

Cryopreservation Extenders Affect Calcium Flux in Bovine Spermatozoa During a Temperature Challenge

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ABSTRACT: Bovine spermatozoa are commercially cryopreserved by diluting the cells in media, known as extenders, followed by slow cooling and freezing. Previous work has shown that this process of cryopreservation alters the cells' ability to control divalent calcium (Ca^{2+}) movement. This study evaluated the effect of a brief exposure to common extenders on bovine spermatozoa during subsequent cooling and rewarming. Three fresh ejaculates from each of three bulls were each split and incubated for 30 minutes at 25°C in milk extender or phosphate-buffered saline (PBS) (control); three other fresh ejaculates from each of three bulls were similarly incubated in egg yolk-Tris extender (EYT) or PBS. Spermatozoa were washed and the fluorescent Ca^{2+} indicator, indo-1 acetoxymethyl ester, was used to monitor the internal Ca^{2+} in the spermatozoa in Ca^{2+} -free PBS over a continuous temperature gradient of 25°C (15 minutes), cooling to 5°C (32 minutes), at 5°C (15 minutes), rewarming to 25°C (25 minutes), and at 25°C (15 minutes). Milk exposure reduced the initial percentage of missing acrosomes and EYT exposure improved the initial viability and acrosome morphology compared to the controls; only milk immediately increased internal Ca^{2+} . The initial rate of Ca^{2+} uptake at 25°C was greater for milk or EYT-exposed sper-

matozoa than controls ($P < 0.05$). During cooling, the rate of Ca^{2+} uptake in all spermatozoa increased ($P < 0.01$), and it continued to increase during the 15 minutes at 5°C. During rewarming to 25°C, the internal Ca^{2+} in all spermatozoa declined. The rate of decline of Ca^{2+} in control exceeded that of EYT-exposed spermatozoa. Addition of 1 mM Ca^{2+} during the final 25°C incubation caused internal Ca^{2+} to increase for 1 minute in all samples. The rate of increase in the milk samples was greater than that of its control ($P < 0.05$). There was no further change over the subsequent 35 minutes except for an increase of free intracellular Ca^{2+} in the EYT control sample. The percentage of either viable or intact spermatozoa had decreased in all samples at the end of the temperature gradient. The brief exposure of spermatozoa to milk and egg yolk increased free intracellular Ca^{2+} in spermatozoa, perhaps initiating the early stages of capacitation. Milk and EYT differed in their effect on Ca^{2+} flux; this result may have implications for fertility.

Key words: Milk, egg yolk-Tris, indo-1 acetoxymethyl ester, cold shock, warm shock, sperm morphology.

J Androl 1995;16:278-285

Although cryopreservation of spermatozoa is successfully used in the bovine industry, more cryopreserved than fresh sperm are needed for successful insemination (Berndtson and Pickett, 1978; Shannon, 1978) due to reduced fertilizing ability. Cold shock and rewarming are two stresses that affect structure and function of spermatozoa. The effects of cold shock might include loss of membrane-selective permeability, change of membrane fluidity (Mack and Zaneveld, 1987; Buhr et al, 1989; Canvin and Buhr, 1989), leakage of cellular components such as phospholipids and proteins, disruption of metabolism, irreversible loss of motility (Watson, 1981; Simpson et al, 1986), and decreased fertilizing ability (Watson, 1981). Cold shock causes severe damage to the acrosome (Han-

cock, 1952; Jones and Martin, 1973; Simpson et al, 1986) and mitochondria (Jones and Martin, 1973) and increased uptake of divalent calcium (Ca^{2+}) by spermatozoa (Kargiannidis, 1976; Simpson et al, 1986, 1987; Robertson et al, 1988, 1990). Rewarming also damages spermatozoa (DeAbreu et al, 1979) by loss of membrane integrity, declining motility and leakage of ATP (Holt et al, 1992), and acrosomal damage (Bamba and Cran, 1985, 1988).

Egg yolk-Tris (EYT) and whole milk are the two common extenders used to preserve semen for artificial insemination in cattle (Foote, 1984). Egg yolk has been shown to protect sperm motility and viability (Phillips and Lardy, 1940), to protect acrosomal and mitochondrial membranes during slow cooling and storage (Jones and Martin, 1973), to enhance the ability of spermatozoa to penetrate zona-free hamster oocytes (Holmgren et al, 1989), to bind to human zona pellucida (Lanzendorf et al, 1992), and to increase the *in vitro* fertilization (IVF) rate of human oocytes (Katayama et al, 1989). It also reduces the accumulation of calcium resulting from cold shock or slow cooling (before 24-hour storage) (Robertson and Watson, 1987).

Ca^{2+} plays an important role in capacitation, hyper-

This research was funded by Semex Canada, NSERC, and CAAB. Y.Z. was the recipient of an Ontario Graduate Scholarship.

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Received for publication October 21, 1994; accepted for publication February 6, 1995.

activation, and the acrosome reaction (Yanagimachi and Usui, 1974; Roldan and Fleming, 1989; Stock and Fraser, 1989; Fraser and McDermott, 1992). Bailey and Buhr (1994) demonstrated a decrease in efficiency of intracellular calcium control mechanisms in bovine spermatozoa following cryopreservation and subsequently showed *in vivo* fertility of cryopreserved bovine spermatozoa to be correlated with free intracellular Ca²⁺ (Bailey et al, 1994). The semen used in that research was all processed in milk extender. The current research was designed to determine if the another common extender, egg yolk-Tris, would affect sperm Ca²⁺ regulation in a similar manner to milk extender, particularly during the thermal challenge of cooling and rewarming. Measurement of the exquisitely balanced Ca²⁺-regulation system could prove a more sensitive measure of the relative impact of these extenders on spermatozoa than the traditional tests of motility, viability by dye exclusion, and acrosomal integrity.

Materials and Methods

All steps were carried out at 25°C, unless otherwise stated.

1. Milk Experiment

Spermatozoa Preparation—Three fresh ejaculates from each of three bulls were collected and donated by United Breeders Inc. (Guelph, Ontario, Canada). Milk was prepared according to the procedures reported by Buhr and Zhao (1992) (homogenized whole milk was heated at 92°C for 10 minutes, cooled, and treated with 250 µg/ml gentamycin, 50 µg/ml tylocin tartrate, 150/300 µg/ml lincomycin and spectinomycin; antibiotics were purchased from Upjohn Company, Orangeville, Ontario, Canada). Glycerol was not included because the normal protocol for freezing in this extender adds glycerolated extender after the semen has cooled to 5°C. Semen was exposed (1:3 v/v) to either calcium-free phosphate-buffered saline (PBS; 125 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 5 mM KCl, 5 mM glucose, pH 7.4; control) or milk for 30 minutes, then washed once through 35% Percoll and the resulting pellet twice through PBS (500 × g, 10 minutes). Sperm concentration was measured with a calibrated spectrophotometer. Loading of the fluorophore into spermatozoa was according to the method of Bailey and Buhr (1994) with minor modifications. Briefly, one aliquot of milk-exposed and one aliquot of PBS-exposed spermatozoa (1 × 10⁸ sperm/ml) were incubated in PBS with 0.1% dimethylsulfoxide (DMSO), 0.025% pluronic F-127, and 5 µM indo-1 acetoxymethyl ester (indo-1 AM, Molecular Probes Inc., Eugene, Oregon) in the dark. Identical aliquots of milk and control spermatozoa were incubated in PBS with 0.1% DMSO and 0.025% pluronic F-127 for 30 minutes in the dark to provide a measure of background fluorescence (blanks; no indo-1 AM). All four samples were washed in PBS (three times at 500 × g, 10 minutes) after incubation and placed in quartz cuvettes at a final concentration of 1 × 10⁷ spermatozoa/ml.

Fluorescence Determination of Ca²⁺—A dual-emission Alphascan spectrofluorometer (Photon Technologies International,

Brunswick, New Jersey) with a four-place thermostatted sample chamber with magnetic stirring was used to measure fluorescence emissions (photon counting) of the sperm samples. Excitation wavelength was 355 nm, and emission wavelengths were set at 405 and 490 nm, which detected maximum intensity of Ca²⁺-bound and unbound indo-1 AM, respectively.

Fluorescence emission from each sample was measured for 1 minute every 5 minutes at 1 point/10 seconds. During data collection, the samples were subjected to a sequence of temperature changes. Cuvettes were kept at 25°C for 15 minutes (0–15 minutes), then cooled from 25°C to 5°C in 32 minutes (16–47 minutes). The cuvettes were kept at 5°C for 15 minutes (48–62 min), rewarmed from 5°C to 25°C in 25 minutes (63–87 minutes), and kept at 25°C for 15 minutes (88–102 minutes). The temperature in the cuvettes was controlled by a circulating solution of 60% ethylene glycol whose temperature was maintained by a programmable temperature-controlled water bath (Bio Cool II, FTS Systems Inc., Stoneridge, New York) fitted with a pump. A pilot experiment measured the temperatures in the cuvettes to achieve this regime.

A solution of CaCl₂ (20 µl; final concentration, 1 mM) was added into each cuvette at 103 minutes, and fluorescence emissions were measured at 10 points/second for 1 minute. Emissions were then measured at 1 point/10 seconds every 5 minutes for 40 minutes.

Microscopy—At the beginning (0 minutes) and the end (147 minutes) of reading fluorescence, samples were taken for evaluation of viability by eosin-nigrosin staining (Mortimer, 1985) and acrosome morphology by differential interference contrast (DIC) microscopy. A total of 100 spermatozoa were counted for each measure. For acrosome morphology, spermatozoa were classified as having intact, abnormal, or missing acrosomes.

Statistical Analyses—The microscopy data were subjected to arcsin transformation. The relative free intracellular Ca²⁺ was calculated as before (Bailey and Buhr, 1994). Variance analysis for the microscopy data and mean free intracellular Ca²⁺ during the different temperature periods was done by the split-plot method, and ejaculates within bulls served as the error term for bull. To analyze the rate of Ca²⁺ uptake by spermatozoa, linear regressions were obtained for each bull and each ejaculate for each of the temperature divisions. Then the split-plot method was used to detect the difference of slopes (the rate) of Ca²⁺ uptake by spermatozoa among bulls, ejaculate, and treatments.

2. EYT Experiment

All procedures were identical to those in the milk experiment except that EYT extender was substituted for milk. The two-step preparation of EYT extender was basically according to the method of Taylor (1991). Stock solution (166.32 ml double-distilled water, 6.056 g Tris (hydroxymethyl aminomethane), 3.4 g citric acid, and 2.5 g fructose was prepared and sterilized the day before use by boiling it in the top of a glass double boiler for 15 minutes. The stock solution was stored at 4°C. EYT extender (84 ml stock solution, 16 ml double-distilled water, 23 ml egg yolk, 50 µg/ml tylosin tartrate, 250 µg/ml gentamicin, 150/300 µg/ml lincospectin and spectinomycin; antibiotics were purchased from Upjohn Company) was prepared on the day of use. As with the milk extender, the EYT was glycerol-free.

3. Determination of Fluorophore Response to Temperature Gradient

To ensure that any detected changes in fluorescence were not due to deterioration of the fluorophore, samples ($n = 4$) of 5 μM indo-1 AM, pentapotassium salt (Molecular Probes) in PBS were subjected to the temperature regime used for spermatozoa, and the fluorescence at 405 and 490 nm was recorded for 1 minute every 5 minutes at 1 point/10 seconds. Linear regression was used to detect the rate of fluorescence change during the different temperature periods, and these calculated slopes were used to convert the corresponding linear slopes obtained for the different temperature periods in the milk and the EYT experiments. These corrected slopes were then analyzed as before for the effects of bull, ejaculate, and treatment on rate of Ca^{2+} uptake.

To ensure that fluorophore had not leaked out of the sperm during the course of the temperature gradient, the total relative amount of indo-1 AM held within the cells was measured at the beginning and the end of the temperature regime. If dye leaked out of the cells during the course of the temperature gradient, the amount of intracellular dye in sperm before the temperature gradient would be greater than the amount of dye in sperm after the temperature gradient. Samples from four ejaculates ($n = 4$) were prepared as those in the milk experiment. To measure the amount of indo-1 AM, the fluorescence was first measured for 10 seconds (data collection rate of 10 points/second) and then either Ca^{2+} (final concentration, 1 mM) or Ca^{2+} plus digitonin (final concentration, 40.2 μM) was added to indo-1 AM-loaded sperm and their blanks while continuing to determine fluorescence. Maximum fluorescence was obtained as the mean of fluorescence from 20 to 40 seconds after addition of Ca^{2+} or Ca^{2+} plus digitonin. The amount of indo-1 AM that could be guaranteed to be intracellular was considered to be the difference between the sperm fluorescence obtained in the first 10 seconds and maximal fluorescence following the addition of digitonin, using sperm receiving Ca^{2+} alone as controls for sperm receiving Ca^{2+} plus digitonin. All data were subjected to arcsin transformation. A two-by-two factorial design was used to test the main effect of buffer and time, and interaction between buffer and time using the transformed data.

Results

Microscopy

Indo-1 AM exposure did not affect the viability or acrosome morphology of spermatozoa in the milk and EYT experiments (data not shown). Milk exposure did not affect the viability or percent intact acrosomes, either at the beginning or at the end of the experiment (Table 1), but it did lower the initial percentage of sperm with missing acrosomes. EYT exposure (Table 2) improved the initial viability and acrosome morphology. As expected, viability and the percentage of cells with intact acrosomes decreased from the beginning to the end of the experiment, with neither treatment differing from controls.

Table 1. Microscopic parameters of milk-extended spermatozoa (%)

	Time	Viability	IA*	AA*	MA*
Milk†	0	60 ± 4¶	69 ± 6¶	5 ± 1¶	23 ± 4‡¶
	End	32 ± 6	8 ± 3	2 ± 1	89 ± 4
PBS†	0	57 ± 4¶	65 ± 4¶	5 ± 1§	30 ± 4¶
	End	32 ± 6	11 ± 4	3 ± 1	86 ± 4

* IA = % of spermatozoa with intact acrosomes, AA = % of spermatozoa with abnormal acrosomes, and MA = % of spermatozoa with missing acrosomes.

† Data are main effects; Milk and PBS represent milk-exposed and PBS-exposed (control) samples.

‡ Values differ from controls at that time, $P < 0.01$.

§ Values differ between time 0 and the end, $P < 0.05$; ¶ $P < 0.01$.

Free Intracellular Ca^{2+}

The initial absolute values of free intracellular Ca^{2+} , calculated by the methods and equation of Grynkiewicz et al (1985) with the dissociation constant for indo-1 AM of 250 nM, were 128.9 ± 8.0 and 143.0 ± 6.1 nM for spermatozoa exposed to PBS and milk, respectively, and 112.3 ± 10.6 and 127.2 ± 13.4 nM for spermatozoa exposed to PBS and EYT, respectively. As indicated in Figure 1, all cells took up Ca^{2+} over time, and by the end of the experiment (after addition of exogenous Ca^{2+}), PBS- and milk-exposed sperm contained 668 ± 38.9 and 787.9 ± 42.7 nM Ca^{2+} , respectively, whereas PBS- and EYT-exposed sperm had 441.2 ± 40.4 and 557.4 ± 50.7 nM Ca^{2+} .

Mean free intracellular Ca^{2+} differed among ejaculates but not bulls in all treatments ($P < 0.05$). Milk-exposed spermatozoa always had a higher mean free intracellular Ca^{2+} than controls (Fig. 1), whereas EYT-exposed spermatozoa differed from controls only after rewarming (Fig. 2).

Slopes indicate the rate of change of free intracellular Ca^{2+} over time, with a positive slope giving the rate of Ca^{2+} uptake and a negative slope giving the rate of loss of free intracellular Ca^{2+} . These linear regression analyses

Table 2. Microscopic parameters of EYT-extended spermatozoa (%)

	Time	Viability	IA*	AA*	MA*
EYT†	0	62 ± 3‡	77 ± 3§¶	2 ± 0¶	21 ± 3§¶
	End	24 ± 3	2 ± 1	1 ± 0	98 ± 1
PBS†	0	57 ± 4¶	52 ± 4¶	2 ± 0	46 ± 5¶
	End	26 ± 3	3 ± 2	1 ± 0	96 ± 1

* IA = % of spermatozoa with intact acrosomes, AA = % of spermatozoa with abnormal acrosomes, and MA = % of spermatozoa with missing acrosomes.

† Data are main effects; EYT and PBS represent EYT-exposed and PBS-exposed (control) samples.

‡ Values differ from controls at that time, $P < 0.05$; § $P < 0.01$.

¶ Values differ between time 0 and the end, $P < 0.01$.

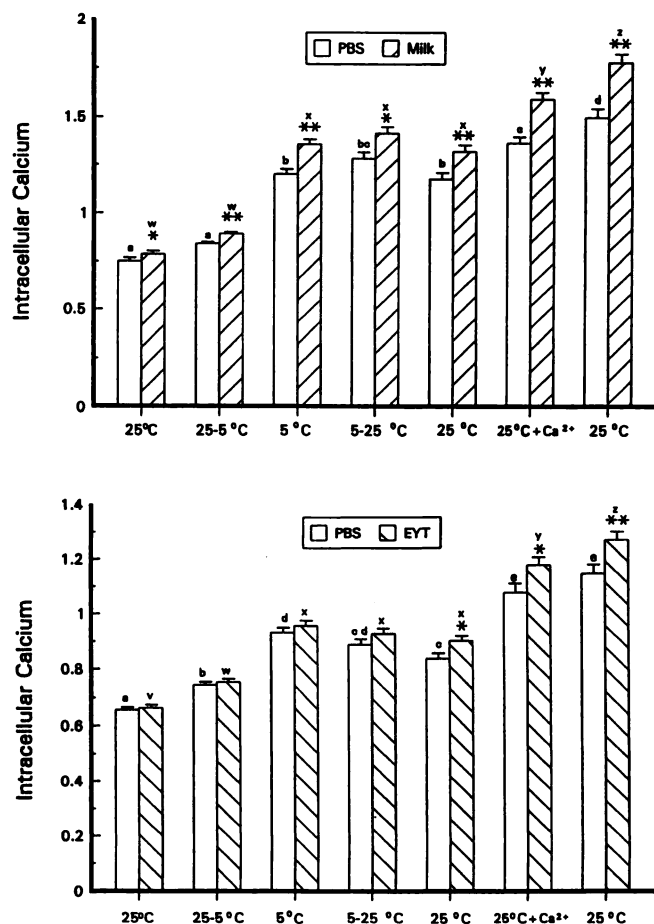


FIG. 1. (Top) Mean free intracellular Ca²⁺ levels (relative Ca²⁺) ± standard error (SE) in PBS- and milk-exposed spermatozoa at different temperature periods. Within a treatment, bars with different superscripts differ ($P \leq 0.05$). *Differs from PBS at that temperature, $P < 0.05$; ** $P < 0.01$.

FIG. 2. (Bottom) Mean free intracellular Ca²⁺ levels (relative Ca²⁺) ± SE in PBS- and EYT-exposed spermatozoa at different temperature periods. Within a treatment, bars with different superscripts differ ($P \leq 0.05$). *Differs from PBS at that temperature, $P < 0.05$; ** $P < 0.01$.

found that bulls differed significantly in initial rates of Ca²⁺ uptake in the EYT experiment and after Ca²⁺ addition in the milk experiment. The rate of Ca²⁺ movement differed among ejaculates from any one bull during some, but not all, parts of the temperature regime for both EYT and milk treatments. All treatment effects were detected by analyses that include bull and ejaculate effects in the analytical model. There were some changes in the intensity of fluorescence of indo-1 AM salt during cooling and rewarming. Correcting sperm fluorescence for these innate fluorescence changes did not alter interpretation of treatment effects, but it did change absolute slope values, and so the rates shown are for the adjusted sperm data. No significant dye leakage occurred during the experiment, indicated by no significant changes in digitonin-released

indo-1 AM, comparing sperm at time 0 to sperm at the end of the temperature regime.

During the initial incubation at 25°C, both milk- and EYT-exposed sperm took up Ca²⁺ at a faster rate than their controls (Figs. 3A, 4A). All control and treated sperm showed similar significant rates of Ca²⁺ uptake during cooling and at 5°C (Figs. 3B,C, 4B,C) and then lost Ca²⁺ during rewarming (Fig. 3D); EYT-exposed sperm lost Ca²⁺ at a slower rate than controls (Fig. 4D). Both milk-exposed and its control sperm continued to lose Ca²⁺ during the time at 25°C, albeit at a much slower rate (Fig. 3E), but EYT and its control maintained steady internal Ca²⁺ concentrations at this time (Fig. 4E). Both controls and treated sperm responded to Ca²⁺ addition with immediate rapid rates of uptake (Figs. 3F, 4F); milk-exposed sperm had a significantly higher rate than their control. In the 40 minutes after addition of Ca²⁺, all sperm except those exposed to milk continued to take up Ca²⁺ at a low, but significant, rate (data not shown).

Discussion

Divalent calcium plays important roles in capacitation, hyperactivation, and the acrosome reaction (AR) that are essential for sperm fertilization. Cold shock and cryopreservation change the ability of spermatozoa to regulate Ca²⁺ (Robertson and Watson, 1986; Bailey and Buhr, 1994). This study documents that cooling and rewarming changed the Ca²⁺-regulating ability of spermatozoa significantly, and a short exposure of spermatozoa to milk or EYT extenders provided little protection to subsequent Ca²⁺-regulating ability.

Cooling the untreated control sperm damaged Ca²⁺ regulation in a partially reversible manner, just as rewarmed plasma membranes reversed the lateral phase separations induced by cooling (de Leeuw et al, 1990, 1991). Available intracellular Ca²⁺ increased during cooling but declined during rewarming and stabilized when the temperature was once again held at 25°C. This reversibility and re-stabilization suggests that the sperm are still capable of regulating Ca²⁺. Bull sperm held continuously at 25°C first took up Ca²⁺ quickly and then more slowly (Bailey and Buhr, 1994). The current control sperm similarly took up Ca²⁺ quickly at the initial 25°C, and more slowly at the later 25°C, further supporting the argument that the regulatory ability had been retained or regained. This resilience to thermal stress may have been enhanced during the 3-hour preparation time, because incubation is known to increase thermal resistance in porcine spermatozoa (Robertson et al, 1988). However, sperm Ca²⁺ regulation did not escape unscathed from thermal shock, because the total intracellular Ca²⁺ after rewarming was higher than initial intracellular levels. Extenders exacerbated this

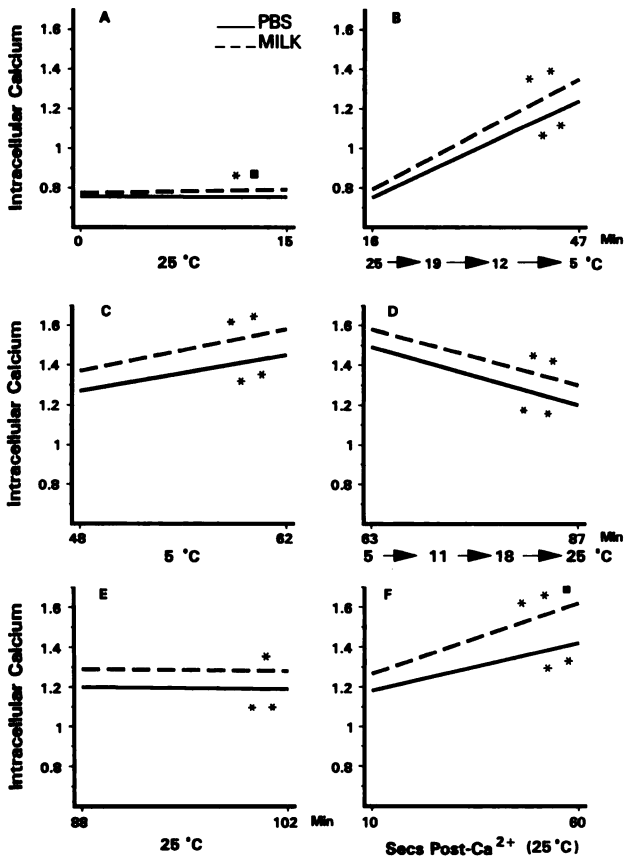


FIG. 3. Change of relative free intracellular Ca^{2+} of ejaculated bovine spermatozoa previously exposed to either milk extender (Milk) or Ca^{2+} -free PBS over the indicated sequential temperature gradient. *Slope differs from 0, $P < 0.05$; ** $P < 0.01$. ■ Slope differs from control (PBS), $P < 0.05$; ■■ $P < 0.01$.

accumulation. The effect of extenders was not detected in the viability or acrosome morphology data, suggesting that Ca^{2+} regulation is a more sensitive indicator of spermatozoal function. Bailey and Buhr (1994) found that cryopreservation in milk extender increased spermatozoal intracellular Ca^{2+} , and Bailey et al, (1994) found that *in vivo* fertility was significantly better when spermatozoal Ca^{2+} regulation following freezing most closely resembled the pre-freeze characteristics. Both extenders tested here increased intracellular Ca^{2+} in the brief exposure time, even though they contain different amounts and types of lipids, proteins, carbohydrates, and minerals that might affect Ca^{2+} regulation in spermatozoa.

During the initial 15 minutes at 25°C , milk exposure increased free intracellular Ca^{2+} and increased the rate of Ca^{2+} uptake, whereas EYT exposure also increased the rate of Ca^{2+} uptake but had no effect on the mean free intracellular Ca^{2+} in spermatozoa during this period. These increases support the contention that milk leaves a rather tightly bound reservoir of Ca^{2+} in and around spermatozoa (Buhr and Zhao, 1992) and suggest that EYT-egg yolk may provide a similar, but less available, pool. The

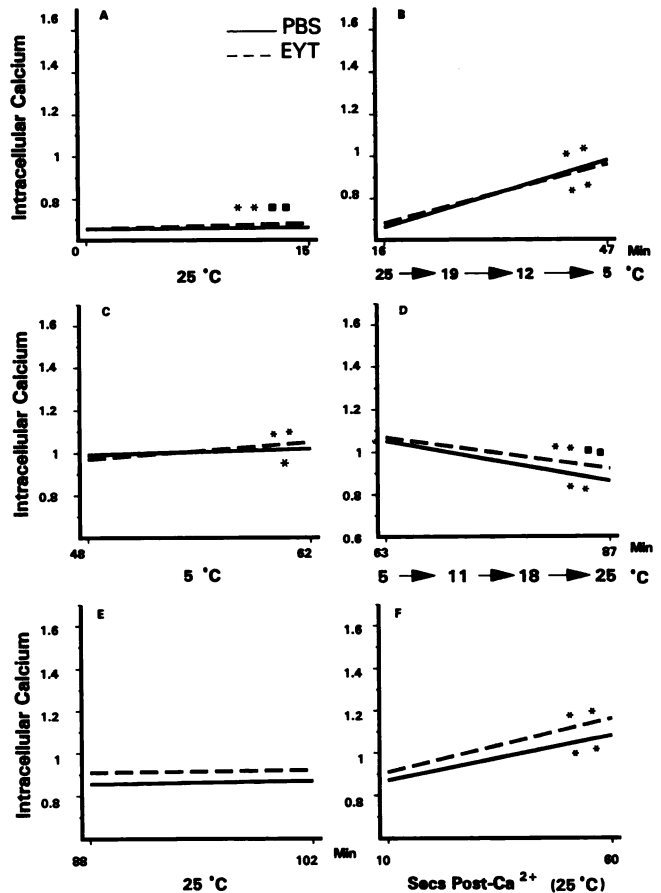


FIG. 4. Change of relative free intracellular Ca^{2+} of ejaculated bovine spermatozoa previously exposed to either EYT or Ca^{2+} -free PBS over the indicated sequential temperature gradient. *Slope differs from 0, $P < 0.05$; ■ $P < 0.01$. +Slope differs from control (PBS), $P < 0.05$; ■■ $P < 0.01$.

enhancement of Ca^{2+} permeability is one of the important characteristics of capacitation (Singh et al, 1978) and could be a useful indicator to verify the occurrence of capacitation (Coronel and Lardy, 1987). Therefore, the greater rate of Ca^{2+} uptake in milk- and EYT-exposed spermatozoa than in controls may indicate enhanced capacitation due to exposure to extenders. Milk exposure had a stronger effect on Ca^{2+} uptake than the EYT exposure. An early capacitation may be detrimental because capacitated spermatozoa were found to be less able to reach the oviduct than uncapacitated spermatozoa after artificial insemination into the uterus (Shalgi et al, 1992). This early capacitation may also shorten the fertilizing life of spermatozoa. Many somatic cells accumulate Ca^{2+} prior to death, and acrosome-reacted sperm will soon die if they do not fertilize. It is quite reasonable to see capacitation and the acrosome reaction as specialized, highly regulated, events in spermatozoal breakdown. The difference in mean free intracellular Ca^{2+} in PBS-exposed samples between the milk and the EYT experiment may be due to differ-

ences between bulls and/or ejaculates, because different bulls were used in the two experiments.

The increase of free intracellular Ca²⁺ in spermatozoa during cooling and at 5°C, and the decrease during re-warming was observed regardless of extenders. The data with indo-1 AM salt showed a decline of the ratio of 405 nm to 490 nm, that is, the opposite effect, and there was no dye leakage after cooling and re-warming. Therefore, the change in spermatozoa Ca²⁺ was not due to a change in dye affinity or to dye leakage. Robertson and Watson (1986) showed that fast cooling caused a greater increase in intracellular Ca²⁺ than slow cooling and removed the ability of spermatozoa to restore free intracellular Ca²⁺ regulation upon re-warming. In this study, the brief exposure to milk or EYT extender neither prevented nor slowed the chilling-induced uptake of free intracellular Ca²⁺. Some protecting components of the extenders, particularly EYT, may have been lost during preparation (Quinn et al, 1980), because phospholipids are loosely associated with sperm membrane (Quinn et al, 1980; Simpson et al, 1987), but clearly sufficient components remained to influence Ca²⁺ uptake at subsequent periods.

During re-warming, the rate of uptake of free intracellular Ca²⁺ in the EYT-exposed spermatozoa was lower than that of controls, perhaps reflecting either a difference in the recovery of the Ca²⁺ pump or warm shock. Re-warming of semen caused loss of membrane integrity (Holt et al, 1992) and acrosomal damage (Bamba and Cran, 1985, 1988). Temperature-induced changes in fluidity, phase transition, and Ca²⁺ pumps have all been considered as potential contributors to loss of the ability of spermatozoa to regulate free intracellular Ca²⁺ (Robertson and Watson, 1986; Buhr et al, 1989; Canvin and Buhr, 1989). Membrane ion pumps are particularly sensitive to lipid-phase transition (Holt and North, 1986). The change of the free intracellular Ca²⁺ during cooling and re-warming may reflect altered activity of ATPase, which may affect the free intracellular Ca²⁺.

The increasing rate of Ca²⁺ uptake in all samples upon addition of exogenous Ca²⁺ in this study is consistent with the uptake of exogenous Ca²⁺ reported by Buhr and Zhao (1992) and Bailey and Buhr (1994). The rapid rise of free intracellular Ca²⁺ in spermatozoa and the high percentage of missing acrosomes at the end of the experiment may indicate that the spermatozoa had undergone true or false acrosome reactions. The incubation and centrifugation might induce capacitation, and the addition of Ca²⁺ then triggers the AR or damages spermatozoa. Washing spermatozoa through a Percoll gradient can hasten capacitation of spermatozoa, possibly by removing adsorbed protein (Berger and Parker, 1989) or removing a surface-associated inhibitory factor (Fraser, 1984, 1990). Ca²⁺ is present in the millimolar range in bovine female reproductive tract fluids (Grippio et al, 1992). Ca²⁺ increased

the percentage of spermatozoa that capacitated and hyperactivated (Suarez et al, 1983, 1984; Cooper, 1984; Courtens et al, 1989; Fraser and McDermott, 1992; Olds-Clarke and Segó, 1992) and initiated the AR of capacitated spermatozoa (Yanagimachi and Usui, 1974; Singh et al, 1978; Hyne and Garbers, 1981; Fraser and McDermott, 1992; Olds-Clarke and Segó, 1992). Bielfeld et al (1990) also reported that the removal of egg yolk after incubation at 5°C, as well as the temperature shock, contributed to enhancement of capacitation and the AR of human spermatozoa. An early AR may be undesirable because an AR before spermatozoa contact unfertilized eggs renders spermatozoa incapable of binding to the zona pellucida (Saling et al, 1979; Huang et al, 1981; Crozet, 1984; Wasarman, 1987). The low viability and high percentage of spermatozoa with missing acrosomes at the end of the experiment confirmed the harmful effects of rapid temperature changes. Perhaps spermatozoa in which the AR has already occurred have a short life; spermatozoa viability wanes soon after the AR (Fleming and Yanagimachi, 1982; Bedford, 1983).

This study has shown that brief exposure to milk or EYT extenders immediately improved spermatozoal viability and/or acrosome morphology and affected subsequent Ca²⁺ movement, perhaps by exchanging components between extender and sperm membrane or by supplying nutrients to spermatozoa. Milk and EYT extenders are both commonly used in the bovine industry, but the complexity of their protective mechanisms and the lack of available parameters that fully represent fertilizing ability of spermatozoa make it difficult to tell which extender is superior. Various studies have found EYT to be better (Senger et al, 1983; Garner et al, 1988), worse (Karabinus et al, 1991; Richardson et al, 1992; Barisic et al, 1994), or equal (Almquist et al, 1954; Foote and Arriola, 1987) to milk extender in protecting sperm quality. Milk- and egg yolk-based extenders differ markedly in their chemical composition, including the types and amounts of lipid, sugar, and buffering agents present. These may result in differential protection for different sperm structures and different membrane domains. Clearly even brief exposure to these substances affects sperm control of critically important intracellular Ca²⁺ stores, in a manner reminiscent of one recently shown to be deleterious to fertility (Bailey et al, 1994). Understanding the mechanism(s) by which extenders function may permit improvement of the fertilizing ability of cryopreserved bull spermatozoa, and it is the subject of ongoing work.

References

- Almquist JO, Flipse RJ, Thacker DL. Diluters for bovine semen. IV. Fertility of bovine spermatozoa in heated homogenized milk and skimmilk. *J Dairy Sci* 1954;37:1303-1307.

- Bailey JL, Buhr MM. Cryopreservation alters the Ca^{2+} flux of bovine spermatozoa. *Can J Anim Sci* 1994;74:45-51.
- Bailey JL, Robertson L, Buhr MM. Relationships among *in vivo* fertility, computer-analysed motility and *in vitro* Ca^{2+} flux in bovine spermatozoa. *Can J Anim Sci* 1994;74:53-58.
- Bamba K, Cran DG. Effect of rapid warming of boar semen on sperm morphology and physiology. *J Reprod Fertil* 1985;75:133-138.
- Bamba K, Cran DG. Effect of rapid warming of bull and rabbit semen. *J Reprod Fertil* 1988;82:501-507.
- Barisic D, Scobey MJ, Johnson DE, Jeyendran RS. The effect of preincubation of human spermatozoa in milk on sperm penetration into zona-free hamster oocytes and on sperm binding to the human zona pellucida. *Fertil Steril* 1994;62:172-175.
- Bedford JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol Reprod* 1983;28:108-120.
- Berger T, Parker K. Modification of the zona-free hamster ova bioassay of boar sperm fertility and correlation with *in vivo* fertility. *Gamete Res* 1989;22:385-397.
- Berndtson WE, Pickett BW. Techniques for the cryopreservation and field handling of bovine spermatozoa. In: Renfret AD, Petricciani JC, eds. *The Integrity of Frozen Spermatozoa*. New York: National Academy of Science; 1978:53-77.
- Biefeld P, Jeyendran RS, Holmgren WJ, Zaneveld LJD. Effect of egg yolk medium on the acrosome reaction of human spermatozoa. *J Androl* 1990;11:260-269.
- Buhr MM, Canvin AT, Bailey JL. Effects of semen preservation on boar spermatozoa plasma membranes. *Gamete Res* 1989;23:441-449.
- Buhr MM, Zhao Y. Milk affects the calcium regulatory ability of bovine spermatozoa. 12th International Congress on Animal Reproduction. The Hague, The Netherlands: ICAR; 1992;1:417-419.
- Canvin AT, Buhr MM. Effect of temperature on the fluidity of boar sperm membranes. *J Reprod Fertil* 1989;85:533-540.
- Cooper TG. The onset and maintenance of hyperactivated motility of spermatozoa from the mouse. *Gamete Res* 1984;9:55-74.
- Coronel CE, Lardy HA. Characterization of Ca^{2+} uptake by guinea pig epididymal spermatozoa. *Biol Reprod* 1987;37:1097-1107.
- Courtens JL, Ekwall H, Paquingnon M, Ploen L. Preliminary study of water and some element contents in boar spermatozoa, before, during and after freezing. *J Reprod Fertil* 1989;87:613-626.
- Crozet N. Ultrastructural aspects of *in vivo* fertilization in the cow. *Gamete Res* 1984;10:241-251.
- DeAbreu RM, Berndtson WE, Smith RL, Pickett BW. Effect of post-thaw warming on viability of bovine spermatozoa thawed at different rates in french straws. *J Dairy Sci* 1979;62:1449-1454.
- de Leeuw FE, Chen H-C, Colenbrander B, Verkleij AJ. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology* 1990;27:171-183.
- de Leeuw FE, Colenbrander B, Verkleij AJ. The role membrane damage plays in cold shock and freezing injury. In: Johnson LA, Rath D, eds. *Boar Semen Preservation II*. Berlin: Paul Parey Scientific Publishers; 1991:97-108.
- Fleming AD, Yanagimachi R. Fertile life of acrosome-reacted guinea pig spermatozoa. *J Exp Zool* 1982;220:109-116.
- Footo RH. Buffers and extenders. What do they do? Why are they important? *Proceedings of the 10th NAAB Technical Conference on AI and Reproduction*. Milwaukee, Wisconsin: NAAB; 1984:62-70.
- Footo RH, Arriola J. Motility and fertility of bull sperm frozen-thawed differently in egg yolk and milk extenders containing detergent. *J Dairy Sci* 1987;70:2642-2647.
- Fraser LR. Mechanisms controlling mammalian fertilization. *Oxford Rev Reprod Biol* 1984;6:174-225.
- Fraser LR. Sperm capacitation and its modulation. In: Bavister B, Cummins J, Rolodan ERS, eds. *Fertilization in Mammals*. Norwell, Massachusetts: Sero Symposium; 1990:141-153.
- Fraser LR, McDermott CA. Ca^{2+} -related changes in the mouse sperm capacitation state: a possible role for Ca^{2+} -ATPase. *J Reprod Fertil* 1992;96:363-377.
- Garner DL, Johnson LA, Allen CH. Fluorometric evaluation of cryopreserved bovine spermatozoa extended in egg yolk and milk. *Theriogenology* 1988;30:369-378.
- Grippio AA, Henault MA, Anderson SH, Killian GJ. Cation concentrations in fluid from the oviduct ampulla and isthmus of cows during the estrous cycle. *J Dairy Sci* 1992;75:58-65.
- Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440-3450.
- Hancock JL. The morphology of bull spermatozoa. *J Exp Biol* 1952;29:445-453.
- Holmgren WJ, Jeyendran RS, Neff MR, Perez-Pelaez M, Zaneveld LJD. Preincubation of human spermatozoa in TEST-yolk medium: effect on penetration of zona-free hamster oocytes and correlation to other semen parameters. *J In Vitro Fert Embryo Transfer* 1989;6:207-212.
- Holt WV, Head MF, North RD. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. *Biol Reprod* 1992;46:1086-1094.
- Holt WV, North RD. Thermotropic phase transitions in the plasma membrane of ram spermatozoa. *J Reprod Fertil* 1986;78:447-457.
- Huang TTF, Fleming AD, Yanagimachi R. Only acrosome-reacted spermatozoa can bind and penetrate into zona pellucida: a study using the guinea pig. *J Exp Zool* 1981;217:286-290.
- Hyne RV, Garbers DL. Requirement of serum factors for capacitation and the acrosome reaction of guinea pig spermatozoa in buffered medium below pH 7.8. *Biol Reprod* 1981;24:257-266.
- Jones RC, Martin ICA. The effects of dilution, egg yolk and cooling to 5°C on the ultrastructure of ram spermatozoa. *J Reprod Fertil* 1973;35:311-320.
- Karabinus DS, Evenson DP, Kaproth MT. Effects of egg yolk-citrate and milk extenders on chromatin structure and viability of cryopreserved bull sperm. *J Dairy Sci* 1991;74:3836-3848.
- Karagiannidis A. The distribution of calcium in bovine spermatozoa and seminal plasma in relation to cold shock. *J Reprod Fertil* 1976;46:83-90.
- Katayama KP, Stehlik E, Roesler M, Jeyendran RS, Holmgren WJ, Zaneveld LJD. Treatment of human spermatozoa with an egg yolk medium can enhance the outcome of *in vitro* fertilization. *Fertil Steril* 1989;52:1077-1079.
- Lanzendorf SE, Holmgren WJ, Jeyendran RS. The effect of egg yolk medium on human sperm binding in the hemizona assay. *Fertil Steril* 1992;58:547-550.
- Mack SR, Zaneveld LJD. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res* 1987;18:375-383.
- Mortimer D. The male factor in fertility part I: semen analysis. In: *Current Problems in Obstetrics, Gynecology and Fertility*. Vol VIII. Chicago: Year Book Medical Publishers, Inc; 1985.
- Olds-Clarke P, Segó R. Calcium alters capacitation and progressive motility of uterine sperm from +/+ and $t^{m22}/+$ mice. *Biol Reprod* 1992;47:629-635.
- Philips PH, Lardy HA. A yolk-buffer pabulum for the preservation of bull sperm. *J Dairy Sci* 1940;23:399-404.
- Quinn PJ, Chow PYW, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *J Reprod Fertil* 1980;60:403-407.
- Richardson GF, Donald AW, Mackinnon CE. Comparison of different techniques to determine the percentage of intact acrosomes in frozen-thawed bull semen. *Theriogenology* 1992;38:557-564.
- Robertson L, Bailey JL, Buhr MM. Effects of cold shock and phospholipase A_2 on intact boar spermatozoa and sperm head plasma membranes. *Mol Reprod Dev* 1990;26:143-149.
- Robertson L, Plummer JM, Watson PF. Prior incubation reduces calcium

- uptake and membrane disruption in boar spermatozoa subjected to cold shock. *Cryo-letters* 1988;9:286-293.
- Robertson L, Watson PF. Calcium transport in diluted or cooled ram semen. *J Reprod Fertil* 1986;77:177-185.
- Robertson L, Watson PF. The effect of egg yolk on the control of intracellular calcium in ram spermatozoa cooled and stored at 5°C. *Anim Reprod Sci* 1987;15:177-187.
- Roldan ERS, Fleming AD. Is a Ca²⁺-ATPase involved in Ca²⁺ regulation during capacitation and the acrosome reaction of guinea pig spermatozoa? *J Reprod Fertil* 1989;85:297-308.
- Saling PM, Sowinski J, Storey BT. An ultrastructural study of epididymal mouse spermatozoa binding to zona pellucidae *in vitro*: sequential relationship to the acrosome reaction. *J Exp Zool* 1979;209:229-238.
- Senger PL, Mitchell JR, Almquist JO. Influence of cooling rates and extenders upon post-thaw viability of bovine spermatozoa packaged in .25- and .5-ml French straws. *J Anim Sci* 1983;56:1261-1268.
- Shalgi R, Smith TT, Yanagimachi R. A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. *Biol Reprod* 1992;46:419-424.
- Shannon P. Factors affecting semen preservation and conception rates in cattle. *J Reprod Fertil* 1978;54:519-527.
- Simpson AM, Swan MA, White IG. Action of phosphatidylcholine in protecting ram sperm from cold shock. *Gamete Res* 1986;15:43-56.
- Simpson AM, Swan MA, White IG. Susceptibility of epididymal boar sperm to cold shock and protective action of phosphatidylcholine. *Gamete Res* 1987;17:355-373.
- Singh JP, Babcock DF, Lardy HA. Increased calcium-ion influx is a component of capacitation of spermatozoa. *Biochem J* 1978;172:549-556.
- Stock CE, Fraser LR. Divalent cations, capacitation and the acrosome reaction in human spermatozoa. *J Reprod Fertil* 1989;87:4763-4778.
- Suarez SS, Katz DF, Meizel S. Changes in motility that accompany the acrosome reaction in hyperactivated hamster spermatozoa. *Gamete Res* 1984;10:253-265.
- Suarez SS, Katz DF, Overstreet JW. Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. *Biol Reprod* 1983;29:1277-1287.
- Taylor J, ed. *Bovine Semen Collection and Processing Techniques*. Regina, Saskatchewan, Canada: Canadian Association of Animal Breeders; 1991:38-45.
- Wassarmam PM. The biology and chemistry of fertilization. *Science* 1987;235:553-560.
- Watson PF. The roles of lipids and protein in the protection of ram spermatozoa at 5°C by egg-yolk lipoprotein. *J Reprod Fertil* 1981;62:483-492.
- Yanagimachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Exp Cell Res* 1974;89:161-174.