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Western Blot Analysis of Proacrosin/Acrosin in Frozen Dog Sperm During *In Vitro* Capacitation

M De los Reyes, G Medina and J Palomino

Animal Reproduction Laboratory, Faculty of Veterinary Sciences, University of Chile, Santiago, Chile

Contents

Acrosin is an acrosomal protease synthesized as an inactive precursor, proacrosin, which is processed via autoproteolysis into active forms alpha- and beta-acrosin. In this paper, a comparative study on the immunoreactivity of acrosin during *in vitro* capacitation of frozen and fresh (control) canine sperm using Western blot analysis is reported. Semen samples were obtained by digital stimulation and ejaculates processed as fresh and frozen samples and then capacitated for 0, 30, 60 and 90 min. At each time period, samples were analyzed with monoclonal antibody C5F10 by Western blot. The antibody specifically recognized, in fresh and frozen/thawed spermatozoa, a 40-, 32- and 27-kDa bands corresponding to proacrosin, alpha- and beta-acrosin, respectively, during capacitation. Western immunoblots showed that the beta-acrosin reactivity in fresh sperm was directly proportional to the time of capacitation, whereas a decreased reactivity of active form of acrosin was observed with frozen-thawed sperm ($p < 0.05$). These results suggest that proacrosin is activated to beta-acrosin earlier in frozen/thawed dog spermatozoa than in fresh dog spermatozoa.

Introduction

Acrosin is the major proteinase with trypsin-like specificity, found in the acrosome of mature spermatozoa. It is stored in the acrosomal compartment in its precursor form, proacrosin (Parrish and Polakoski 1979). The zymogen proacrosin can self-catalyze its own conversion into the active forms of acrosin during acrosome reaction (Hardy et al. 1991). The exocytotic process of acrosome reaction releases its acrosomal content of hydrolytic enzymes, such as acrosin, at the proper time and place in order to digest the zona matrix, thus facilitating penetration of the sperm through the zona pellucida and its subsequent fusion with the oocyte oolema (Moreno et al. 2002). Additionally, this event results in significant remodelling of the sperm surface because the outer acrosomal and anterior head plasma membranes vesiculate and are lost from the sperm, exposing the inner acrosomal membrane (Barros et al. 1996).

Acrosin activity and acrosome morphology are indices of the efficacy of cryopreservation of spermatozoa as a freezing technique may render the acrosome dysfunctional (Froman et al. 1987). Although freezability of the dog spermatozoa is high, the longevity after thawing is considerably brief which may be due in part to acrosomal damage or changes related to premature capacitation (Peña et al. 2004), which in turn significantly affects fertilizing ability. There have been few reports on acrosin in dog sperm; however, in previous studies, we found that the proportion of dog spermatozoa with acrosomal damage and acrosin loss was greater in frozen/thawed than in fresh spermatozoa (Cortés et al. 2006). Considering that freezing and thawing process affect sperm membranes, especially over acrosome region (Cortés et al. 2006), this fact can alter functional mechanisms involved in acrosome reaction inducing an effect on proacrosin activation into acrosin. Kawakami et al. (1999) reported that acrosin activity in fresh dog sperm increased in association with sperm capacitation, but it is unknown if the same occurs in frozen/thawed samples. Thus, the aim of this work was to analyze proacrosin/acrosin system by Western blotting in fresh and frozen/thawed dog sperm during different times of *in vitro* capacitation.

Materials and Methods

Materials

All the chemicals were purchased from Sigma (St Louis, MO, USA). Monoclonal antibody against acrosin was purchased from Biosonda (Santiago, Chile; Cat No. AMC-ACRO-C5F10-AS).

Semen processing

A total of six ejaculates were obtained from three fertile dogs by manual stimulation. Sperm-rich fraction of each ejaculate (considered as one experimental replicate) was liquated into two fractions and centrifuged in Tris buffer. The pellet of one fraction was diluted in Fert-talp medium and cultured for capacitation during 0, 30, 60 and 90 min at 20°C (fresh control samples) and the pellet of the other fraction was diluted in TRIS-citrate fructose-freezing extender, and frozen in liquid nitrogen at −196°C as described previously (De los Reyes et al. 2006). After thawing, sperm were cultured in Fert-talp medium for capacitation during the same periods.

Protein extraction and Western blot

At each time of culture for capacitation, fresh and frozen /thawed sperm extracts were made by homogenizing the sperm in buffer containing 1% Triton X-100, NaCl 1 M, EDTA 1 mM, PMSF (phenylmethylsulphonyl fluoride) 10 μg/ml, Tris-HCl 20 mM pH 7.0 and then centrifuged for 10 min at 10 000 × ***g***. The proteins of acid-soluble extracts from the canine spermatozoa were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions according to standard techniques reported by Laemmli (1970), and then transferred onto nitrocellulose paper by

Western blotting at 100 V for 1.5 h. Blots were blocked with 2% BSA in PBS, pH 7.4 for 1 h and incubated overnight at 4°C with monoclonal antibody C5F10 (dilution 1 : 1000) against human acrosin. In a previous study, using immunofluorescence techniques, we showed that this anti-acrosin serum reacted strongly with dog proacrosin as well as with forms of active dog acrosin (Cortés et al. 2006). The blots were then washed with 0.1% PBS-Tween 20 and then incubated for 1 h at room temperature with 1 : 5000 of rabbit anti-mouse antibody conjugated to peroxidase. Protein bands were visualized with NBT/BCIP substrate (Nitro blue tetrazolium, 300 pg/ml; Bromo-chloro-indolyl phosphate-p-toluidine salt, 150 pg/ml; in 0.1 M Tris, pH 9.5). Molecular masses of separated proteins were estimated by comparison with positions of a molecular-mass standard run in parallel.

Acrosomal staining was specific because no signal was obtained when the C5F11 antibody was not included in the procedure (data not shown).

Statistical analysis

The immunoblots from each experimental replicate were scanned using a HP Scanjet 3970 scanner (Hewlett-Packard, Palo Alto, CA, USA). Quantification of Western blot bands was performed by densitometry analysis using the GEL-PRO ANALYZER V3.1 program (Media Cybernetics Inc., Silver Springs, MD, USA). The program automatically identifies lanes and bands, and graphs the optical density. The amount of dark pixels (optical density) in the same area for each band was determined and expressed as integrated optical density. The results are presented as the means of six separate experiments.

Density values were compared by ANOVA (Statistical Analysis System, SAS Institute, Cary, NC, USA), including the effects of spermatozoa treatment, duration of culture and their interaction. Significant differences among means were studied using Tukey tests, considering with $p \leq 0.05$.

Results

A Western blot analysis in both frozen/thawed and fresh dog spermatozoa probed with C5F10 antibody recognized proacrosin and acrosin active forms (alpha and beta) as bands which appear to be specific as no label was detected in samples incubated without the antibody.

A band of 40 kDa corresponding to proacrosin and two low-molecular weight bands corresponding to other molecular forms of active acrosin: alpha-acrosin of 32 kDa and beta-acrosin of 27 kDa, were detected in fresh and frozen/thaw dog spermatozoa during each time of culture (Fig. 1a, b). Proacrosin pattern did not change much throughout capacitation time in both fresh and frozen samples; however, the densities of proacrosin bands were higher ($p < 0.05$) in fresh sperm in comparison to frozen/thawed spermatozoa. The effects of sperm treatment and time of capacitation and their interaction on acrosin reactivity were significant ($p < 0.05$). Freezing at −196°C caused a significant

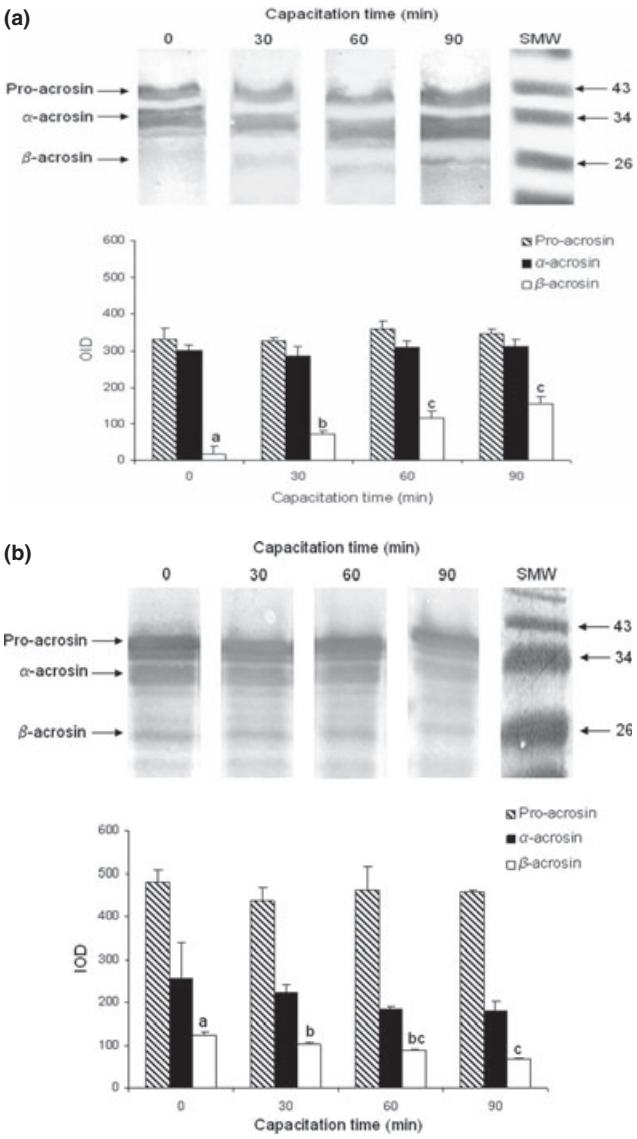

Fig. 1. Western Blots analysis of canine: (a) Fresh and (b) Frozen/thawed sperm proacrosin/acrosin in acid extracts probed with human anti-acrosin C5F10 followed by 1/5000 diluted peroxidase-conjugated rabbit anti- mouse antibody. Molecular masses were estimated according positions to standards molecular weight (SMW). Integrated optical densities (IOD) of each band throughout capacitation time are expressed in graphical formats below each Western blot. The effects of sperm treatment and time of capacitation and their interaction on acrosin reactivity were significant ($p < 0.05$).

($p < 0.05$) effect in the amount of the enzyme present in active form throughout the time of culture, resulting in a change in beta-acrosin pattern.

Beta-acrosin band in frozen sperm was strongest at the beginning of culture and this reactivity decreased over the time; in contrast, beta-acrosin reactivity in fresh control spermatozoa was lowest at the beginning of incubation and increased with time ($p < 0.05$).

Discussion

The acrosome reaction triggers a process during which proacrosin is converted into different active forms of acrosin. This conversion was analyzed in this study by

Western immunoblotting, with the use of a specific monoclonal antibody C5F10 against human proacrosin/acrosin, which in previous experiments at our laboratory, demonstrated cross-reactivity with dog proacrosin/acrosin system.

It has been shown previously that the proacrosin activation passes through an instable intermediate form, alpha-acrosin, which is subsequently converted into the more stable form of active beta-acrosin which is thought to contribute further to the acrosomal zona dispersion (Hardy et al. 1991; Barros et al. 1996). In our previous report using non-capacitated dog sperm, we were able to localize this enzymatic system in both fresh and frozen/thawed spermatozoa; however, beta-acrosin was observed only in frozen sperm samples, suggesting that proacrosin was activated into alpha- and beta-acrosin in frozen/thawed spermatozoa (Cortés et al. 2006). In the present study, Western blots analysis showed that the antibody recognized bands of 40, 32 and 27 kDa in either fresh or frozen/thawed spermatozoa, corresponding to proacrosin, alpha-acrosin and beta-acrosin, respectively. Comparatively, immunoblots bands of proacrosin did not show significant difference between each capacitation period in frozen and fresh samples; however, the density of these bands was higher in fresh sperm in comparison to frozen/thawed sperm, which could be associated to conversion of proacrosin into active forms in frozen samples.

Beta-acrosin reactivity was different among periods in both types of sperm. In fresh samples, the immunoblot bands corresponding to beta-acrosin indicate a minor reactivity of the antibody at the first periods of incubation, which increased with time. The increasing reactivity of beta-acrosin in fresh sperm may be related to the capacitation process, because possibly more proacrosin and alpha-acrosin were being activated and converted into beta-acrosin throughout the time. Although in some species like rabbit, the proacrosin–acrosin system remains largely as proacrosin during capacitation and acrosome reaction (Sillerico et al. 1996), in fresh dog sperm acrosin activity, measured with light spectrophotometry, increased in association with capacitation (Kawakami et al. 1999).

In contrast, in frozen/thawed sperm monoclonal antibody C5F10 detected a major immunoblot band corresponding to beta-acrosin at the beginning of incubation, and the density of this band decreased markedly over capacitation time. Several reports employing biochemical techniques have provided evidence that cryopreservation causes alterations in spermatozoa resembling premature capacitation, or at least acrosomal exocytosis, which accounts for the reduced fertility (Watson 2000; Peña et al. 2004). Our recent study showed that the proportion of acrosomal reacted or abnormal acrosomes (characterized by a fenestration of the membranes covering the acrosome region of the sperm head) were more often seen in frozen/thawed sperm in comparison with fresh samples (Palomino and De los Reyes 2009).

The process of capacitation and the acrosome reaction imply an influx of calcium (Brewis et al. 2001) and the variation in acrosin activity seems to be dependent on intracellular calcium concentration, as it was observed in boar and bull (Rosatti et al. 2003). A substantial rise in the intracellular calcium has been also shown in thawed dog spermatozoa (Strom-Holst et al. 1998). In this regard, acrosomal membranes are altered during the freezing and thawing process inducing an effect on acrosin activity.

The consequences of activation of proacrosin to active acrosin are the binding and penetration to the zona glycoproteins (Moreno et al. 2002); in fact, a significant correlation between alterations in acrosin proteinase activity and abnormal fertilization rate in human *in vitro* fertilization procedures has been reported (De Jonge et al. 1993). In addition, it has also been shown that enhanced acrosin activity in sperm is associated with normal fertility in bovine (De los Reyes and Barros 2000). Thus, the different reactivity of beta-acrosin either with fresh or frozen/thawed dog sperm could have an implication to its fertilizing ability. Our previous studies on canine gamete interactions have indicated that the time course of penetration through the zona pellucida appears to be faster in frozen/thawed dog spermatozoa than in fresh sperm; but fresh sperm can retain their fertilizing ability longer and therefore, penetrate more oocytes over time in comparison with frozen/thawed spermatozoa (Palomino and De los Reyes 2009; De los Reyes et al. 2009).

In conclusion, these results suggest that proacrosin is converted to beta-acrosin earlier in frozen/thawed than in fresh dog spermatozoa, which could explain in part the reduced fertilizing competence of those sperm over time.

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Author contributions

MDLR designed the study, coordinated the roles of the authors, performed analysis, and wrote the manuscript. JP was responsible for western blot experiments and performed the statistical analysis and GM was responsible for semen collection, semen processing and participated in the laboratory protocols. All authors read and approved the final manuscript.

Conflicts of interest

The authors have declared no conflicts of interest.

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Author's address (for correspondence): Monica De los Reyes, Animal Reproduction Laboratory, Faculty of Veterinary Sciences, University of Chile, PO Box 2, Correo 15, Santiago, Chile. E-mail: mdlreyes@uchile.cl