Proacrosin/acrosin quantification as an indicator of acrosomal integrity in fresh and frozen dog spermatozoa

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Abstract

The scope of the present study was to evaluate the presence and activation of proacrosin/acrosin as a tool to determine the acrosomal status of fresh and frozen/thawed dog spermatozoa. Monoclonal antibody C5F11, directed against human acrosin, cross-reacted with dog spermatozoa and labeled the acrosome of both fresh and frozen/thawed dog spermatozoa. Frozen/thawed spermatozoa had a lesser proportion of labeled spermatozoa than fresh spermatozoa ($P < 0.05$). When live spermatozoa were labeled with soybean trypsin inhibitor conjugated with Alexa 488 (SBTI-Alexa 488), the proportion of acrosome-labeled fresh spermatozoa was less than frozen/thawed spermatozoa ($P < 0.05$). By using Western blots and enzymatic activity, frozen/thawed spermatozoa had a greater proportion of active acrosin than fresh spermatozoa. In addition, beta 1,4-galactosyl-transferase (GalT), a plasma membrane bound protein, remained attached to frozen/thawed spermatozoa. Proacrosin is activated during freezing/thawing of dog spermatozoa, and that proacrosin/acrosin may be a good indicator of acrosomal integrity of frozen/thawed spermatozoa.

Keywords: Spermatozoa; Acrosome; Freezing; Fertilization; Acrosome reaction; Syntaxin

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1. Introduction

Mammalian spermatozoa are unique amongst secretory cells in the sense that their sole purpose is to fertilize the oocyte. If fertilization does not occur then the spermatozoa are committed to die. In the same context, the spermatozoon had ideal cellular characteristics for this purpose, possessing a specific organelle to successfully perform such functions (Barros et al., 1996; Ramalho-Santos et al., 2002). The acrosome is a secretory vesicle covering about two-third of the nuclear surface. The acrosome contains a number of enzymes that are believed to help the spermatozoa cross the egg zona pellucida (Tulsiani et al., 1998). The acrosome secretory process, properly named the acrosome reaction (AR), is a highly regulated process because it can only occur on the surface of the zona pellucida thus allowing sperm to cross this barrier (Evans and Florman, 2002; Ramalho-Santos et al., 2002). The component of the zona pellucida, a glycoprotein named ZP3 and the hormone progesterone, are the most likely candidates to have a relevant physiological role in this process (Wassarman, 1999). However, the mechanism through which spermatozoa actually induce the acrosome reaction is still questionable. It is important to note that a premature AR can impair the fertilization rate and decrease the outcome of in vitro and in vivo embryo production of relevant species.

Upon the AR, many components and enzymes are released into the extracellular milieu and several inactive enzymes, like proacrosin, are processed to the most stable conformation (Howes and Jones, 2002; Moreno and Barros, 2000). Proacrosin activation in vitro involves an autocatalytic cleavage between Arg23 and Val24 at the amino terminal, resulting in a linked two-chain molecule. The newly generated 23-amino acid fragment ("light chain") remains bridged by disulphide bonds to the rest of the molecule ("heavy chain"). This 49 kDa form is enzymatically active and constitutes alpha-acrosin (α-Acr) (Baba et al., 1989). The second and third cleavages take place at the carboxyl-terminal between Lys363 and Arg367 and between Arg322 and Pro323, respectively. These cleavages lead to a sequential loss of 18- and 43-residue fragments (Baba et al., 1989; Moreno and Barros, 2000). These last processing events result in the formation of a stable 36 kDa enzymatically active form named beta-acrosin (β-Acr) (Schleuning et al., 1976; Topfer-Petersen and Cechova, 1990). Multiple studies have shown a correlation between the levels of proacrosin and acrosin activity with the fertilizing potential of human spermatozoa (Shimizu et al., 1997). In addition, proacrosin/acrosin is sequentially released from the acrosome under capacitating conditions. Thus, localization of proacrosin/acrosin in the acrosome is an indicator of the acrosome reaction and/or of membrane integrity in several species.

Freezing promotes several changes related to capacitation and sperm senescence that may modify sperm function (Pena and Linde-Forsberg, 2000; Pena et al., 2003). In addition, freezing is a process that can potentially seriously damage the sperm plasma and/or acrosomal membranes (Burgess et al., 2001; Nishizono et al., 2004; Rasul et al., 2001). Therefore, the aim of the present study was to evaluate whether membrane damage induced by freezing/thawing can induce proacrosin activation in dog spermatozoa.

2. Materials and methods

2.1. Reagents

All the reagents were purchased from Sigma (Sigma Co., St. Louis, MO) unless otherwise specified. Antibody against mouse beta 1,4-galactosyl-transferase (GalT) was a gift from Dr. Barry Shur (Department of Cell Biology, Emory University School of Medicine, CO), monoclonal antibodies against acrosin were purchased from BIOSONDA (Santiago, Chile; Cat no. AMC-ACRO-C5F10-AS).

2.2. Semen sample collection

Six ejaculates were collected from three healthy fertile dogs of mixed breeding (two samples from each dog, aged 2–6 years) and examined within 1 h after collection. The samples were collected by digital manipulation in fractions (pre-secretion, sperm-rich fraction, sperm-poor fraction, and sperm-free fraction). The sperm-rich fraction was used for the experiments. Sperm quality assessment including evaluation of sperm concentration, sperm motility, and assessment of morphological alterations were performed as previously described (Rota et al., 1999). All ejaculates were of sufficient quality, as judged by conventional semen parameters. After collection and quality estimation, spermatozoa were washed from seminal fluids by centrifugation in a Tris-medium. The pellet was used for protein extraction or acrosome imaging.

2.3. Freezing and thawing of dog semen samples

Freezing was performed as follows: the sperm-rich fraction of each ejaculate was extended in a Tris–fructose–egg yolk freezing medium containing 2.4 g Tris, 1.4 g citric acid, 0.8 g glucose, 0.06 g Na-benzylpenicillin and 0.1 g streptomycin sulphate solubilized in 100 ml of distilled water (De los Reyes et al., 2002). Spermatozoa were adjusted at a concentration of 200×10^6 spermatozoa/ml and then loaded into 0.25 ml straws (L'Aigle Cedex, France) and frozen at a rate of 30 to −196 °C/min.

Thawing was performed as described by Parrish et al. (1988) with slight modifications: straws were taken out of liquid nitrogen and placed in a water bath at 60 °C for 8 s. Then, 0.25 ml of thawed semen was layered on 500 μl Tris-medium in Eppendorf tubes. The mixture was then centrifuged at 500 rpm for 5 min. The final pellet was re-suspended in sperm-TALP (Parrish et al., 1988) or in Tris-medium.

2.4. Acrosome imaging

Spermatozoa were plated on poly-L-lysine coated coverslips. After 5–10 min, these were fixed for 5–24 h in PBS containing 4% *p*-formaldehyde. Spermatozoa were then permeabilized with 100% cold methanol for 10 min and then re-hydrated with 1% Triton X-100 in PBS for 1 h. These preparations were blocked with PBS containing 1% bovine serum albumin (PBS-BSA) for 1 h in a humid chamber at room temperature and then incubated with a primary antibody, either a mouse monoclonal antibody directed against proacrosin/acrosin

(C5F11, working concentration 0.2 mg/ml) or with a rabbit polyclonal antibody against beta 1,4-galactosyl-transferase (working concentration 0.1 mg/ml). The primary antibody was incubated overnight at 4 °C and then washed twice for 3 min in 0.2% PBS-Tween. The samples were incubated for 1 h at 37 °C in a humidity chamber with a secondary goat anti-mouse antibody conjugated with Alexa 488 or goat anti-rabbit antibody conjugated with Alexa 594 (Molecular Probes, Eugene, OR), both antibodies were used in a dilution of 1/100. The samples were then rinsed three times and mounted with Vectashield (Vector, Burlingame, CA).

Soybean trypsin inhibitor conjugated to Alexa 488 (SBTI-Alexa 488, Molecular Probes, Eugene, OR) was added to a live sperm suspension to a final concentration of 1 μg/ml. The suspension was incubated for 15 min at 39 °C and then observed in an epifluorescence microscope (Optiphot-2, Nikon, Japan) with a 460–500 nm (excitation) and 510–560 nm (barrier) filter.

2.5. Protein extraction and Western blot

Sperm extraction was made by homogenizing a sperm pellet in buffer A (1% Triton X-100, NaCl 1 M, EDTA 1 mM, PMSF 10 μg/ml, Tris–HCl 20 mM pH 7.0), and then centrifuged for 10 min at 10,000 rpm. The sample was run on a 12% polyacrylamide gel (SDS-PAGE) under reducing and denaturant conditions, and then transferred to Nitrocellulose at 100 V for 1.5 h. Nitrocellulose was blocked with 2% BSA in PBS, pH 7.4, and then incubated overnight at 4 °C with a mouse anti-human acrosin antibody (C5F11, dilution 1:1000). After extensive washing with PBS plus 0.05% Tween 20 (PBS-Tween), the membrane was incubated with a rabbit anti-mouse antibody conjugated to peroxidase (KPL, Gaithersburg, MD) diluted 1:10,000 in PBS-BSA for 1 h at room temperature. Protein bands were revealed using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

2.6. Acrosin activity

Acrosin activity was measured spectrophotometrically at 25 °C by following the hydrolysis of *N*-benzoyl-larginine-ethyl-ester (BAEE) and by the addition of 50 or 100 μl of the enzyme solution, at 253 nm. The assays were performed in 3 ml volumes, using a substrate mixture of 50 mM Tris, 50 mM $CaCl_2$, and 500 μM BAEE at pH 8.0. A molar absorption difference of 1150 M^{-1} cm^{-1} was used to convert changes in optical density to micromoles of BAEE hydrolyzed (Pena and Linde-Forsberg, 2000). One international unit of activity was defined as that amount of acrosin hydrolyzing 1 μmol BAEE/min at 25 °C.

2.7. Statistical analysis

All statistical analysis were performed using the SPSS for Windows Release 11.5.0 Standard Version, from Lead Technologies Inc. Significant differences were determined when $P < 0.05$, when tested using the Bonferroni statistical test. Results are expressed as a mean ± standard deviation (S.D.) (Bailey, 1995).

3. Results

3.1. Acrosome imaging in live and fixed dog spermatozoa

The initial goal was to determine whether or not two monoclonal antibodies against human proacrosin could cross-react with fresh dog spermatozoa. The results showed that monoclonal antibody C5F11 (Fig. 1(A and D)) gave a bright signal over the acrosome of fresh dog spermatozoa. Frozen/thawed spermatozoa gave a similar pattern; however, the percentage of labeled spermatozoa was less than for fresh spermatozoa (Fig. 2, $P < 0.05$). SBTI-Alexa 488 (Fig. 1(B and E)) gave a pattern that appeared as two domains rather than one, as occurred with the anti-acrosin antibody (Fig. 1(A and D)). Acrosomal staining was specific because no signal was obtained when the C5F11 antibody was not included in the procedure (data not shown). SBTI-Alexa 488 labeled 12% of fresh dog spermatozoa (Figs. 1(B) and 2). Whereas 48% of frozen/thawed spermatozoa were labeled with SBTI-Alexa 488, this number being significantly greater than for fresh spermatozoa (Fig. 2; $P < 0.05$). In addition, it appeared as though the label of SBTI-Alexa 488 in frozen/thawed spermatozoa was stronger than with fresh spermatozoa.

These results strongly suggest that frozen/thawed spermatozoa had lost most of the acrosin probably due to acrosome damage during the freezing or thawing process. To assess

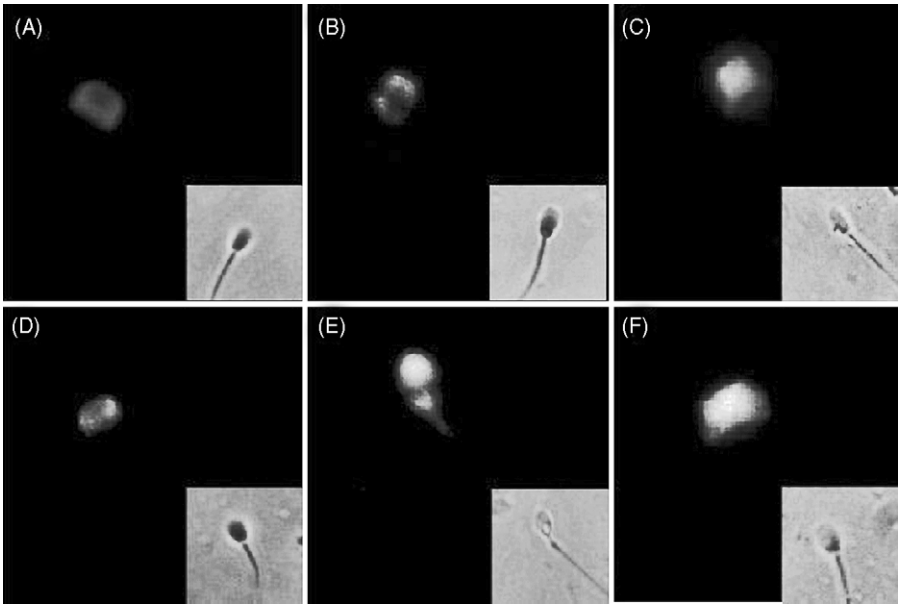

Fig. 1. Imaging of fresh and frozen/thawed dog spermatozoa using anti-proacrosin/acrosin antibodies. Staining patterns for the monoclonal anti-proacrosin/acrosin antibody C5F11 (A and D), SBTI-Alexa 488 probe (B and E), and galactosyl-transferase (C and F) on dog spermatozoa. Fresh spermatozoa (A–C), frozen/thawed spermatozoa (D–F). The pattern obtained with SBTI-Alexa 488 appears as two domains rather than one as seen with anti-acrosin (A and D) and GalT antibodies (C and F). Magnification ×400.

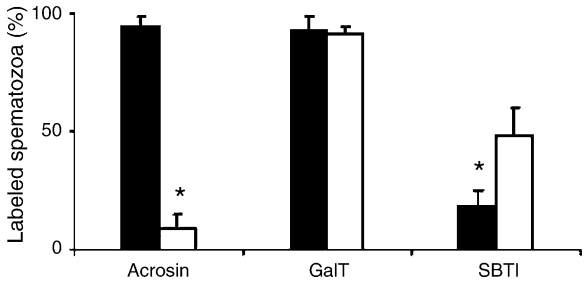

Fig. 2. Quantification of immunolabeling of fresh and frozen/thawed spermatozoa. The percentage of frozen/thawed spermatozoa (white bars) labeled with the anti-acrosin antibody was significantly less than that of fresh spermatozoa (black bar). No differences were detected between samples with the anti-GalT antibody. The percentage of fresh spermatozoa (black bar) labeled with SBTI-Alexa 488 was significantly lower to frozen/thawed ones (white bars). Asterisk (*) indicates $P < 0.05$ between fresh and frozen/thawed samples. At least 100 spermatozoa were counted for each determination, $N = 3$.

the plasma membrane, antibodies against beta 1,4-galactosyl-transferase, a sperm surface plasma membrane protein involved in gamete recognition and in the AR were used (Pena et al., 2003). The results showed that the anti-GalT antibody gave a strong signal over the entire acrosome in both fresh and frozen/thawed spermatozoa (Fig. 1(C and F)). Quantification of the proportion of labeled spermatozoa showed that there was no difference between both sperm populations (Fig. 2). As a control, no signal was detected when the samples where incubated without the primary antibody (data not shown). These results suggest that most GalT remains attached to the acrosome in frozen/thawed dog spermatozoa.

3.2. Proacrosin activation in frozen/thawed dog spermatozoa

To determine the status of proacrosin, enzymatic activity in both fresh and frozen/thawed spermatozoa was assayed. Acrosin activity of frozen/thawed samples was higher than in fresh spermatozoa ($P < 0.05$; Fig. 3). After in vitro activation in Tris–HCl pH 8.0 for 30 min, fresh and frozen/thawed samples were not different in acrosin activity (Fig. 3). Interestingly,

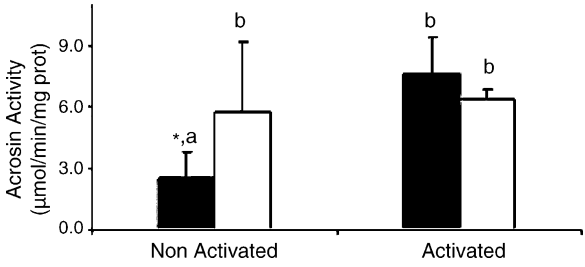

Fig. 3. Acrosin specific activity in fresh and frozen/thawed spermatozoa. Acrosin specific activity in frozen/thawed spermatozoa (white bar) was significantly greater than in fresh spermatozoa (black bar) in non-activated samples ($P < 0.05$). An increase in acrosin activity occurs upon activation (activated), allowing the fresh samples to match the activity found in non-activated frozen spermatozoa. The increase in acrosin activity was not detected in frozen/thawed samples. Different letters indicate significant differences, $P < 0.05$, $N = 3$.

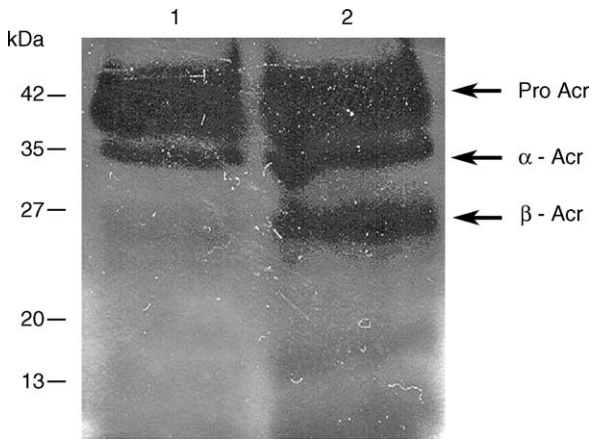

Fig. 4. Detection of proacrosin and acrosin in fresh and in frozen/thawed samples. Proacrosin is present in both fresh (lane 1) and frozen/thawed spermatozoa (lane 2). Alpha-acrosin was detected as a band of 36 kDa and beta-acrosin as a band of 27 kDa only present in frozen/thawed spermatozoa (lane 2).

acrosin activity increased approximately three-fold in fresh samples, but the same procedure using frozen/thawed samples did not indicate a significant increase in acrosin activity (Fig. 3). These results imply that all acrosin in frozen/thawed spermatozoa was already activated, as opposed to fresh spermatozoa were almost all acrosin was as proacrosin. If total content of acrosin as the enzyme activity reached after in vitro activation is considered (i.e. 100%), it was determined that 67% of the observed activity was as proacrosin, and 33% as active acrosin in fresh spermatozoa. Frozen/thawed spermatozoa, however, showed only a 10% increase in acrosin activity after in vitro activation. Thus, only 10% of the observed enzymatic activity was as proacrosin and 90% was as acrosin.

Western blots showed that the antibody C5F11 recognized a band of 40 and 36 kDa in fresh spermatozoa, corresponding to proacrosin and alpha-acrosin, respectively (Fig. 4, lane 1). In frozen/thawed spermatozoa, however, the antibody C5F11 recognized proacrosin, alpha-acrosin and a band of 27 kDa corresponding to beta-acrosin (Fig. 4, lane 2). These results strongly suggest that proacrosin was activated into alpha- and beta-acrosin in frozen/thawed spermatozoa.

4. Discussion

It has been reported that freezing and/or thawing of mammalian spermatozoa induces capacitation-like changes such as enzyme activation, plasma membrane rupture, and an increase in intracellular calcium concentration (Bravo et al., 2005; Fuller and Whittingham, 1997; Schembri et al., 2002). The major finding was that there was activation and release of proacrosin/acrosin in frozen/thawed spermatozoa without loss of plasma membrane proteins like GalT. In addition, we showed that SBTI-Alexa 488 can be a reliable and efficient process to evaluate acrosome integrity in both fresh and frozen/thawed spermatozoa samples. A

monoclonal antibody against human acrosin was used in the present study. The antibody C5F11 has been an excellent resource to study the acrosome reaction in human, rabbit, bull, and hamster (Yunes et al., 1992; Valdivia et al., 1994; Ramalho-Santos et al., 2000; Moreno et al., 1998). This antibody labels the acrosome of both fresh and frozen/thawed dog spermatozoa. This label appears to be specific because no signal was detected when only the secondary antibody was used. In addition, in Western blot assays the antibody recognized proacrosin and both its activated forms (alpha- and beta-acrosin). Moreover, this antibody recognizes the polysulphate binding domain of acrosin that is highly conserved among different species. Therefore, even though this antibody was built against human acrosin, the antibody C5F11 cross-reacts with dog acrosin and can be used as a reliable resource to study acrosomal function in dog spermatozoa. Freezing and thawing likely impacts the acrosomal membrane to the extent that more proacrosin/acrosin is available to interact with the antibodies. In this context, antibodies to human acrosin also equally stain proacrosin and alpha-acrosin which may intensify the signal and overestimate the quantity of acrosin molecules that are present in the spermatozoa acrosome. The amount of acrosin in the acrosome was not estimated by fluorescence intensity; however, we quantified the percentage of labeled spermatozoa no matter how intense, and in this way we overcame this problem.

After the AR, proacrosin is converted to alpha-acrosin, which remains bound to the acrosome. Alpha-acrosin is then auto-catalytically converted into beta-acrosin that is released to the extracellular milieu (Baba et al., 1989; Hermans et al., 2003; Howes and Jones, 2002). In vivo processing and activation of proacrosin occurs gradually and even after 4–6 h of incubation, it is possible to detect a small proportion of proacrosin/acrosin in the acrosome of human and bovine spermatozoa (Barros et al., 1992; Kim et al., 2001). Therefore, the proportion of spermatozoa lacking proacrosin/acrosin and/or the extent of proacrosin activation is an accurate indicator of the AR. The proportion of dog spermatozoa with acrosomal damage and acrosin loss was significantly greater in frozen/thawed than in fresh spermatozoa in the present study. These results suggest that acrosin, probably in the form of beta-acrosin, is lost from the acrosome during or after either freezing and/or thawing. If this is the case, it is speculated that the loss of proacrosin/acrosin from the acrosome can be a result of the activation of proacrosin into acrosin. This hypothesis was confirmed by Western blot, which showed that fresh dog spermatozoa has two bands of 40 kDa protein corresponding to proacrosin and a faint band corresponding to alpha-acrosin, which may have resulted from some activation during extraction or by proacrosin activation induced by the acrosome reaction. Most of the acrosin detected in frozen/thawed spermatozoa, however, corresponded to alpha- and beta-acrosin. How is proacrosin activated in frozen/thawed dog spermatozoa? Previous studies have shown that frozen/thawed bovine spermatozoa accumulates more calcium than fresh sperm samples, most likely because of plasma membrane alterations induced during the cryopreservation procedure (Collin et al., 2000). However, proacrosin activation does not depend on the physiological concentrations of Ca^{2+}, Mg^{2+} or Zn^{2+}, yet strongly relies on intra-acrosomal pH (Meizel and Deamer, 1978; Brown and Hartree, 1978; Brown and Harrison, 1978). Thus, it is possible that plasma membrane damage, in frozen/thawed dog spermatozoa, allows an increase in the intracellular pH, which in turn promotes proacrosin activation and eventually the release of beta-acrosin from the acrosome. This would explain the greater acrosin activity and the lesser proportion

of anti-acrosin label found in frozen/thawed spermatozoa in comparison to fresh spermatozoa.

The GalT mediates fertilization in mice by binding to specific O-linked oligosaccharide ligands on the egg coat glycoprotein ZP3 (Miller et al., 2002). Before binding to the egg, sperm GalT is masked by epididymal-derived glycosides shed from the sperm surface during capacitation. After binding the egg, sperm-bound oligosaccharides on ZP3 induce the acrosome reaction by receptor aggregation, presumably involving GalT (Miller et al., 2002; Wassarman, 1999; Wassarman et al., 2001). Previous studies have shown that the antibody against mouse GalT recognizes this enzyme in the acrosome of mouse, cattle, pig, rabbit, rat, and guinea pig spermatozoa (Larson and Miller, 1997). In addition, no signal was detected when samples were incubated only with the secondary antibody or with the pre-immune serum. Thus, it is highly probable that the signal observed in dog spermatozoa using this antibody is specific to GalT (Miller et al., 2002; Wassarman, 1999; Wassarman et al., 2001). It has been shown that GalT stain is lost during the acrosome reaction. It is hypothesized that if frozen/thawed dog spermatozoa had lost their acrosome, a decrease in the proportion of GalT labeled spermatozoa would occur. However, in the present study the proportion of fresh spermatozoa labeled with GalT is similar to frozen/thawed spermatozoa. The most probable explanation for these results in the present study is that despite acrosome fracture, only a small portion of the acrosome is lost during this process, thus immunofluorescence does not reach this level of resolution. It is, however, possible that despite membrane fusion and acrosome protease activation, a portion of the plasma membrane of the acrosome still remains after the AR is finished. In this scenario, GalT could remain in acrosome-reacted spermatozoa and would, therefore, not be a reliable marker of acrosome damage or the acrosome reaction. Similarly, Larson and Miller (2000) showed that GalT is not a reliable marker of fertilization ability in bulls.

Therefore, results of the present study indicate that the extent of the proacrosin activation and/or the percentage of spermatozoa labeled with an acrosin antibody can be an effective method to evaluate acrosomal damage of frozen/thaw dog spermatozoa. In addition, evidence suggests that small fractures in the plasma membrane and/or in the outer acrosomal membrane can induce the proacrosin activation.

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