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Immunolocalization of proacrosin/acrosin in bovine sperm and sperm penetration through the zona pellucida

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Abstract

The aim of the present work was to immunolocalize acrosin in bull spermatozoa incubated for up to 6 h in capacitating culture medium (TALP-heparin), in order to study the kinetics of its release during the acrosome reaction and in vitro sperm penetration. Six replicates from semen of one bull were used. Acrosin was localized by the silver-enhanced immunogold technique using anti-bovine acrosin monoclonal antibody ACRO-C2E5. Spermatozoa thus showed the presence of acrosin only at the acrosomal region. Four different patterns were seen: (1) no labeling: (2) intense labeling on the rim of the portion of the acrosome; (3) diffuse label over the entire acrosomal region; and (4) intense label over the entire acrosomal region. Spermatozoa incubated in capacitating medium for 4 h showed that unlabeled (pattern 1) spermatozoa decreased from 72% to 28% difference that was found to be significant ($p < 0.05$). Patterns 3 and 4 increased from about 10% to 20–29%, ($p < 0.05$). With further incubation (4–6 h), pattern 1 increased while patterns 3 and 4 decreased differences were not significant ($p > 0.05$). The incidence of pattern 2 did not change through the whole incubation period. Sperm penetration through the zona pellucida of in vitro matured bovine oocytes (57%) or empty zonae pellucida (70.5%) increased ($p < 0.05$) as a function of sperm incubation time in capacitating medium. The presence of acrosin, as determined by the silver-enhanced immunogold technique, was highly correlated with sperm

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penetration of in vitro mature bovine oocyte ($r = 0.98$) and cryopreserved zonae pellucidae ($r = 0.93$) ($p < 0.01$).

Keywords: Cattle, male reproduction; Sperm characteristics; Proacrosin/acrosin; Zona pellucida

1. Introduction

Interaction with and penetration of the spermatozoa into the zona pellucida is one of the key events during mammalian fertilization. This is a species-specific cell recognition process. The acrosome reaction is a prerequisite for the penetration of the zona pellucida and for the fusion of the spermatozoa with the oocyte plasma membrane (Barros and Berrios, 1977; Wassarman, 1992; Yanagimachi, 1994). The acrosome reaction allows the release of the acrosomal contents, including a serine-proteinase, acrosin. Acrosin has been demonstrated in spermatozoa of different species, and it has been shown in the bovine species that its proteolytic activity was associated to specific sperm proteinases (Srivastava et al., 1965).

Acrosin is stored as the zymogen form, proacrosin, in the acrosomal compartment of the spermatozoon (Polakoski and Parrish, 1977; Harrison et al., 1982; Huneau et al., 1984). It has been reported that proacrosin can self-catalyze its own conversion into acrosin by an intrazymogen mechanism during the acrosome reaction (Kennedy and Polakoski, 1981; Urch, 1991). However, zona pellucida glycoproteins are also involved in this process (Wassarman, 1987; Florman and First, 1988; Leyton and Saling, 1989; Jones, 1990; Eberspaecher et al., 1991; Wassarman, 1994).

Proacrosin/acrosin has been shown to be involved in the acrosomal matrix dispersion (Dumbar et al., 1985; Jones, 1990; Jones et al., 1988) and in binding and penetration through the zona pellucida (Bleil and Wassarman, 1988; Tesarik et al., 1990; Jones, 1991; Sillerico et al., 1996; Crosby et al., 1998). Nevertheless, the role of this serine protease in sperm penetration continues to be debated. Rabbit eggs treated with wheat germ agglutinin (WGA), which makes them resistant to digestion by trypsin and acrosin, can be successfully fertilized when transferred to oviducts of a previously inseminated female (Bedford and Cross, 1978). Highly purified preparations of acrosin obtained from ram sperm failed to remove any of the investment from the sheep egg (Brown, 1982). On the other hand, there is experimental evidence that supports the involvement of acrosin in sperm penetration through the zona pellucida, that is the demonstration that the zona pellucida is the natural substrate of sperm acrosin (Urch, 1986; Urch and Patel, 1991). On the other hand, it has also been shown that purified acrosin can digest the zona pellucida of eggs from different mammalian species (Elce et al., 1986; Urch, 1991). It has also been reported that anti-acrosin monoclonal antibodies can inhibit in vivo (Dudkiewicz, 1983) and in vitro (De Ioannes et al., 1990; Barros et al., 1993) fertilisation. Similarly, trypsin inhibitors such as soybean trypsin inhibitor (SBTI) can inhibit fertilisation (Stambaugh et al., 1969; Zaneveld et al., 1971; Saling, 1981; Elce et al., 1986; De Ioannes et al., 1990). Recent studies have shown that rabbit perivitelline spermatozoa retain residual proacrosin/acrosin in the equatorial and postacrosomal region (Valdivia et al., 1994), and these spermatozoa can fertilize freshly ovulated rabbit eggs (Valdivia et al, 1999).

The presence of proacrosin/acrosin system during sperm capacitation, acrosome reaction and fertilization has not been demonstrated in the bovine species. The presence of this enzymatic system in bull spermatozoa at different times during capacitation and the acrosome reaction was studied in order to determine the relationship of the proacrosin/acrosin system on the inner acrosomal membrane to the ability of spermatozoa to cross the zona pellucida.

2. Materials and methods

2.1. Spermatozoa

Frozen bovine semen from one ejaculate of one bull was used. For each replicate, two 0.25 ml straws, with approximately 20×10^6 spermatozoa were thawed by placing the straws in water bath at 40°C for 15 s; 0.25 ml of thawed semen were layered under 1 ml of sperm-TALP (Parrish et al., 1988; De los Reyes et al., 1996) in plastic tubes. After 1 h of swim up through sperm-TALP at 39°C, the top 0.8 ml of media from each tube was removed and pooled and then centrifuged at $300 \times g$ for 10 min. This procedure was done two times. The final pellet of spermatozoa was resuspended in sperm-TALP without glucose containing 10 μg/ml of heparin (Sigma, St. Louis, MO) to a final sperm concentration of 1×10^6 spermatozoa/ml. The samples were incubated at 39°C in an atmosphere of 5% CO_2 for a period up to 6 h.

2.2. Immunocytochemistry

The presence of the proacrosin/acrosin system was determined by the silver-enhanced immunogold technique as described by Barros et al. (1992). Briefly, the spermatozoa were fixed in 4% paraformaldehyde for 20 min, washed in PBS-BSA and non-specific binding sites blocked for 30 min with buffer supplemented with 5% goat blood serum (Towbin et al., 1979). Then, the samples were incubated for 1 h with anti-bovine acrosin monoclonal antibody ACR-C2E5 (dilution 1/50, Elce et al., 1986), in a humid chamber at 37°C. The samples were then incubated with a second antibody, an anti-mouse IgG tagged with 1 nm colloidal gold particles. The label produced by each gold particle was amplified with a silver solution for 15 min. In this way, each gold particle becomes a nucleating site for silver that forms a black precipitate that it is visible with the light microscope (Leunisses et al., 1989). As a control, only the second antibody was used. Six experiments were carried on and on each sperm sample over 200 spermatozoa were studied.

2.3. Collection and in vitro maturation of bovine oocytes

Cow ovaries collected at the local slaughterhouse were brought, within 2 h, to the laboratory in thermal bottle with a 0.9% NaCl solution maintained at 37°C. Follicles of a

diameter from 2 to 6 mm were aspirated with 10-ml disposable syringe with an 18-gauge needle. Cumulus-intact oocytes were washed three times in a Hepes–TALP solution (Parrish et al., 1988). For in vitro maturation, the cumulus oocytes complex (COC) were incubated in TCM-199 (Earl's salt, Life Technologies®, Gaithersburg, MD; USA) with 10% heat-inactivated fetal calf serum (FCS), 0.2 mM of sodium pyruvate, 40 μg/ml of Folltropin-V®, 1.5 IU/ml hCG, 1 μg/ml 17 β-estradiol and 50 μg/ml of gentamycin sulfate, in tissue cultured capsules (Falcon® 3001, Beckton Dickinson and Co., Lincoln Park, NJ, USA), at 39°C, in 5% CO_2 for 24 h. The culture medium was passed through a 0.22-μm pore size (Micro Filtration Systems®, Dublin, CA, USA) before adding estradiol (De los Reyes et al., 1996).

2.4. Bovine zona pellucida preparation

In order to have a permanent supply of bovine zonae pellucidae, bovine ovaries collected from slaughterhouse were minced and the oocytes were released into BWW medium (Biggers et al., 1971) containing 0.3% bovine serum albumin (BSA, fraction V, Sigma), 0.1 mM soybean, 10 μg/ml lima bean trypsin, (Sigma) inhibitors. The released bovine zonae were cryopreserved in a final 2 M solution of dimethyl sulfoxide (DMSO) and stored at −40°C according to the technique described by Overstreet et al. (1980). Before using, the empty zonae were thawed at a room temperature (21°C) and then they were washed in Hepes–TALP solution (Parrish et al., 1988).

2.5. Experimental series

2.5.1. Immunolocalization of acrosin

At hourly intervals after the start of sperm incubation, samples were obtained from the incubation capsule and the percent of progressive motility was estimated and samples were prepared for the silver-enhanced immunogold technique. For the observation with the scanning electron microscope, the slides containing spermatozoa were critically point dried with CO_2, sputtered with a palladium–gold target and studied with a Jeol JSM 25 SII scanning electron microscope (Barros et al., 1984). Sperm samples prepared with the silver-enhanced immunogold technique were also evaluated with light microscope. Both with the scanning electron (Fig. 1) and light microscopes (Fig. 2), at least 200 spermatozoa were studied and then the percentages of spermatozoa displaying label at different times during incubation were determined.

2.5.2. Gamete interaction

In vitro matured cow oocytes and cryopreserved empty zonae were inseminated in vitro with bull spermatozoa preincubated for different lengths of time (0 to 6 h). This is because the proportion of spermatozoa with modified acrosomes (as evaluated by the silver-enhanced immunogold technique) was higher with longer incubation times. The gamete coincubation (sperm-oocytes and sperm-zonae) was done in 50 μl drops of Fert-TALP medium (Parrish et al., 1988), by mixing 10 oocytes with 10^6 sperm/ml,

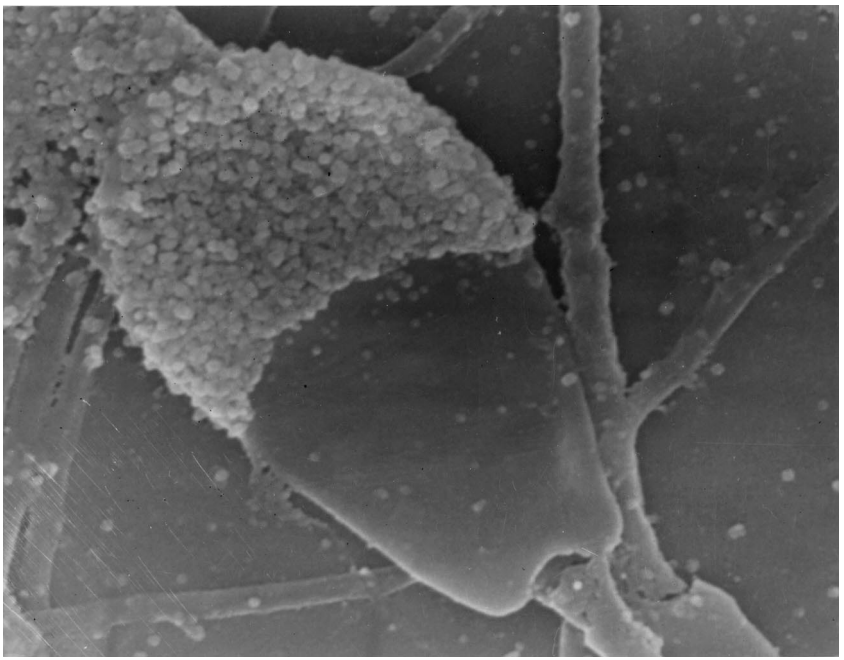

Fig. 1. Bull spermatozoa treated with the silver-enhanced immunogold technique, using anti-bovine acrosin monoclonal antibodies. This technique produces a black precipitate where acrosin is located and it is visible with the scanning electron microscope. Note the particulate precipitate over the entire acrosomal region.

and 10 zonae with 10^6 sperm/ml, at 39°C in an atmosphere of 5% CO_2 and with high humidity for 18 h.

2.6. Examination for zona penetration

At the end of the incubation period, the eggs and zonae were washed, fixed with aceto ethanol (1:3) (Leibfried et al., 1989) or 2% glutaraldehyde (Barros et al., 1984) before being evaluated for evidence of sperm penetration with phase-contrast microscope (Fig. 2) or with the scanning electron microscope, respectively.

2.7. Statistical analysis

The result of six replicates from frozen–thawed semen from one bull preincubated 0 to 6 h in capacitating medium was evaluated with analysis of variance. For the comparison of the means, the Student–Newman–Keuls Test (SNKT) was used. Correlation between presence of acrosin, as determined by the silver-enhanced immunogold technique, and sperm penetration of in vitro mature bovine oocyte and cryopreserved

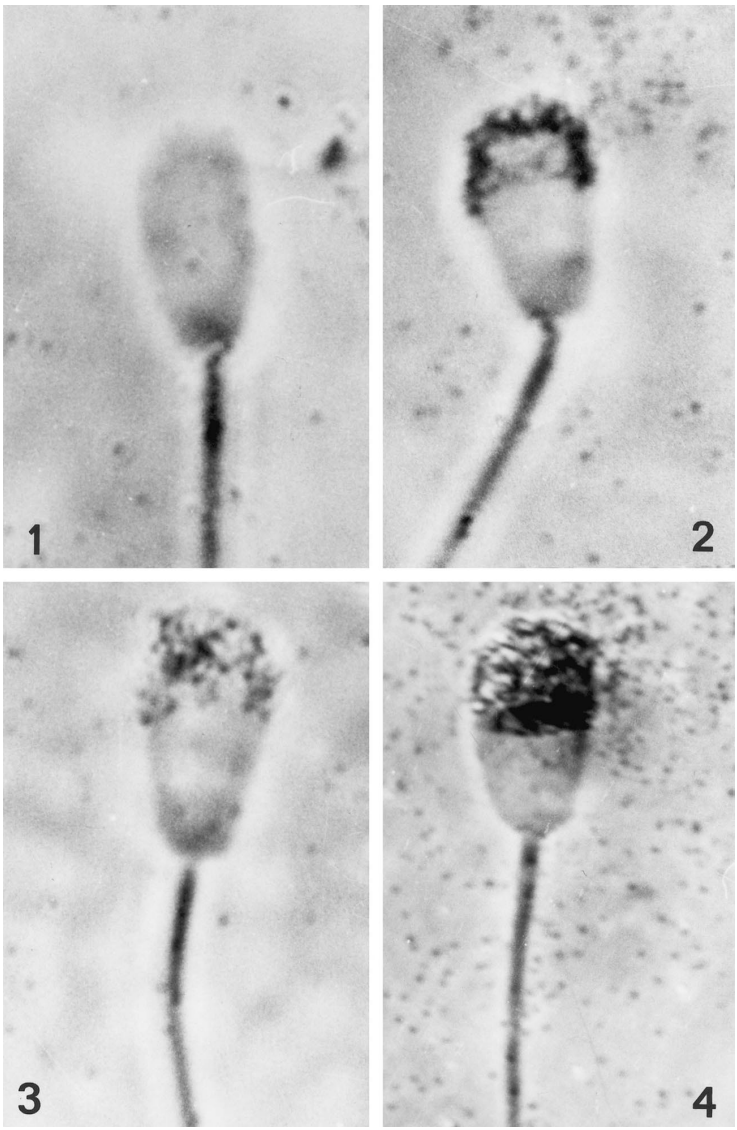

Fig. 2. Bull spermatozoa treated with the silver-enhanced immunogold technique, using anti-bovine acrosin monoclonal antibodies. This technique produces a black precipitate where acrosin is located and it is visible with the light microscope. Patterns of proacrosin/acrosin immunolocalization in bull spermatozoa during capacitation. (1) No labelling; (2) intense labelling on the rim of the acrosome; (3) diffuse label over the acrosomal region; (4) intense label over the entire acrosomal region.

zonae pellucidae were done through Pearson's Correlation Coefficient (Sokal and Rohlf, 1981).

3. Results

During the first 3 h of incubation, sperm viability as assayed by sperm motility, did not decline significantly ($p > 0.05$). Progressive motility decreased after 6 h of incubation from 71.4% at the onset to 53% at the end of the incubation period ($p < 0.05$).

The use of the silver-enhanced immunogold technique using the monoclonal antibody ACRO-C2E5 raised against bovine acrosin allowed us to immunolocalized, with the light microscope, this enzyme on the acrosomal region of non-permeabilized bull spermatozoa. That is due to the fact that each colloidal gold particle tagged to the antibody, acts as a nucleating site for silver thus producing a black precipitate on the acrosomal region visible with the light microscope. Neither the postacrosomal area nor the tail showed label. The negative controls, without the first antibody (C2E5), did not show label.

Sperm treated by the silver-enhanced immunogold technique were visualized as: (1) no labeling; (2) intense labeling on the rim of the acrosome; (3) diffuse label over the entire acrosomal region; and (4) intense label over the entire acrosomal region (Figs. 1 and 2).

The proportions of the immunolabeling patterns were different through the incubation period in capacitating medium (Fig. 3). There was a decrease of unlabeled sperm from 71% at the onset of incubation to 28% after 4 h ($p < 0.05$), and then, a slight increase at 6 h (33%). Patterns 3 and 4 increased ($p < 0.05$) from about 10% at the onset to 20–22% at 4 h followed by a decrease at 6 h. On the other hand, pattern 2 showed continuous increase over the 6-h observation period.

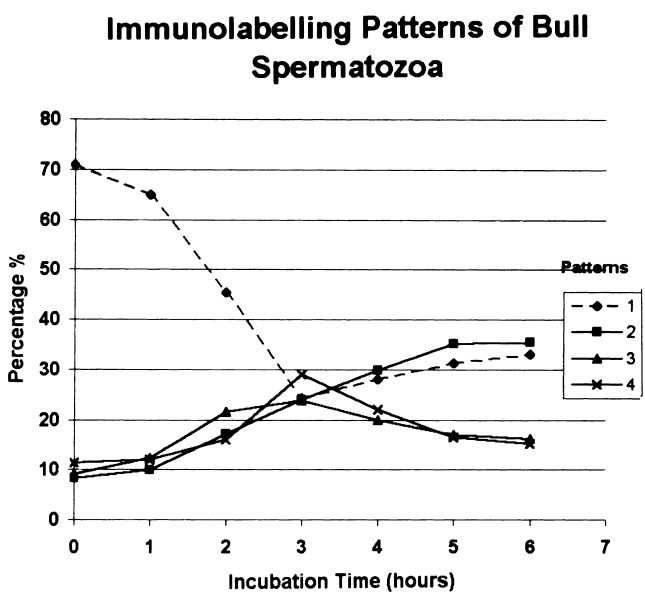

Fig. 3. Percentages of bull spermatozoa with different patterns of immunolabeling of proacrosin/acrosin after different times of incubation in capacitating medium.

The percentages of oocytes and zonae penetrated (Figs. 4 and 5) by sperm preincubated 0 to 6 h in capacitating medium is shown in Fig. 6. A linear increase of sperm penetration rates was observed during gamete interaction when the sperm were preincu-

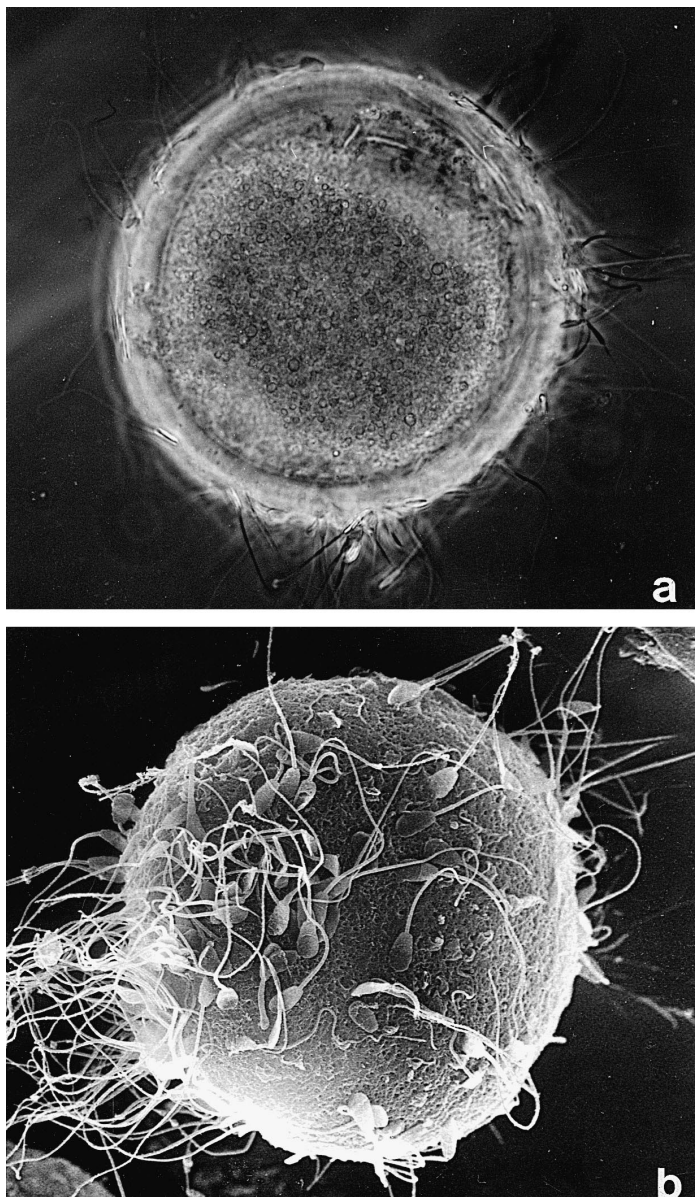

Fig. 4. Cow oocytes inseminated in vitro with preincubated bull spermatozoa. (a) Phase-contrast microscopic micrograph. Note the presence of spermatozoa in the perivitelline space. (b) Scanning microscopic micrograph where one can see the many spermatozoa bound to the outer surface of the zona pellucida.

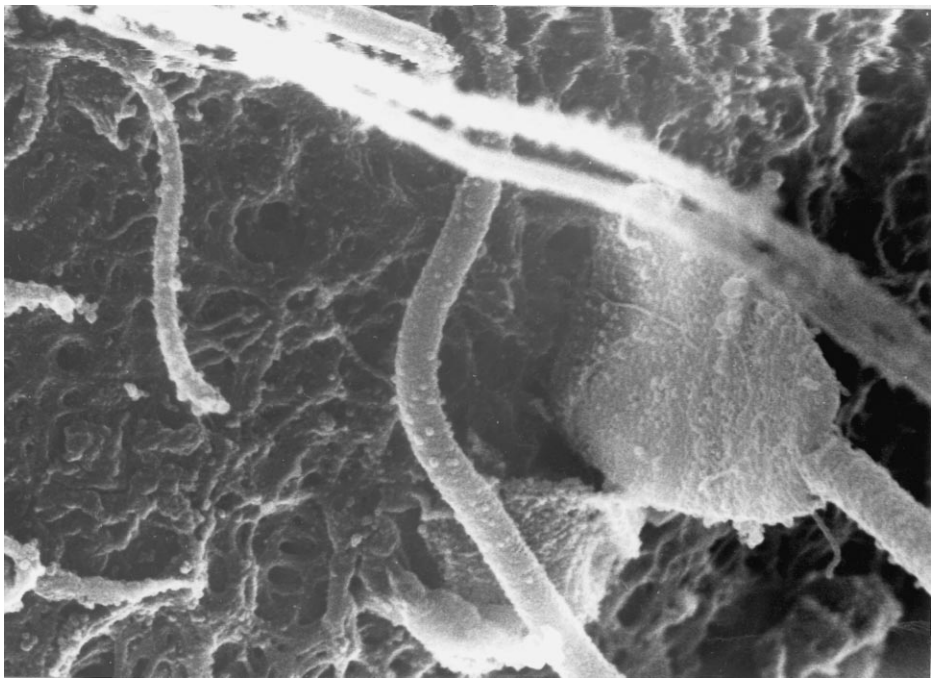

Fig. 5. Scanning microscopic micrograph of bovine sperm penetrating the zona pellucida.

bated 0 to 4 h in capacitating medium ($p < 0.05$). Bull sperm preincubated for 4 h penetrated higher proportion of oocytes and zonae, 57% and 70%, respectively, than those spermatozoa preincubated for 0, 1, 2, 3, 5 and 6 h. These differences in percent

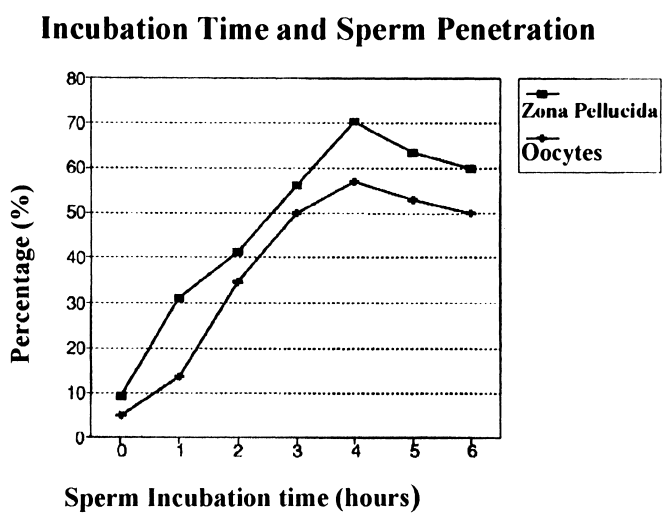

Fig. 6. Time course of capacitation of bull sperm as measured by penetration rates of bovine oocytes and empty zonae.

penetration proved to be significantly different ($p < 0.05$). Very little penetration was achieved at the onset of incubation and as well as the sperm preincubation time increased beyond 4 h, the penetration rates of both oocytes and zonae decreased.

The increase in sperm immunolabeling and sperm penetration were both dependent on the period of time sperm were exposed to capacitating medium. There was a high correlation between presence of acrosin, as determined by the silver-enhanced technique (percentage of immunolabed sperm) (patterns 2, 3 and 4) and the sperm penetration rates; for oocytes ($r = 0.98$; $p < 0.01$) and for empty zonae ($r = 0.93$; $p < 0.01$), giving a similar pattern of penetrability.

4. Discussion

The acrosome reaction is a fine regulated process that is a requisite of sperm penetration through the zona pellucida during fertilisation. It is a receptor-mediated process regulated by ligands in the zona pellucida (Bleil and Wassarman, 1986, 1988; Florman and First, 1988). One of the three glycoproteins that form the zona pellucida, ZP3, is the sperm ligand that accounts this process in the mouse (Florman et al., 1984). After the induction of the acrosome reaction, the acrosomal matrix is exposed and this first binding between the sperm and the zona pellucida is lost. Nevertheless, the penetrating spermatozoa establish a second type of interaction, named secondary binding. This interaction is maintained during sperm penetration through the zona pellucida and it is account by another zona pellucida glycoprotein, ZP2 (Bleil et al., 1988; Mortillo and Wassarman, 1991, Wassarman, 1994). On the other hand, it has been postulated that the sperm protein responsible of this secondary binding is the proacosin/acrosin system (Jones, 1991; Jones et al., 1988; De Ioannes et al., 1990; Töpfer-Petersen et al., 1990; Urch and Patel, 1991).

In this study, proacrosin/acrosin was immunolocalized in bull sperm using the anti-bovine acrosin monoclonal antibody ACR C2E5. Furthermore, we determined the kinetics of its release was evaluated by the silver-enhanced immunogold technique. The immunolabeling patterns were similar to those seen in guinea pig, human and hamster (Barros et al., 1992), and rabbit (Valdivia et al., 1994), suggesting that the different immunolabeling patterns could represent the different states of sperm acrosome reaction.

It is important to note that in pattern 1 in which no label was detected could represent two different types of spermatozoa. One type could be those spermatozoa in which the acrosome still remains non-reacted, therefore the antibody has not been able to interact with its antigens:acrosin. The other type of spermatozoa could be those in which the whole acrosome has reacted and the antigen:acrosin has become dissolved or eliminated from the acrosomal region. The percent of spermatozoa displaying pattern 1, decreased from the start of incubation up to 4 h and then increased; this could mean that 71% of unlabeled sperm at the onset of incubation could represent mainly those spermatozoa that have not undergone the acrosome reaction. On the other hand, spermatozoa displaying patterns 2, 3 and 4 in which the label appear on the rim of the sperms head and on the whole acrosomal area increased from time zero up to 4 h of incubation. Thus,

the slight increase of pattern 1 after 4 h could represent spermatozoa that have completely reacted their acrosomes with no acrosin left on the surface of the inner acrosomal membrane. The observed increase throughout the incubation of spermatozoa displaying pattern 2 is consistent with increasing numbers of spermatozoa undergoing the acrosome reaction. This finding would also imply that after the occurrence of the acrosome reaction in part of the sperm population, acrosin would remain associated to the acrosomal region.

It has been reported previously, that 4-h exposure of bovine sperm to capacitating milieu with heparin appears to be required to cause capacitation and the acrosome reaction (Parrish et al., 1986, 1988; Ax and Lenz, 1987; Florman and First, 1988; Gliedt et al., 1996). This agrees with our results, because the highest percentages of immunolabeling were observed around 3 and 4 h of incubation, which suggests an acrosome-reacted sperm population.

In golden hamster spermatozoa, it has been shown that as a result of the acrosome reaction, most proacrosin/acrosin is lost from the acrosomal cap (Capote et al., 1990; Barros et al., 1992); while in human, guinea pig (Crosby and Barros, 1990; Barros et al., 1992) and rabbit (Valdivia et al., 1994), it has been shown that it is possible to detect proacrosin/acrosin on the outer surface of the inner acrosomal membrane, even long after the occurrence of the acrosome reaction. Results suggest that the same occur in bull spermatozoa, since proacrosin/acrosin, as shown by pattern 2 of immunolabeling, remained associated to the sperm head over the 6-h incubation period.

As the time of sperm incubation increased up to 4 h, gamete interaction resulted in an increase in the percentage of cow oocytes and cryopreserved zona penetrated. It has been suggested that acrosin is involved with sperm penetration through the zona pellucida, and loss of acrosin from the acrosomal cap would be concomitant with a loss of the ability of sperm to cross the zona. In another study, preincubation of hamster spermatozoa for prolong periods of time resulted in reduced or non-immunolabeling, due to a loss of acrosin from the sperm head along with a loss of the ability of sperm to penetrate the zona pellucida (Barros et al., 1990, 1992).

In spite of the evidence indicating that acrosin is the enzyme responsible for the digestion of the zona pellucida, there are three other serine proteases present in the mammalian spermatozoon acrosome (Akama et al., 1994; Kohno et al., 1998) that could also be participating in spermatozoa penetration through the zona pellucida. One of these enzymes, acrosin-like, shares 80% homology with acrosin but it can be extracted only in the presence of detergent. The role of acrosin has been challenged by experiments in which mice carrying a targeted mutation of the acrosin gene (Baba et al., 1994) were shown to be fertile, and although they were able to fertilise homologous oocytes in vitro, it took them 30 min longer than the controls. Moreover, it has been shown that in in vitro fertilisation assays in which wild type spermatozoa compete with those lacking a functional acrosin gene, only wild type spermatozoa were able to fertilise the oocytes (Adham et al., 1997). These authors concluded that spermatozoa lacking the acrosin gene have a selective disadvantage when they are in competition with those having the active gene. Taken together, these results may suggest that during normal fertilisation the acrosin gene, and maybe the acrosin-like gene (Akama et al., 1994), are required for successful fertilisation (Valdivia et al., 1999).

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