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Proacrosin activation mechanisms in capacitated and frozen-thawed boar spermatozoa

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ABSTRACT. The main objective of the current study was to explore the different activation mechanisms of capacitation and freeze-thawed spermatozoa. Using SDS-PAGE and Western blotting, the conversion process of boar proacrosin during freeze-thawing and capacitation of spermatozoa was analyzed. The results revealed that capacitated spermatozoa exhibited a greater fluorescence area than that of the freeze-thawed spermatozoa, which were smaller than those of the fresh group. Fresh spermatozoa displayed 45- and 35-kDa protein bands, while those of freeze-thawed andcapacitated spermatozoa displayed 45-, 35- and 28-kDa bands. In summary, these data indicate that proacrosin is activated, thus becoming α- and β-acrosins and a 28-kDa protein during capacitation and freeze-thawing.

Key words: Boar sperm; Proacrosin; Freeze-thaw; Capacitation; SDS-PAGE; Western blotting

INTRODUCTION

Low fertilization rates of freeze-thawed spermatozoa are thought to be caused by damage to the spermatozoa (Bailey et al., 2000), including capacitation-like changes, which have been referred to as cryocapacitation (NagDas, 1992). Reports have shown that the sperm acrosome contains as many as 24 enzymes, including proteases and phospholipases; among them, acrosin is the most relevant to fertilization (Barros et al., 1996). Studies have provided evidence that acrosin primarily exists (85-90%) as proacrosin in ejaculated semen (Cormier and Bailey, 2003). The boar proacrosin (55 and 53 kDa) is automatically activated to become α-acrosin (45-49 kDa), which is then transformed into mature β-acrosin (34-38 kDa) (Moos et al., 1993a). Acrosin is capable of recognizing and penetrating the zona pellucida. However, damaged spermatozoa experience decreased fertilization capabilities (Barros et al., 1996). Therefore, the objective of the current study was to explore the transformation mechanism of proacrosin during the freeze-thawing process to clarify the underlying mechanisms affecting capacitation-like and capacitation spermatozoa. This study could provide a theoretic basis for improving technologies for freeze-thawing semen

MATERIAL AND METHODS

Boar semen

Semen was provided by the Hanji Husbandry and was collected from 8 Duroc boars using a manual semen collection method.

Reagents

All reagents were purchased from Sigma (Beingjing, China), except where indicated otherwise. Anti-acrosin (ACR-2) and HRP-goat-anti-mouse IgG h+1 were purchased from Shanghai Youning Biotech. The prestained protein marker, NC membrane, DAB color development solution, and other immunoblotting reagents were obtained from Beijing Colourful Biotechnology.

Frozen solution preparation

The frozen solution was made by mixing the following: 2420 mg Tris, 1480 mg citrate, and 1100 mg glucose in 100 mL triple distilled water. Frozen dilution solution A was made by freshly adding 20% (v/v) egg yolk to the base frozen solution. Frozen dilution solution B was made by freshly mixing 9% (v/v) glycerol into frozen dilution solution A.

Capacitation solution

The capacitation solution (100 mL) contained 661 mg NaCl, 22.3 mg KCl, 110.2 mg $CaCl_2$, 55 mg sodium pyruvate, and 200 mg caffeine. The solution was filtered through a 0.22-μm filter and stored at -4°C.

Antibody dilution

The antibody dilution solution was prepared by mixing 500 μL Tween 20 with 1000

mL PBS and then filtered. The primary antibody was diluted by mixing with a dilution solution at 1:50 for fluorescent staining; it was further diluted (1:4) for Western blotting and stored at 4°C in the dark. Secondary antibody (HRP-goat-anti-mouse IgG h+l) was prepared by diluting to 1:5000 and maintained in the dark.

Freezing and thawing

For the freezing process, racks were set 3 cm above the surface of liquid nitrogen for 10 min; then, small tubes were set horizontally on the rack, covered with a lid (allowed to steam for 10 min), and then rapidly set into liquid nitrogen.

For the thawing process, tubes were removed from liquid nitrogen and immediately put into a water bath at 37°C for 60 s. After thawing, the tubes were blotted dry and cut from the sealed end.

Spermatozoa capacitation

The washing solution mPBS and capacitation solution were warmed by setting at room temperature for 20 min. Semen samples were diluted (1:1) in mPBS and centrifuged for 5 min at 500 rpm. The pellet was suspended in 2 mL capacitation solution containing 20 µg/mL heparin and incubated for 2 h in a 5% CO_2 incubator at 39°C and a 45° angle. The swim-up method was used.

Protein extraction

Pretreated semen was spun at 150 rpm for 10 min at 4°C. A volume of 6 mL sperm supernatant was washed with filtered water and then spun at 150 rpm for 10 min to remove spermatogonia. A volume of 6 mL supernatant was spun again at 3000 rpm for 10 min; the pellet was resuspended in 3 mL cold PBS (0.1 mL/L, pH 7.4, 0.7% NaCl), combined, and spun at 3000 rpm for 10 min at 4°C. The pellet was again washed with 6 mL cold PBS, and the supernatant was removed with centrifugation at 3000 rpm for 10 min. The preparation was washed twice with Tris-HCl buffer (0.1 M, pH 7.4, 0.7% NaCl, 1 mM EDTA, 0.5 mM PMSF). Finally, spermatozoa were suspended in 1 mL Tris-HCl buffer. The membrane protein was extracted by mixing with an extraction buffer containing 1% Triton X-100 and shaking for 1.5 h in a water bath. The membrane proteins were collected with the supernatant after centrifugation at 5000 rpm for 15 min.

Statistical analysis

Data were analyzed with SPSS 11.5. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Indirect fluorescent staining of proacrosin incapacitation and capacitation-like spermatozoa

Figure 1 shows the fluorescence that was observed in the spermatozoa from 3 treatment groups. Spermatozoa in the capacitation group exhibited a larger area of fluorescence than those in the freeze-thawed group. Spermatozoa in the fresh group exhibited the largest area of fluorescence. These results show that proacrosin in the freeze-thawed group was par-

tially damaged, and proacrosin was partially activated during capacitation.

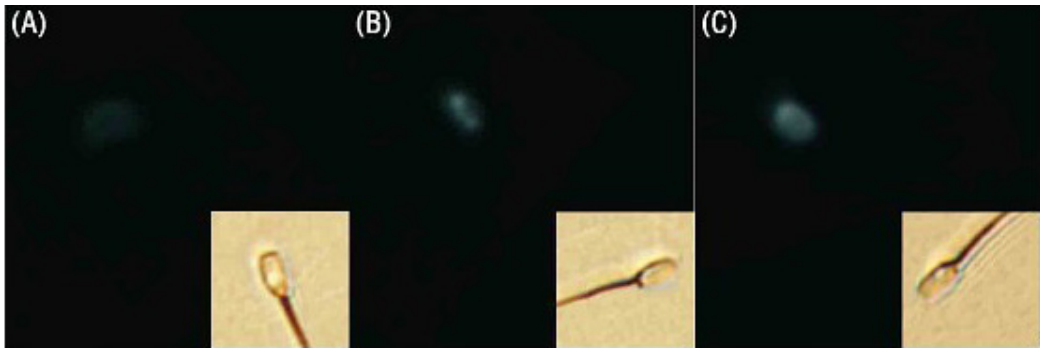

Figure 1. Fluorescent staining of proacrosin. **A.** Fresh spermatozoon. **B.** Frozen-thawed spermatozoon. **C.** Capacitation spermatozoon.

SDS electrophoresis analysis of capacitation and capacitation-like spermatozoa

SDS-PAGE analysis indicates that proteins in all treatment groups were isolated. The fresh spermatozoan group contained proteins ranging from 55 to 95 kDa (Figure 2). The proteins were distributed evenly in both the capacitation and freeze-thawed groups. New bands were produced and some bands disappeared. These results indicate the occurrence of notable changes in protein species and levels in the treatment group when compared to those in the control groups.

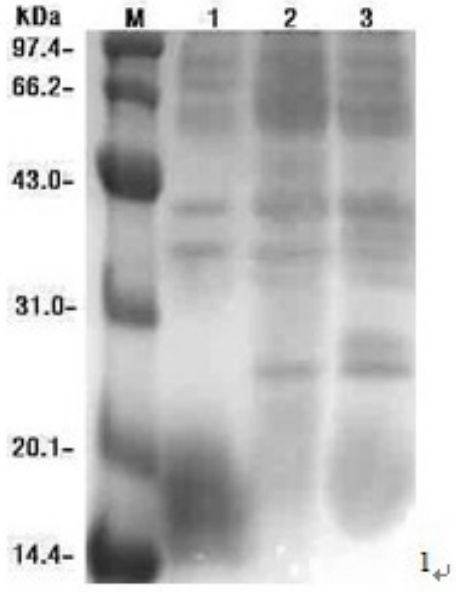

Figure 2. Electrophoresis analysis of proacrosin. *Lane M* = protein standard; *lane 1* = fresh spermatozoa; *lane 2* = frozen-thawed spermatozoa; *lane 3* = capacitation spermatozoa.

Western blot analysis of capacitation and capacitation-like spermatozoa

Samples of fresh, freeze-thawed, and capacitation spermatozoa were separated using SDS-PAGE, and then analyzed using Western blot analysis. Figure 3 shows that fresh spermatozoa exhibited 45- and 35-kDa bands, but the freeze-thawed and capacitation spermatozoa exhibited 45-, 35-, and 28-kDa bands. This result indicates that proacrosin was partially activated to α-acrosin and β-acrosin and a 28-kDa protein.

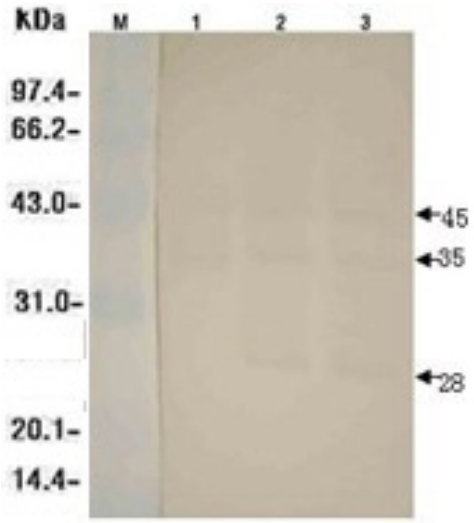

Figure 3. Westem blot analysis of proacrosin. *Lane M* = protein standard; *lane 1* = fresh spermatozoa; *lane 2* = frozen-thawed spermatozoa; *lane 3* = capacitation spermatozoa.

DISCUSSION AND CONCLUSION

The capacitation-like and capacitation spermatozoa exhibited a high degree of similarity; however, significant differences were found in the acrosin levels between the diluted semen and freeze-thawed semen when analyzed according to the method described by Kennedy et al. (1989). The level of acrosin is positively correlated with *in vitro* fertilization rates (Kennedy et al., 1989). The authors analyzed fresh, capacitation and freeze-thawed boar spermatozoa via SDS-PAGE and Western blotting and displayed them with anti-acrosin monoclonal and enzyme-linked antibodies. The results showed that the fresh spermatozoa were expressed at 45 and 35 kDa. In addition, the freeze-thawed and capacitation spermatozoa expressed 45-, 35- and 28-kDa proteins. These results indicate that proacrosin was partially activated to α-acrosin and β-acrosin in the freeze-thawed and capacitation spermatozoa.

A 28-kDa protein was observed in the freeze-thawed and capacitation spermatozoa. Hardy et al. (1991) first showed the existence of a 28-kDa protein in guinea pig spermatozoa. This protein is widely hydrolyzed by acrosin, and the process is closely associated with the disappearance of the acrosomal matrix. Studies have shown that the 28-kDa protein binds with proacrosin and 49-kDa α-acrosin, but not with the 36-kDa β-acrosin, thus maintaining the integrity of the acrosomal matrix and controlling acrosin release in the acrosomal reaction (Moos et al., 1993b). However, this mechanism requires further study.

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