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Sperm Calcium Levels and Chlortetracycline Fluorescence Patterns are Related to the In Vivo Fertility of Cryopreserved Bovine Semen

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ABSTRACT: Cryopreserved bovine semen is less fertile than fresh semen for reasons that have not been fully elucidated. Cryopreservation is known to disrupt the sperm plasma membrane and it induces premature capacitation of a sperm subpopulation, which may be a result of the increased internal calcium levels after thawing. To test the hypothesis that sperm intracellular calcium level is correlated with in vivo fertility, we used the fluorescent calcium indicator, indo-1, and flow cytometry to assess intracellular calcium levels in frozen-thawed sperm from bulls of varying degrees of fertility. We also tested a second hypothesis that the physiological status of sperm, as assessed by the chlortetracycline (CTC) fluorescent assay, is correlated with fertility. As detected by indo-1 fluorescence, the intracellular calcium level is negatively correlated with bull fertility immediately after thawing ($P = .0362$; n = 3 ejaculates from each of 10 animals). Moreover, there was a significant difference between the 3 most and least fertile bulls over 4 hours of incubation ($P < .05$; n = 3 ejaculates per bull). Finally, there was a positive correlation between sperm displaying the CTC acrosome reaction pattern and fertility ($P = .0014$; n = 3 ejaculates from each of 10 bulls).

Key words: Freezing, thawing, acrosome reaction, capacitation, indo-1, flow cytometry.

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Cryopreservation techniques over the past 40 years have permitted long-term conservation of bovine semen (Hammerstedt et al, 1990), however, cryopreservation reduces semen quality and affects about half of the sperm (Garner et al, 1997; Bailey et al, 2000). Methods such as cooling, cryoprotectant penetration, freezing, storage, and thawing affect the lipid architecture of the plasma membrane (de Leeuw et al, 1990) and metabolism (Hammerstedt et al, 1990). These ultrastructural changes affect the ability of cells to control calcium flux across their plasma membranes. Bailey and Buhr (1994) demonstrated that calcium levels are higher in cryopreserved sperm than they are in fresh semen. It is well-established that calcium plays an important role in the capacitation process of bovine sperm (Handrow et al, 1989; Parrish et al, 1999). A recent, interesting report demonstrated that a sperm subpopulation is capacitated as a result of cryopreservation (ie, *cryo-capacitation*) even prior to artificial insemination (Cormier et al, 1997).

Clearly, regulation of calcium plays an important role during capacitation and the acrosome reaction. On the basis that calcium regulation plays an important role during capacitation and acrosome reaction (Spungin and Breitbart, 1996; Parrish et al, 1999), both of which are necessary for successful fertilization, it is reasonable to speculate that intracellular sperm calcium levels are associated with semen fertility. Therefore, the general hypothesis of this study is that the level of intracellular calcium of cryopreserved bovine sperm is correlated to in vivo fertility of bulls. Because cryopreservation alters sperm membrane integrity, thereby favoring calcium uptake, we used flow cytometry and a fluorescent intracellular calcium probe, indo-1, to assess whether bulls of low fertility have higher calcium levels than sperm from bulls with higher fertility. To test a second hypothesis that that the percentage of capacitated sperm upon thawing is also correlated with fertility, we used chlortetracycline (CTC) fluorescence as an assay of sperm capacitation (Saling and Storey, 1979; Ward and Storey, 1984; Cormier et al, 1997).

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Materials and Methods

Bulls

Bulls of known fertility were housed at the Centre d'insémination artificielle du Québec (St-Hyacinthe, PQ, Canada). In vivo fertility was assessed as 60- to 90-day nonreturn

rates (NRRs), which were adjusted with the use of a linear statistical model to include the effects of cow age, month of insemination, herd, technician, and price of semen (Bailey et al, 1994). These fertility values were converted to a scale of −9 to +9 for the least and most fertile sires, respectively, with 0 being the breed average, and are referred to as *adjusted NRR.*

Sperm Preparation

Each ejaculate was cryopreserved in egg yolk-Tris-glycerol extender according to standard industry procedures (Cormier et al, 1997). For each experiment, 250 μL straws (25×10^6 sperm/straw) were thawed in a 37°C water bath for 60 seconds.

Thawed semen samples were diluted in modified Tyrodes HEPES-buffered medium (Talp-H; 100 mM NaCl, 3.1 mM KCl, 10 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 0.4 mM EDTA, 21.6 mM sodium lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 40 mM HEPES, 1 mM pyruvate, 1 mg/mL polyvinyl alcohol). The sample was washed twice by centrifugation ($370 \times g$ for 10 minutes at room temperature). The sperm pellet was then resuspended in Talp-H and the concentration was adjusted to 50×10^6 sperm/mL. A 1 mM stock solution of indo-1AM (Molecular Probes, Eugene, Ore) in dimethylsulfoxide plus 10% pluronic F-127 (Molecular Probes) was added to a tube containing an equal volume of Talp-H as a sample for a final concentration of 2.5 μM in 25×10^6 sperm/mL. Sperm were shielded from light and incubated with indo-1AM for 30 minutes at room temperature. After incubation, the sample was washed once in Talp-H and then in modified Tyrodes bicarbonate-buffered medium with bovine serum albumin (Talp-BSA; 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 0.4 mM EDTA, 21.6 mM sodium lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM pyruvate, 6 mg/mL BSA [fatty acid free, fraction V, Sigma Chemical Company, St Louis, Mo]; Parrish et al, 1988), then adjusted to a concentration of 50×10^6 sperm/mL. For Experiment 1 (comparison of calcium levels of sperm from high and low fertility bulls), the sperm sample was divided into 2 identical aliquots, treated with or without 10 μg/mL heparin, and incubated for up to 4 hours (38.5°C, 5% CO_2). For Experiment 2 (correlations between calcium level or CTC fluorescent patterns and bull fertility), sperm were ready for evaluation of calcium level without further incubation.

Evaluation of Intracellular Calcium Level

To prevent sperm agglutination during flow cytometry, protamine (20 μg/mL; Sigma) was added to a tube containing 980 μL Talp-BSA and 20 μL sperm (final concentration of 1×10^6 sperm/mL) 1 minute prior to cytometry.

Indo-1 fluorescence from the sperm was measured using an EPICS ELITE ESP (Beckman-Coulter, Miami, Fla) with an He-Cd laser (Omnichrome Model 100). The flow cytometer detected violet fluorescence (indo-1 bound to calcium) with a 440 DL filter (381 nm wavelength) and blue fluorescence (calcium-free indo-1) with a 550 DL filter (525 nm wavelength). The relative intracellular calcium level of each sperm was expressed as the ratio of violet/blue fluorescence (Grynkiewicz et al, 1985). The flow of sperm through the quartz flow cell occurred at a rate of about 300 sperm/s. Data on at least 30 000 sperm were collected at each reading and analyzed with flow cytometry application software.

Chlortetracycline Assay

The CTC assay was slightly modified from that described by Ward and Storey (1984). The CTC stock solution containing 750 μM CTC-HCl (Sigma), 130 mM NaCl, 5 mM L-cysteine, 20 mM Tris acid (pH 7.8) was prepared daily, wrapped in foil to protect against light, and stored at 4°C until required. Ten microliters of sperm suspension were mixed with 15 μL of CTC solution on a slide at room temperature. Then, 0.3 μL of 12.5% glutaraldehyde in 2.5 M Tris base was added as a fixative. Samples (in duplicate) were covered with coverslips and stored in the dark at 4°C. A total of 200 sperm per slide were observed within 24 hours using a Nikon microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). Sperm were evaluated according to 1 of 3 CTC staining patterns (Fraser et al, 1995): fluorescence over the entire head (precapacitated cells, pattern F), fluorescence-free band in the postacrosomal region (capacitated cells, pattern B) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells, pattern AR).

Experiment 1: Comparison of Calcium Levels of Sperm From High- and Low-Fertility Bulls

Indo-1–loaded sperm were incubated for up to 4 hours in Talp-BSA (38.5°C, 5% CO_2) either with or without heparin (10 μg/mL), which is known to promote bull sperm capacitation (Parrish et al, 1988). Calcium measurements were performed hourly from 0 to 4 hours on sperm from 3 highly fertile bulls (adjusted NRRs of 3.8 to 4.7) and on samples from bulls of very poor fertility (adjusted NRRs of −4.1 to −8.2). Three different ejaculates from each male were assayed.

Experiment 2: Correlations Between Ca^{2+} Level or CTC Fluorescent Patterns and Bull Fertility

In addition to the semen tested in Experiment 1, sperm from 3 ejaculates of each of 4 bulls of intermediate fertility levels were prepared for calcium determination as described earlier (n = 3 ejaculates from each of 10 bulls of fertility ranging from 4.7 to −8.2 adjusted NRR). However, calcium measures were performed on sperm after thawing without either additional incubation or the inclusion of heparin (equivalent to Hour 0 in Experiment 1). The CTC fluorescence assay was performed on each sample immediately prior to calcium determination.

Statistical Analyses

Homogeneity of variance among the samples was tested and confirmed on intracellular calcium levels and on CTC pattern data according to the general linear model procedure (SAS Institute, 1990). For Experiment 1, orthogonal contrasts were performed to determine the difference between the 3 most and 3 least fertile bulls according to their relative intracellular calcium level expressed in channel number (mean of at least 30 000 sperm per reading). For Experiment 2, regression analyses were carried out on the relative calcium levels of sperm from all ejaculates of the 10 bulls as well as on the CTC assay data.

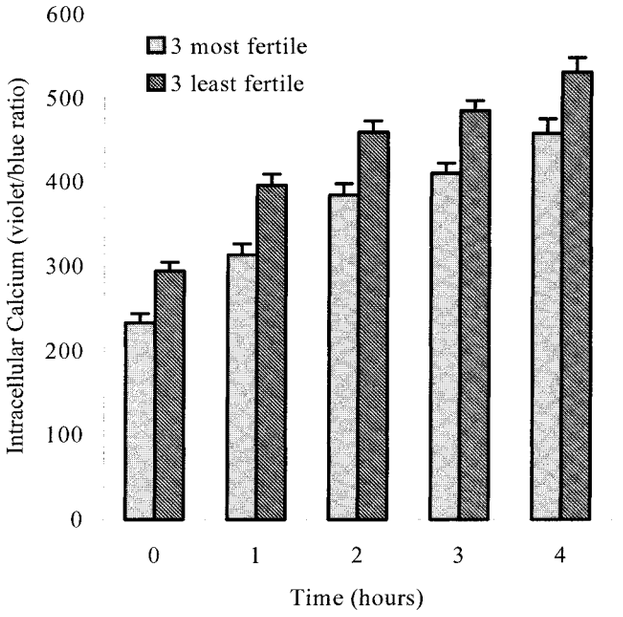

Figure 1. Comparison of high- and low-fertility bulls on the basis of their sperm calcium levels (n = 3 ejaculates from each of 3 highly or poorly fertile bulls according to their adjusted NRR; values are means ± SE). Sperm intracellular calcium concentrations for bulls with high and low fertility are estimated at 35 and 59 nM, respectively at Hour 0, and 148 and 206 nM, respectively at Hour 4 using the calculation described by Grynkiewicz et al (1985). Sperm were loaded with indo-1 AM, resuspended in Talp-BSA, and heparin (or saline) was added at Hour 0. Sperm were incubated with or without heparin (38.5°C, 5% CO_2) for up to 4 hours. At least 30 000 sperm were analyzed for each calcium measure. Heparin had no overall effect on Ca^{2+} level ($P = .1644$), so these data were pooled within each fertility level. Sperm from the lower-fertility bulls contained more intracellular $Ca2^{+}$ than did those from higher-fertility bulls at all times of incubation ($P < .05$).

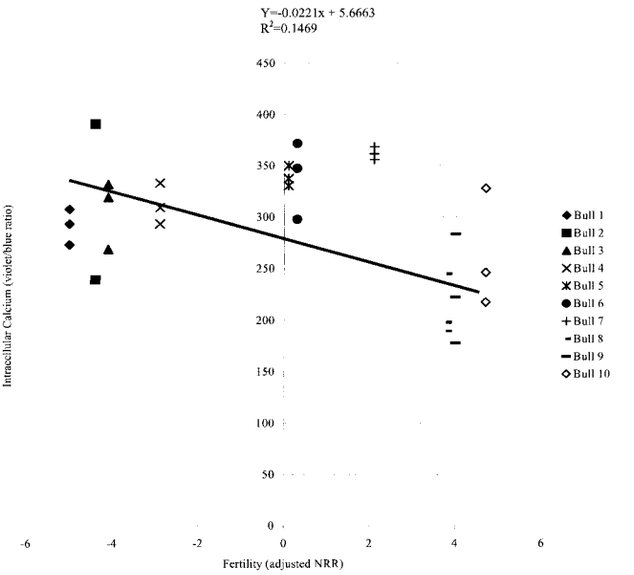

Figure 2. Correlation between relative intracellular calcium levels of cryopreserved sperm immediately after thawing and in vivo fertility rates of 10 different bulls (n = 3 ejaculates per bull). Relative calcium level is expressed as violet/blue indo-1 fluorescence ratio and fertility as adjusted NRR. At least 30 000 sperm per repetition were evaluated for their intracellular calcium levels, which is negatively correlated with bull fertility ($R^2 = 0.1469$; $P = .0362$).

Results

Experiment 1: Comparison of Calcium Levels of Sperm from High- and Low-Fertility Bulls

The effect of a 4-hour incubation in Talp-BSA with or without heparin of cryopreserved sperm on relative calcium level (violet/blue ratio) was tested on 2 different groups of bulls according to their fertility (adjusted NRR). Figure 1 shows the intracellular calcium levels of sperm from each group at each hour of incubation as detected by indo-1. Sperm from low-fertility bulls contain more calcium than do sperm from high-fertility bulls ($P < .002$). The 0-hour intracellular calcium concentrations of sperm from high- and low-fertility bulls are estimated to be 35 and 59 nM, respectively using the calculation described by Grynkiewicz et al (1985) and assuming the K_d of indo-1 to be 250 nm. At 4 hours, the relative calcium levels correspond to cytosolic calcium concentrations of approximately 148 nM for sperm from high-fertility sires and 206 nM for sperm from poor-fertility bulls.

There was no overall effect of heparin on calcium level ($P = .1644$; Figure 1). When individual times were analyzed separately, the inclusion of heparin with the sperm during incubation did not affect internal calcium levels from 0 to 3 hours ($P > .05$); however, after 4 hours of incubation, heparin-treated sperm contained higher levels of calcium than did heparin-free sperm ($P = .0497$).

Experiment 2: Correlations Between Ca^{2+} Level or CTC Fluorescent Patterns and Bull Fertility

The hypothesis that there is a relationship between the relative intracellular calcium level (assessed as the violet/blue ratio of indo-1) of sperm and sire fertility in vivo was tested on 10 bulls with adjusted NRRs ranging from −8.2 to 4.7. In Experiment 1, calcium levels differed in sperm from bulls of high or low fertility rates immediately after thawing and preparation (Hour 0), irrespective of the presence or absence of heparin. Therefore, in the second experiment, calcium levels were only assessed in Talp-BSA (ie, no heparin) after thawing and without further incubation. Figure 2 represents the correlation between relative intracellular calcium levels and sire fertility; sperm calcium level is negatively correlated with the adjusted NRRs of bulls ($P = .0362$).

The relationship between the percentages of sperm demonstrating the various CTC patterns and bull fertility was also evaluated. The average percentages of sperm displaying the various CTC fluorescence patterns for all bulls are shown in Figure 3. The AR pattern is positively

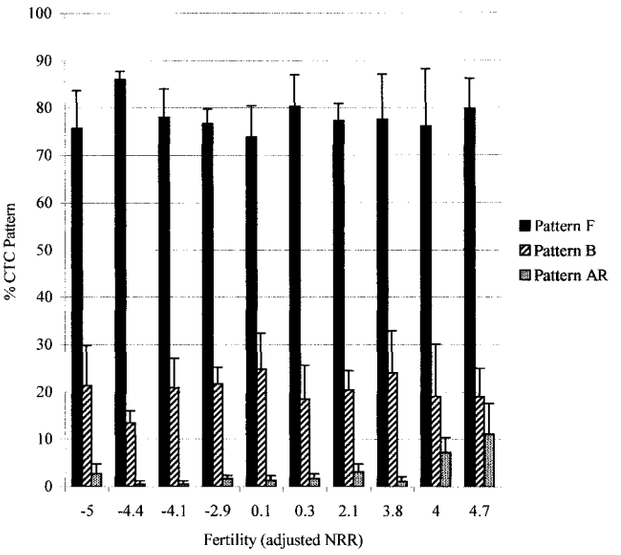

Figure 3. Distribution of the various CTC fluorescence patterns of sperm from 10 bulls of varying fertility levels immediately after thawing (means ± SD, n = 3 ejaculates per bull). See text for description of the CTC patterns.

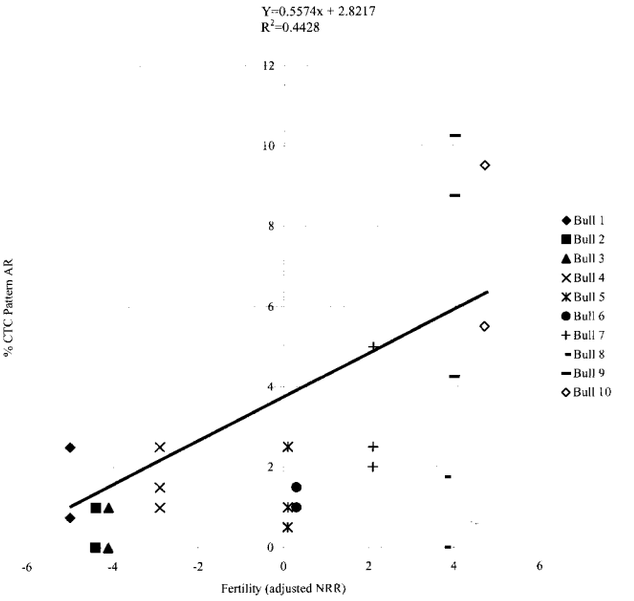

Figure 4. Correlation between the percentages of cryopreserved sperm displaying the CTC fluorescent pattern AR immediately after thawing and the in vivo fertility of 10 bulls. The percentage of sperm displaying CTC pattern AR is positively related to adjusted NRR (R^2 = 0.4428; P = .0015).

correlated to the adjusted NRR of the 10 bulls ($P < .05$). Therefore, in contrast to the correlation between intracellular calcium level and fertility, there is a much higher percentage of acrosome-reacted sperm in bulls with good fertility rates (Figure 4). None of the other CTC patterns appeared to be related to sire fertility ($P > .05$).

Discussion

The current study clearly demonstrates that relative intracellular calcium levels of cryopreserved sperm are inversely correlated with in vivo bull fertility. These results suggest that after freezing and thawing, sperm from poorly fertile bulls cannot regulate intracellular calcium levels as well as sperm from bulls of good fertility. A plausible explanation is that sperm from low-fertility bulls were damaged even prior to cryopreservation. Consequently, more calcium was already accumulated before freezing, although this suggestion is unable to be investigated because fresh semen is not available from these sires. An alternative explanation for the correlation between sperm calcium levels and bull fertility is that sperm from bulls of low fertility have more fragile and less functional plasma membranes after cryopreservation compared with sperm from high-fertility sires. Although fresh semen from low-fertility sires may be highly fertile, during cryopreservation, sperm appear to be more susceptible to undergoing membrane alterations that favor calcium influx. It has previously been shown that sperm have difficulty regulating internal calcium levels after cryopreservation (Bailey and Buhr, 1994). Bovine sperm that have been previously frozen and thawed accumulate more calcium than fresh sperm, most likely because of plasma membrane alterations induced during the cryopreservation procedure (Buhr et al, 1989; de Leeuw et al, 1991; Parks and Graham, 1992). Taken together with the present data, therefore, sperm calcium levels are inversely related to the fertility of frozen-thawed semen such that low calcium levels favor fertilization in vivo.

Previous work has shown that cryopreserved bovine sperm can control calcium entry and that this regulation is correlated with in vivo fertility (Bailey et al, 1994). From this we hypothesized that the calcium levels of live sperm, as detected by flow cytometry, are correlated with bull fertility in vivo. The data obtained in Experiment 1 demonstrated that 1) initial calcium levels at Hour 0 are sufficiently different to discriminate among the sperm from low- and high-fertility bulls, and 2) heparin does not affect sperm calcium levels from 0 to 3 hours (Figure 1). Therefore, for Experiment 2, the post-thaw calcium levels of sperm (without heparin or further incubation) were assessed for the 10 bulls. The relative calcium level of sperm was found to correlate with the adjusted NRR of the bulls (P = .0362; R^2 = 0.1469). The low determination coefficient is likely attributable to the 4 bulls with intermediate levels of fertility (adjusted NRRs of −2.9 to +2.1; Figure 2). It is interesting to note that the sperm calcium levels of these bulls approximate those observed for poor-fertility bulls, although the very good and very

poor fertility groups remain easily distinguishable (as in Figure 1). The rather low R^2 may be partially explained by the fact that different ejaculates were used to establish the NRR for the current experiments. Attribution of NRRs was carried out when the bulls were undergoing young sire testing on ejaculates processed 2–3 years prior to semen collection for the present study. It is not impossible that variations of their fertilizing potential have occurred over this time; testing recent ejaculates for sperm calcium content could reveal an even better correlation with adjusted NRRs than are reported in Figure 2. Alternatively, it is tempting to speculate that after cryopreservation, a high percentage of sperm from bulls of intermediate fertility have dysfunctional plasma membranes such that calcium levels are elevated, approaching those of sperm from bulls with very low adjusted NRRs. However, a more profound level of damage, which may or may not be related to calcium influx, occurs in the sperm of the latter bulls. Consequently, the fertilizing capacity of these cells is further reduced compared with sperm from intermediate sires. Nevertheless, a significant correlation between intracellular sperm calcium levels and bull fertility is evident, providing a rapid, easy method to discriminate the fertilizing potential among individuals or ejaculates.

Because only cells retaining indo-1 are evaluated with flow cytometry, it is assumed that all the sperm assessed were viable (with intact, functional plasma membranes). Therefore, the relation between bull fertility and sperm calcium levels may be attributed to the level of sublethal, ultrastructural damage to the plasma membrane. Compared with sperm from high-fertility bulls, the sperm from less fertile bulls could have plasma membranes that are disrupted to a greater level of dysfunction. The importance of membrane structure to subsequent fertility is demonstrated by observations that sperm plasma membrane integrity is compromised in infertile men but not in fertile men, as assessed by the hypo-osmotic swelling test (HOS test) and eosin-Y staining (Ramirez et al, 1992).

Furthermore, because of adenosine triphosphate (ATP) depletion and reduced ion pump function, cryopreservation is believed to depolarize sperm plasma membranes (Hammerstedt et al, 1990). Such a depolarization may prime voltage-sensitive calcium T-channels (Florman et al, 1998), which would lead to a premature calcium influx in a cell. Assuming that sperm from low-fertility bulls are more susceptible to membrane alterations at cryopreservation, it would be logical to observe higher relative calcium levels in these sperm. It is also possible that a putative Ca^{2+}-ATPase (Parrish et al, 1999) on the plasma membranes of sperm from low-fertility bulls is rendered dysfunctional by the cryopreservation protocol. Consequently, cytoplasmic calcium would be retained in the sperm instead of being pumped to the extracellular surroundings.

Although excess intracellular calcium is associated with sperm necrosis (Robertson et al, 1990), many events experienced by the sperm en route to fertilization in vivo necessitate proper calcium regulation. Sperm binding to oviductal epithelium is dependent on calcium (Lapointe and Sirard, 1996). Hyperactivated motility involves fluctuations in intraflagellar calcium levels (Suarez and Dai, 1995). Sperm regulation of cytosolic and acrosomal calcium levels is of critical importance during capacitation (Parrish et al, 1999). The ability of sperm to undergo the zona pellucida-induced acrosome reaction is also dependent on exquisitely controlled calcium elevations (Florman et al, 1998). Therefore, it is evident that any anomaly in the capacity of sperm to appropriately control calcium regulation would hamper their ability to fertilize at one or more of these events.

It has previously been demonstrated that heparin induces capacitation of bovine sperm in vitro (Parrish et al, 1986; Parrish et al, 1988). In Experiment 1, sperm incubated in the presence of heparin did not display differences in their calcium levels for all incubation times combined ($P > .05$), which supports other observations that cryopreserved bull sperm do not respond to heparin (Cormier et al, 1997). However, Parrish et al (1999) reported that the intracellular calcium levels in the head regions of bovine sperm treated with heparin also begin to rise after 3 hours of incubation. Indeed, in Experiment 1, when the data are statistically analyzed at each hour, a difference appears at 4 hours of incubation such that heparin-treated sperm accumulated more calcium than the heparin-free controls ($P = .0497$).

Cormier et al (1997) demonstrated that cryopreservation induces partial or complete capacitation of bull sperm. In this study, we used the CTC assay (Ward and Storey, 1984) to indicate the physiological status of sperm. In bull sperm, CTC pattern B is indicative of capacitation, whereas pattern AR indicates that sperm have undergone the acrosome reaction, and pattern F reveals uncapacitated sperm (Fraser et al, 1995). In this study, these 3 patterns were evaluated in the sperm of 10 bulls. There was no correlation with the proportion of sperm displaying either CTC patterns B or F and bull fertility ($P > .05$). We found it surprising that the acrosome-reacted state (CTC pattern AR) was correlated with the fertility rate of bulls immediately after thawing ($P = .0015$; Figure 4). Bulls of high fertility displayed a greater percentage of acrosome reaction than did sires with lower fertility. This correlation may possibly reflect the responsiveness of the sperm from the more fertile bulls, as has been observed in human sperm (Fuse et al, 1993). Cryopreserved bovine sperm have previously been shown to be less able to physiologically respond to heparin than fresh sperm can (Cormier et al, 1997). Alternatively, because our CTC assay did not include a cell viability mark-

er, dead cells were undoubtedly assessed and may have confounded our results, although our finding that the AR pattern relates to fertility in vivo remains interesting. Recently, Thundahil et al (1999) used the CTC assay in conjunction with ethidium homodimer-1 to label dead cells and found the proportion of live pattern F (uncapacitated) sperm to be correlated to bull field fertility.

In conclusion, this study provides evidence that sperm calcium levels are related to bull fertility. Calcium regulation by sperm from sires of low fertility appears to be deficient because their post-thaw relative intracellular calcium level is higher than it is in bulls of good fertility. Further investigation concerning the mechanisms of calcium entry in relation to cryopreservation and sperm function will hopefully lead to the improvement of the fertility of cryopreserved semen from bulls as well as from other mammals.

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