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Capacitation inducers act through diverse intracellular mechanisms in cryopreserved bovine sperm

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Abstract

The effect of various capacitation inducers, i.e. heparin, superoxide anion, bicarbonate, adenosine, and caffeine, and their role in intracellular mechanisms involved in capacitation, were studied in cryopreserved bovine sperm. Capacitation was determined by epifluorescence chlortetracycline, protein tyrosine phosphorylation, and the ability of capacitated sperm to undergo an acrosome reaction and fertilize *in vitro* matured oocytes. Participation of membrane adenylate cyclase and protein kinases (protein kinase A, protein kinase C, and protein tyrosine kinase) was evaluated indirectly (with specific inhibitors). Involvement of reactive oxygen species (ROS) was determined with scavengers of superoxide anion, hydrogen peroxide, or nitric oxide. Percentages of capacitated (27–29%) and acrosome-reacted sperm (23–26%) did not differ ($P > 0.05$) among various capacitation inducers. Significantly higher rates of IVF were obtained with heparin (43%) or bicarbonate plus caffeine (45%), when compared with control samples (17%). Adding the membrane adenylate cyclase inhibitor diminished capacitation rates with heparin (8%) or adenosine (10%). There was differential protein kinase participation in response to inducers; protein kinase inhibitors diminished cleavage rates in heparin-capacitated sperm relative to controls. There were differences between and within the studied inducers in protein tyrosine phosphorylation patterns. We inferred that capacitation in cryopreserved bovine sperm was promoted through diverse pathways. Mechanisms triggered by heparin, or caffeine plus bicarbonate-induced capacitation, involved activation of intracellular pathways to optimize fertilizing capability of cryopreserved bovine sperm.

Keywords: In vitro fertilization; Heparin; Caffeine; Adenosine; Superoxide anion

1. Introduction

Unlike sperm of many species, mammalian sperm are unable to fertilize oocytes immediately after ejaculation. *In vivo*, they must undergo capacitation in the female reproductive tract, in which they acquire fertilizing capability (reviewed by Yanagimachi) [1]. Capacitation is associated with protein tyrosine phosphorylation [2,3], which is modulated via a cAMP-dependent pathway in many species, including cattle [4], and is regulated by several signal transduction elements that involve protein kinase A (PKA) [5], protein kinase C (PKC) [6], and protein tyrosine kinase (PTK) [7]. The cAMP is generated by the enzyme, adenylate cyclase (AC). There are controversial reports regarding the nature of the AC responsible for regulating the fertilizing potential of sperm. Although several authors have detected the presence and modulation of sperm AC activity in response to various agents known to affect somatic transmembrane AC [8–13], many others have

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not [14–16]. The transmembrane AC activity is regulated by G proteins in response to extracellular ligands [17]. In contrast, soluble AC is associated with various intracellular organelles [18] and is regulated by bicarbonate and calcium [19–21].

In vitro capacitation of bovine sperm can occur in media supplemented with the glycosaminoglycan heparin, which binds to bovine seminal plasma (BSP) proteins [22]. When BSP proteins were linked to the capacitation factors, they potentiated sperm capacitation induced by these molecules [23]. Furthermore, BSP proteins induced changes in the sperm plasma membrane by stimulating the efflux of cholesterol and phospholipids [24,25]. Heparin stimulated intracellular increases of calcium, pH and cAMP, which seemed necessary to start the signaling pathway concomitant with capacitation [4]. Similarly, bicarbonate, adenosine, caffeine, and reactive oxygen species (ROS) induced capacitation. For instance, bicarbonate played a key role in capacitation of human [26], hamster [27,28], and mouse sperm [2]. Furthermore, bicarbonate also induced PKA-dependent changes in the lipid architecture of the sperm plasma membrane [29], due to phospholipid scrambling [30]. In cattle, it stimulated the acrosome reaction and the penetrability of sperm [31]. The cAMP phosphodiesterase (PDE) is the only known enzyme that metabolizes cAMP to 5′-AMP [32]. Several isoforms of PDE are present in mature sperm and have important functional significance during capacitation and fertilization [33]. Caffeine is a non-selective inhibitor of PDE [34], which increases cAMP levels [35]. Caffeine increased bovine sperm capacitation, as determined by the chlortetracycline epifluorescence assay [36], and acting synergistically with heparin, induced capacitation and/or the acrosome reaction in sperm, and stimulated *in vitro* fertilization of cattle oocytes [37]. Adenosine promotes capacitation in mouse sperm by interacting with membrane receptors and increasing the intracellular availability of cAMP [10]. Superoxide anion, hydrogen peroxide, and nitric oxide can activate membrane targets to trigger the intracellular mechanisms involved in sperm capacitation, including protein tyrosine phosphorylation [7,38,39]. A low concentration of hydrogen peroxide induced sperm capacitation by activating AC and producing cAMP, as demonstrated in bovine sperm [40]; it occurs in various cellular systems where ROS stimulate AC activity [41,42].

Glycosaminoglycan heparin is considered a physiological capacitation inducer in bovine sperm. It acts through specific ligands in the plasma membrane by activating intracellular pathways that increase cAMP; however, the molecular events underlying these processes are poorly understood. Compounds that increase cAMP by different intracellular mechanisms provide a model to elucidate the capacitation signaling pathways of bovine cryopreserved sperm. The objective of the present study was to investigate the effects of various inducers on bovine sperm capacitation, evaluated with a chlortetracycline epifluorescence assay, changes in protein tyrosine phosphorylation patterns, and the ability of capacitated sperm to undergo an acrosome reaction and fertilize *in vitro* matured oocytes. Intracellular mechanisms triggered by various compounds were evaluated through involvement of protein kinases (e.g., PKA, PKC, PTK), and by ROS participation.

2. Materials and methods

2.1. Materials

Unless specified, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample preparation

The experiments were performed with pellets of frozen semen from four purebred Holstein bulls (4–5 y old), of proven fertility. To prepare semen pellets, each ejaculate was diluted in a buffer containing 0.2 M Tris, 0.06 M sodium citrate, 0.13 M glycine, and 0.06 M fructose, supplemented with 20% egg yolk and 7% glycerol (final pH 6.6). Sperm suspensions were cooled to 5 °C in 90 min. The content was mixed by inversion, frozen into pellets of 0.1 mL in dry ice at −76 °C, and stored in liquid nitrogen (−196 °C).

Semen pellets from each bull were thawed in Tyrode's Albumin Lactate Pyruvate (TALP) medium without calcium or bovine serum albumin (BSA) [43] at 37 °C in a 1:2 ratio. After equilibration (10 min), samples were centrifuged at 300 × g for 5 min to separate seminal plasma and freezing extender. The pellet was resuspended in the same medium and centrifuged as described earlier. Sperm were then suspended in a TALP medium containing 2 mM $CaCl_2$ and 6 mg/mL BSA (concentration, 1.5×10^7 sperm/mL).

2.3. Evaluation of sperm motility

For each experiment, progressive motility was evaluated by the same observer using light microscopy under × 400 magnification (with a thermal stage at 37 °C).

2.4. Capacitation induction

Sperm suspensions were incubated in the presence of various capacitation inducers: bicarbonate (10–100 mM), caffeine (0.5–10 mM), adenosine (1–100 μM), heparin (10 U/mL) [43], or superoxide anion, generated by the *in vitro* system of xanthine (0.05 mM), xanthine oxidase (5 mUI/mL), and catalase (100 μg/mL) (X-XO-C) [44]. All incubations were performed in TALP medium, except for bicarbonate, which was incubated in a modified TALP medium without bicarbonate and for which the osmolarity was adjusted to 330 mOsm by changing the concentration of NaCl (125 mM NaCl, 3.1 mM KCl, 0.35 mM NaH_2PO_4, 10 mM Hepes, 1.1 mM $MgCl_2$, 1 mM sodium pyruvate, and 21.6 mM sodium lactate). In all treatments, the final pH of capacitation media was carefully adjusted to 7.4. Aliquots of the various treatments were incubated at 38 °C under 5% CO_2 in humidified air for 45 min; this promoted capacitation in cryopreserved bovine sperm [45,46]. Optimal concentrations of bicarbonate, caffeine and adenosine as capacitation inducers were established by determining the lowest concentration that induced the highest sperm capacitation rates (evaluated by CTC assay), without affecting progressive motility.

2.5. Determination of capacitation

2.5.1. Chlortetracycline epifluorescence assay

Sperm capacitation was evaluated through modifications in chlortetracycline fluorescence patterns, as described by Fraser et al [47], using an epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) at ×400 magnification. Two hundred sperm were examined and designated as one of the following: F-pattern (intact sperm), where the fluorescence was detected over the whole region of the sperm head; B-pattern (capacitated sperm), where the fluorescence was detected in the sperm head, except in the post-acrosomal region; and AR-pattern (acrosome reacted sperm), with no head fluorescence. The percentages of the B and AR patterns were obtained by subtracting from the values obtained in the control and treated samples, the ones obtained at zero time, in order to account for sperm destabilized during the freezing-thawing process.

2.5.2. Induction and evaluation of an acrosome reaction

The ability of capacitated sperm to undergo an acrosome reaction was assessed as follows: samples of frozen-thawed sperm capacitated with optimal concentrations of the inducers were incubated with bovine follicular fluid (30% (v/v) [48] for 15 min in the same capacitating conditions. The acrosome reaction was evaluated by a combined technique of differential-interferential contrast microscopy and a supravital stain Trypan Blue [44]. Sperm (200/sample) were assessed at ×1000 magnification (Carl Zeiss Jenamed 2 microscope, Jena, Germany). The percentage of acrosome reacted sperm was evaluated by counting the percentage of live acrosome reacted sperm in each treatment from which the one obtained at zero time was substracted, in order to exclude cells destabilized during the freezing-thawing process.

2.5.3. Determination of protein tyrosine phosphorylation

Protein tyrosine phosphorylation was performed by SDS-page and blotting. Samples of frozen-thawed sperm incubated with capacitation inducers at optimal concentrations were centrifuged for 5 min at 600 × g to remove the capacitation medium; sperm were resuspended in phosphate buffered saline (PBS; final concentration 400 × 10^6 sperm/mL). Sodium orthovanadate (0.2 mM) was added to aliquots (115 μL) of sperm suspension and the samples were centrifuged (6 min, 11 190 × g, 4 °C), to obtain a sperm pellet that was resuspended in the sample buffer (62.5 mM TRIS-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol) [49] without β-mercaptoethanol, and heated for 5 min at 100 °C. The sperm suspension was recentrifuged (30 min, 11 190 × g, 4 °C) and 5% (v/v) β-mercaptoethanol was added to the supernatant. Following that, the sperm concentration was evaluated to ensure that equivalent aliquots were processed, and samples were immediately frozen (−18 °C) until electrophoresis was performed. Aliquots of sperm proteins were heated for 5 min at 95 °C. Equivalent aliquots of treated sperm were loaded on 12% SDS-polyacrylamide gels (6 × 10^6 sperm, 16 μL per lane). Separated proteins were transferred electrophoretically to nitrocelulose membranes, with the efficiency of transfer determined by staining with 0.5% (w/v) Red Ponceau in 1% (v/v) acetic acid. Nonspecific protein binding sites on membranes were blocked with 5% (w/v) dry non-fatty milk in TRIS-buffer saline (TBS: 25 mM TRIS-HCl, 150 mM NaCl) for 1 h at room temperature. Membranes were washed 1 × 15 min, and 2 × 5 min with fresh TTBS (0.1% (w/v) Tween 20, 20 mM TRIS-HCl, and 136 mM NaCl). Blots were incubated for 1 h at room temperature with an anti-phosphotyrosine antibody (Clone 4G10, Upstate, Lake Placid, NY, USA) diluted 1:1000, in 3% (w/v) dry non-fatty milk in TTBS, washed 1 × 15 min, 2 × 5 min with TTBS, and then incubated for 1 h at room temperature with peroxidase conjugated

goat anti-mouse antibody (Inmuno-Star Goat Anti-mouse HRP conjugate, Biorad, Hercules, CA, USA) diluted 1:1000, in 3% (w/v) dry non-fatty milk in TTBS. Finally, blots were washed again 1 × 15 min, 4 × 5 min with TTBS. Labeled tyrosine phosphoproteins were visualized using a chemiluminescence detection kit (ECL, Amersham Biosciences, Piscataway, NJ, USA). The quantification of the tyrosine phosphorylated proteins was performed in the bands corresponding to 30, 45, and 66 Kd, chosen as capacitation markers. The photographic film was scanned and quantified by measuring the intensities of each digitalized band using Image J software (Version 1.240, National Institute of Health) [50]. The intensities of protein tyrosine phosphorylation were measured by converting them into peaks and were expressed as arbitrary units (AU). These units were calculated as the peak area of the band of the phosphoprotein visualized by chemiluminescence over the peak area of the same lane previously stained by Red Ponceau.

2.5.4. *In vitro fertilization (IVF)*

2.5.4.1. Recovery of cumulus-oocyte complexes and in vitro maturation. Bovine ovaries were obtained from an abattoir within 30 min after slaughter and kept warm (30 °C) during the 2 h journey to the laboratory. Ovaries were washed in saline containing 100,000 U/L penicillin and 100 mg/L streptomycin. Cumulus-oocyte complexes (COCs) were recovered by the aspiration of antral follicles (2 to 5 mm in diameter); only oocytes completely surrounded by a compact and multilayered cumulus oophorus were used. Groups of 50 cumulus oocyte-complexes (COCs) were cultured in 500 μL 199 medium (Earle′s salts, L-glutamine, 2.2 mg/mL sodium bicarbonate, GIBCO, Grand Island, NY, USA), supplemented with 50 μg/mL gentamicin sulfate and 5% (v/v) fetal bovine serum (FBS; GIBCO), under mineral oil at 39 °C in 5% CO_2 in humidified air, for 22 h. *In vitro* maturation was performed without the addition of gonadotrophins in order to avoid the capacitation effect of expanded cumulus, as demonstrated by Gutnisky et al [51].

2.5.4.2. IVF procedure. The ability of the capacitated sperm to fertilize *in vitro* matured oocytes was assessed as follows: after 45 min of incubation of the thawed sperm, in the presence of capacitating inducers at optimal concentrations, samples were centrifuged at 300 × g for 5 min and resuspended in FERT TALP (IVF medium) [43]. Fertilization was performed in 500 μL FERT TALP under mineral oil at 39 °C in 5% CO_2, in humidified air for 20 h (final concentration of 1×10^6 motile sperm/mL). Zygotes were denuded of the remaining cumulus cells by repeated pipetting and incubated in 500 μL of *in vitro* culture medium (IVC)-mSOF, consisting of mSOF [52] supplemented with 30 mL/L essential amino acids (GIBCO), 10 mL/L non-essential amino acids (GIBCO), 2 mM L-glutamine, 6 g/L BSA and 5% (v/v) FBS (GIBCO), under mineral oil at 39 °C in 90% N_2: 5% CO_2: 5% O_2, and 100% humidity for 24 h. The proportion of the cleaved oocytes was determined under an inverted microscope, by evaluating the number of embryos with two or more blastomeres. An additional cohort of 10 oocytes from each replicate was maintained through the fertilization procedure, without exposure to the sperm, to test for parthenogenesis.

2.6. *Effect of membrane adenylate cyclase inhibitor on sperm capacitation*

Sperm were incubated in TALP medium supplemented with heparin, bicarbonate, caffeine, adenosine or the X-XO-C system (at optimal concentrations) at 39 °C under 5% CO_2, in humidified air for 45 min, in the presence or absence of 2′5′dideoxiadenosine (100 μM), a specific membrane AC inhibitor [12].

2.7. *Effect of protein kinases inhibitors on sperm capacitation*

Sperm were incubated, as described earlier, in the presence or absence of PKA, PKC or PTK inhibitors: H89 (50 μM) [4], bisindolmaleimide I (BM; 100 nM) [53], or genistein (GE; 370 μM) [54], respectively.

2.8. *Effect of ROS scavengers on capacitation induced by bicarbonate, caffeine, or adenosine*

Sperm were incubated, as described earlier, with bicarbonate, caffeine, or adenosine at optimal concentrations in the presence or absence of superoxide dismutase (SOD; 0.5 mg/mL) [44], catalase (CAT; 100 μg/mL) [55], or hemoglobin (HB; 40 μg/mL) [56] scavengers of superoxide anion, hydrogen peroxide, or nitric oxide, respectively.

2.9. *Statistical analysis*

Percentages of motility, capacitated and acrosome reacted sperm of each treatment were expressed as mean ± SEM. Percentages were arcsine-transformed, to normalize distributions. Data for motility, capacitated, and acrosome reacted sperm were analyzed by one-way ANOVA (totally random experimental design), with a Bonferroni test for comparisons among treatments. Arbitrary units (mean ± SEM) of phospho-

Table 1
Effect of various concentrations of bicarbonate, caffeine or adenosine on percentages of progressively motile bovine sperm.

Control samples	Bicarbonate (mM)							
	10	20	30	40	50	60	80	100
48 ± 5[a]	43 ± 6[a]	38 ± 4[a]	40 ± 7[a]	36 ± 3[a]	24 ± 4[b]	15 ± 3[b]	5 ± 2[c]	3 ± 1[c]
Control samples	Caffeine (mM)							
		0.5	1	2.5	5	7.5	10	
34 ± 4		40 ± 9	33 ± 8	32 ± 7	35 ± 5	35 ± 5	41 ± 7	
Control samples	Adenosine (μM)							
	1	5	10	20	30	50	100	
40 ± 6	45 ± 7	43 ± 8	44 ± 7	49 ± 9	52 ± 10	46 ± 7	48 ± 11	

N = 4, mean ± SEM. Values are mean ± SEM of four experiments performed with semen samples from different bulls.
[a–c] Within a row, concentrations without a common superscript differed in the proportion of progressively motile sperm ($P < 0.05$).

rylation were normalized by log transformation and analyzed by two-way ANOVA (capacitation inducer, phosphoprotein molecular weight, and their interactions), using posterior contrasts for comparisons among capacitation treatments or phosphoprotein molecular weight). The cleavage rate among treatments was compared using Chi-square analysis. The P value used to determine significance in all tests was 0.05.

3. Results

3.1. Determination of optimal concentration of capacitation inducers

3.1.1. Bicarbonate

Sperm motility had a decreased dependence on the concentration used, diminishing from the concentration of 50 mM ($P < 0.05$; Table 1). Based on a CTC assay, the percentage of capacitated sperm (B-pattern) increased in a dose-dependent manner, whereas the AR-pattern increased from a concentration of 60 mM ($P < 0.05$; Fig. 1A).

3.1.2. Caffeine

The addition of caffeine did not significantly modify sperm motility (Table 1). There was a progressive increase of the percentage of sperm displaying CTC B-pattern in response to increasing concentrations of caffeine; this was significant (relative to controls) from a concentration of 7.5 mM of caffeine (Fig. 1B).

3.1.3. Adenosine

Adenosine in the capacitation medium did not significantly affect sperm motility (Table 1). Adenosine increased ($P < 0.05$) the percentage of capacitated sperm when compared with the control samples; maximal responses were obtained with concentrations between 10 and 30 μM (Fig. 1C). The optimal concentrations of bicarbonate (30 mM), caffeine (7.5 mM) and adenosine (10 μM) as capacitation inducers were used for further assays.

3.2. Comparative effect of various in vitro capacitation inducers

3.2.1. Chlortetracycline epifluorescence assay

Sperm motility was not significantly affected by inducers. The percentage of the CTC B-pattern significantly increased when compared to the control samples, without significant differences among the evaluated inducers (Table 2).

3.2.2. Induction of acrosome reaction

Sperm motility only significantly decreased in the caffeine capacitated sperm (37 ± 4%) compared with control samples (53 ± 3%). With all capacitation inducers, there was a significant increase in the percentage of acrosome reacted sperm (relative to the control), with no significant differences among treatments (Table 2).

3.2.3. Protein tyrosine phosphorylation

The pattern of phosphoproteins (measured as arbitrary units) differed within each capacitation inducer. There were no significant differences among 30, 45, and 66 Kd phosphoproteins in heparin, caffeine, or superoxide anion-capacitated sperm. Control or bicarbonate-treated samples had a lower intensity of the 30 Kd band ($P < 0.05$). Adenosine had significant differences between bands 30 and 66 Kd. When comparing each phosphorylated protein among the capacitation inducers, the band corresponding to the protein of 30 Kd was more intense in heparin-capacitated sperm, having the lowest levels in bicarbonate-capacitated sperm or control samples ($P < 0.05$). There were no significant differences between the studied inducers or even control samples for 45 or 66 Kd phosphoproteins (Fig. 2).

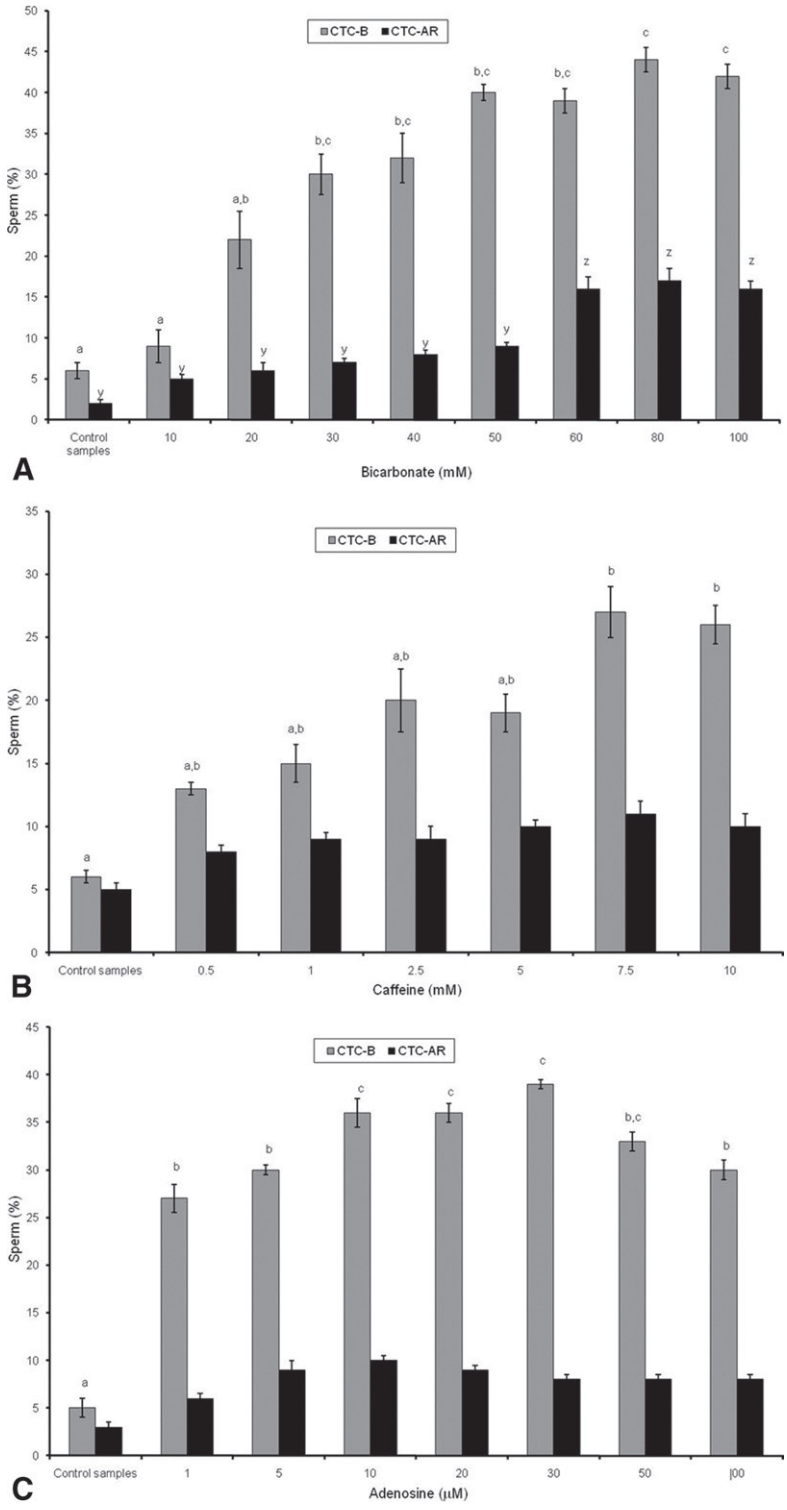


Fig. 1. Effect of bicarbonate, caffeine or adenosine (Fig. 1A, 1B, and 1C, respectively), on CTC patterns of bovine sperm. N = 4, mean ± SEM, CTC-B, capacitated sperm; CTC-AR, acrosome reacted sperm.

[a–c] Concentrations without a common superscript differed in the proportion of capacitated sperm ($P < 0.05$).

[y,z] Concentrations without a common superscript differed in the proportion of acrosome reacted sperm ($P < 0.05$).

Table 2
Comparative effect of *in vitro* capacitation inducers on percentages of CTC B-pattern and live acrosome reacted bovine sperm.

	Control samples	Heparin	Bicarbonate	Caffeine	Adenosine	Superoxide anion
CTC B-pattern (%)	6 ± 1^{a}	27 ± 2^{b}	29 ± 1^{b}	27 ± 1^{b}	28 ± 2^{b}	28 ± 3^{b}
Live acrosome reacted sperm (%)	3 ± 0^{a}	23 ± 2^{b}	24 ± 3^{b}	25 ± 5^{b}	25 ± 4^{b}	26 ± 3^{b}

N = 4, mean ± SEM. Control samples = without inducer, heparin 10 IU/mL, bicarbonate 30 mM, caffeine 7.5 mM, adenosine 10 μM, superoxide anion = xanthine 0.05 mM + xanthine oxidase 5 mIU/mL + catalase 100 μg/mL. All samples were incubated with follicular fluid (30% v/v) as an acrosome reaction inducer.
[a,b] Within a row, inducers without a common superscript differed in the proportion of capacitated or live acrosome reacted sperm ($P < 0.05$).

3.2.4. Effect of various capacitation inducers on cleavage rates

The addition to the IVF medium of sperm previously treated with optimal concentrations of bicarbonate, adenosine or caffeine did not significantly affect cleavage rates with respect to the control. Moreover, the cleavage rate diminished with the addition of sperm treated with exogenous superoxide anion (generated by X-XO-C) to the IVF medium ($P < 0.05$). Heparin supplementation significantly increased the cleavage rate ($P < 0.05$; Fig. 3A). A similar cleavage rate as that obtained with heparin was obtained with a combination of bicarbonate plus caffeine ($P < 0.05$; Fig. 3B).

3.3. Effect of 2′5′ dideoxyadenosine on capacitation induced by various compounds

Percentages of progressive sperm motility were between 40 and 60%, which was not significantly different between sperm incubated with or without 2′5′dideoxyadenosine. Only heparin or adenosine-induced capacitation was prevented (measured by CTC assay) by the presence of 2′5′dideoxidenosine in the capacitation medium (Table 3). Similar results were observed by the induction of a true acrosome reaction in heparin-capacitated sperm treated with the inhibitor (data not shown). When this inhibitor was included in the TALP medium containing heparin as the capacitation inducer, it did not decrease the cleavage rate (38%), being comparable to heparin (40%; $P > 0.05$).

3.4. Effect of protein kinase inhibitors on capacitation induced by various compounds

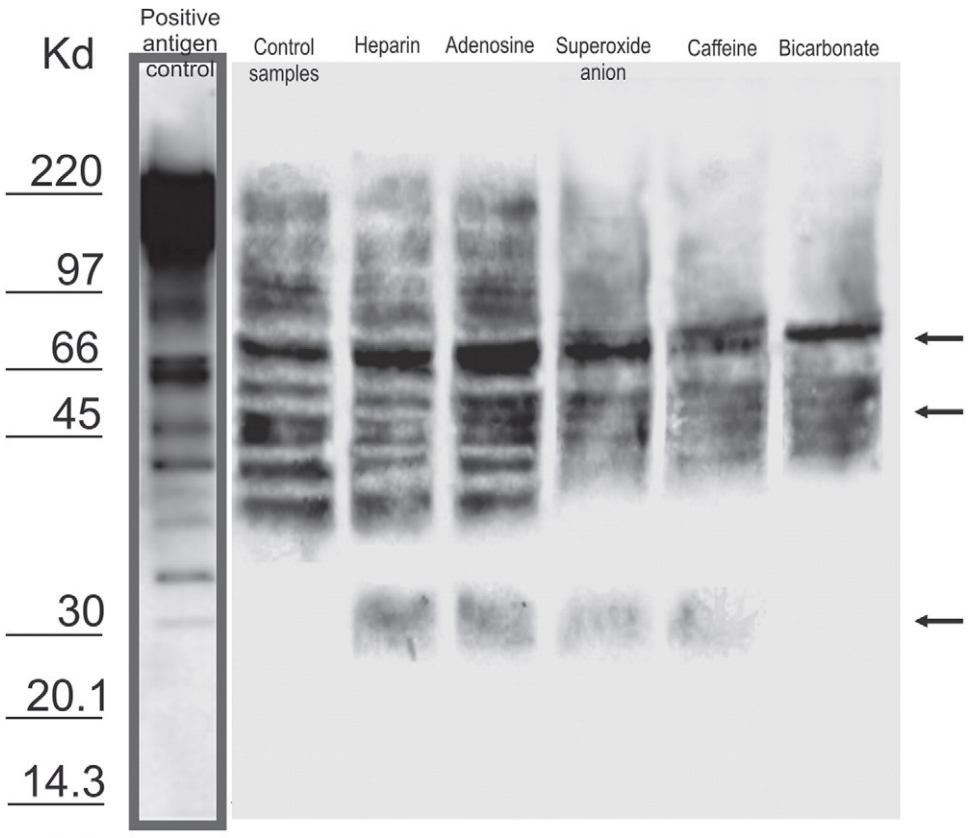
Sperm motility significantly decreased when H-89 was included in the capacitation medium containing caffeine or superoxide anion (33 ± 2% and 34 ± 2% with caffeine or superoxide anion, respectively, versus 47 ± 1% in control samples; $P < 0.05$). The inclusion of H-89 in the capacitation medium prevented the capacitation of sperm incubated with all inducers, thereby diminishing the percentages of the CTC B-pattern relative to the control ($P < 0.05$; Table 3). Samples containing caffeine or superoxide anion as capacitation inducers and BM significantly decreased sperm motility (33 ± 1% and 31 ± 1% with caffeine or superoxide anion, respectively) as compared to controls (46 ± 2%; $P < 0.05$). The presence of BM significantly diminished the percentages of the CTC-B pattern in the samples containing heparin, superoxide anion or caffeine ($P < 0.05$; Table 3). Sperm motility was not significantly different between sperm incubated with or without GE, with respect to control samples (47 ± 2%). However, GE prevented ($P < 0.05$) capacitation (evaluated by the CTC assay) of sperm incubated with heparin, caffeine, or superoxide anion (Table 3). The decrease in capacitation rates with the inducers in the presence of H-89, BM or GE was confirmed by inducing the acrosome reaction; there was a significant decrease in the percentages of acrosome reacted sperm with the kinase inhibitors used (data not shown). When these protein kinase inhibitors were added to the TALP medium containing heparin, cleavage rates diminished (12 to 18%) and were similar to control samples (17%; $P > 0.05$).

3.5. Participation of reactive oxygen species in capacitation induced by various compounds

Progressive motility ranged from 40 to 55% (not significantly different within sperm incubated with or without each compound). Sperm treated with bicarbonate, caffeine, or adenosine were capacitated even in the presence of SOD, CAT, a combination of SOD plus CAT, or HB (percentages of CTC-B pattern ranged from 41 to 48%).

4. Discussion

Sperm capacitation is an obligatory biochemical modification (within the female reproductive tract) prior to sperm binding to the zona pellucida and undergoing an acrosome reaction. Although several compounds are known to induce *in vitro* capacitation, hep-



Arbitrary units of phosphorylation

30 Kda	0.0102 ± 0.0072^{ay}	0.2385 ± 0.0097^{by}	0.1908 ± 0.0057^{bcy}	0.1517 ± 0.0251^{cy}	0.1572 ± 0.0225^{cy}	0.0197 ± 0.0098^{ay}
45 Kda	0.1845 ± 0.0246^{az}	0.2148 ± 0.0674^{ay}	0.2137 ± 0.0244^{ayz}	0.1788 ± 0.0269^{ay}	0.3108 ± 0.0547^{ay}	0.1757 ± 0.0336^{az}
66 Kda	0.2180 ± 0.0216^{az}	0.2606 ± 0.0158^{ay}	0.2676 ± 0.0149^{az}	0.2384 ± 0.0357^{ay}	0.2585 ± 0.0586^{ay}	0.2231 ± 0.0382^{az}

Fig. 2. Effect of various capacitation inducers on protein tyrosine phosphorylation in cryopreserved bovine sperm. Control samples = without inducer, heparin 10 IU/mL, bicarbonate 30 mM, caffeine 7.5 mM, adenosine 10 μM; superoxide anion = xanthine 0.05 mM + xanthine oxidase 5 mIU/mL + catalase 100 μg/mL. The result shown is representative of five independent replicates. Arbitrary units of 30, 45, or 66 Kd proteins (marked with arrows) were obtained as described (in Materials and methods).

[a–c] Within a row, values without a common superscript differed ($P < 0.05$).

[y,z] Within a column, values without a common superscript differed ($P < 0.05$).

arin is most commonly used to capacitate bovine sperm [43]. In cryopreserved bovine sperm, the addition of heparin in fertilization media improved *in vitro* embryo production [57]. In the present study, capacitation with optimal concentrations of bicarbonate, caffeine, or adenosine achieved the same capacitation level, as determined with the CTC assay (Table 2), of heparin or superoxide anion, both known inducers of *in vitro* capacitation in cryopreserved bovine sperm. As changes in the concentration of cAMP have been linked to heparin-dependent capacitation in bovine sperm [58,59], it is not surprising that all the studied inducers that produce cAMP by different mechanisms accomplished the same rates of capacitation. Although the exact mechanism by which CTC exhibits the various patterns has not been elucidated, it has been suggested that this fluorescent stain evaluates events of sperm capacitation associated with changes in the plasma membrane structure [60]. Despite the different intracellular mechanisms triggered by the studied inducers, they could be producing similar cellular changes related to capacitation.

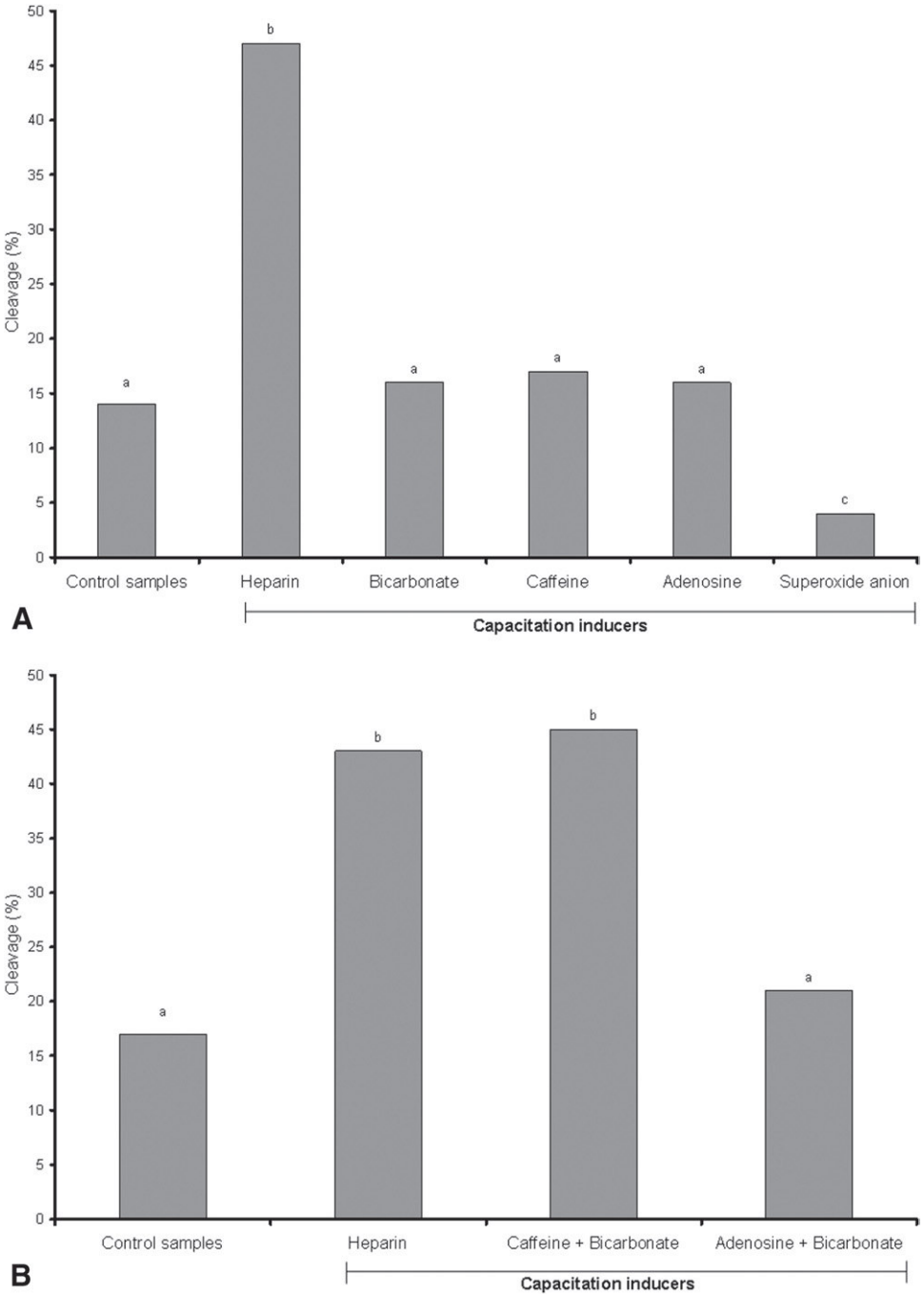


Fig. 3. Effect of individual or combination sperm capacitation inducers (Fig. 3A and 3B, respectively) on rates of early cleavage of bovine oocytes (N = 90 to 120 for each treatment in four replicates).

Control samples = without inducer, heparin 10 IU/mL, bicarbonate 30 mM, caffeine 7.5 mM, adenosine 10 μM; superoxide anion = xanthine 0.05 mM + xanthine oxidase 5 mIU/mL + catalase 100 μg/mL, bicarbonate 30 mM + caffeine 7.5 mM, bicarbonate 30 mM + adenosine 10 μM.

[a–c] Values without a common superscript differed ($P < 0.05$).

Follicular fluid induces an acrosome reaction in human [61] and bovine capacitated sperm [48]. This exocytotic event results in the release of hydrolytic enzymes and the exposure of new membrane domains, both of which are essential for fertilization [62]. Progesterone is present in follicular fluid [63] and induces

Table 3
Effect of membrane adenylate cyclase or protein kinase inhibitors on percentages of CTC B-pattern bovine sperm.

Inhibitor	None	DD	H89	BM	GE
Heparin	29 ± 1[a]	8 ± 1[b]	8 ± 2[b]	10 ± 2[b]	10 ± 1[b]
Bicarbonate	29 ± 3[a]	27 ± 2[a]	8 ± 1[b]	29 ± 1[a]	31 ± 2[a]
Caffeine	27 ± 2[a]	27 ± 3[a]	14 ± 1[b]	18 ± 3[b]	9 ± 2[c]
Adenosine	28 ± 2[a]	10 ± 2[b]	11 ± 2[b]	27 ± 2[a]	30 ± 3[a]
Superoxide anion	28 ± 3[a]	25 ± 3[a]	9 ± 2[b]	10 ± 1[b]	7 ± 3[b]

N = 4, mean ± SEM. Sperm were capacitated with heparin 10 IU/mL, bicarbonate 30 mM, caffeine 7.5 mM, adenosine 10 μM or superoxide anion (xanthine 0.05 mM + xanthine oxidase 5 mIU/mL + catalase 100 μg/mL) in the absence or presence of 100 μM of 2′5′dideoxiadenosine (DD), 50 μM of H-89, 100 nM of BM I, or 100 μg/mL of GE.
[a–c] Within a row, inducers without a common superscript differed in the proportion of capacitated sperm ($P < 0.05$).

an acrosome reaction in cryopreserved bovine sperm through intracellular mechanisms which are dependent of the voltage-dependent calcium channel [64]. Similar percentages of acrosome reacted sperm obtained with capacitation inducers (Table 2) were attributed to the effect of progesterone on activation of calcium channels, thereby increasing the intracellular calcium necessary for the exocytotic process in a similar manner with all studied inducers. Although some BSP proteins appeared to be beneficial for sperm functions (e.g. the interaction between BSP proteins and heparin that promoted capacitation), others seemed detrimental, particularly for sperm cryopreservation [65]. In that regard, the cholesterol and phospholipid effux from sperm, mediated by BSP proteins, was stimulated in a time- and concentration-dependent manner [24,25]; therefore, continuous exposure of sperm to seminal plasma which contains the BSP proteins, caused continuous cholesterol and phospholipid removal from the sperm membrane, which can render the sperm very sensitive to storage (in either liquid or frozen states [65]). The cryopreservation of bovine sperm was performed in the presence of seminal plasma. The prolonged contact between the sperm and seminal plasma could produce reorganization and/or destabilization of the membrane, thereby triggering some unknown signal transduction pathways.

Conversely, when sperm previously capacitated with various inducers were tested in IVF, cleavage rate was significantly increased only when the sperm were incubated with heparin; the other inducers (bicarbonate, caffeine or adenosine) did not increase fertilization rates as compared to the control. Moreover, *in vitro* capacitation with superoxide anion produced a decrease in cleavage rates (Fig. 3A). It is known that freezing and thawing of sperm affect the lipid architecture of the sperm plasma membrane [66] and their metabolic activity [67]. Membrane destabilization affected its fluidity and made it more permeable, thus allowing free calcium to enter the cell, which stimulated capacitation-like changes (cryocapacitation) [68]. The CTC technique or the induction of an acrosome reaction, the usual assays used to evaluate capacitation, could be conditioned by these ultrastructural changes in the cryopreserved sperm. The modifications produced in the plasma membrane by BSP proteins, cryopreservation, or both, could be conditioning the surface expression of sperm receptors that interact with the *zona pellucida*, thereby altering the capability of the sperm to acquire the intracellular requirements neccesary for fertilization. Although all studied inducers might complete the acrosome reaction, only heparin could complete mechanisms required for fertilization.

Cleavage rates were similar when sperm were capacitated *in vitro* with heparin or bicarbonate plus caffeine (Fig. 3B). In bovine sperm, treatment with caffeine, in a short-term incubation, activated the calcium permeable cation channels in the plasma membrane and stimulates hyperactivation, whereas long-term incubation produced capacitation and protein tyrosine phosphorylation, indicating that caffeine subsequently functioned as a phosphodiesterase inhibitor [69]. The greater IVF rate with bicarbonate plus caffeine may be explained by bicarbonate activating soluble AC and thereby increasing cAMP, whereas caffeine suppressed cAMP hydrolysis and increased intracellular calcium concentrations, thereby stimulating hypermotility. As a consequence, the synergistic effect of both inducers could produce intracellular conditions required for cumulus and *zona pellucida* penetration, as well as fertilization.

Soluble AC, as the predominant, if not only source of cAMP in sperm, was predicted to be responsible for changes in cAMP content induced by seminal plasma, oviduct fluids, or IVF media [70]. Soluble AC activity is modulated by bicarbonate [19]. Based on evaluation of the participation of various capacitation inducers in intracellular mechanisms related to the involvement of

different AC (membrane or soluble), we inferred that the membrane AC was only activated with compounds (e.g. heparin and adenosine) that bound to specific receptors, as demonstrated by the inhibition of capacitation with a specific membrane AC inhibitor (2′5′ dideoxyadenosine; Table 3). As this inhibitor failed to inhibit the capacitation when bicarbonate was used, this compound would be inducing capacitation by activating soluble AC in cryopreserved bovine sperm. Capacitation with superoxide anion, generated *in vitro* by the X-XO-C system, was not inhibited by the presence of the membrane AC inhibitor, indicating that it could have other membrane targets associated with its action on sperm. Interestingly, adding 2′ 5′ dideoxyadenosine to the capacitation medium failed to diminish the cleavage rate when heparin was used as a sperm capacitation inducer. Mouse sperm, deficient in soluble AC, were unable to fertilize zona-intact eggs, but did fertilize zona-free oocytes, indicating that gamete fusion did not require soluble AC [71]. Since our present results were in accordance with these findings, we inferred the participation of membrane and soluble ACs in fertilization of bovine oocytes.

Different protein kinase inhibitors were used to evaluate intracellular mechanisms triggered by the increase of cAMP produced by capacitation inducers. Although the requirement of PKA and PTK in the capacitation-associated tyrosine protein phosphorylation is well established, it implicated the role of other kinases and transduction pathways that act between the early events of capacitation and the late phosphorylation of proteins [72,73]. Based on reduced capacitation in sperm incubated with both heparin or superoxide anion in the presence of H-89, BM or GE, we inferred the participation of PKA, PKC and PTK in bovine sperm capacitation, as reported by O′Flaherty et al [74]. Caffeine-induced capacitation would also require PKA, PKC and PTK participation; meanwhile, bicarbonate- or adenosine-dependent capacitation would only activate PKA (Table 3). These results, taken together, suggested that differential protein kinase participation would not be conditioned by membrane or soluble AC activation, indicating that increase in cAMP would be produced by different signaling pathways dependent on the capacitation inducer. In accordance, cleavage rates diminished when each protein kinase inhibitor was added to sperm incubated with heparin. These results confirmed that PKA, PKC, and PTK were necessary for bovine sperm treated with heparin could complete processes related to the acquisition of fertilizing ability.

Protein tyrosine phosphorylation may actually be a consistent indicator of intracellular changes associated with capacitation. There are two main parallel pathways that produce these phosphorylation events, one involving cAMP/PKA and phosphorylated linked PKA substrates, and the other related to the extracellular signal regulated kinase (ERK) pathway, with phosphorylation of ERK kinase-like proteins and proteins carrying the threonine-glutamate-tyrosine motif [75]. *In vitro* capacitation of ejaculated bovine sperm was correlated with tyrosine phosphorylation of proteins of 40–120 kd [4]. In the absence of a key marker of protein tyrosine phosphorylation associated with capacitation in bovine sperm, bands corresponding to 30, 45, and 66 Kd were chosen as capacitation markers. There was a thick band of protein (30 kd) in sperm incubated with heparin, adenosine, caffeine and superoxide anion (Fig. 2), which was also reported by Cormier et al [76] in heparin-capacitated cryopreserved bovine sperm. Despite a lack of differences in the intensity of the bands corresponding to the proteins of 45 or 66 Kd among the studied inducers, there was a higher intensity in 30 Kd protein following the use of heparin, as compared to the other inducers. Since there were quantitative differences in phosphorylated proteins among and within capacitation inducers, we inferred there was involvement of diverse signaling pathways, leading to protein tyrosine phophorylation.

Redox activity appeared to be directly involved in sperm capacitation [77]. That the addition of ROS scavengers failed to block capacitation induced by bicarbonate, caffeine, or adenosine, we inferred that the action of these inducers did not include ROS participation. Superoxide anion [44] and nitric oxide [56] participate in heparin-induced capacitation in bovine cryopreserved sperm, whereas hydrogen peroxide induced capacitation in ejaculated bovine sperm [40]. The differential ROS participation observed with caffeine, bicarbonate, and adenosine with respect to heparin, could be contributing to the diverse intracellular pathways triggered by capacitation inducers.

Although the same capacitation and true acrosome reaction levels were achieved by the studied inducers (caffeine, bicarbonate, adenosine, heparin, or superoxide anion), different intracellular mechanisms of sperm capacitation of cryopreserved bovine sperm appeared to be involved. Differences in IVF rates, protein tyrosine phosphorylation patterns, and the differential participation of adenylate cyclases, protein kinases, and reactive oxygen species in the capacitation promoted by the evaluated inducers would confirm this suggestion. The findings of this study provided new knowledge regard-

ing intracellular requirements for capacitation. Determination of these molecular events would help to optimize *in vitro* conditions to use cryopreserved bovine sperm for fertilization.

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