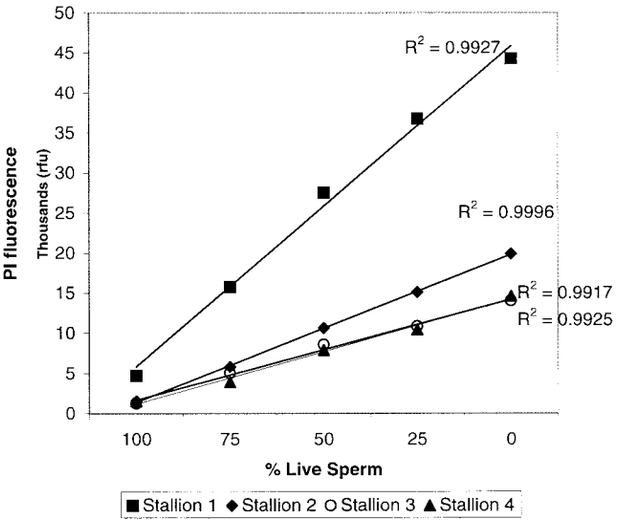

Figure 1. Linear regression plot for PI fluorescence (arbitrary units) and the proportion of viable spermatozoa. Spermatozoa were killed by freezing and thawing and added to live spermatozoa to obtain various proportions of live spermatozoa. Fluorescence of PI was determined by a fluorescence microplate reader. Each line represents spermatozoa from an individual stallion. Overall regression equation was Y = 10.766 − 0.097 × X; R^2 = 0.927, where Y = PI fluorescence and X = % live spermatozoa.

Statistical Analysis

Data were analyzed by analysis of variance, and differences between individual means were compared with the Fisher protected least significant difference (Statview; SAS Institute, Cary, NC). In some experiments, data were normalized to control values prior to analysis. Data are presented as least-squares means ± SEM.

Results

Validation of Propidium Iodide Staining for Viability

Propidium iodide fluorescence was linearly related to the percentage of viable spermatozoa (Y = 10.766 − 0.097 × X; R^2 = 0.927). For individual stallions, blank-corrected PI fluorescence had regression coefficients of greater than 0.99 (Figure 1). Changes in osmolality did not (P = .5) affect PI fluorescence of digitonin-permeabilized, frozen-thawed spermatozoa (data not shown).

Experiment 1

When osmolality of TALP was altered by adjusting NaCl content, motility of spermatozoa decreased (P < .01) at isosmolar ± 100 mOsm (Figure 2). Although reduction in osmolality decreased the membrane integrity of spermatozoa as measured by exclusion of PI, increases in osmolality did not affect membrane integrity through the

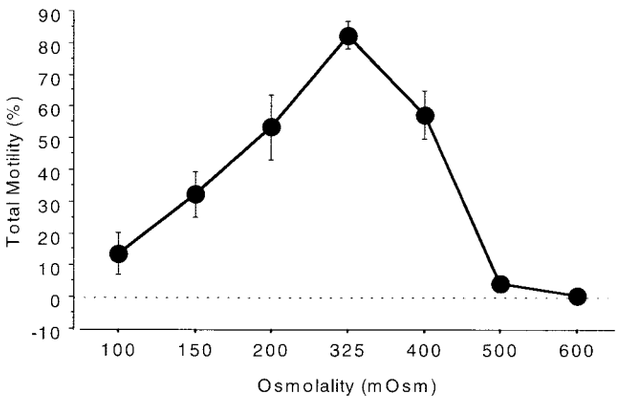

Figure 2. Effect of osmolality on motility of equine spermatozoa. Spermatozoa were separated via Percoll gradient centrifugation and resuspended in TALP with osmolality adjusted by changing [NaCl]. Sperm were incubated for 10 minutes (22°C), and motility was determined via CASA. Data are least squares means ± SEM. (n = 5 experiments, 3 stallions).

range of osmolalities tested (Figure 3). When equine spermatozoa were exposed to anisosmotic conditions and then abruptly returned to near isosmotic conditions, motility declined but did not differ from that observed when spermatozoa were maintained at anisosmotic conditions (Figure 4).

Experiment 2: Effects of Glycerol Addition and Removal

The addition of increasing concentrations of the permeant cryoprotectant, glycerol, decreased the motility of equine spermatozoa (Figure 5). When spermatozoa that were equilibrated with glycerol were abruptly returned to near

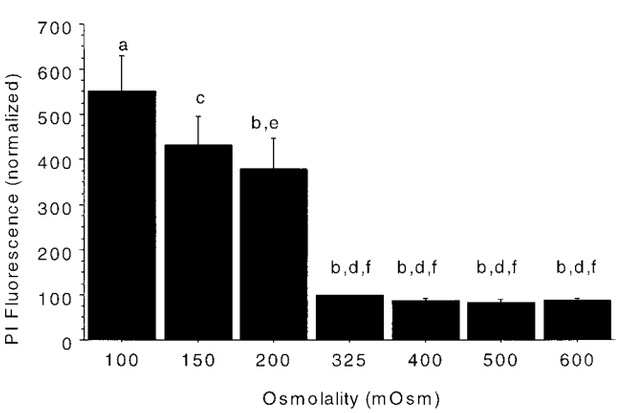

Figure 3. Membrane integrity of equine spermatozoa after exposure to different anisosmotic conditions as determined by exclusion of PI. Spermatozoa were separated by Percoll gradient centrifugation and resuspended in TALP with osmolality adjusted by altering [NaCl]. Spermatozoa were incubated for 10 minutes at 22°C and loaded with PI. Fluorescence was determined by microplate reader (excitation = 550 nm; emission = 635 nm) in triplicate wells of a 96-well plate. Data are normalized to 325 mOsm. Values with different superscripts (a,b; c,d; e,f) differ ($P < .01$). Data are least squares means ± SEM. (n = 5 experiments, 3 stallions).

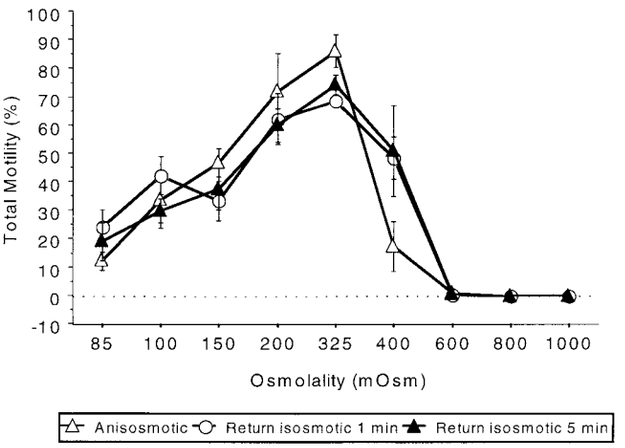

Figure 4. Effect of anisosmotic conditions on motility of equine spermatozoa and subsequent change in motility after dilution to near isosmotic conditions at 1 and 5 minutes of incubation. Spermatozoa were separated via Percoll gradient centrifugation and resuspended in TALP with osmolality adjusted by reducing [NaCl] in hypotonic media or adding sucrose in hypertonic media. Sperm were incubated for 10 minutes (22°C), and motility was determined (anisosmotic). Sperm were subsequently diluted in isosmotic TALP, and motility was recorded at 1 and 5 minutes after return to near isosmotic conditions. Data are least squares means ± SEM. (n = 4 experiments, 3 stallions).

isosmotic conditions by dilution, there was a significant reduction ($P < .0001$) in motility for all concentrations of glycerol tested (Figure 5). Motility did not differ between 5 and 10 minutes following re-equilibration of spermatozoa into isosmotic conditions. Mitochondrial membrane potential was not significantly altered by increased osmolality associated with increased glycerol concentration (Figure 6). When spermatozoa were abruptly returned to near isosmotic conditions, however, there was a significant decline ($P < .0001$) in mitochondrial

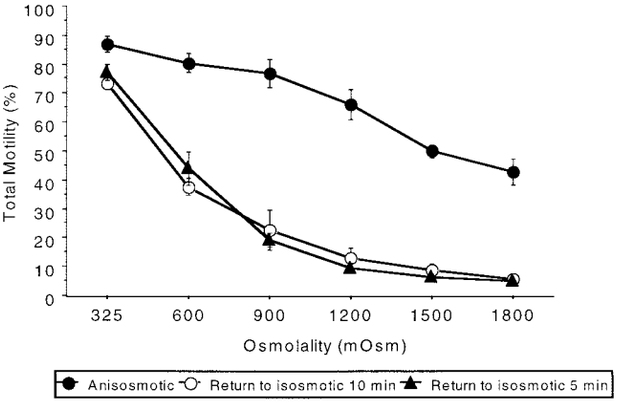

Figure 5. Effect of glycerol addition and removal on motility of equine spermatozoa. Osmolality of TALP was adjusted by the addition of glycerol. Sperm were incubated for 10 minutes (22°C) and motility was determined (anisosmotic). Sperm were subsequently diluted in isosmotic TALP and motility was recorded at 5 and 10 minutes after return to near isosmotic conditions. Data are least squares means ± SEM. (n = 5 experiments, 4 stallions).

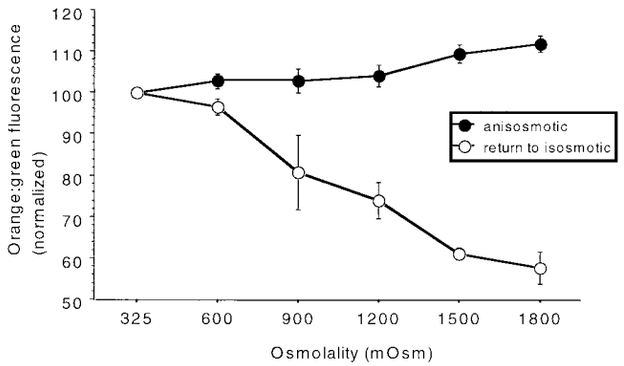

Figure 6. Effect of glycerol addition and removal on mitochondrial membrane potential of equine spermatozoa. Osmolality of TALP was adjusted by the addition of glycerol. Sperm were incubated for 10 minutes (22°C), and sperm were loaded with JC-1 (anisosmotic). Additional aliquots of sperm were subsequently diluted in isosmotic TALP, and loaded with JC-1. After 20 minutes of incubation, samples were loaded into 96-well microplates in triplicate wells. Orange (emission = 595) and green (emission = 535) fluorescence of JC-1 loaded sperm were determined via a microplate reader (excitation = 485). Data are ratio of orange:green fluorescence normalized to 325 mOsm. Data are least squares means ± SEM. (n = 4 experiments, 4 stallions).

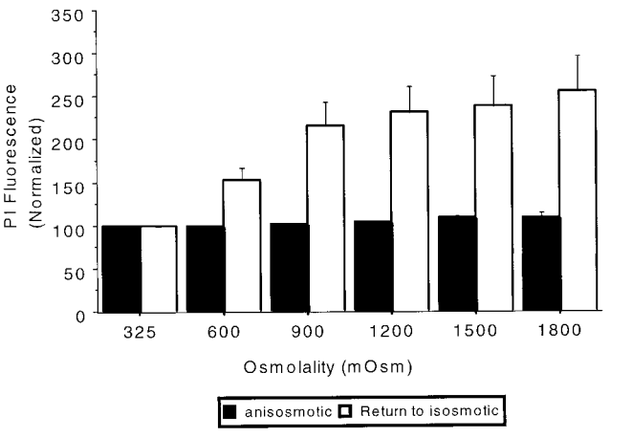

Figure 7. Effect of glycerol addition and removal on membrane integrity of equine spermatozoa. Osmolality of TALP was adjusted by the addition of glycerol. Sperm were incubated for 10 minutes (22°C), and sperm were loaded with PI (anisosmotic). Additional aliquots of sperm were subsequently diluted in isosmotic TALP, and loaded with PI. After a 5-minute incubation, samples were loaded into 96-well microplates in triplicate wells. Fluorescence of PI-loaded sperm was determined via a microplate reader (excitation = 550 nm; emission = 635 nm). Data are normalized to 325 mOsm. Data are least squares means ± SEM. (n = 7 experiments, 4 stallions).

membrane potential (Figure 6). Exposure of spermatozoa to increasing glycerol concentrations increased ($P < .01$) the uptake of PI at greater than 1500 mOsm (Figure 7); however, return of equine spermatozoa to near isosmotic conditions significantly ($P < .005$) increased the uptake of PI compared with spermatozoa maintained at anisosmotic conditions at all osmolalities greater than 325 mOsm (Figure 7). The percentage of live, acrosome-intact spermatozoa did not decrease during exposure to increased concentrations of glycerol; however, the abrupt removal of glycerol by returning spermatozoa to near isosmotic conditions significantly reduced ($P < .01$) the percentage of live, acrosome-intact spermatozoa (Figure 8). Changes in the percentage of live, acrosome-intact spermatozoa were due entirely to changes in PI exclusion because the percentage of acrosome-intact spermatozoa was not significantly affected by changes in osmolality or the subsequent removal of glycerol by dilution (data not shown).

Experiment 3: Effect of Addition and Removal of 1.0 M Cryoprotectants

There was no significant ($P > .1$) effect of cryoprotectant on the uptake and fluorescence of PI in digitonin-permeabilized, frozen-thawed spermatozoa (data not shown). There were significant ($P < .0001$) effects of treatment (1.0 M cryoprotectant or return to isosmotic conditions), and cryoprotectant (glycerol, EG, DMSO, PG, or control) on viability and motility as well as a significant ($P < .05$) treatment × cryoprotectant interaction on viability (Figures 9 and 10). The addition of 1.0 M glycerol decreased viability, whereas the addition of 1.0 M DMSO improved viability compared with the control (Figure 9). The abrupt removal of all cryoprotectants decreased viability compared with control (Figure 9). The decrease in viability was comparable for DMSO, EG, and PG, and was significantly lower than the decrease in viability observed after the abrupt removal of glycerol (Figure 9). The addition or removal of all cryoprotectants significantly (*P*

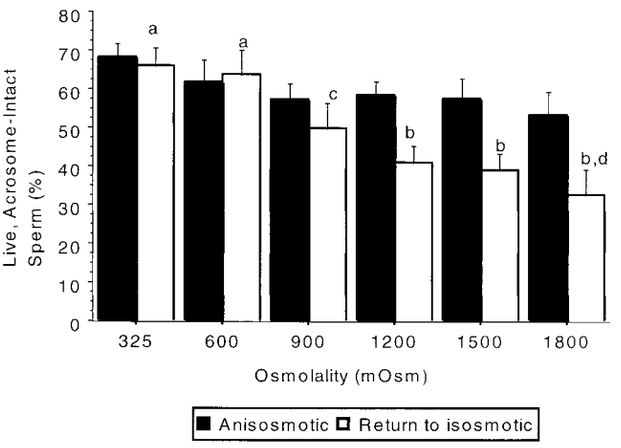

Figure 8. Effect of glycerol addition and removal on viability and acrosomal integrity of equine spermatozoa. Osmolality of TALP was adjusted by the addition of glycerol. Sperm were incubated for 10 minutes (22°C), and sperm were stained with FITC-PSA and PI (anisosmotic). Additional aliquots of sperm were subsequently diluted in isosmotic TALP and loaded with FITC-PSA and PI (return to isosmotic). Fluorescence of stained sperm was determined via flow cytometry and the percentage of live, acrosome-intact sperm was determined. Values with different superscripts (a,b: $P < .01$; c,d: $P < .05$) differ. Data are least squares means ± SEM. (n = 6 experiments, 3 stallions).

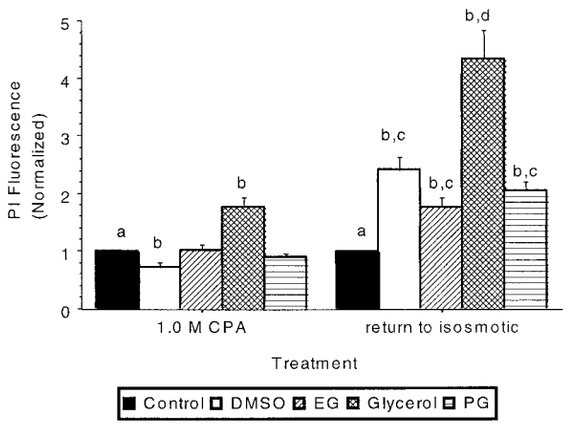

Figure 9. Effect of addition and removal of 1.0 M cryoprotectants (CPA) on membrane integrity of equine spermatozoa. Osmolality of TALP was adjusted by the addition of 1.0 M DMSO, EG, glycerol, or PG. Sperm incubated in TALP alone served as the control. Sperm were incubated for 10 minutes (22°C), and sperm were loaded with PI (anisosmotic). Additional aliquots of sperm were subsequently diluted in isosmotic TALP, and loaded with PI. After a 5-minute incubation, samples were loaded into 96-well microplates in triplicate wells. Fluorescence of PI-loaded sperm was determined via a microplate reader. Data are normalized to 325 mOsm. Within treatments (1.0 M CPA or return to isosmotic), values with different superscripts (a,b; c,d) differ ($P < .05$). Data are least squares means ± SEM. (n = 10 experiments, 4 stallions).

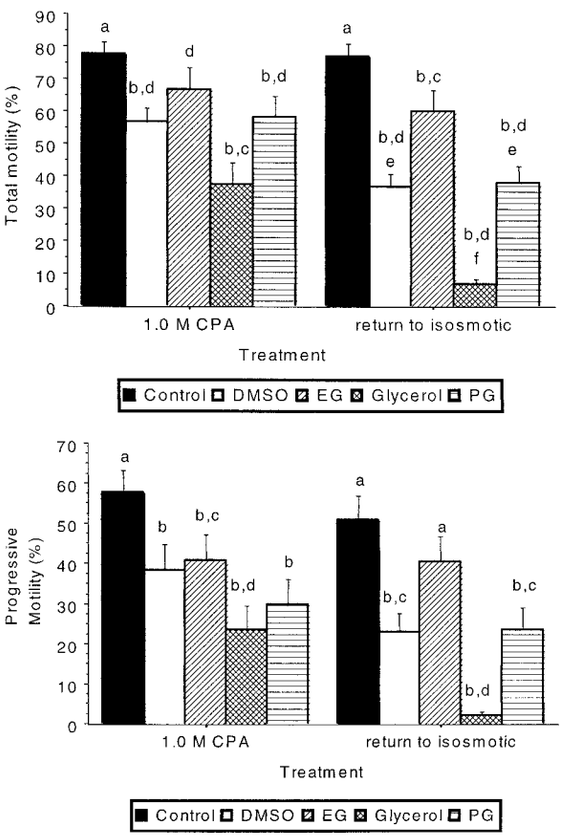

Figure 10. Effect of addition and removal of 1.0 M cryoprotectants on motility of equine spermatozoa. Osmolality of TALP was adjusted by the addition of 1.0 M DMSO, EG, glycerol, or PG. Sperm incubated in TALP alone served as the control. Sperm were incubated for 10 minutes (22°C), and motility was determined by CASA. Additional aliquots of sperm were subsequently diluted in isosmotic TALP, and motility was determined by CASA. Within treatments, values with different superscripts (a,b; c,d) differ ($P < .05$). Data are least squares means ± SEM. (n = 10 experiments, 4 stallions).

$< .05$) reduced total motility (Figure 10, top) compared with control. Likewise, the addition or abrupt removal of 1.0 M cryoprotectants reduced ($P < .01$) both total and progressive motility compared with control for all cryoprotectants except EG, which did not differ from control (Figure 10, bottom). Of the cryoprotectants tested, the abrupt removal of glycerol after returning cells to isosmotic conditions resulted in the greatest ($P < .01$) decrease in total and progressive motility (Figure 10). In contrast, the abrupt removal of EG resulted in the lowest ($P < .01$) decline in total and progressive motility of all cryoprotectants tested. Based on FITC-PSA and PI staining, abrupt removal of glycerol resulted in the largest decline in viable, acrosome-intact spermatozoa (Figure 11).

Discussion

Osmotic Tolerance of Equine Spermatozoa

Equine spermatozoa appear to have a limited osmotic tolerance compared with spermatozoa of mouse (Willoughby et al, 1996) or human (Gao et al, 1995) and appear similar to boar spermatozoa, which also have a very limited osmotic tolerance (Gilmore et al, 1998). Total motility of equine spermatozoa fell to less than 50% at isosmolal ± 100 mOsm and declined to less than 10% at 100 and 500 mOsm. Although motility declined rapidly under hyperosmotic conditions, there was no change in plasma membrane integrity through 600 mOsm, similar to reports in spermatozoa of human (Gao et al, 1995), boar (Gilmore et al, 1996), bull (Liu and Foote, 1998), and mouse (Willoughby et al, 1996). The decline in sperm motility noted under hyperosmotic conditions did not appear to be related to changes in ionic strength because similar declines were noted when either NaCl or sucrose was used as the osmolyte. Because integrity of the plasma membrane was not altered under the range of hyperosmotic conditions tested, it appears that the alteration in sperm motility must be due to other factors.

In contrast to hyperosmotic conditions, both motility and plasma membrane integrity decreased under hyposmotic conditions. similar to reports in other mammalian spermatozoa (Gao et al, 1995; Gilmore et al, 1996; Willoughby et al, 1996). For equine spermatozoa, the reduction in sperm viability appeared linear in the range of 100–325 mOsm, which is well above the critical osmolality of 47 mOsm estimated previously for equine sper-

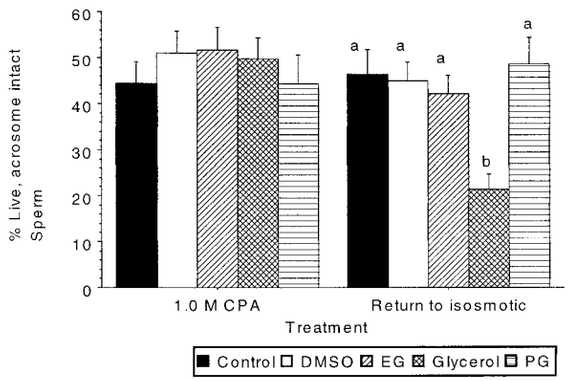

Figure 11. Effect of addition and removal of 1.0 M cryoprotectants on viability and acrosomal integrity of equine spermatozoa. Osmolality of TALP was adjusted by the addition of 1.0 M DMSO, EG, glycerol, or PG. Sperm incubated in TALP alone served as the control. Sperm were incubated for 10 minutes (22°C), and sperm were stained with FITC-PSA and PI (anisosmotic). Additional aliquots of sperm were subsequently diluted in isosmotic TALP, and also loaded with FITC-PSA and PI (return to isosmotic). Fluorescence of stained sperm was determined via flow cytometry, and the percentage of live, acrosome-intact sperm was determined. Within treatments, values with different superscripts (a,b) differ ($P < .01$). Data are least squares means ± SEM. (n = 6 experiments, 3 stallions).

matozoa (Noiles et al, 1992). In an earlier study, the volume of equine spermatozoa increased linearly on the basis of spermatocrit measurements between 300 to 100 mOsm, and there was a rapid loss of viable spermatozoa between 100 and 50 mOsm (Lagares et al, 2000). Together, these studies suggest that critical osmolality of equine spermatozoa is approximately 50 mOsm, and that equine spermatozoa may act as linear osmometers within this range of osmotic pressure. Measurement of sperm volume changes in response to changes in osmolality will be needed to confirm this hypothesis for equine spermatozoa.

From these studies, it appears that damage to the equine sperm plasma membrane occurs as a result of osmotic stress at osmolalities above that at which the cell has reached its maximal volume. Under hyposmotic conditions, at least part of the reduction in motility appeared related to damage of the plasma membrane, as reflected by an increased uptake of PI. In addition to lysis of the cell under hyposmotic conditions, damage to the plasma membrane has been related to a high water flux through the plasma membrane under hyposmotic conditions (Muldrew and McGann, 1990). Whatever damage was induced after exposure to hyposmotic stress was not reversible after return of equine spermatozoa to isosmotic conditions because motility did not improve subsequent to reestablishing normal osmolality. This result is similar to that reported in boar spermatozoa (Gilmore et al, 1998); however, in both mouse and human spermatozoa, motility improved subsequent to return to isosmotic conditions (Gao et al, 1995; Willoughby et al, 1996). The difference in response subsequent to return of sperm to isosmotic conditions may reflect differences in the relative osmotic tolerance of sperm from these species.

Although the basis for species differences in osmotic tolerance is not well defined, previous studies with equine spermatozoa indicate that resistance to osmotic stress is dependent upon a ouabain-sensitive Na^+/K^+ ATPase and an amiloride-sensitive Na^+/H^+ antiporter (Caiza de la Cueva et al, 1997a,b). These ion pumps appear to play an important role in the maintenance of normal intracellular osmolality in equine sperm, and inhibition of these pumps reduced the tolerance of equine spermatozoa to changes in osmotic pressure (Caiza de la Cueva et al, 1997a).

In addition to the active movement of ionic constituents, there is increasing evidence that spermatozoa may possess selective water channels that facilitate movement of water across the sperm membrane at a greater rate than can be accounted for by diffusion alone. Spermatozoa generally have a very high water permeability (Noiles et al, 1993; Liu et al, 1995) that appears to be mediated by the presence of water channels (Curry et al, 1995; Liu et al, 1995; Ishibashi et al, 1997; Sitaramam and Sauna 2000). Evidence for the nature of these water channels (aquaporins) in spermatozoa has been lacking (Liu et al, 1995); however, an aquaporin has recently been identified in rat testis and spermatozoa, and appears to serve as a channel that allows permeation of both water and glycerol (Ishibashi et al, 1997). Although the functional significance of a high water permeability through specific channels in spermatozoa has not been well defined, there is a suggestion on an evolutionary basis that such mechanisms may be important in the function of spermatozoa from aquatic species with external fertilization (Sitaramam and Sauna, 2000). Whatever the evolutionary basis for these water channels, the high water permeability of spermatozoa directly effects their sensitivity to osmotic stress induced during the addition and removal of permeable solutes such as glycerol.

Effect of Addition and Removal of Glycerol on Equine Sperm Function

The addition of increasing concentrations of the permeable solute, glycerol (600–1800 mOsm), resulted in a decline in sperm motility and viability; however, the reduction in motility was not as great as that observed when osmolality was increased by the addition of nonpermeable solutes. This observation is consistent with earlier studies in bull (Liu and Foote, 1998), mouse, boar, and human spermatozoa (Gao et al, 1995) and reflects the ability of glycerol to reach equilibrium across the plasma membrane.

The rapid, one-step removal of glycerol from equine spermatozoa by dilution to near isosmotic conditions resulted in a marked decline in both motility and plasma

membrane integrity for all concentrations of glycerol tested. Similar reductions in sperm motility have been noted after the rapid removal of glycerol from human (Gao et al, 1995; Gilmore et al, 1997) and mouse (Phelps et al, 1999) spermatozoa. In contrast, changes in human sperm viability subsequent to the rapid, one-step removal of 1.0 or 1.5 M glycerol did not appear as marked as those noted after the addition and removal of 1.0 M glycerol from equine spermatozoa in the present study (Gao et al, 1993, 1995).

The origin of osmotic stress after the rapid removal of molar concentrations of glycerol has been reported for many cell types (Gao et al, 1997) and is related to differences in the relative permeability of glycerol and water across the sperm plasma membrane. After exposure to increasing concentrations of a permeable solute such as glycerol, spermatozoa shrink due to a loss of water and then swell as water and the permeating solute move into the cell. When glycerol-loaded spermatozoa are placed into an isosmotic media, cells initially swell as water enters the cell and then shrink as water and the solute move out of the cell. The differential movement of water and glycerol across the sperm plasma membrane appears to account for the rapid decline in both motility and viability in spermatozoa after the rapid removal of glycerol by dilution.

Changes in motility of spermatozoa after addition and removal of glycerol in the present study may have been associated with disruption of plasma membrane integrity (as determined by PI uptake) as well as to a disruption or loss of mitochondrial membrane potential (as measured by JC-1 fluorescence). Willoughby et al (1996) reported that increasing osmolality did not reduce uptake of rhodamine 123 by murine spermatozoa; however, return to isosmotic conditions resulted in a posthyperosmotic stress with a significant reduction in R123 uptake. Therefore, it appears that damage to the plasma membrane along with disruption of the mitochondria may be associated with the decline in motility noted with osmotic stress.

Effect of Addition and Removal of 1.0 M Cryoprotectants

Our results demonstrate a deleterious effect of rapid addition and an even greater effect of rapid removal of 1.0 M concentrations of cryoprotectants on equine sperm viability and motility. These findings are similar to those reported previously in human (Gilmore et al, 1995, 1997) and mouse (Phelps et al, 1999) spermatozoa. Of the cryoprotectants tested, EG appeared to have the least detrimental effect on sperm viability and motility, whereas glycerol had the greatest deleterious effect on these parameters compared with spermatozoa maintained in isosmolar TALP. This observation is also consistent with previous work in mouse (Phelps et al, 1999) and human (Gilmore et al, 1997) spermatozoa. A differential effect on rapid removal of EG and glycerol is consistent with the measured permeabilities of these cryoprotectants as reported in human (Gilmore et al, 1995), boar (Gilmore et al, 1998), and mouse (Phelps et al, 1999) spermatozoa. In those species in which permeability estimates are available, the permeability of EG is approximately 1.5–3 times that of glycerol.

It is interesting that the results observed with DMSO appeared to differ from those predicted from relative permeability of this cryoprotectant in other species. DMSO was approximately 10 times less permeable than EG in human spermatozoa (Gilmore et al, 1995) and 2 times less permeable in boar spermatozoa (Gilmore et al, 1998). However, in the present study, DMSO and PG were intermediate between glycerol and EG in posthyperosmotic stress measured by changes in either motility or viability. There appears to be considerable species difference in the relative permeability of cryoprotectants and determination of these parameters for equine spermatozoa along with estimates of their temperature dependence will be important in determining improved methods for addition and removal of cryoprotectants during cyropreservation of equine sperm.

Summary—Relevance to Sperm Cryopreservation

Although the experiments reported here dealt only with unfrozen equine spermatozoa, the results of these studies provide new information relative to osmotic tolerance of equine spermatozoa, as well as the relative effects of rapid addition and removal of various cryoprotectants from equine spermatozoa. As reported for other species, the rapid addition, and more importantly, the rapid removal of glycerol from equine spermatozoa, resulted in marked decline in viability, motility, as well as mitochondrial membrane potential. Of the four cryoprotectants evaluated, glycerol appeared to induce the greatest osmotic stress, whereas DMSO and PG were intermediate, and EG caused the least osmotic damage subsequent to rapid removal of the cryoprotectant. These data suggest that alternative cryoprotectants such as EG should be considered as cryoprotectants for equine spermatozoa and that continued research is required in order to gain a better understanding of osmotic stress as it applies to cryopreservation of spermatozoa in the horse.

References

Amann RP, Pickett BW. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *J Equine Vet Sci.* 1987;7: 145–173.

Barker CAV, Gandier JCC. Pregnancy in a mare resulted from frozen epididymal spermatozoa. *Can J Comp Med Vet.* 1957;21:47–51.

Bavister BD. A consistently successful procedure for in vitro fertilization of Golden hamster eggs. *Gamete Res.* 1989;23:139–158.

Caiza de la Cueva FI, Pujol MR, Rigau T, et al. Resistance to osmotic stress of horse spermatozoa: the role of ionic pumps and their relationship to cryopreservation success. *Theriogenology.* 1997a;48:947–968.

Caiza de la Cueva FI, Rigau T, Bonet S, Miro J, Briz M, Rodriguez-Gil JE. Subjecting horse spermatozoa to hypoosmotic incubation: effects of ouabain. *Theriogenology.* 1997b;47:765–784.

Caiza de La Cueva F, Rigau T, Pujol R, Piedrafita J, Rodraiguez-Gil JE. Resistance to hyperosmotic stress in boar spermatozoa: the role of the ionic pumps and the relationship with cryosurvival. *Anim Reprod Sci.* 1997c;48:301–315.

Curry MR, Kleinhans FW, Watson PF. Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. *Cryobiology.* 2000;41:167–173.

Curry MR, Millar JD, Watson PF. The presence of water channel proteins in ram and human sperm membranes. *J Reprod Fertil.* 1995;104:297–303.

Curry MR, Watson PF. Osmotic effects on ram and human sperm membranes in relation to thawing injury. *Cryobiology.* 1994;31:39–46.

Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. *Biol Reprod.* 1993;49:112–123.

Gao DY, Liu J, Liu C, et al. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Hum Reprod.* 1995;10:1109–1122.

Gao D, Mazur P, Critser JK. Fundamental cryobiology of mammalian spermatozoa. In: Karow AM, Critser JK, eds. *Reproductive Tissue Banding: Scientific Principles*. San Diego, Calif: Academic Press; 1997:263–328.

Gao DY, Mazur P, Kleinhans FW, Watson PF, Noiles EE, Critser JK. Glycerol permeability of human spermatozoa and its activation energy. *Cryobiology.* 1992;29:657–667.

Gilmore JA, Du J, Tao J, Peter AT, Critser JK. Osmotic properties of boar spermatozoa and their relevance to cryopreservation. *J Reprod Fertil.* 1996;107:87–95.

Gilmore JA, Liu J, Gao DY, Critser JK. Determination of optimal cryoprotectants and procedures for their addition and removal from human spermatozoa. *Hum Reprod.* 1997;12:112–118.

Gilmore JA, Liu J, Peter AT, Critser JK. Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. *Biol Reprod.* 1998;58:28–36.

Gilmore JA, McGann LE, Liu J, Gao DY, Peter AT, Kleinhans FW, Critser JK. Effect of cryoprotectant solutes on water permeability of human spermatozoa. *Biol Reprod.* 1995;53:985–995.

Gravance CG, Garner DL, Baumber J, Ball BA. Assessment of equine sperm mitochondrial function using JC-1. *Theriogenology.* 2000;53:1691–1703.

Ishibashi K, Kuwahara M, Gu Y, et al. Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J Biol Chem.* 1997;272:20782–20786.

Lagares MA, Petzoldt R, Sieme H, Klug E. Assessing equine sperm-membrane integrity. *Andrologia.* 2000;32:163–167.

Liu C, Gao D, Preston GM, McGann LE, Benson CT, Critser ES, Critser JK. High water permeability of human spermatozoa is mercury-resistant and not mediated by CHIP28. *Biol Reprod.* 1995;52:913–919.

Liu Z, Foote RH. Osmotic effects on volume and motility of bull sperm exposed to membrane permeable and nonpermeable agents. *Cryobiology.* 1998;37:207–218.

Muldrew K, McGann LE. Mechanisms of intracellular ice formation. *Biophys J.* 1990;57:525–532.

Noiles EE, Mazur P, Benker FW, Kleinhaus FW, Amann RP, Critser JK. Critical osmolarity, water and glycerol permeability coefficient determination for equine spermatozoa. *Biol Reprod.* 1992;46(suppl 1):95.

Noiles EE, Mazur P, Watson PF, Kleinhans FW, Critser JK. Determination of water permeability coefficient for human spermatozoa and its activation energy. *Biol Reprod.* 1993;48:99–109.

Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, Roche JF. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. *Theriogenology.* 1997;48:299–312.

Phelps MJ, Liu J, Benson JD, Willoughby CE, Gilmore JA, Critser JK. Effects of Percoll separation, cryoprotective agents, and temperature on plasma membrane permeability characteristics of murine spermatozoa and their relevance to cryopreservation. *Biol Reprod.* 1999;61:1031–1041.

Sitaramam V, Sauna ZE. What does a common channel for electrolytes and non-electrolytes in the sperm mean? *J Theor Biol.* 2000;206:419–428.

Vidament M, Dupere AM, Julienne P, Evain A, Noue P, Palmer E. Equine frozen semen: freezability and fertility field results. *Theriogenology.* 1997;48:907–917.

Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev.* 1995;7:871–891.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60–61:481–492.

Watson PF, Kunze E, Cramer P, Hammerstedt RH. A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. *J Androl.* 1992;13:131–138.

Wilhelm KM, Graham JK, Squires EL. Effects of phosphatidylserine and cholesterol liposomes on the viability, motility, and acrosomal integrity of stallion spermatozoa prior to and after cryopreservation. *Cryobiology.* 1996;33:320–329.

Willoughby CE, Mazur P, Peter AT, Critser JK. Osmotic tolerance limits and properties of murine spermatozoa. *Biol Reprod.* 1996;55:715–727.