A Differential Mechanism Is Involved During Heparin- and Cryopreservation-Induced Capacitation of Bovine Spermatozoa

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ABSTRACT

After ejaculation, mammalian spermatozoa must undergo capacitation to fertilize. Capacitation of bovine spermatozoa occurs in vitro in medium supplemented with heparin. Semen cryopreservation is an important tool for assisted reproduction, although the fertility of frozen-thawed spermatozoa is reduced, possibly due to precocious capacitation-like changes that are known to occur. Our purpose was to clarify the mechanisms involved in bull sperm cryocapacitation induced by cryopreservation. Our general hypothesis is that the signaling pathways that lead to capacitation are triggered by the cryopreservation procedure. Ejaculated bovine semen was divided into two aliquots and diluted in extender; one was then kept fresh, whereas the second was cryopreserved. Western blots of extracted sperm proteins with anti-phosphotyrosine antibody showed that capacitation, induced by either heparin in fresh sperm or cryopreservation (cryocapacitation), is associated with a differential profile of phosphotyrosine-containing proteins. Immunolocalization of phosphotyrosine-containing proteins in the fresh and cryopreserved spermatozoa showed that, after thawing, cryocapacitated sperm displayed labeling over the acrosomal region, whereas for fresh sperm, this labeling appeared after 5-h incubation with heparin. The chlortetracycline assay and the ability of the sperm to undergo the lysophosphatidylcholine-induced acrosome reaction were used to confirm that a subpopulation of cryopreserved sperm is capacitated at thawing, irrespective of heparin inclusion. Since glucose is known to inhibit heparin-induced capacitation, the semen extender was modified to include glucose as a means of inhibiting cryocapacitation; however, cryocapacitation was not prevented according to the chlortetracycline assay and profile of phosphotyrosine-containing sperm proteins.

INTRODUCTION

After ejaculation, mammalian spermatozoa undergo an obligatory maturational process called capacitation either in vivo during transit through the female genital tract [1, 2] or in vitro in defined media [3]. Capacitation allows spermatozoa to undergo the zona pellucida-induced acrosome reaction (AR) and fertilize oocytes. Although the molecular mechanisms of capacitation are not completely elucidated, many studies have demonstrated an involvement of numerous structural and biochemical modifications in spermatozoa, such as changes in membrane composition and fluidity [4–6], increased intracellular calcium [7–9], cytoplasmic alkalization [10, 11], activation of ion channels [12, 13], and generation of reactive oxygen species [14, 15]. Capacitation is associated with protein tyrosine phosphorylation [8, 16–18], which is modulated via a cAMP-dependent pathway in many species, including the mouse, human, and bull [8, 19, 20].

Capacitation of bovine spermatozoa can occur in vitro in medium supplemented with a glycosaminoglycan, heparin (HEP) [21]. The putative mechanism is that HEP functions as a ligand for a receptor localized in the sperm plasma membrane, but such a receptor is as yet uncharacterized. This HEP, which is bound to the spermatozoa, appears to stimulate 1) the intracellular elevation of calcium, pH, and cAMP, which seem to be necessary to initiate the signaling pathway concomitant with capacitation [20–23]; and 2) the removal of seminal plasma proteins adsorbed to the plasma membrane, which are considered to be inhibitors of capacitation [24, 25]. In vitro, glucose inhibits HEP-induced capacitation of bovine spermatozoa via mechanisms that affect both cAMP metabolism and intracellular pH [10, 22, 26]. Moreover, the tyrosine phosphorylation of sperm proteins during HEP-induced capacitation was inhibited by the presence of glucose [20].

Semen cryopreservation is an important tool for assisted reproduction, although the fertility of frozen-thawed spermatozoa is reduced due to sublethal damage that is not completely understood [27, 28]. However, it has been recently recognized that cryopreservation procedures (dilution, cooling, freezing/thawing) induce capacitation-like changes in spermatozoa [28, 29]. Indeed, recent studies have reported similarities between the changes associated with capacitation and cryoinjury, such as plasma membrane reorganization and fluidization, and calcium influx to the spermatozoa [30]. Therefore, partially or fully cryopreserved spermatozoa demonstrate capacitation-like behavior revealed by a greater proportion of chlortetracycline (CTC) fluorescent pattern B (capacitated cells) [31] and their ability to undergo the AR or fertilize oocytes in vitro [30, 32–35].

This cryocapacitation is thought to be partly responsible for the reduced fertility of frozen-thawed bull semen. The purpose of the present study is to characterize the mechanisms implicated in bull sperm capacitation induced by cryopreservation. Cryopreserved sperm demonstrate elevated intracellular calcium levels, and this cation is an important molecule in signaling events; calcium increases are associated with capacitation, hyperactivation, and the AR.
Therefore, our general hypothesis was that the signaling pathway that leads to capacitation is triggered by cryopreservation. Specifically, we speculated that immediately after thawing cryopreserved spermatozoa would display a tyrosine phosphoprotein pattern similar to that of fresh cells having undergone capacitation. Therefore, we identified a specific subset of phosphoproteins involved in both HEP capacity and cryocapacitation. We then localized the distribution of these phosphotyrosine-containing proteins in fresh and cryopreserved spermatozoa. Finally, we also tested the hypothesis that cryocapacitation could be prevented by cryopreserving the semen with glucose.

**MATERIALS AND METHODS**

**Collection and Preparation of Spermatozoa**

Fresh semen from Holstein bulls of proven fertility was collected using an artificial vagina at the Centre d’Insémination Artificielle du Québec (St-Hyacinthe, Quebec, Canada). Only ejaculates with sperm motility of more than 60% and concentration of more than 3 × 10⁶ cells/ml were used for subsequent manipulations.

Each ejaculate was divided into two samples corresponding to two sperm pretreatments. The first was diluted in incomplete egg yolk-Tris extender without glycerol at 38°C (EYT); one part of the ejaculated semen mixed with one part of the EYT (fresh-extended spermatozoa). The second sample was diluted in complete egg yolk-Tris extender with glycerol (EYTG) [20], then frozen in 0.25-ml straws containing 15–20 × 10⁶ cells/ml and stored as per normal industry procedures (cryopreserved spermatozoa). Fresh-extended semen was transported to the laboratory in an insulated thermos at 23°C within 2 h of collection. One week later, frozen semen straws were transported to the university in a dry shipper and stored at −196°C in a liquid-nitrogen cryostat. Spermatozoa were thawed by plunging straws into a water bath (37°C) for 1 min.

All samples of spermatozoa were washed twice by centrifugation (280 × g, 10 min, 25°C) with noncapacitating medium, pH 7.4–7.45 (modified Tyrode Hepes-buffered medium [NCM] [21] containing 0.1% [wt/vol] polyvinyl alcohol [PVA]; average molecular weight, 30,000–70,000 Da; Sigma, St. Louis, MO). The pellets of spermatozoa were resuspended in capacitating medium, pH 7.4 at 38.5°C (modified Tyrode bicarbonate-buffered medium [CM] [21] containing 0.6% [wt/vol] fatty acid free BSA, 1% [wt/vol] glucose, and 1 mM pyruvate) (capacitated medium [CM]). Further manipulations were performed on the capacitated spermatozoa unless otherwise stated.

**Preparation of Sperm Proteins and Immunoblotting**

The profiles of phosphotyrosine-containing proteins from fresh and cryopreserved spermatozoa were identified using SDS-PAGE and immunoblotting [20]. Before (0 h) and after 2.5 and 5 h of incubation in CM with or without HEP, aliquots of 1 × 10⁶ cells from sperm suspensions were centrifuged (16,000 × g) at room temperature in a modified phosphohosphate-buffered saline (m-PBS), pH 7.4 (2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 5.5 mM glucose, and 1 mM pyruvate) (15–20 × 10⁶ cells/ml) for 10 min, 25°C, then centrifuged (250 × g, 10 min, 25°C), and the sperm pellets were incubated overnight at 4°C in m-PBS containing 2% [wt/vol] BSA to block nonspecific binding sites. After centrifugation (250 × g, 10 min, 25°C), the sperm pellets were resuspended 1:10 in m-PBS. A 20-µl sperm suspension was smeared onto a slide and dried, then sperm were permeabilized in absolute ethanol for 5 min. Fixed, permeabilized cells were incubated with α-PY antibody (diluted 1:10 in TBS) for 1 h at room temperature. Fluorescein isothiocyanate (FITC)-conjugated GAM IgG (Zymed Laboratories, South San Francisco, CA) was used as secondary antibody (diluted 1:10 in TBS) for a 45-min incubation in the dark at room temperature. Excess antibodies were removed by plunging 4–5 times in TBS. For negative controls, the α-PY antibody was replaced by m-PBS. Coverslips were mounted with 90% (vol/vol) glycerol. Phosphotyrosine fluorescence was observed under blue-violet illumination using an upright epifluorescent microscope.

One hundred spermatozoa per slide (from two independent experiments) were classified according to one of three fluorescence patterns that were observed: 1) a short line or triangle of fluorescence in the equatorial segment (pattern S), 2) uniform fluorescence over the entire acrosome (pattern A), and 3) fluorescence both at the equatorial segment and the anterior acrosome (pattern AS).

**Functional Status of Spermatozoa**

In vitro capacitation of bovine spermatozoa was evaluated using the CTC fluorescence assay as described by Collin et al. [38]. Before and after 5 h of incubation in CM with or without HEP, aliquots of 1 × 10⁶ cells from sperm suspensions were centrifuged (16,000 × g) at room temperature in a modified phosphohosphate-buffered saline (m-PBS), pH 7.4 (2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 5.5 mM glucose, and 1 mM pyruvate) supplemented with 0.2 mM Na₂VO₄. Sperm proteins were extracted in a nonreducing solubilization buffer (2% SDS; 62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 1% bromphenol blue; and 1 mM thugstic acid; Sigma) by heating for 5 min at 100°C, then centrifuging (16,000 × g) for 3 min. Supernatants were added to β-mercaptoethanol (final concentration, 5%), heated for 5 min at 100°C, and centrifuged (16,000 × g) for 1 min. Solubilized proteins were stored at −180°C until separation on 10% SDS-PAGE minigels [36], then electrotransferred onto PVDF membranes (Poly-screen; NEN Life Science Products, Boston, MA) using an Electrophoretic Blotting System (C. B. S. Scientiﬁc Company, Inc., Del Mar, CA). Electrophoretic protein transfers were performed in blotting buffer (25 mM Tris, 192 mM glycéline, and 20% methanol, pH 8.3–8.5) at 400 mA constant for 4 h at 4°C.

Non-specific binding sites were saturated by soaking membranes with 5% [wt/vol] skim milk in PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 5.5 mM glucose, and 1 mM pyruvate) (15–20 × 10⁶ cells/ml) for 1 h at room temperature. Blots were incubated with the primary monoclonal anti-phosphotyrosine (α-PY) antibody (clone 4G10; UBL, Lake Placid, NY) diluted 1:10,000 in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) for 1 h at room temperature, then washed 3 times (10 min per wash) in TBS containing 0.1% [vol/vol] Tween 20 (T-TBS) to remove excess α-PY antibody. Peroxidase-conjugated goat anti-mouse (GAM) IgG (Bio-Rad, Mississauga, Ontario, Canada) was used as the secondary antibody and incubated with the blots at a dilution of 1:15,000 in TBS for 45 min. Excess GAM IgG was removed by washing the blots 3 times (10 min per wash) in T-TBS. Phosphotyrosine-containing bands were detected with an enhanced chemiluminescence kit (Amersham, Baie d’Urfe, Quebec, Canada) according to the manufacturer’s instructions.

**Immunolocalization**

Localization of phosphotyrosine-containing proteins was determined using an indirect immunofluorescence assay as described by Tardif et al. [37]. Before and after 5 h of incubation in CM with or without HEP, aliquots of 1 × 10⁶ cells were centrifuged (250 × g, 10 min, 25°C), fixed in 2% [vol/vol] formaldehyde for 1 h at 4°C, and centrifuged again (250 × g, 10 min, 25°C), and the sperm pellets were incubated overnight at 4°C in m-PBS containing 2% [wt/vol] BSA to block nonspecific binding sites. After centrifugation (250 × g, 10 min, 25°C), the sperm pellets were resuspended 1:10 in m-PBS. A 20-µl sperm suspension was smeared onto a slide and dried, then sperm were permeabilized in absolute ethanol for 5 min. Fixed, permeabilized cells were incubated with α-PY antibody (diluted 1:10 in TBS) for 1 h at room temperature. Fluorescein isothiocyanate (FITC)-conjugated GAM IgG (Zymed Laboratories, South San Francisco, CA) was used as secondary antibody (diluted 1:10 in TBS) for a 45-min incubation in the dark at room temperature. Excess antibodies were removed by plunging 4–5 times in TBS. For negative controls, the α-PY antibody was replaced by m-PBS. Coverslips were mounted with 90% (vol/vol) glycerol. Phosphotyrosine fluorescence was observed under blue-violet illumination using an upright epifluorescent microscope.

One hundred spermatozoa per slide (from two independent experiments) were classified according to one of three fluorescence patterns that were observed: 1) a short line or triangle of fluorescence in the equatorial segment (pattern S), 2) uniform fluorescence over the entire acrosome (pattern A), and 3) fluorescence both at the equatorial segment and the anterior acrosome (pattern AS).

**Experiment with Glucose**

The potential of glucose to inhibit cryocapacitation was evaluated by diluting fresh semen with modified EYTG extender (m-EYTG; 69 mM fructose was fully replaced by glucose). Each ejaculate was divided into two samples: the first was diluted in standard EYTG (control pretreatment) and the second was diluted in m-EYTG (glucose pretreatment). After cooling, the pretreated semen was frozen in 0.25-ml straws that contained 15–20 × 10⁶ cells/ml and stored as per normal industry procedures. Cryopreserved spermatozoa were thawed and prepared as previously described in Materials and Methods. Briefly, after thawing and without further in-
The homogeneity of variances among treated samples was confirmed using the Levene test and residue plots (General Linear Model [GLM] and Plot procedures, respectively; SAS Institute Inc., Cary, NC, 1990). Raw data from the immunolocalization experiment were modified using arcsine transformation. The localization and relative distribution of phosphotyrosine-containing proteins to the fresh-extended and cryopreserved spermatozoa immediately after thaw and during the 5 h of incubation with or without HEP, confirmed with immunoblots performed using the same ejaculate from the same bull (Fig. 2).

The 56-PP, which progressively appears in the presence of HEP when fresh-extended spermatozoa were incubated in CM for up 5 h, was already evident in cryopreserved spermatozoa before incubation in capacitating conditions for 9 of 18 ejaculates from the different bulls tested (Figs. 1 and 2). For 2 of 18 ejaculates from different bulls with cryopreserved spermatozoa, the intensity of 56-PP was slightly enhanced over time in the presence of HEP, indicating some variability among bulls to respond to HEP after thaw. Furthermore, this 56-PP in cryopreserved spermatozoa at the start of incubation (0 h) appears to be the same tyrosine phosphoprotein appearing in fresh-extended cells after 5 h of incubation with or without HEP, confirmed with immunoblots performed using the same ejaculate from the same bull (Fig. 2).

The 56-PP was specifically phosphorylated on tyrosine residues, since the signal disappears when blots were incubated with the secondary antibody alone (data not shown). In contrast, a major band (at approximately M₉ 35 000, 35-PP) was always immunodetected in cryopreserved spermatozoa immediately after thaw and during the 5 h of incubation with or without HEP (Fig. 1B), even though its intensity decreased over time in the absence of HEP. This high intensity of tyrosine phosphorylation was never observed in fresh-extended spermatozoa before incubation in capacitating conditions, indicating some variability among bulls to respond to HEP after thaw. Furthermore, this 56-PP in cryopreserved spermatozoa at the start of incubation (0 h) appears to be the same tyrosine phosphoprotein appearing in fresh-extended cells after 5 h of incubation with or without HEP, confirmed with immunoblots performed using the same ejaculate from the same bull (Fig. 2).

The localization and relative distribution of phosphotyrosine-containing proteins to the fresh-extended and cryo-

**RESULTS**

**Profile of Phosphotyrosine-Containing Proteins**

The profile of phosphotyrosine-containing proteins associated with capacitation, induced by either HEP or cryopreservation, in fresh-extended and frozen-thawed spermatozoa is shown in Figure 1A and B, respectively. After 0, 2.5, and 5 h of incubation in CM with or without HEP, tyrosine-phosphorylated proteins of M₉ 21 500–220 000 were immunodetected in both fresh-extended and cryopreserved spermatozoa. However, these cells did not display exactly the same tyrosine phosphoprotein profile. Specifically, the intensity of several proteins (approximately M₉ 50 000–220 000) from fresh-extended spermatozoa was enhanced over time in presence of HEP (Fig. 1A). Moreover, two specific phosphotyrosine-containing proteins (M₉ 56 000, 56-PP, and 114 000, 114-PP) were systematically detected after 5 h of incubation with HEP when most of this fresh-extended sperm population was capacitated, according to the CTC pattern B. Conversely, there was no increase in the intensity over time with or without HEP for nearly all of the phosphotyrosine-containing proteins in cryopreserved spermatozoa (Fig. 1B); the intensity of labeling even tended to decrease in the absence of HEP.
Preserved spermatozoa before and after 5 h of incubation in CM with or without HEP are shown in Fig. 3A and C. Since the percentages of both A and AS patterns indicated the localization of tyrosine phosphoproteins to the acrosomal region, these data were pooled for statistical analysis and now termed as pattern AS (Fig. 3C). Even at 0 h of incubation, a differential distribution of phosphotyrosine-containing proteins was evident between fresh-extended and cryopreserved spermatozoa (Fig. 3A and C). Most fresh-extended spermatozoa displayed immunofluorescence at the equatorial segment (pattern S) compared with a major subpopulation of cryopreserved cells that exhibited phosphotyrosine labeling at the acrosomal region, particularly pattern AS. Indeed at 0 h, 15 times more cryopreserved than fresh-extended cells exhibited fluorescence at the acrosomal region, pattern AS, irrespective of HEP inclusion (Fig. 3C; \( P < 0.01 \)). In contrast, 3.6 times more fresh-extended than cryopreserved cells displayed a small amount of fluorescence at the equatorial segment (pattern S, \( P < 0.01 \)). The presence of HEP had no effect on the initial percentage of immunofluorescence patterns for both fresh-extended and cryopreserved spermatozoa (\( P > 0.01 \)).

After 5 h of incubation with HEP, a subset of phosphoproteins was redistributed to the acrosomal region of fresh-extended spermatozoa (Fig. 3A and C; patterns A and AS). This redistribution was only detected in a minor subpopulation when fresh-extended spermatozoa were incubated without HEP. In contrast, there was no apparent change in the distribution of tyrosine phosphoproteins in the cryopreserved spermatozoa, since a major subpopulation of these cells continued to display immunofluorescence at the acrosomal region (pattern AS). The redistribution of tyrosine phosphoproteins was time dependent for fresh-extended but not for cryopreserved cells, as indicated by the increase in the percentages of pattern AS only in fresh-extended spermatozoa, regardless of HEP inclusion (Fig. 3C; \( P = 0.0001 \)). There was no change in the percentage of pattern AS cells in cryopreserved spermatozoa following the 5 h incubation.
of incubation with or without HEP (P > 0.01). Nevertheless, the prevalence of immunofluorescence pattern AS in fresh-extended cells was also HEP related compared with those cryopreserved, regardless of incubation time (P = 0.0376), since the percentage of pattern AS increased 2-fold when fresh-extended spermatozoa were pretreated with HEP (P < 0.01). Conversely, there was no increase in the percentage of pattern AS exhibited by cryopreserved spermatozoa with or without HEP (P > 0.01).

Immunofluorescence was also detected in flagella of fresh-extended and cryopreserved spermatozoa for all treatment groups. In the absence of α-PY antibody (CTRL−, negative controls), neither fresh-extended nor cryopreserved spermatozoa displayed any specific fluorescence patterns (Fig. 3A).

Determination of Capacitation by the CTC Assay

Capacitation (according to the CTC pattern B) of fresh-extended and cryopreserved spermatozoa before and after 5 h of incubation is shown in Figure 4. At 0 h, following HEP inclusion and before incubation in CM, three times more cryopreserved than fresh-extended spermatozoa exhibited pattern B (P < 0.05). The presence of HEP had no effect on the initial percentage of capacitation of both fresh-extended and cryopreserved spermatozoa (P > 0.05).

During the 5 h of incubation in CM, all treated spermatozoa underwent capacitation as indicated by a time-dependent increase in the percentages of CTC pattern B, regardless of HEP dose (P = 0.0001). Despite the fact that three times fewer fresh-extended than cryopreserved spermatozoa exhibited pattern B at 0 h (P < 0.05), similar rates of capacitation (CTC pattern B) were achieved after 5 h (P > 0.05). However, this increase over time was HEP related only in fresh-extended spermatozoa (P = 0.0001). Therefore, the percentage of capacitated cells increased 3.6-fold from 0 to 5 h for fresh-extended spermatozoa pretreated with HEP compared with any other treatment groups incubated with or without HEP. This effectiveness of HEP to induce capacitation in fresh-extended spermatozoa was evident regardless of incubation time (P = 0.0001), such that two times more of these cells exhibited CTC pattern B in the presence of HEP compared with those without HEP (P < 0.05). Conversely, there was no difference in the percentage of pattern B cells in cryopreserved spermatozoa with or without HEP (P > 0.05).

To ensure that differences in functional status were not related to a subpopulation of dead spermatozoa, sperm viability (before and after 5 h of incubation with or without HEP) was determined using eosin-nigrosin staining [34]. The percentages of viable cells were similar for both fresh-extended and cryopreserved spermatozoa (90% ± 5% vs 81% ± 8% at time 0 h and 73% ± 14% vs 69% ± 12% at time 5 h, respectively; P = 0.0612).

Determination of Initial Capacitation Status by LPC Induction

The LPC-induced AR rates (indicating capacitated cells) of fresh-extended and cryopreserved spermatozoa before incubation in CM with or without HEP are shown in Figure 5. Statistical analysis indicated that HEP inclusion had no effect on the ability of spermatozoa to undergo the LPC-induced AR (P = 0.5082). Therefore, these data were pooled in a second statistical model.

The LPC-induced AR in all treatments (P < 0.01). Fur-
thermore, sperm pretreatments in combination with presence of LPC significantly affected the percentage of AR ($P = 0.0011$) such that 2.3 times more cryopreserved than fresh-extended spermatozoa underwent the LPC-induced AR ($P < 0.05$). Conversely, there was no significant difference in the percentages of AR achieved in the absence of LPC (spontaneous AR) between fresh-extended and cryopreserved spermatozoa ($P > 0.05$).

### Inhibition of Cryocapacitation by Glucose

The distribution of the various CTC patterns (F, B, or AR) of frozen-thawed spermatozoa cryopreserved with extender containing either fructose (EYTG) or glucose (m-EYTG) before incubation in CM with or without HEP is displayed in Figure 6. There was no effect of HEP inclusion on the percentage of the various CTC patterns ($P = 0.3745$), so these data were pooled in the following statistical analysis.

Cryopreservation of spermatozoa with m-EYTG extender instead of EYTG did not affect the distribution of CTC patterns immediately after thaw, as indicated by the similar percentages of pattern F, B, and AR exhibited for control and glucose pretreated spermatozoa ($P = 0.2238$). Therefore, there was no significant decrease in the percentage of glycerol-containing spermatozoa displaying pattern B (capacitated cells according to CTC) compared with the control.

Sperm viability after thaw (0 h) was not affected by cryopreservation with m-EYTG, HEP inclusion, or the m-EYTG × HEP interaction ($P = 0.2316$). The percentages of viable cells as determined by eosin-nigrosin [34] were 83% ± 5% and 77% ± 2% for control and glucose pretreated spermatozoa, respectively, regardless of the presence of HEP.

The profiles of phosphotyrosine-containing proteins from frozen-thawed spermatozoa, cryopreserved with extender containing either fructose (EYTG) or glucose (m-EYTG), before incubation in CM with or without HEP are shown in Figure 7. Immediately after thaw, there were no major differences between the phosphotyrosine-containing protein profiles for spermatozoa cryopreserved with EYTG or m-EYTG. Indeed, almost all the tyrosine phosphoproteins previously described herein for frozen-thaw spermatozoa are still apparent, and their intensity is not reduced as we hypothesized. Consequently, sperm cryopreservation using m-EYTG extender instead of EYTG did not affect the premature appearance of the tyrosine phosphoproteins associated with cryocapacitation.

### DISCUSSION

#### Different Tyrosine Phosphoprotein Profiles for Fresh-Extended and Cryopreserved Spermatozoa

The results obtained in this study show that capacitation of bovine spermatozoa, induced either by HEP (physiological capacitation) or cryopreservation (cryocapacitation), is associated with a differential profile of phosphotyrosine-containing proteins. The phosphotyrosine-containing protein profile exhibited by fresh-extended spermatozoa (in EYT extender without glycerol) during capacitation induced by HEP (Fig. 1A) is similar to that previously described [20]. Interestingly, cryopreserved spermatozoa, which are known to be cryocapacitated [34], do not display the identical tyrosine phosphoprotein profile (Fig. 1B). A subset of phosphotyrosine-containing proteins is immunodetected at 0 h, and no major increase in the intensity of these proteins over time is observed, even in conditions that are used to induce capacitation in fresh spermatozoa (CM with HEP). Moreover, the signal tends to decrease for some tyrosine phosphoproteins during the 5-h incubation period in the absence of HEP. These data support the hypothesis that tyrosine phosphoproteins somehow mediate capacitation because they are evident during both HEP-induced capacitation and cryocapacitation of bovine spermatozoa [20]. However, it appears that cryocapacitation involves a different regulatory mechanism of protein tyrosine phosphorylation than HEP. Lane et al. [41] recently found that high-density lipoproteins lead to capaci-
Cryocapacitation and Tyrosine Phosphorylation

It was suggested that both HEP and high-density lipoproteins mediate capacitation but via different mechanisms. Moreover, it was previously shown that subpopulations of cooled or cryopreserved spermatozoa are competent to fertilize oocytes even without HEP [34]. Therefore, it is plausible that the initial tyrosine phosphorylation level of target proteins in frozen-thawed spermatozoa is sufficient to initiate an intracellular signaling pathway associated with HEP-mediated capacitation. We did not assess the direct effect of glycerol on the tyrosine phosphoprotein profiles of fresh sperm since we have previously examined bull sperm physiology in each step of the cryopreservation procedure (dilution with EYT, glycerol addition, cooling, freezing), and we did not find that glycerol supplementation altered sperm function as assessed by internal calcium levels and a vitality assay [42]. Furthermore, we have recently shown that the inclusion or exclusion of glycerol does not affect the quality of fresh ram spermatozoa [43]. Cooling and freezing are clearly the stresses that trigger the capacitation-like changes to the spermatozoa.

Two phosphotyrosine-containing proteins (56-PP and 114-PP) appeared to be modulated by HEP when fresh-extended spermatozoa were incubated in CM for up to 5 h (Fig. 1A), but only 56-PP was already evident in cryopreserved spermatozoa at 0 h from the same ejaculate (Fig. 2). This initial tyrosine phosphorylated state in frozen-thawed spermatozoa could reflect capacitation-like changes that mimic the early membrane modifications associated with physiological capacitation. Visconti et al. [23] hypothesized that the fluidization of sperm plasma membranes, which occurs during capacitation and favors calcium influx, stimulates adenylate cyclase to initiate protein tyrosine phosphorylation. Furthermore, following cryopreservation, frozen-thawed bovine spermatozoa cannot properly regulate their intracellular calcium levels [44]. Since it is well established that calcium plays an important role in bull sperm capacitation [7, 45] and because this cation remains elevated in cryopreserved spermatozoa [44], it is tempting to speculate that the plasma membrane reorganization and destabilization provoked by cryopreservation are sufficient to trigger the intracellular signaling cascade associated with physiological capacitation. Consequently, a subpopulation of cryopreserved spermatozoa does not require HEP to undergo capacitation.

The fact that 56-PP is not systematically detected immediately after thaw for all bulls tested (9 of 18 ejaculates from different bulls) seems to indicate some interindividual variability. Likewise, the occasional appearance of specific tyrosine phosphoproteins after 5 h with HEP, such as 114-PP, could reflect a differential capability of frozen-thawed spermatozoa to respond to HEP during in vitro capacitation. These observations are consistent with current knowledge of semen cryopreservation, since it is well known that different ejaculates from the same or different bulls can respond differently to cooling and/or freezing and thawing and subsequent fertility in vivo [29]. To explain the differences observed in the intracellular calcium levels of bulls of varying fertility, Collin et al. [38] proposed that during cryopreservation, spermatozoa from sires of low fertility are more susceptible to membrane alterations, which could favor calcium influx and is correlated to poor fertility. Therefore, it is reasonable to suppose that the degree of sperm cryoinjury, such as membrane alterations and calcium influx, differs among ejaculates and bulls, which would influence their subsequent HEP responsiveness.

The Redistribution of Tyrosine Phosphoproteins

Indirect immunofluorescence reveals that before incubation in CM, most cryopreserved spermatozoa exhibits a characteristic distribution of phosphotyrosine-containing proteins identical to that of fresh-extended spermatozoa after HEP-induced capacitation, as indicated by pattern AS (Fig. 3A and C). This result, together with the others described in the present study, provides novel evidence that frozen-thawed spermatozoa are cryocapacitated. To our knowledge, this is the first report of the distribution of phosphotyrosine-containing proteins in bovine spermatozoa. Likewise, this experiment shows that the redistribution of tyrosine phosphoproteins to the acrosomal region of fresh-extended spermatozoa appears to be mediated by HEP during physiological capacitation (Fig. 3C). Similar experiments were conducted with mouse and human spermatozoa [14, 46], although the phosphotyrosine labeling was principally restricted to the flagella rather than to the acrosomes of capacitated spermatozoa; thus, it was proposed that the redistribution of the tyrosine phosphoproteins might be involved in hyperactivation in these species. More recently, tyrosine phosphoprotein distribution in the head region has also been reported for boar spermatozoa [37]. The presence of tyrosine phosphoproteins in the head region is appropriate, given the current theory that these proteins are involved in capacitation [20, 23]. The current study did not uncover any major changes in the phosphotyrosine labeling for the flagellum during the 5 h of incubation with or without HEP for either fresh-extended or cryopreserved spermatozoa. Therefore, it is suggested that the redistribution of phosphotyrosine-containing proteins to the acrosomal region is related to the capacitation process. This redistribution may be attributed to the head plasma membranes and/or the cytoskeletal reorganization that accompanies both capacitation [4, 6] and cryopreservation [29]. Although the prevalence of pattern AS in cryopreserved spermatozoa remains unchanged after 5 h of incubation with or without HEP, it does not exclude the likelihood that the window of tyrosine phosphoprotein redistribution to the acrosomal region progresses during incubation to a secondary subpopulation of these cells providing a new pool of functionally capacitated spermatozoa that may have actually responded to HEP in a regulated manner.

Existence of Cryocapacitation

A subpopulation of the frozen-thawed spermatozoa is effectively cryocapacitated before incubation under conditions that support in vitro capacitation according to the CTC and LPC-induced AR data. A higher percentage of cryopreserved than fresh-extended spermatozoa exhibits CTC pattern B at 0 h, supporting previous reports in the mouse [32], bull [34], ram [35], and boar [30]. Moreover, a greater proportion of cryopreserved than fresh-extended spermatozoa is able to undergo LPC-induced AR in the same conditions, also supporting the hypothesis that spermatozoa are prematurely capacitated immediately after thaw. The initial damage associated with cryopreservation seems sufficient to facilitate and/or provoke the ability of frozen-thawed spermatozoa to undergo functional capacitation.

Glucose Does Not Inhibit Cryocapacitation

According to the proportion of spermatozoa displaying the CTC pattern B and the profile of phosphotyrosine-con-
taining proteins before incubation in CM, cryopreservation of bull semen in EYTG extender that includes glucose instead of fructose does not prevent premature capacitation-like behavior of frozen-thawed spermatozoa (Figs. 6 and 7). First, the failure of glucose to inhibit the cryocapacitation process provides additional evidence that capacitation induced by cryopreservation is regulated by a different mechanism than HEP. Parrish et al. [10] demonstrated that HEP-induced capacitation is inhibited by the inclusion of 5 mM glucose. Using the same conditions, Galantino-Homer et al. [20] showed that glucose also prevented the appearance of protein tyrosine phosphoproteins in bovine spermatozoa. Taken together, these previous observations support the present hypothesis that cryocapacitation involves a different regulatory mechanism. It is possible that the lipoproteins in egg yolk facilitate the nonregulated cryocapacitation [34]. However, since our fresh controls are also suspended in ETY extender and did not demonstrate such nonregulated capacitation, cryocapacitation is not likely to be solely due to egg yolk components. It is possible that the nonregulated cryocapacitation arises following the osmotic and mechanical stresses to the plasma membrane during freezing and thawing [47]. The plasma membrane is the primary site of sperm cryoinjury [28, 48] and undergoes various modifications during cryopreservation, including changes to fluidity [49], phospholipid profiles, and phospholipid distribution [50, 51]. The increased permeability to calcium observed in cryopreserved sperm [44, 52] might simply be a direct consequence of the mechanical damage to the sperm plasma membrane. High levels of calcium in thawed sperm may trigger capacitation-like signal transduction pathways that lead to protein tyrosine phosphorylation in a non-regulated manner that is independent of internal pH. Therefore, if the presence of glucose in the extender does not restrict internal calcium levels, the cascade toward capacitation might not be tempered even by maintaining a low internal pH. Indeed, similar findings have been reported regarding the functional capacitation of boar spermatozoa induced by cooling to 5°C [53]. The authors report comparable but not identical changes to sperm membrane fluidity and tyrosine phosphoprotein profiles of capacitated and cooled boar sperm and conclude that, although cooling induces capacitation-like modifications to the spermatozoa, they are a by-passing of regulated (physiological) capacitation.

Regarding the ineffectiveness of glucose during the cryopreservation procedures, Parrish et al. [10] observed that glucose acidified the intracellular pH (pHₐ) of spermatozoa during a 5-h incubation period in conditions that support capacitation (39°C under 5% CO₂ in a defined medium). Considering that cryopreserved spermatozoa are diluted in EYTG extender and cooled for approximately 4 h (not in capacitating conditions), it would be interesting to assess sperm pHₐ during this initial cryopreservative step to determine whether the anticipated inhibition of sperm alkalization occurs in sperm cryopreserved in m-EYT. If pHₐ does not rise, it would indicate that glucose maintains sperm pHₐ as reported [10] but that other biological events, such as calcium influx, override the inhibiting effect of glucose on tyrosine phosphoprotein appearance [20] and capacitation.

Conclusions

The present findings suggest different mechanisms for HEP-induced capacitation of fresh bull spermatozoa and cryocapacitation. The latter is not inhibited by glucose, and the regulation of capacitation by protein tyrosine phosphorylation differs in frozen-thawed spermatozoa compared with fresh-extended cells. The 56-PP appears to be a dependable marker for HEP-induced capacitation in noncryopreserved spermatozoa. Interestingly, the distribution of the tyrosine phosphoproteins appears to be concentrated on the sperm head, which is consistent with their involvement in capacitation. Regarding the frozen-thawed spermatozoa, the existence of a subpopulation cryocapacitated is evident as revealed by the distribution of the phosphotyrosine-containing proteins, the CTC pattern B, and the LPC-induced AR immediately after thaw. A better understanding of the events involved in both capacitation and cryopreservation could lead to a substantial improvement of the quality of frozen-thawed semen, which is commonly used in animal- and human-assisted reproduction.

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