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Brief Note

Proacrosin-acrosin activity in capacitated and acrosome reacted sperm from cryopreserved bovine semen

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ABSTRACT: Acrosin activity is associated with normal fertility in human and bovine spermatozoa. The aim of the study was to determine the variation of the enzyme activity in the proacrosin-acrosin system in capacitated and acrosome reacted cryopreserved bovine sperm. Enzyme activity was assessed spectrophotometrically using N-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as specific substrate for acrosin at pH 8. Capacitation with heparin and quercitin failed to induce conversion of proacrosin to acrosin. An increase in acrosin activity produced by the presence of progesterone, in a dose-dependent manner, was related with the induction of true acrosome reaction. The total level of acrosin activity registered showed that 96% of acrosin of capacitated sperm samples and control is present in the zymogen form. Moreover, progesterone is capable of duplicating the level of active enzyme, indicating that enzyme activity changes are related to acrosome reaction, suggesting that only a minor proportion of the total of proacrosin-acrosin system is required in the exocytotic process on cryopreserved bovine sperm.

Introduction

The spermatozoa of many animal species contain in the apical region of the head a secretory vesicle called the acrosome. The exocytotic process of the acrosome reaction consists in a terminal morphological alteration that must occur prior to penetrating the extracellular coat of the eggs (Florman and Babcock, 1991; Kopf and Gerton, 1990). The glycosaminoglycan heparin, present in the female genital tract (Lee and Ax, 1984), binds to bull spermatozoa as a typical receptor ligand interaction, promoting capacitation (Ax and Lenz, 1987; Parrish *et al*., 1988). Heparin induces capacitation and increases intracellular calcium concentration through the activation of a voltage calcium channel type L (60%) (Córdoba *et al.,* 1997). Quercitin, a specific inhibitor of calcium ATPase present in the plasma membrane (Fraser *et al*, 1995), induces capacitation and an intracellular calcium increase at a similar level to heparin treatment in bovine sperm (Córdoba *et al.*, 2001). In turn, progesterone elicits a large and rapid increase in calcium concentration and acrosome reaction in non-capacitated cryopreserved bovine spermatozoa (Córdoba and Beconi, 2001), but has no effect on non-capacitated human spermatozoa (Blackmore and Lattanzio, 1991; O´Toole *et al.,* 1996).

Several acrosomal proteases have been detected in various species, among which acrosin is the most studied enzyme (Chen *et al.*, 1991). In bovine sperm the presence of acrosin, as determined by the silver enhanced immunogold technique, has been highly correlated with sperm penetration of *in vitro* mature oocytes

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and cryopreseved zona pellucida (De los Reyes and Barros, 2000). It is synthesized as an inactive precursor, proacrosin, and processed via (auto)proteolysis into active form(s) (Suter and Habenicht, 1998). The proacrosin-acrosin system has a significant role in retaining acrosome-reacted sperm on the zona pellucida (ZP) surface for starting ZP penetration in mouse (Howes and Jones, 2002). Specific domains within the acrosomal matrix of bovine spermatozoa participate to maintain a particulate proacrosin pool and to regulate proacrosin/acrosin release (NagDas *et al.*, 1996). Proacrosin-acrosin conversion is markedly inhibited by calcium in a dose dependent, reversible manner; on the other hand, calcium exerts a stimulatory effect on the hydrolytic activity of acrosin in ejaculated bovine sperm (NagDas, 1992). In ram spermatozoa, it has been demonstrated that acid phosphatase and acrosin activities are modulated by ultra-low temperatures and storage duration (Tsekova *et al*., 1986). It has also been shown that enhanced acrosin activity in sperm is associated with normal fertility in human and bovine (De Jonge *et al.*, 1993; De los Reyes and Barros, 2000). Likewise, low acrosin activity in human sperm correlates with low fertility rates (Koukoulis *et al*., 1989; Palencia *et al*., 1996). Mechanisms regulating hydrolase release from mammalian spermatozoa are poorly understood. The aim of this study was to determine the variation of acrosin activity in cryopreserved bovine spermatozoa in heparin or quercitin induced capacitation and progesterone induced acrosome reaction.

Materials and Methods

Sperm suspension

Spermatozoa from frozen-thawed bovine semen (pellets) of proven fertility were used. The percentage of cells with progressive motility was evaluated at 37ºC by light microscopy, after 10 minutes thawing in 3FMB medium (125 mmoles NaCl l^{-1}, 10 mmoles KCl l^{-1}, 2.0 mmoles $MgCl_2$ l^{-1}, 2.5 mmoles sodium pyruvate l^{-1}, 20 mmoles lactate l^{-1} and 20 mmoles HEPES l^{-1}), without the addition of bovine serum albumin (BSA) or calcium (Shimizu *et al*., 1993). After motility evaluation, samples were centrifuged at 600 *g* during 5 min and resuspended in 3FMB with 2.1 mmoles calcium chloride l^{-1} and 6 mg ml^{-1} BSA, to carry out the different treatments related to the induction of capacitation and acrosome reaction, at a final concentration of 4 x 10^7 sperm / ml.

Progressive motility

Sperm vigor and progressive motility were evaluated by light microscopy at 38ºC. Samples presenting 60% average of progressive motility and a vigor of 3-4 were considered suitable for experiments.

Sperm capacitation

Samples were capacitated in the presence of 60 µg ml^{-1} heparin for 15 min at 38ºC in 3FMB medium with 2.1 mmoles calcium chloride l^{-1} and 6 mg ml^{-1} BSA (Córdoba *et al.*, 1997).

Ca^{2+} ATPase inhibition

Samples were treated with 50 µM quercitin, a specific inhibitor of calcium ATPase, for 15 min at 38ºC in FM3B medium with 2.1 mmoles calcium chloride l^{-1} and 6 mg ml^{-1} BSA (Córdoba *et al*., 2001).

Induction of the acrosome reaction

Acrosome reaction was induced by 0.5, 1 and 3 µM of progesterone in order to study progesterone dose dependent effect on acrosin activity, by incubation at 38ºC for 10 min in FM3B medium with 2.1 mmoles calcium chloride l^{-1} and 6 mg ml^{-1} BSA (Córdoba and Beconi, 1999).

Epifluorescence chlortetracycline assay

Percentages of capacitated and acrosome reacted spermatozoa were determined by the chlorotetracycline epifluorescent technique (CTC) (Fraser *et al.*, 1995). Zero time and incubation time controls for the different treatments were carried out. The percentage of capacitated and reacted spermatozoa obtained at zero time was subtracted from values registered for the incubation control and treated samples. This procedure was adapted to offset the percentage of spermatozoa showing a capacitated or acrosome reacted patterns due to damage during freezing-thawing.

Glutaraldehyde was used as staining fixative at a final concentration of 0.1g $10^{-2}ml^{-1}$. Samples were examined at 400x magnification using 410 nm excitation by epifluorescence microscopy. Three chlorotetracycline patterns were observed in the bovine species: 1) intact, non-capacitated spermatozoa, with a fluorescent head; 2) intact, capacitated spermatozoa, with a band lacking fluorescence in the post-acrosomal region; and 3) with

reacted acrosome, presenting low fluorescence in the whole cell except for a band in the equatorial segment. In all of them, fluorescence was observed in the intermediate segment of the spermatozoa.

Sperm viability

An aliquot of the sperm suspension from different treatments was incubated with an equal volume of 0.25% trypan blue in F3MB during 15 min at 37ºC, centrifuged at 600 *g* for 10 min to remove excess stain and then fixed with 5% formaldehyde in PBS. Samples were observed using light microscopy.

Differential-interferential optical contrast microscopy (DIC)

Acrosomes from the different sperm samples stained with Trypan blue were evaluated by differential-interferential optical contrast microscopy (DIC) to determine the presence of reacted acrosomes in live and dead spermatozoa. The percentage of true acrosome reaction (TAR) was determined by counting 200 spermatozoa. From the TAR value obtained in the different treatments, the AR value at zero time was subtracted, in order to rule out spontaneous acrosome reaction not due to the treatment (O´Flaherty *et al*., 1999).

Sperm concentration

Sperm count was carried out by hematocytometry in a Neubauer chamber.

Evaluation of the proacrosin-acrosin system

BAPNA (N-α-benzoyl-DL-arginine *p*-nitroanilide) was used as a specific substrate for acrosin (NagDas, 1992). The activation of proacrosin to acrosin was inhibited using a solution of HCl (10 M). A solution of 0.2 M Tris buffer was used to measure acrosin activity at pH 8.

A solution of HCl was added to the control incubated at 38ºC. Aliquots were taken from each sample at zero time and after incubation to determine the percentage of capacitation and acrosomal integrity by CTC and DIC techniques, respectively. Again, a solution of HCl was added to all samples, except for the control and the total acrosin ones. Samples were then homogenized and sonicated for 6 min at 4ºC, followed by centrifugation at 14,000 rpm at 4ºC during 10 min. A solution of 0.2 M buffer Tris and 100 µl of BAPNA (100 mM) was added to each supernatant, which were then incubated for 3 min at room temperature (Kennedy *et al.*, 1989). All the samples had their incubation controls in order to determine the real effect of the different capacitation and acrosomal reaction inducers.

Sample absorbance (Ab) was recorded at 410 nm. Activity was expressed in mIU/10^6 sperm. One unit of acrosin is defined as the quantity of enzyme that hydrolyses 1 µmol of BAPNA in one minute at 25ºC. Acrosin activity was calculated as mIU:

$$\text{mIU of acrosin}/10^6\text{ sperm} = \frac{[\text{Ab sample} - \text{Ab control}] \times 10^6}{9.9\ \text{mM}^{-1} \times \text{cm}^{-1} \times 3\ \text{min} \times 10^6\ \text{sperm} \times \text{vol. of cuvette}}$$

The absorbance of control samples (Ab control) was assessed using 500 µl of F3MB medium with Ca^{2+} and BSA, 500 µl HCl, 1 ml Tris and 100 µl BAPNA.

Statistical analysis

Data were expressed as mean values ± SD. The significance of results was evaluated by an ANOVA test and means were compared using Schefee test. A $P < 0.05$ value was regarded as statistically significant.

Results

In the presence of heparin or quercitin, acrosin activity of the sperm suspension was similar to that scored in the control samples ($P > 0.05$). Percentages of capacitated spermatozoa obtained by heparin or quercitin induction, assessed by CTC staining, were signicantly different respect to control values ($P<0.05$). (Table 1).

TABLE 1.

Percentage of capacitated sperm and Acrosin activity (mIU/10^6 sperm)

	Capacitated spermatozoa (%) (CTC)	Acrosin Activity (mIU/10^6 sperm)
Control	3.77 ± 1.75 *	0.55 ± 0.06 [a]
Heparin	27.78 ± 1.62 **	0.51 ± 0.07 [a]
Quercitin	28.11 ± 2.08 **	0.51 ± 0.02 [a]

CTC stain and acrosin activity controls are used to evaluate the capacitation induction and acrosin activation during 15 minutes of incubation, respectively.Data are expressed as means ± SD (n=9 for each treatment). Different letters and symbols denote a significant difference ($P < 0.05$).

To establish whether progesterone is involved in acrosin activation in non-capacitated sperm, acrosin activity was determined using increasing steroid concentrations (0.5, 1 and 3 μM). A significant increase in acrosin activity was observed vs. controls, leveling off at 1μM progesterone concentration. In sperm samples treated with progesterone, the percentage of acrosome reaction (CTC) and true acrosome reaction (DIC-Trypan Blue) were significantly different respect to controls, reaching a plateau at 1 μM concentration (P<0.05). (Table 2).

Proacrosin was totally activated in order to determine total acrosin in sperm, obtaining a mean value of 15.48 ± 1.10 mIU/10^6 sperm. The percentage of enzyme activated by capacitation and acrosome reaction inducers was related to total acrosin content. Significant differences were observed in sperm suspension treated with progesterone vs heparin, quercetin and control samples (P<0.05) (Fig. 1).

Discussion

Acrosin, a sperm acrosomal protease, has an essential role in the fertilization process and has been proposed as an indicator of sperm quality. As capacitation and acrosomal reaction are required for fertilization, it was interesting to establish whether these processes take

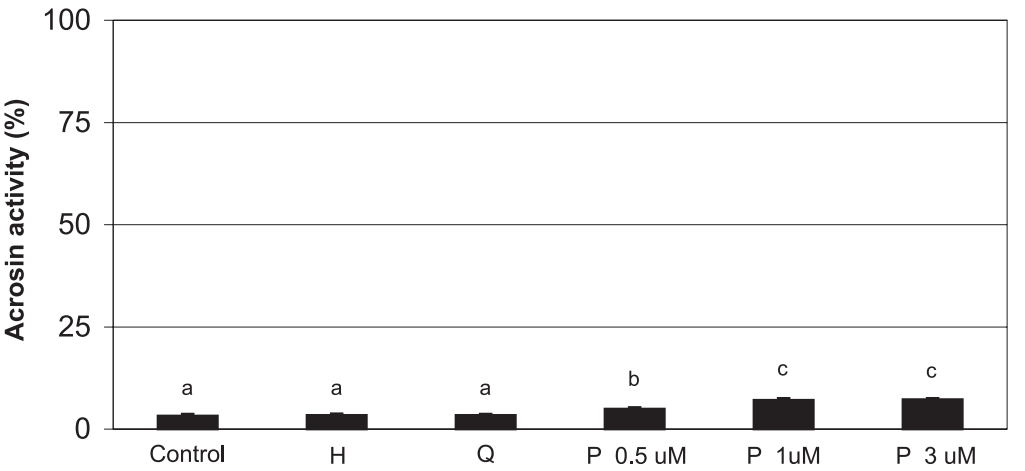

FIGURE 1. Percentage of activated acrosin respect to the total acrosin sperm. (H) heparin, (Q) quercitin, and (P) progesterone. The control percentage is used to evaluate the acrosin activation during the incubation time. Data are expressed as means ± SD (n=9 for each treatment). Different letters denote a significant difference (P < 0.05).

TABLE 2.

Percentage of acrosome-reacted (CTC), true acrosome reaction (DIC / Trypan Blue) and Acrosin activity (mIU/10^6)

Progesterone (μM)	Acrosome reaction (%)	True acrosome reaction (%)	Acrosin Activity (mIU/10^6sperm)
Control	1.44 ± 1.83 *	1.33 ± 1.63 ^{a}	0.57 ± 0.02 ^{o}
0.5	15.22 ± 3.34 **	14.33 ± 3.36 ^{b}	0.70 ± 0.05 ^{oo}
1.0	20.11 ± 3.46 ***	20.00 ± 3.46 ^{c}	1.08 ± 0.13 ^{ooo}
3.0	22.57 ± 4.10 ***	21.66 ± 4.16 ^{c}	1.14 ± 0.14 ^{ooo}

CTC, DIC- Trypan blue stain controls and acrosin activity control are used to evaluate the acrosome reaction induction and acrosin activation, respectively during 10 minutes of incubation. Data are expressed as means ± SD (n=9 for each treatment). Different letters or symbols denote a significant difference (P< 0.05).

part in the activation of proacrosin-acrosin system in cryopreservated bovine sperm.

Interaction of solubilized Zona Pellucida Glycoproteins (ZPGs), heparin and Acrosome Reaction Substance of Starfish (ARIS) with boar and human sperm indicates that ZPGs and ARIS but not heparin bind to a related domain on the proacrosin surface and induce enzyme activation (Moreno *et al.*, 1999). In accordance to these results our data suggest that the conversion of proacrosin to acrosin was not induced by heparin or quercitin in capacitation on cryopreserved bovine sperm. These results, however, contrast with the acrosin activation that has been observed during capacitation in cauda epididymal hamster spermatozoa (Meizel and Mukerji, 1976).

The increase in intracellular free calcium previously described supports proacrosin activation in the acrosome reaction of guinea pig sperm (Green, 1978). In non-capacitated cryopreserved bovine spermatozoa, progesterone induces acrosome reaction with a large intracellular calcium concentration increase (Córdoba and Beconi, 2001). In contrast the steroid has no effect in noncapacitated human sperm (Blackmore and Lattanzio, 1991; O'Toole *et al*., 1996). The increase in acrosin activity caused by progesterone in a dose-dependent manner and its relationship with true acrosome reaction induction suggests that the proacrosin-acrosin system may be modified by progesterone through steroid-induced cellular mechanisms, that may include the variation in intracellular calcium.

The variation of enzyme activity observed after progesterone treatment compared to heparin or quercitin, suggests that enzyme activation in cryopreserved spermatozoa takes place in association with the acrosome reaction rather than capacitation process. In previous reports, similar levels of intracellular calcium reached with heparin or quercitin have been lower than the one obtained with progesterone alone (Córdoba *et al.*, 1997; Córdoba and Beconi, 2001); in our data the different activities of acrosin registered with capacitation and acrosome reaction inductors suggest that the variation in acrosin activity seems to be dependent on intracellular calcium concentration, as it was observed in boar sperm (Parrish and Polakoski, 1978).

A decrease in the proportion of proacrosin-acrosin system capable of being activated, as a result of cryopreservation, was observed in bovine sperm. Cryopreservation may inactivate acrosin or cause acrosin to be lost from the acrosome even in spermatozoa surviving the process (Palencia *et al.*, 1996). Although premature capacitation has been reported in cryopreserved bovine sperm (Cormier *et al.*, 1997), the low level of sperm capacitation observed in controls compared to heparin and quercitin capacitation levels confirms that these inducers are not involved in the acrosin zymogen activation.

Our data show that the total level of acrosin registered in sperm samples undergoing the frozen-thawing process, remains in the zymogen form. Furthermore, progesterone is capable of duplicating the level of active enzyme, indicating that changes are closely related to true acrosome reaction rather than to capacitation, in contrast to that in rabbit sperm, in which the proacrosin-acrosin system remains largely as proacrosin during capacitation and acrosome reaction (Sillerico *et al*., 1996).

In conclusion, this study suggests that a minor proportion of acrosin is required in the sperm exocytotic process, and cryopreserved bovine sperm maintain sufficient proacrosin capable of being activated by the acrosome reaction.

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