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Cryopreservation in Different Concentrations of Glycerol Alters Boar Sperm and Their Membranes

MARY M. BUHR,* PAUL FISER,† JANICE L. BAILEY,*‡ AND ELIZABETH F. CURTIS*

*From the *Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada; and †Centre for Food Animal Research, Ottawa, Ontario, Canada.*

ABSTRACT: To test the hypothesis that glycerol would concomitantly affect sperm membrane structure and the function of the intact cells, boar semen (4 ejaculates from 4 boars) was cryopreserved in an egg yolk extender with 0%, 2%, 4%, or 8% glycerol in 0.5-mL straws using previously derived optimal cooling and thawing rates. Increasing glycerol concentrations increased spermatozoal progressive motility immediately after thawing and after 2 hours at 43°C, but decreased the percentage of sperm with normal acrosomal morphology. The mathematical products of the motility and acrosomal integrity scores (MOT × NAR index) were low in 0% and 8% glycerol, and significantly higher in 2% and 4% glycerol. The fluidity of sperm-head plasma membranes, a measure of molecular interaction, was assessed with the lipid probes *trans*-parinaric acid and *cis*-parinaric acid (tPNA, cPNA), during a 2.5-hour incubation with or without 1 mM Ca^{2+}. Membrane fluidity detected by each probe differed significantly, indicating the presence of at least 2 domains whose constituent molecules had unique dynamics. Behavior of each domain was radically altered by cryopreservation. Increasing glycerol concentration caused a variably faster loss of fluidity in the cPNA domain, and had highly variable effects on fluidity change over time in the tPNA domain. Normal acrosomal ridge (NAR) and the MOT × NAR index correlated significantly with the fluidity of the more mobile cPNA domain (±1 mM Ca^{2+}), supporting the hypothesis of an interrelationship of glycerol concentration during cryopreservation with sperm membrane structure and cell function. The MOT × NAR index may be a useful guide in choosing optimal cryoprotectant concentrations.

Key words: Swine, sperm motility, acrosome, membrane fluidity.
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Cryopreservation of boar sperm results in lower conception rates and smaller litters after artificial insemination compared to insemination with fresh semen (Johnson et al, 1981), despite doubling the number of sperm (Pursel and Johnson, 1975). Among the many factors that influence the fertility of frozen-thawed semen of different species are the nature of the cryoprotectant (Wilmut and Polge, 1977a), thawing temperature, sperm concentration (Pace et al, 1981), and variations in methodology (Pursel et al, 1978; Cochran et al, 1984). The effects of cooling velocity and cryoprotectant concentration (Wilmut and Polge, 1977a; Almlid and Johnson, 1988) on postthaw fertility have been investigated in several species (Cochran et al, 1984; Fiser and Fairfull, 1984), including the boar (Almlid et al, 1987). The important interaction between freezing rate and glycerol concentrations has also been studied in boar sperm (Fiser and Fairfull, 1984, 1990; Fiser et al, 1993).

Prediction of the fertilizing potential of fresh or frozen mammalian semen is usually based on in vitro assessment. However, there is no single in vitro test of frozen boar semen whose results correlate well with, let alone accurately predict, fertility. Postthaw motility (Wilmut and Polge, 1977b) or acrosomal integrity alone (Hammit et al, 1990) are poorly correlated with fertility. It has been suggested that cryopreservation (defined as the dilution, cooling, and freezing and thawing process) damages the plasma membrane of the sperm head, causing molecular changes that interfere with fertilization (Buhr et al, 1989, 1994; Bailey et al, 2000). In mouse (Wolf et al, 1986) and bull sperm (de Curtis et al, 1986; Buhr et al, 1993), the membrane covering the anterior and posterior portions of the head, although continuous, has local domains with unique properties. Ultrastructural differences within the plasma membrane overlying the anterior of the head have been found (Peterson et al, 1987; Harrison et al, 1996), and chilling induces a site-specific rearrangement of membrane ultrastructure, suggesting that there are domains with different temperature sensitivities in sperm membranes (de Leeuw et al, 1990; Buhr et al, 1994) as in platelets (Crowe et al, 1999). Domains exist in boar sperm membranes (Buhr and Pettitt, 1996; Ladha et al,

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Correspondence to: Dr Mary M. Buhr, Department of Animal and Poultry Science, University of Guelph, Guelph, ON, Canada N1G 2W1.

‡Current address: Centre de recherche en biologie de la reproduction, Département des sciences animales, Université Laval, Quebec, PQ, Canada G1K 7P4.

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1997) and the various major components of the cryopreservation extender act and interact to alter molecular relationships within these domains, which are measurable as a change in fluidity of the domain (Pettitt and Buhr, 1998).

Knowing that the presence or absence of glycerol in a cryopreservation medium affected membrane domains in boar sperm (Pettitt and Buhr, 1998), the present study hypothesized that glycerol would alter domain fluidity in conjunction with altered function of intact sperm. Boar sperm were frozen and thawed with different concentrations of glycerol, but otherwise under the optimal conditions previously derived (Fiser and Fairfull, 1990; Fiser, 1991; Fiser et al, 1993). The objectives were to compare and contrast spermatozoal motility and acrosomal morphology obtained after thawing with the presence, nature, and dynamic properties of domains in the head plasma membranes isolated from these sperm.

Materials and Methods

Semen Collection and Processing

All animals were cared for according to the guidelines of the Canadian Council of Animal Care. Four ejaculates were collected using the gloved-hand technique from each of 4 mature Yorkshire boars, with an interval of 4–7 days between collections for any 1 boar. The ejaculates were fractionated visually, collecting the sperm-rich fraction into an insulated 220-mL beaker covered with cheesecloth. The volume of the sperm-rich fraction was measured, and sperm concentration was determined using a spectrophotometer previously calibrated according to hemocytometer counts (79 ± 4 mL; 687 ± 21 × 10^6 spermatozoa/mL [mean ± SEM]). Motility was determined as for thawed semen (see below). The individual sperm-rich fractions were processed as described by Pursel and Johnson (1975), diluting after centrifugation and removal of the supernatant with BF5 diluent (Pursel and Johnson, 1975) to a volume of 5 mL/tube (12 × 10^8 spermatozoa/mL) and cooling to 5°C over 2 hours. Further processing followed the optimal method previously developed for boar sperm (Fiser and Fairfull, 1990; Fiser, 1991; Fiser et al, 1993). Each of the ejaculates was then further diluted with equal parts of one of the glycerolated BF5 diluents that contained 0%, 4%, 8%, or 16% glycerol (v/v), resulting in final glycerol concentrations of 0%, 2%, 4%, or 8%, respectively. The semen was then loaded into 0.5-mL plastic straws that were color-coded to identify the glycerol concentration. The straws were cooled at 30°C/min to −60°C in a programmable freezer (Cryomed 900-4 controller, 972-3 freezing chamber; Thermo Forma, Marietta, Ohio), then plunged into liquid nitrogen for storage.

Fresh semen (4 ejaculates from each of 4 boars) was obtained from different boars, as the time required to transport semen between the investigators' institutions precluded use of the same boars as for the frozen semen. Collection procedures were similar. The fresh semen was subjected to filtration through a double layer of Miracloth (Calbiochem, La Jolla, Calif) to remove fine, gelatinous particles prior to the first centrifugation.

Thawing of Semen and Assessment of Spermatozoa

Semen samples were thawed by immersing the straws for 8 seconds, with gentle agitation, in water at 60°C. To assess motility and acrosomal integrity, the dried straws were emptied into test tubes containing 2 mL of 35°C BTS (Beltsville Thawing Solution; Pursel and Johnson, 1975), and the sperm in BTS were allowed to equilibrate for 5 minutes. Motility (percentage progressively motile, %PM) and kinetic rating (rated 1–5: 1 = very slow, 5 = vigorous) were evaluated blind in aliquots diluted with 35°C BTS placed on a 35°C microscope stage. For evaluation of acrosomal integrity, 0.5 mL of semen was fixed in 0.5 mL of 1% glutaraldehyde; at least 100 acrosomes per sample were examined by phase-contrast microscopy and classified (Pursel and Johnson, 1972). A thermal stress test was performed by incubating the samples at 43°C for 2 hours in BTS and then measuring motility (%PMT).

Membrane Preparation

All procedures were performed at 20°C unless otherwise stated. Semen samples were thawed as described above, the sperm pooled in 50-mL polycarbonate tubes, diluted 1:1 (v:v) with 50 mM Tris-0.25 M sucrose pH 7.4, and centrifuged (2500 × *g* for 10 minutes). The supernatant was discarded and pelleted sperm were gently resuspended in Tris-sucrose to a final volume of 160 mL. Resuspended sperm were layered on oil (a 1:1 mixture of 550 and 1107 silicone oils (Canvin and Buhr, 1989). All subsequent procedures were identical to those used to prepare membranes from fresh sperm, as previously described (Canvin and Buhr, 1989). Briefly, the sperm-oil gradient was centrifuged (2500 × *g* for 10 minutes), and the resulting pellet was washed twice and centrifuged (2500 × *g* for 10 minutes). The washed sperm were subjected to nitrogen cavitation followed by differential centrifugation. Protein concentration of the resultant

Table 1. *Functional characteristics of boar sperm**

Glycerol (%)	%PM	%PMT	NAR	%PM × NAR	%PMT × NAR
0	6.0 ± 3.7†	3.1 ± 2.0†	58.6 ± 12.5†	357 ± 57†	181 ± 12†
2	23.8 ± 11.1‡	11.9 ± 9.3‡	46.7 ± 14.8†‡	1107 ± 132‡	561 ± 102‡
4	41.0 ± 6.2§	19.3 ± 5.6§	36.2 ± 13.1‡	1499 ± 164‡	693 ± 64‡
8	36.9 ± 7.9§	11.8 ± 7.6‡	11.7 ± 7.8§	429 ± 107†	121 ± 26†

* %PM indicates percentage of progressively motile sperm after thawing; %PMT, percentage of progressively motile sperm after 2 hours at 43°C; NAR, percentage of sperm with normal acrosomes; NS, not significant, $P > .10$.

†,‡,§ Within a column, means with different superscripts differ ($P < .01$).

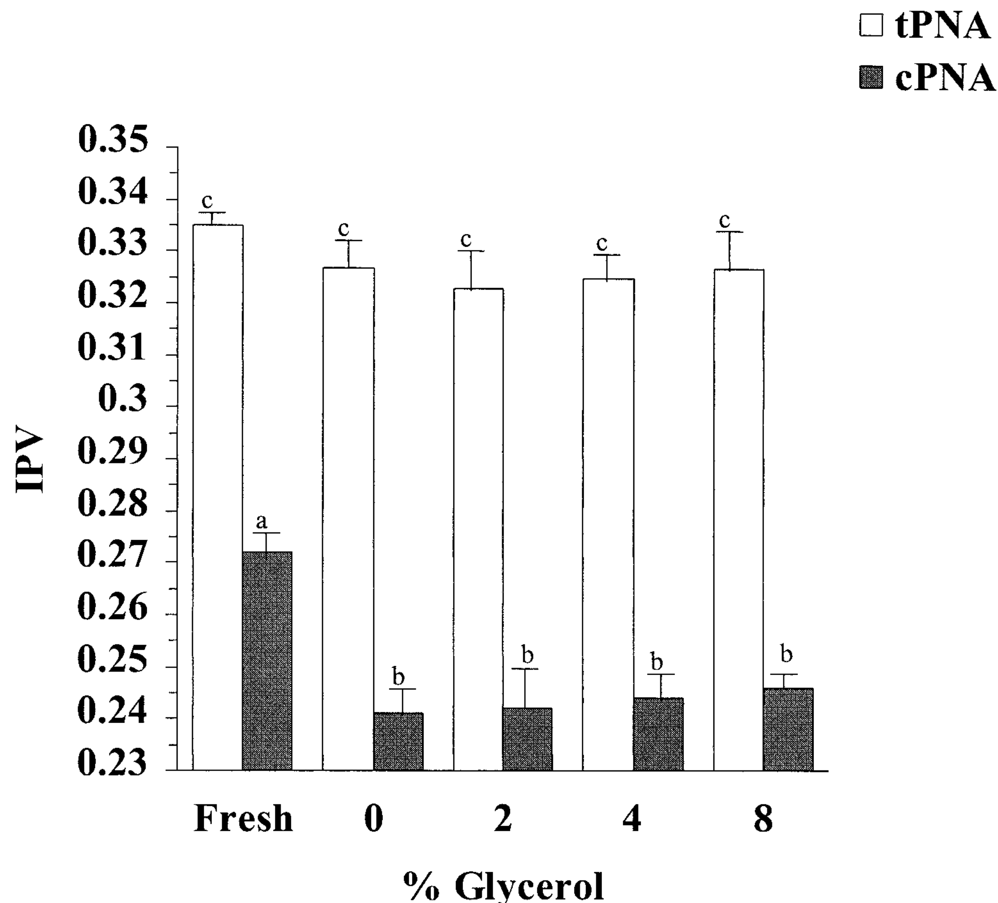


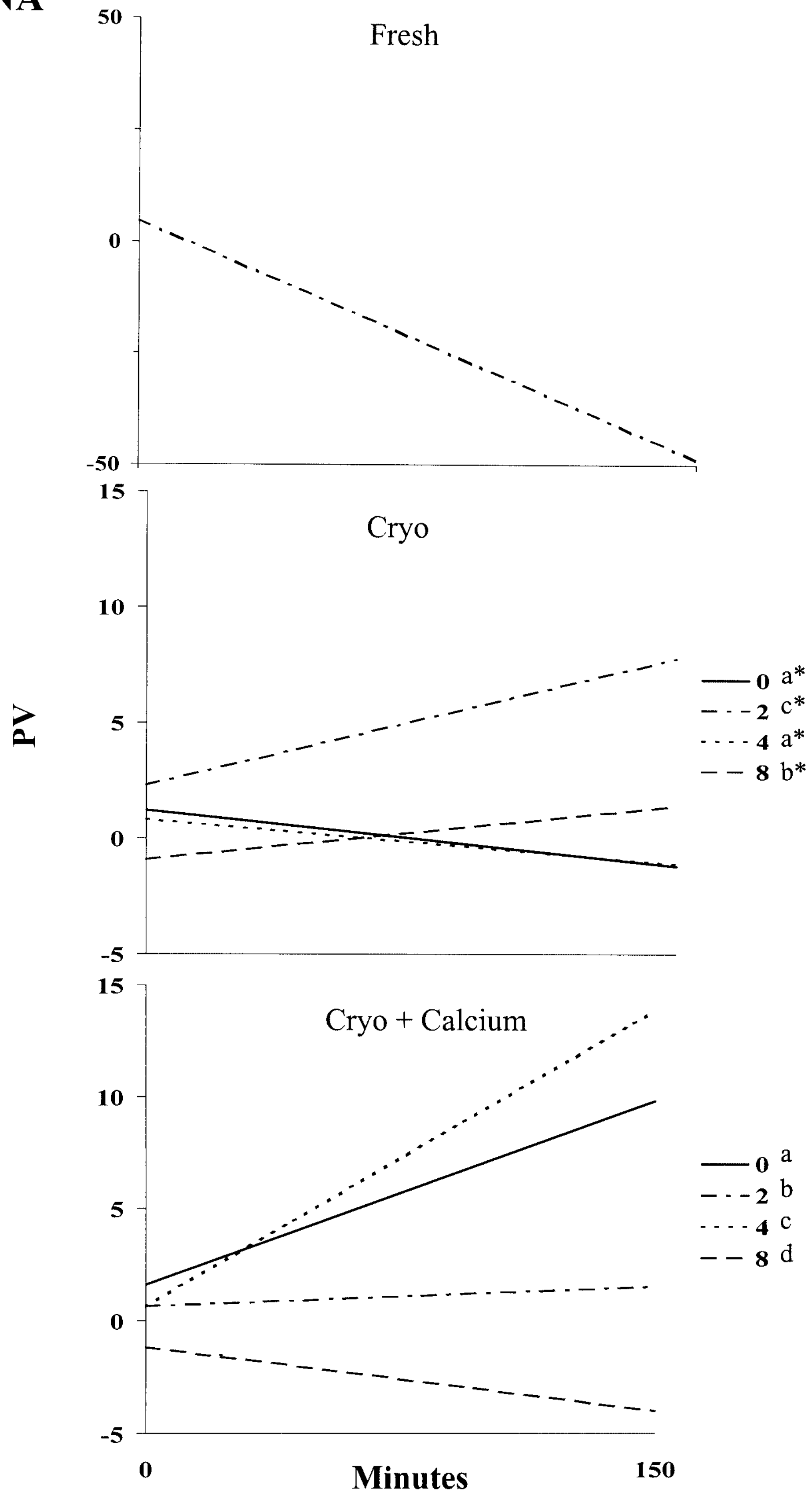
Figure 1. Initial fluidity (IPV) of membranes isolated from boar spermatozoa either immediately after semen collection (fresh; 4 ejaculates from 4 boars) or after the sperm were cryopreserved in the indicated concentration of glycerol (4 ejaculates from 4 boars). Fluidity is measured as polarization values (PV) produced by cPNA and tPNA, which insert into the bulk membrane lipid and gel phase membrane lipids, respectively. Polarization values are inversely related to fluidity, so higher polarization values indicate less fluid domains. [a,b,c]Values with different superscripts differ ($P \leq .005$); cPNA differs from tPNA ($P \leq .0001$)

sperm-head plasma membrane fraction was assayed by the method of Bradford (1976) using bovine gamma globulin (Sigma Chemical Company, St Louis, Mo) as a standard. The membrane sample was immediately divided into 4 equal portions (50 μg protein/mL, final concentration) for fluorescence polarization analysis. Each portion was tested using 1 μM of probe *cis*-parinaric acid (cPNA) or *trans*-parinaric acid (tPNA; both from Molecular Probes, Eugene, Ore) in the presence or absence of 1 mM Ca^{2+} (final concentration). The tPNA molecule has a preferential affinity for the more ordered areas of a membrane, whereas cPNA inserts into all areas almost equally well (Sklar, 1980; Welti and Silbert, 1982; Pettitt and Buhr, 1998). Polarization in each of the 4 portions was determined every 5 minutes for 140 minutes using an SLM 8000 polarizing spectrofluorometer with a T-format, with excitation at 315 nm and emission at 410 nm, or using a 370-nm cutoff filter. Excitation and emission slit widths were 16 nm. Temperature was maintained at 20°C with a thermostat in a circulating water bath.

Statistical Analyses

The %PM, %PMT, and the percentage of spermatozoa with normal acrosomal ridge (NAR) were arc-sin transformed and then assessed using ANOVA and least squares procedures (Statistical Analysis Systems [SAS], 1985) to analyze the effects of glycerol. A general linear model (GLM) that included the effects of glycerol, boar, ejaculate within boar (used as the error term for boar effect), and boar × glycerol interaction was used to evaluate treatment effects. Orthogonal linear contrasts with a single degree of freedom were used to compare the effects of glycerol concentrations.

Differences in initial fluidity, measured by the initial polarization value, were assessed by ANOVA after determining that the data were normally distributed. To test for changes in fluidity over time, the initial polarization value (IPV) of each sample was set to zero, and all subsequent polarization values in the same sample were similarly adjusted (Canvin and Buhr, 1989). The adjusted data were subjected to linear regression analysis (SAS, 1985). Linear, quadratic, and cubic equations were fitted to the regressions and the equation with the highest R^2 value was selected as the best model for the data. The effects of Ca^{2+}, probe, or preservation procedure on both the slopes and intercepts were established with the Predicted Differences Procedure of SAS, using the pooled data sets from the appropriate membrane preparations.


tPNA
Fresh
Cryo
Cryo + Calcium
PV
Minutes
50
0
-50
15
10
5
0
-5
0
150
0 a*
2 c*
4 a*
8 b*
0 a
2 b
4 c
8 d

Pearson correlation coefficients were used to test for correlations of fluidity characteristics (initial fluidities, slopes, and intercepts) with the NAR and percentage motile values. Specific morphology and motility characteristics of thawed sperm (%PM, %PMT, NAR) were further evaluated for associations with fluidity parameters using a GLM analysis in which boar, glycerol, and the fluidity parameter of interest were the independent variables, and morphology/motility values were the dependent variables. To test the a priori assumption that a combination of morphology and motility values would be more informative than the individual values, the transformed %PM and %PMT values for each sample were multiplied by the corresponding NAR and the products (%PM $\times$ NAR; %PMT $\times$ NAR) were tested in the GLM model.

Results

The %PM after thawing was independent of boar or ejaculate ($P > .05$), but was strongly affected by the concentration of glycerol used as cryoprotectant ($P \leq .01$; Table 1); glycerol concentration had no detectable effect on the rated vigor of motility (data not shown). The %PM immediately after thawing increased with increasing glycerol concentration up to 4%, with no apparent further change at 8%. Motility after the thermal stress (%PMT) similarly increased with glycerol concentration up to 4% and was decreased at 8%, but the differences between 2% and 8% were not significant ($P > .05$).

Normal acrosomal morphology in cryopreserved sperm varied among boars and among glycerol levels (Table 1). The proportion of damaged acrosomal caps was dependent on glycerol concentration ($P \leq .01$), whereas the proportion of spermatozoa with loose or missing caps was dependent on both boar ($P \leq .05$) and glycerol concentration.

The initial fluidity of membranes from both fresh and frozen spermatozoa differed among probes (Figure 1), indicating the presence of different domains, with the cPNA domain being more fluid (lower IPV) than the tPNA domain ($P < .0001$). Ca^{2+} had no effect on the initial fluidity (data not shown), but did allow detection of differences among boars ($P \leq .01$); boars did not differ in the absence of Ca^{2+}. Cryopreservation of whole sperm made the cPNA domain more fluid initially (lower IPV, Figure 1, $P < .005$), regardless of glycerol concentration, and tended ($P = .08$) to fluidize the tPNA domain for sperm frozen in 0% or 4% glycerol.

The fluidity of membranes from fresh sperm changed significantly over time in a linear manner (Figures 2 and 3), after accounting for significant variation among boars. In membranes from fresh sperm, with or without Ca^{2+}, the tPNA domain became more fluid over time (decreasing polarization value) and the cPNA domain became less fluid (increasing polarization value). Cryopreservation of the whole sperm significantly affected the nature, the rate, or both, at which fluidity changed in both domains, after accounting for boar-to-boar differences. Glycerol concentration also differentially affected the 2 domains (Figures 2 and 3). Fluidity change in the tPNA domain was significantly affected by Ca^{2+}, but whether Ca^{2+} caused an increase or loss of fluidity was dependent on the concentration of glycerol used to cryopreserve the sperm (Figure 2). The cPNA domain always became less fluid over time (significant positive slope; Figure 3), with the cPNA domain from sperm frozen in 2% glycerol losing fluidity more quickly than those frozen in 4% or 8% glycerol, regardless of the presence of Ca^{2+}.

Several specific fluidity characteristics were correlated with morphology/motility values. Correlations of membrane fluidity with sperm function varied somewhat with the different concentrations of glycerol, but the fluidity of the cPNA domain was consistently correlated with aspects of acrosomal morphology. First, the NAR of sperm frozen in 4% glycerol tended to be positively correlated (Pearson correlation coefficients; $P \leq .077$, $R > .92$) with the initial fluidity of the cPNA domain and tended to be negatively correlated ($P < .087$, $R = -.91$) with the slope of the same domain. The correlation of this NAR was highly significant ($P = .0003$; $R = .99$) with the slope of the cPNA domain in the presence of Ca^{2+}. As would be expected for these sperm, the number with loose or missing acrosomes had the opposite, and significant, relationship with the cPNA domain. Second, GLM analysis confirmed that the rate of change of fluidity in the overall head plasma membrane (Table 2; slope with cPNA probe) in the presence or absence of Ca^{2+} was correlated with changes in NAR and %PM, whereas cPNA + Ca^{2+} tended to be associated with %PMT alone. Third, the mathematical combinations %PM $\times$ NAR and %PMT $\times$ NAR were strongly correlated with the cPNA domain dynamics, with %PMT $\times$ NAR also tending to be correlated with the dynamics of the tPNA gel domain (Table 2).

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Figure 2. Fluidity changes in the head plasma membranes isolated from fresh or cryopreserved (Cryo) boar spermatozoa (4 ejaculates from each of 4 boars); 0 or 1 mM Ca^{2+} was added at time 0 to membranes from cryopreserved sperm. Fluidity was measured every 5 minutes as the polarization values (PV) of the membrane probe tPNA, which seeks out the more ordered membrane domains. Data are displayed as the mathematical slope from linear regression analysis; all data produced slopes that were significantly linear. A negative slope indicates increasing fluidity; a positive slope indicates decreasing fluidity. [a,b,c] Within a panel, lines with different letters have significantly different slopes, intercepts, or both; all lines differ significantly from fresh. *Significantly different from calcium.

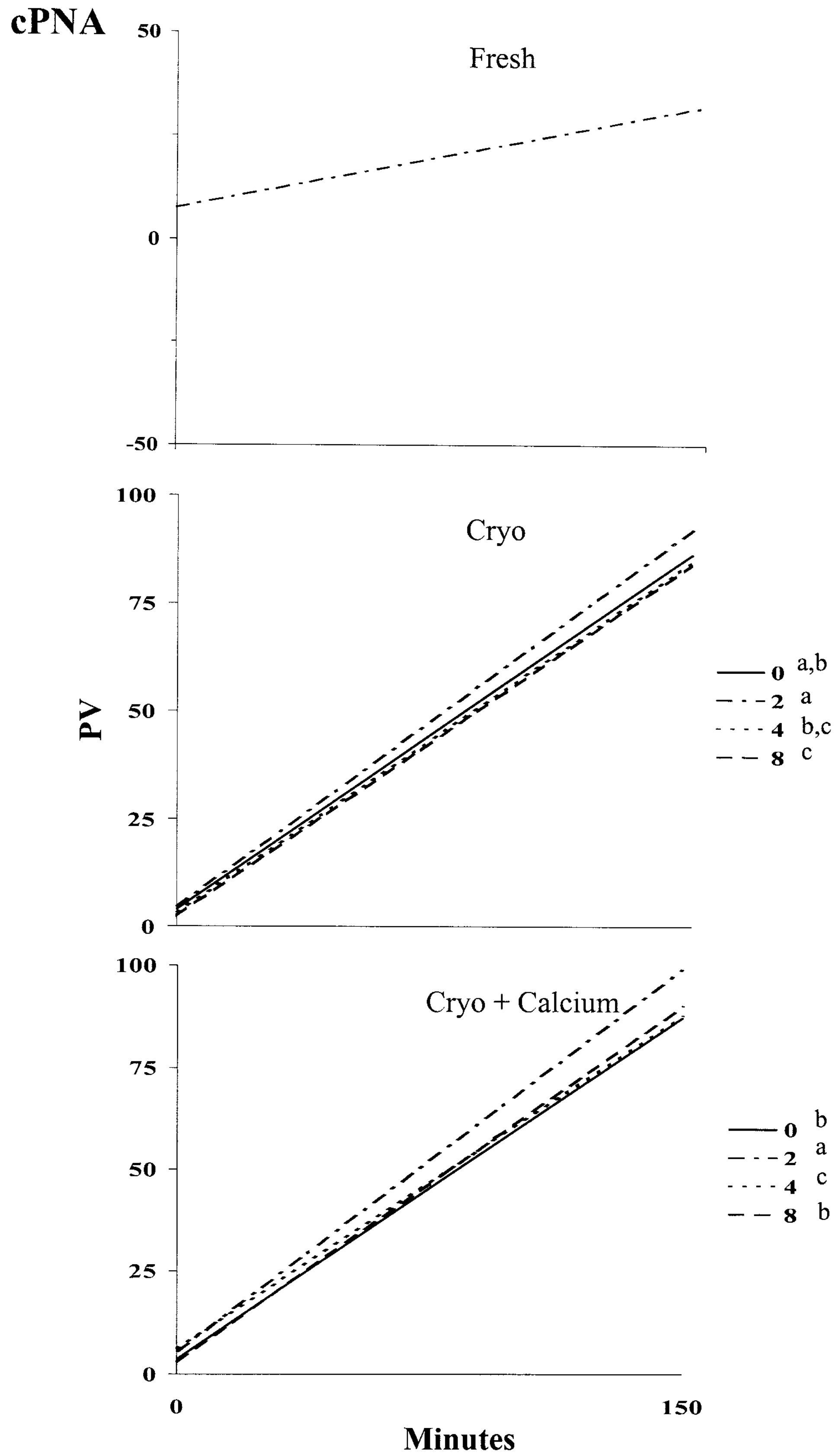
cPNA
Fresh
50
0
-50
100
Cryo
75
50
25
0
PV
0 a,b
2 a
4 b,c
8 c
100
Cryo + Calcium
75
50
25
0
0 b
2 a
4 c
8 b
0
150
Minutes

Table 2. *Significance (P value from type III general linear models analysis) indicating relationship between the rate of change in fluidity (slope, see Figures 2 and 3) of the indicated domain and the functional characteristics of the whole sperm**

Domain	%PM	%PMT	NAR	%PM × NAR	%PMT × NAR
cPNA	0.029†,§	NS	0.007†,§	0.0032§	0.057§
cPNA + Ca^{2+}	0.003†,§	0.087§	0.051†,‡	0.0069‡,§	0.001§
tPNA	0.044‡,§	NS	NS	NS	0.069†,§
tPNA + Ca^{2+}	NS	0.068‖	NS	NS	0.065‡,§

* %PM indicates percentage motile sperm after thawing; %PMT, percentage of progressively motile sperm after 2 hours at 43°C; NAR, percentage of sperm with normal acrosomes; NS, not significant, $P > .10$.

†,‡ Individual boars a significant component (†, $P < .05$; ‡, $P < .1$).

§,‖ Glycerol concentration a significant component (§, $P < .001$; ‖, $P < .01$).

Discussion

Membranes isolated from the heads of fresh and cryopreserved boar sperm have domains with different molecular dynamics, indicated by both different initial states of fluidities and different patterns of change in fluidity with time, confirming and expanding previous results (Pettitt and Buhr, 1998; Harrison and Miller, 2000). Different domains have been recognized in electron micrographs of fixed boar sperm membranes (Peterson et al, 1987) and probes used in the present study have allowed detection of different domains in membranes from bovine (Buhr et al, 1993) and porcine sperm (Buhr and Pettitt, 1996). The current study demonstrates that the concentration of glycerol used to freeze whole sperm alters the dynamics of specific domains in the head plasma membranes of the thawed sperm.

In addition to the unique responses of the various membrane domains to the concentrations of glycerol tested, a dramatic difference was found between the behavior of membranes from fresh and frozen sperm. The effects of whole-sperm cryopreservation on cell membranes are only slightly ameliorated by the presence of glycerol. Glycerol does not completely prevent the effects of cryopreservation on sperm morphology and function (Wilmut and Polge, 1977b; Almlid and Johnson, 1988), and whereas it may ameliorate the damage of chilling, it cannot prevent damage attendant on rewarming (Medrano and Holt, 1996). Although the fresh semen was obtained from different boars than the cryopreserved semen, comparing them is valid, first because the fresh and control cryopreserved semen showed similar domain dynamics with that obtained from different boars in a split ejaculate fresh-frozen comparison (Pettitt and Buhr, 1998). Second, all conclusions about differences between fresh and frozen sperm in the current study are based on statistical analyses that evaluate treatment differences after having accounted for any boar differences present. Third, the same research group performed all assessments of sperm function and membrane dynamics in fresh and cryopreserved sperm. Finally, the effect of glycerol on the cellular and membrane functions of cryopreserved sperm was determined after eliminating fresh sperm from the data set.

No one concentration of glycerol maximized all the functional parameters of the cryopreserved sperm. Concentrations of glycerol that best preserve one sperm characteristic after thawing are seldom optimal with respect to others. Wilmut and Polge (1977a,b) noted that maximal postthaw motility and acrosome integrity were achieved with different concentrations of glycerol, but neither of these concentrations preserved maximal fertility. Almlid and Johnson (1988) froze boar semen in straws and found that each of 3 measures for sperm quality (motility, normal acrosomes, and plasma membrane integrity) were best when a different concentration of glycerol had been used for freezing. This lack of consistency among cell parameters as to the optimal concentration of glycerol was also evident in the present study.

An assumption of the current study, that a combination of whole-sperm characteristics would be more indicative of overall sperm ''health'' than individual characteristics, was based on previous work (Ericsson et al, 1993; Fiser et al, 1993; Pettitt and Buhr, 1998). Therefore, the values of percent of progressively motile sperm were multiplied by the NAR in each sample. The derived index (MOT × NAR) was highest for sperm cryopreserved with 2% and 4% glycerol, agreeing with our earlier conclusion that 3% glycerol would be an effective concentration for cryopreservation of boar sperm (Fiser et al, 1993). The behavior of the more ordered areas of the membrane (tPNA domain) was infrequently correlated with whole sperm characteristics, emphasizing the uniqueness of the dynamics in this more limited domain within the sperm head membrane. GLM analysis found that the rate at which fluidity of the overall membrane (cPNA domain) decreased over time was slightly but significantly associated

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Figure 3. Fluidity changes in the head plasma membranes isolated from fresh or cryopreserved (Cryo) boar spermatozoa (4 ejaculates from each of 4 boars); 0 or 1 mM Ca^{2+} was added at time 0 to membranes from cryopreserved sperm. Fluidity was measured every 5 minutes as the polarization values (PV) of the membrane probe cPNA, which freely enters all membrane domains. Data are displayed as the mathematical slope from linear regression analysis; all data produced slopes that were significantly linear. A negative slope indicates increasing fluidity; a positive slope indicates decreasing fluidity. Calcium had no significant effect on slopes. [a,b,c] Within a panel, lines with different letters have significantly different slopes, intercepts, or both; all lines differ significantly from fresh.

with NAR and %PM, and strongly and significantly associated with the combined index. This concurs with findings linking the behavior of membrane domains with function of cryopreserved sperm (Muller et al, 1999). Pearson correlations were significantly negative, clarifying that the slower the membranes lost fluidity, the more sperm had normal acrosomes. Because cPNA inserts equally well into all areas of membrane lipid, these statistical associations clearly suggest that the physiologic dynamics of the overall membrane are linked to important aspects of sperm function.

This study has shown that boar sperm cryopreserved in straws at optimal rates of cooling and warming in the presence of different concentrations of glycerol sustain different alterations in motility, morphology, and head membrane fluidity. Domains in the membranes of boar sperm have different molecular dynamics, different responses to the presence of various concentrations of glycerol in cryopreservation media, and Ca^{2+} affects the membrane domains differently. The behavioral and morphological characteristics of whole sperm appear to be reflected in, and perhaps related to, molecular interactions within the head membranes.

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