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Heparin- and superoxide anion-dependent capacitation of cryopreserved bovine spermatozoa: Requirement of dehydrogenases and protein kinases

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Abstract
Capacitation is part of an oxidative process necessary for bovine spermatozoa to acquire fertilizing capacity. This process includes the generation of reactive oxygen species (ROS) and the participation of protein kinases such as A (PKA), C (PKC) and tyrosine kinase (PTK). A redox status is required to support both sperm motility and capacitation. Our aim was to determine the requirement of lactate dehydrogenase C4 (LDH-C4) and isocitrate dehydrogenase (NADP-ICDH) and of protein kinases in cryopreserved bovine sperm capacitation. The presence of inhibitors of both LDH-C4 and NADP-ICDH prevented the heparin-induced capacitation. H89, GF109203X or genistein blocked capacitation triggered by heparin or the superoxide $\left(O_2^{-\cdot}\right)$ generator system xanthine–xanthine oxidase–catalase (XXOC) suggesting the requirement of PKA, PKC and PTK in this process. Taken together these results suggest that LDH-C4 and NADP-ICDH contribute with the redox status to support bovine sperm capacitation and that PKA, PKC and PTK are involved in different mechanisms induced by different inducers that lead bovine spermatozoa to be capacitated.

Keywords: *Reactive oxygen species, spermatozoa, redox status, dehydrogenases, protein kinases*

Introduction

Mammalian spermatozoa must accomplish a preparation period called "capacitation" in order to fertilize a mature oocyte. This process involved morphological and biochemical changes including the generation of reactive oxygen species (ROS) and the participation of different protein kinases such as protein kinase A (PKA), C (PKC) and tyrosine kinase (PTK) [1–3]. Superoxide anion $\left(O_2^{-\cdot}\right)$ is produced and involved in bovine sperm capacitation [4–6]. Moreover, the xanthine–xanthine oxidase–catalase system (XXOC) that generates $O_2^{-\cdot}$ was able to induce capacitation at the same levels than heparin, the regular capacitation inducer in bovine spermatozoa [5,6]. PKA was associated with protein tyrosine phosphorylation in bovine spermatozoa incubated under capacitating conditions [7]. PKC activity was detected in bovine spermatozoa and it was suggested its participation in both capacitation and acrosome reaction in bovine spermatozoa [8–12].

Redox activity appears to be directly involved in motility [13], and capacitation [14,15]. Several enzymatic activity that have a crucial role in sperm metabolism require NAD(P)H, and the source of these reducing equivalent production is not yet established. Lactate dehydrogenase C4 (LDH-C4) is an isoenzyme specific of testis and spermatozoa from severa l species [16,17], included the bovine [18]; it is located in the cytosol [15,19], mitochondria [20], and the plasma membrane of bovine spermatozoa [15,21]. Since this isoenzyme plays an important role in the energetic metabolism, it was associated with sperm fertilizing capacity [22]. A role for the LDH-C4 as responsible of cytosolic lactate generation to supply NADH required for bovine sperm capacitation it was

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suggested [15]. Recently, it was confirmed the participation of LDH-C4 in sperm capacitation, by using the mouse as a model [23].

Candidates to supply NADPH required for the sperm redox status are the NADP-dependent isocitrate dehydrogenase (NADP-ICDH) and/or glucose-6-P dehydrogenase (G6PDH). NADP-ICDH was detected in the cytosol of spermatozoa and epididymal cells in rats and has greater activity than the respective mitochondrial isoenzyme NADH-dependent [24]. G6PDH was detected and implicated to contribute in the generation of the redox status in human spermatozoa [13]. Although, in bovine spermatozoa G6PDH was not detected [25].

The aim of the present study was to determine the requirement of dehydrogenases such as LDH-C4 and NADP-ICDH and of protein kinases in the heparin- and the $O_2^{-\cdot}$-induced capacitation in cryopreserved bovine spermatozoa.

Materials and methods

Materials

All chemical reagents employed were of the highest commercially available purity. Chlortetracycline (CTC), superoxide dismutase (SOD; from bovine erythrocyte), catalase (from bovine liver), xanthine, xanthine oxidase (from butter milk, grade I), β-nicotinamide adenine dinucleotide phosphate (NADP), bovine serum albumin (BSA), cysteine, sodium oxamate and sodium oxalomalate (α-hydroxy-β-oxalosuccinic acid, sodium salt), GF109203X and genistein were supplied by Sigma Co. (St Louis, MO, USA). Heparin (from porcine intestinal mucosa) and H89 were purchased from Calbiochem Co (La Jolla, CA, USA).

Semen freezing

Semen was collected by means of an artificial vagina from four pedigree Holstein bulls (4–5 years old) of proven fertility. The bulls belong to a controlled program of artificial insemination and were maintained under uniform nutritional conditions and management during the period of research. For all ejaculates progressive motility was greater than 70% and the percentage of abnormal spermatozoa was less than 20%. Ejaculates from each bull were obtained once a week during 12 weeks and used for cryopreservation. Semen from each bull were diluted in a buffer containing Tris (0.20 mM), citrate (0.06 mM), glycine (0.12 mM), fructose (0.06 mM), 20% egg yolk and 7% glycerol at a 2:1 ratio. Final concentration was within $3.0–4.5 \times 10^7$ sperm/ml. A slow cooling curve at 5°C (1°C/min) was performed, and the semen was then equilibrated at 5°C for a further 90 min. It was frozen at −76°C on dry ice, and the pellets were preserved at −196°C in liquid nitrogen.

Sample preparation

Frozen semen samples were thawed for 1 min in Tyrode's albumin lactate pyruvate (TALP) medium containing 99 mM NaCl, 3.1 mM KCl, 0.35 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Hepes, 1.1 mM $MgCl \cdot 6H_2O$, 25 mM $NaHCO_3$, 1 mM sodium pyruvate, 21.6 mM sodium lactate, at pH 7.4 and 36°C [26]. It was then equilibrated for 5 min followed by centrifugation at 600*g* for 5 min to separate the seminal plasma and buffer. The pellet thus obtained was washed in the same medium and centrifuged as stated above. The final pellet was resuspended in TALP medium containing 2 mM $CaCl_2$ and 6 mg/ml BSA, to a final concentration of 1.5×10^7 spermatozoa/ml. Aliquots for different treatments were incubated at 38°C (bovine body temperature) under 5% CO_2 in humidified air for 45 min. Sperm concentration was carried out in a Neubauer chamber (Reichert Buffalo, NY, USA). For each experiment, the progressive motility was evaluated 10 min after thawing and after each treatment by the same observer using light microscopy under ×400 magnification with a thermal stage at 37°C.

For each experiment, the effect of all the compounds used at the indicated concentrations was tested on sperm viability nor the progressive motility. Percentages of viability and progressive motility were 55–65 and 45–60%, being not significant differences between spermatozoa incubated without or with each compound.

Determination of capacitation

Percentage of capacitated spermatozoa was determined by the chlortetracycline (CTC) epifluorescence assay [27]. By using this technique it can distinguish three fluorescent pattern corresponding to different sperm sub-populations in the same sample: (1) F (fluorescent), corresponding to intact non-capacitated sperm displaying fluorescence throughout their surface, (2) B (capacitated), intact capacitated spermatozoa that lost fluorescence in the post-acrosomal region and (3) AR (acrosome reacted), spermatozoa with a reacted acrosome that lost fluorescence in the post-acrosomal and acrosomal regions, expressing fluorescence only in the midpiece. The CTC solution was prepared daily by adding 500 μM CTC to a buffer containing 130 mM NaCl, 5 mM cysteine and 20 mM Tris (Trizma base), pH 7.8. Sperm samples of about 500 μl were mixed with an equal volume of the CTC solution and fixed by the addition of 0.1% glutaraldehyde. Slides were examined at ×400 magnification by using an epifluorescence microscope (Carl Zeiss Jenamed 2, Jena, Germany). The percentage of capacitated (pattern B) spermatozoa was obtained by subtracting from the values obtained in the control and treated samples at zero time in order to rule out cells destabilized during the freezing–thawing process.

In order to confirm that spermatozoa were capacitated after the treatment with heparin, cells were incubated with 100 μg/ml lysophosphatidylcholine (LPC) for 15 min to induce the acrosome reaction [12,26]. The percentages of spermatozoa showing the pattern B (capacitated) after capacitation with heparin was 39 ± 5. The percentage of AR was 41 ± 6. There was a positive correlation between these two patterns ($r = 0.80$, $p < 0.05$). This experiment was performed three times with sperm samples from four different bulls ($n = 12$).

Determination of NADP-isocitrate dehydrogenase and glucosa-6-P-dehydrogenase

For the enzymatic determination, frozen sperm pellets were thawed in a 1:3 dilution in TALP medium, pH 7.4, without calcium and BSA, at 36°C, and were centrifuged at 600*g* for 5 min to separate the seminal plasma and the semen extender. Pellet was resuspended in the same fresh TALP medium and was centrifuged again under the same conditions. The final pellet was resuspended in deionized water at 4°C and sonicated at 100 W in 50% cycle using a VibraCell sonicator (Sonics & Materials model 600W) during 5 min for sperm samples to G6PDH; for NADP-ICDH, sperm suspension was sonicated with 25% cycle during 4 min. The sperm extracts were centrifuged at 20,000*g* for 20 min at 4°C for G6PDH, and at 100,000*g* for 60 min at 4°C for NADP-ICDH. Enzymatic activities were determined spectrophotometrically by the change of the absorbance of NADP using a UV-160 A Shimadzu recording spectrophotometer (Shimadzu Corporation, Tokyo, Japan). The enzymatic activity of each enzyme was expressed as units/10^{10} spermatozoa. NADP-ICDH activity was determined by measuring the absorbance change recorded at 340 nm, using 3 mM DL-isocitrate and 0.5 mM NADP in a 70 mM Tris–HCl buffer (pH 7.5, at 37°C) containing 1 mM $MnCl_2$, 8 mM $MgCl_2$, and 20 mM citrate, according to Brooks (1978) [24]. An enzymatic unit was considered as the amount of enzyme producing the generation of 1 μM of NADPH/min. Glucose-6-P dehydrogenase activity was determined in washed spermatozoa incubated in a buffer containing 40 mM glycine (pH 7.5) with 12.5 mM $MgCl_2$, 1.5 mM $NADP^+$, 12 mM maleymide and 1.05 mM glucose-6-P according to Kornber and Horecker (1955) [28] and Lohr And Waller (1974) [29]. An enzymatic unit was considered as the amount of enzyme producing the generation of 1 μM of NADPH/min.

Effect of oxidoreductases inhibitors on bovine sperm capacitation

Spermatozoa were incubated in TALP medium supplemented with 2 mM Cl_2Ca and 6 mg/ml BSA, with 10 IU/ml heparin for 45 min at 38°C under 5% CO_2 in humidified air [4], in the presence and absence of different concentrations of sodium oxamate (NaOx) or sodium oxalomalate (NaOM) inhibitors of LDH-C4 [15], and NADP-ICDH, respectively [30]. After determination of the minimum inhibitory concentration, the effect of these inhibitors were tested also in spermatozoa capacitated with 0.05 mM xanthine, 5 mIU/ml xanthine oxidase and 100 μg/ml catalase (X–XO–C system that generates $O_2^{\cdot-}$) for 45 min [5].

Effect of superoxide dismutase on capacitation induced by heparin or superoxide anion in the presence of caffeine

Bovine spermatozoa were incubated in TALP medium supplemented with 2 mM Cl_2Ca and 6 mg/ml BSA, without or with 10 IU/ml heparin or with 0.05 mM xanthine, 5 mIU/ml xanthine oxidase and 100 μg/ml catalase in the absence or the presence of 0.5 mM caffeine [31], and 100 μg/ml SOD for 45 min.

Effect of protein kinases inhibitors on bovine sperm capacitation

Spermatozoa were incubated with heparin or the X–XO–C system during 45 min in the absence or the presence of different protein kinases inhibitors: 50 μM H89, a PKA inhibitor [7,12]; 100 nM GF109203X (GF) (inhibitor of PKC) [9,12], or 100 μg/ml genistein [12,32].

Statistical analysis

Percentages of capacitation obtained by CTC assay, presented as means ± standard deviation (SD), were analyzed by ANOVA and Bonferroni tests. A $p < 0.05$ value was regarded as statistically significant.

Results

In order to study the participation of oxidoreductases in the capacitation process, different concentration of specific inhibitors (NaOx and NaOM for LDH-C4 and NADP-ICDH, respectively) were added to the sperm suspension in the presence of heparin. There was a complete prevention of capacitation when NaOx was present at concentration equal or higher than 1 mM (Figure 1). Sodium oxalomalate (NaOM) at concentration equal or higher than 5 mM also prevented the capacitation induced by heparin (Figure 2). When XXOC was used as capacitation inducers, 1 mM NaOx and 5 mM NaOM failed to block capacitation (Figure 3).

NADP-ICDH activity was detected in bovine spermatozoa and it was inhibited by 5 mM of NaOM (Table I). Confirming previous results found by Hamerstedt (1975) [25], bovine spermatozoa do not contain detectable G6PDH activity (Table I).

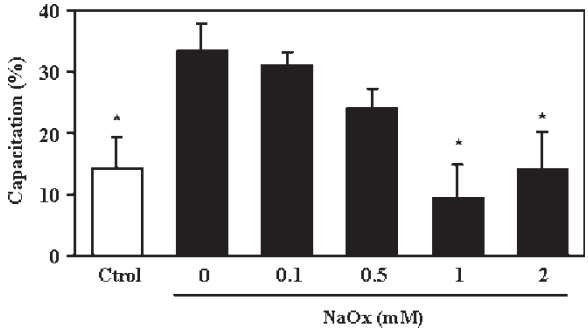

Figure 1. Effect of the LDH-C4 inhibitor on the heparin-induced sperm capacitation. Spermatozoa were incubated in TALP medium supplemented with 6 mg/ml BSA and 2 mM $CaCl_2$ alone (white bars) or with 10 IU/ml heparin (black bars) in the presence of different concentrations of sodium oxamate (NaOx). Values are mean ± SD of four experiments performed with semen samples from different bulls. *Values lower than all others ($p < 0.05$).

Spermatozoa treated with heparin and caffeine were capacitated even in the presence of SOD, a $O_2^{-\cdot}$ scavenger that blocks capacitation in bovine spermatozoa (Figure 4) [4,5]. Caffeine did not promote capacitation in spermatozoa incubated in TALP medium alone, confirming previous results [31] that the presence of this phosphodiesterase inhibitor alone is not sufficient to induce capacitation.

Because $O_2^{-\cdot}$ is involved in bovine sperm capacitation [6], and this ROS was postulated as a signal transduction element [33], we investigated the possible kinases associated with $O_2^{-\cdot}$ that lead to capacitation. Heparin- or XXOC-induced capacitation was prevented by H89, GF, or genistein, inhibitors of PKA, PKC and PTK, respectively (Figure 5).

Discussion

In this study, we suggest the requirement of LDH-C4 and NADP-ICDH as well as protein kinases such as PKA, PKC and PTK in bovine sperm capacitation induced by heparin or by the exogenous addition of $O_2^{-\cdot}$.

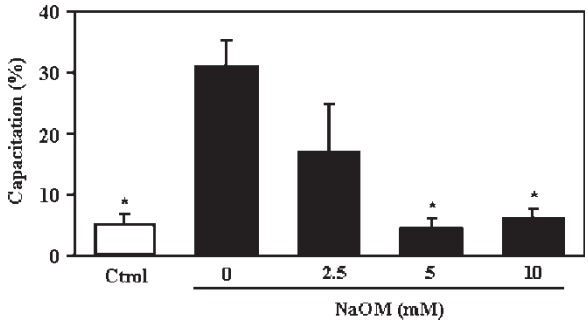

Figure 2. Effect of the NADP-ICDH inhibitor on the heparin-induced sperm capacitation. Spermatozoa were incubated in TALP medium supplemented with 6 mg/ml BSA and 2 mM $CaCl_2$ alone (white bars) or with 10 IU/ml heparin (black bars) in the presence of different concentrations of sodium oxalomalate (NaOM). Values are mean ± SD of four experiments performed with semen samples from different bulls. *Values lower than all others ($p < 0.05$).

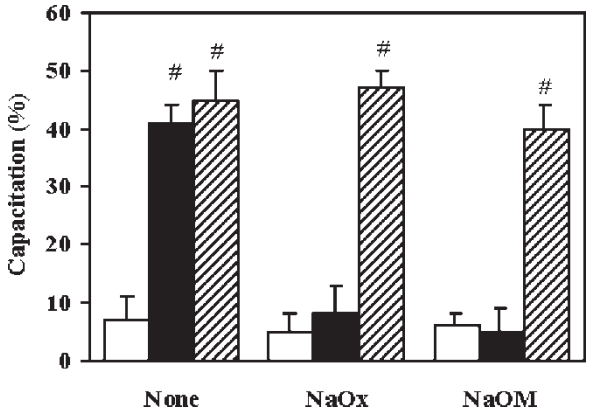

Figure 3. Effect of oxidoreductases inhibitors in capacitation induced by heparin or superoxide anion. Spermatozoa were incubated in TALP medium supplemented with 6 mg/ml BSA and 2 mM $CaCl_2$ alone (white bars) or with 10 IU/ml heparin (black bars) or 0.05 mM xanthine, 5 mIU/ml xanthine oxidase and 100 μg/ml catalase (stripped bars) in the absence (none) or presence of 1 mM sodium oxamate (NaOx) or 5 mM sodium oxalomalate (NaOM). Values are mean ± SD of four experiments performed with semen samples from different bulls. #Values higher than all others ($p < 0.05$).

Capacitation is part of an oxidative process where ROS generation is one of its associated-early events [2–4]. In bull spermatozoa, the generation of $O_2^{-\cdot}$ by an oxidase was demonstrated in both capacitation and acrosome reaction [6,9,12]. The prevention of capacitation by the specific inhibitors of LDH-C4 and NADP-ICDH (Figures 1 and 2) indicates the participation of these oxidoreductases in this process. These results confirmed our previous study where a role of LDH-C4 in the capacitation process was suggested [15]. Due to mammalian spermatozoa required a redox status to support motility and capacitation [13–15], both LDH-C4 and NADP-ICDH may contribute with the redox status required to support bovine sperm function.

Superoxide dismutase prevented the capacitation induced by heparin [5,6], suggesting that $O_2^{-\cdot}$ is involved in the mechanisms that lead cryopreserved bovine spermatozoa to be capacitated. The XXOC system (generator of $O_2^{-\cdot}$) induce capacitation in bovine spermatozoa [5,6]. It is known that ROS can induce capacitation by activating adenylyl cyclase [34–36], which generates cAMP activating the PKA pathway [37,38]. Galantino-Homer et al. (1995) suggested the participation of PKA in the capacitation-associated protein tyrosine phosphorylation in bovine spermatozoa [7]. In the present study, H89 prevent the capacitation induced by both heparin or the X–XO–C system suggesting a role of PKA in the $O_2^{-\cdot}$-induced sperm capacitation (Figure 4). Caffeine inhibits the phosphodiesterase activity [39], increasing cAMP concentration, motility and respiration [40], and reverses the glucose-dependent inhibition of capacitation induced by heparin in bovine spermatozoa [31] and the *in vitro* fertilization bovine oocytes

Table I. Activites of NADP-isocitrate and glucose-6-P dehyrogenases in bovine spermatozoa.

		Enzymatic activity (units/10^{10} cells)		
	n	Without inhibitor	With inhibitor	Inhibition (%)
NADP-isocitrate ehydrogenase	5	0.96 ± 6.3	0.48 ± 0.13	50 ± 10*
Glucose-6-P dehydrogenase	8	<0.0001	n.d.	n.d.

n.d., not determined. Values are mean ± SD of five and eight experiments performed with semen samples from different bulls. *Inhibition obtained by the presence of sodium oxalomalate.

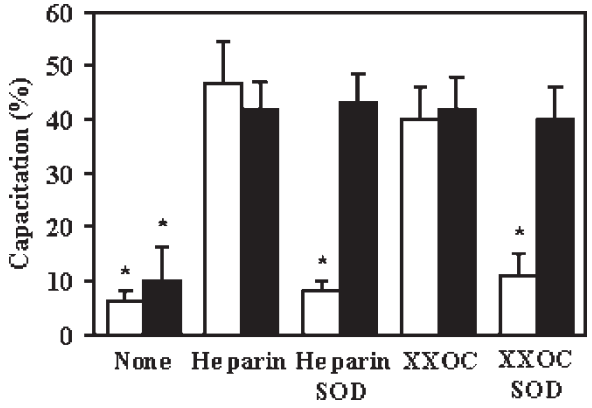

Figure 4. Effect of caffeine on capacitation induced by heparin or superoxide anion in the presence of superoxide dismutase. Spermatozoa were incubated in TALP medium supplemented with 6 mg/ml BSA and 2 mM $CaCl_2$ alone (none), with 10 IU/ml heparin (black bars) or 0.05 mM xanthine, 5 mIU/ml xanthine oxidase and 100 μg/ml catalase (XXOC) without or with 100 μg/ml superoxide dismutase (SOD), in the absence (white bars) or the presence (black bars) of 0.5 mM caffeine. Values are mean ± SD of four experiments performed with semen samples from different bulls. *Values lower than all others ($p < 0.05$).

[41]. The lack of SOD to block heparin or $O_2^{-\cdot}$-dependent capacitation when caffeine is present in the incubation medium suggest that the action of $O_2^{-\cdot}$ is upstream the production of cAMP, probably by activating adenylyl cyclase as was observed in human spermatozoa [34].

Although the participation of PKC in capacitation is controversial, some evidence support its role in this process [9,42]. Moreover, this kinase seems to be involved in the activation of phospholipase D and the F-actin formation [10]. The diminution in the percentages of capacitation in spermatozoa incubated with both heparin or the X–XO–C system in the presence of GF109203X (inhibitor of PKC), indicates the participation of this kinase in bovine sperm capacitation.

It is known that protein tyrosine phosphorylation is associated with capacitation in several species including the bovine [7,37,38,43]. Genistein was able to prevent the capacitation of spermatozoa incubated with heparin or the $O_2^{-\cdot}$ generator system (X–XO–C) (Figure 4). These results confirm the participation of PTK in the capacitation process.

Taken together these results suggest that LDH-C4 and NADP-ICDH contribute with the redox status to support bovine sperm capacitation, and the requirement of PKA, PKC and PTK in mechanisms triggered by heparin and $O_2^{-\cdot}$, that lead bovine spermatozoa to achieve capacitation.

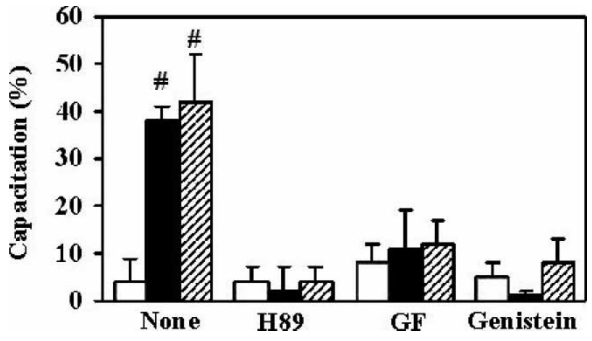

Figure 5. Effect of protein kinases inhibitors on capacitation induced by heparin or superoxide anion. Spermatozoa were incubated in TALP medium supplemented with 6 mg/ml BSA and 2 mM $CaCl_2$ alone (white bars), with 10 IU/ml heparin (black bars) or 0.05 mM xanthine, 5 mIU/ml xanthine oxidase and 100 μg/ml catalase (stripped bars) in the absence (none) or presence of 50 μM H89, 100 nM GF109203X (GF), or 100 μg/ml genistein. Values are mean ± SD of four experiments performed with semen samples from different bulls. #Values higher than all others ($p < 0.05$).

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