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Sperm passage through the egg coats

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Studies were performed on sperm penetration through the egg coats in two different experimental models: mammalian motile flagellated spermatozoon and shrimp (Rhynchocinetes typus), non-flagellated, non motile spermatozoon.

In mammals, the association of the spermatozoon to the outer surface of the zona pellucida induces the acrosome reaction and exposes acrosin, a serine protease, that has been shown to be involved in mammalian sperm penetration through the zona pellucida. The zona pellucida is a specific and natural substrate for acrosin and its hydrolysis and fertilization can be inhibited by antiacrosin monoclonal antibodies. Moreover, in in vitro fertilization experiments, soybean trypsin inhibitor (SBTI) added before or after insemination significantly inhibits fertilization.

The use of a silver enhanced immunogold technique has shown in the golden hamster spermatozoa that after the acrosome reaction and detachment of the acrosomal cap, most of proacrosin/acrosin is lost from the sperm head. The loss of acrosin parallels the loss of the sperms ability to cross the zona pellucida.

Rabbit perivitelline spermatozoa can fertilize freshly ovulated rabbit eggs and 26% of these spermatozoa retain residual acrosin in the equatorial and postacrosomal region. However, using the same procedure, 100% of hamster perivitelline spermatozoa showed no detectable proacrosin/acrosin.

In the crustacean Decapod Rhynchocinetes typus, the spermatozoon is able to cross the egg coats by means of the tip of the rigid spike that seems to exert a lytic activity upon the coats. A trypsin-like proteinase activity was isolated from spermatozoa. Trypsin inhibitors, such as soybean trypsin inhibitor, p-aminobenzamidine and PMSF, have inhibitory effects on the activity of this enzyme, as well as on sperm penetration through the egg coats.

All the above evidence strongly supports the involvement of acrosin in sperm penetration through the mammalian zona pellucida and a trypsin-like proteinase on R. typus sperm penetration.

Key words: *Acrosin, acrosome, egg, egg coats, spermatozoon*

INTRODUCTION

Eggs are physically (mechanically), chemically and biologically protected by one or few layers of egg coats. These coats play key roles in fertilization and indeed most of the sperm-egg interactions take place between sperms and egg coats. Upon fertilization, some coats are dispersed under the influence of lytic proteins from the sperm, while others are modified by products from the egg, to form embryo coats that partly block polyspermy. We will discuss the effect of the spermatozoon upon the egg coats during fertilization in mammals and in the crustacean *Rhynchocinetes typus*.

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MAMMALS

Acrosome Reaction

The mammalian spermatozoa must undergo the acrosome reaction as a prerequisite for fertilization; as a result of this reaction, the acrosome enzymes are exposed and enable the spermatozoa to cross the egg coats, particularly the zona pellucida. The reaction involves vesiculation between the plasma and the outer acrosomal membranes, except in that portion covering the posterior region of the acrosome, the equatorial segment (Barros *et al*, 1967).

Oviduct and follicular fluid, both homologous and heterologous, have the ability to induce the acrosome reaction, suggesting that the acrosome reaction can occur as a result of sperm incubation in either of those fluids. Other natural fluids, such as liquid egg white and aqueous humour, are not able to induce the acrosome reaction (Barros and Austin, 1967). Using an *in vitro* fertilization system of hamster eggs, it was found that many acrosomal caps were bound to the outer surface of the zona pellucida. This finding suggested that the acrosome reaction occurred (or at least was completed) at the surface of the zona pellucida (Franklin *et al*, 1970). It was also concluded that hamster spermatozoon binds to the zona surface through the acrosome, then detaches from it to pass through the thickness of the zona, the acrosomal cap remaining bound to the outer surface of the zona pellucida.

The above results brought forward the issue of the site where the acrosome reaction should occur for a successful fertilization. This was addressed using the *in vitro* fertilization system with hamster eggs. Hamster spermatozoa preincubated in a blood serum fraction for different time periods were added to cumulus-free hamster oocytes. It was found that the rate of acrosome reaction increased, while the rate of fertilizaton decreased with time. It was concluded that a long preincubation altered the sperm ability to cross the zona pellucida: when the zona pellucida was enzymatically removed, the same spermatozoa were able to fertilize 100% of the zona-free oocytes. It was inferred that the loss of the sperm ability to bind and to cross the zona was due to a premature acrosome reaction that took place far from the zona surface (Barros *et al*, 1973).

The association of hamster spermatozoa with the zona pellucida at different times after the onset of sperm preincubation was studied with the scanning electron microscope. The observations revealed that when gametes were mixed at the start of sperm preincubation, the spermatozoa that bound to the zona through the acrosome showed a successful penetration (Fig 1). On the other hand, with long-preincubated spermatozoa, the association of sperm with the zona pellucida was only partial and spermatozoa failed to penetrate leaving only sperm tracks at the zona surface (Fig 2) or passing tangential to the zona (Fig 3). When sperm preincubation lasted for 6 or more hours no binding was observed and no eggs were fertilized (Barros *et al*, 1984). From these results it was concluded that after the acrosome reaction, acrosin would be exhausted and/or inactivated, being unable to help the spermatozoon to digest its way through the thickness of the zona pellucida. However, at the time no direct evidence was presented to prove such hypothesis.

Zona Pellucida

The mammalian zona pellucida is composed of sulphated glycoproteins formed during oogenesis (Dunbar *et al*, 1980). In the mouse, the zona pellucida is formed by three glycoproteins, namely ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (80 kDa); it has been shown that the carbohydrate moiety of ZP3 is the ligand for a receptor located on the sperm plasma membrane, since treatment with pronase or endo-N-acetylgluco-saminidase F does not destroy the receptor activity (Bleil and Wassarman, 1980a,b; Greve and Wassarman, 1985; Florman *et al*, 1984; Wassarman, 1987a,b; Vásquez *et al*, 1989). It has also been reported that the polypeptide chain is responsible for the induction of the acrosome reaction (Florman *et al*, 1984; Florman and Wassarman 1985; Wassarman, 1990). This acrosome reaction induction property of the zona pellucida has been

reported in mouse (Wassarman *et al*, 1986; Kligman *et al*, 1991; Leyton and Saling, 1989), hamster (Cherr *et al*, 1986; Yoshimatsu and Yanagimachi, 1988; Uto *et al*, 1988; Yunes *et al*, 1993), rabbit (O'Rand and Fisher, 1987), bovine (Florman and First, 1989a,b) and human (Nagae *et al*, 1986; Cross *et al*, 1988). The zona pellucida of fertilized eggs loses its ability to bind spermatozoa and to induce the acrosome reaction (Bleil and Wassarman, 1983, 1986) probably due to the zona reaction elicited by the cortical granule breakdown (Barros and Yanagimachi, 1971, 1972).

In the mouse, it has been shown that during the association of the spermatozoon with the zona pellucida, the former interacts through a receptor located on its plasma membrane with a ligand present on ZP3 (Bleil and Wassarman, 1986, 1988). After the induction of the acrosome reaction the receptor for ZP3 would be released along with ZP3 and, in this way, the sperm would remain bound to the zona through a second receptor located on the inner acrosomal membrane that interacts with ZP2 (Mortillo and Wassarman, 1991).

Evidence has been presented that mouse ZP3 has the ability to aggregate a zona pellucida receptor present at the sperm plasma membrane overlying the acrosome (Saling *et al*, 1990). Glycopeptides from the zona pellucida bind to spermatozoa, but they do not induce the acrosome reaction. However, treatment with anti-ZP3 IgG induces the acrosome reaction to the same extent as exposure to the whole zona pellucida. A 95 kDa protein has been identified as a putative receptor for the zona pellucida and its aggregation would result in the acrosome reaction (Leyton and Saling, 1989; Leyton *et al*, 1992; Saling *et al*, 1991).

In the mouse, a sperm surface ß-1,4 galactosyl transferase has been also suggested to mediate fertilization by binding oligosaccharide residues in the zona pellucida. In this context, some authors have shown that sperm galactosyl transferase specifically recognizes those oligosaccharides on ZP3 that possess sperm-binding activity but that it does not interact with other zona pellucida glycoproteins. After initial binding, ZP3 aggregates a receptor, probably galactosyl transferase, which activates the acrosome reaction (Miller *et al*, 1991; Shur, 1993).

The sperm receptor for the zona pellucida has been found to be present in all the sperm plasma membranes overlying the acrosome. Therefore, when the acrosome reacts and the sperm plasma membrane surrounding the acrosome is no longer present, the sperm loses its ability to bind to the zona pellucida (Bleil and Wassarmann, 1986; Miller *et al*, 1991; Shur, 1993) and to cross it (Barros *et al*, 1973, 1984). However, guinea pig spermatozoa with reacted acrosome bind and penetrate guinea pig zona pellucida. In this regard, it is worth to mention the experiments which showed that rabbit spermatozoa recovered from the perivitelline space were able to bind and penetrate the zona pellucida of other unfertilized rabbit eggs (Kuzan *et al*, 1984). These findings brought forward many questions, namely: Is sperm penetration due to the action of the acrosomal enzyme acrosin, and -as a corollary of this- is the loss of acrosin from the spermatozoon responsible for the loss of sperm penetration ability? Finally, is there a relationship between the acrosome reaction and the ability to bind and cross the zona pellucida?

Acrosin

The involvement of the serine endoprotease acrosin in sperm penetration through the zona pellucida has been amply discussed (see Urch, 1986, for discussion). The presence of zona lysins was postulated before the experimental demonstration of the presence of acrosomal enzymes (Srivastava *et al*, 1965a, b). The zona pellucida is a normal substrate for acrosin (Urch *et al*, 1985) and heat-solubilized zona pellucida is a competitive inhibitor of BANA (Benzoyl-DL-Arginine-beta-Naftilamide), a specific substrate for acrosin (Urch, 1986). It has been also demonstrated that acrosin may act as a matrix degrading proteinase, since acrosin can degrade proteolytically fibronectin and type IV collagen (Plan-chenault *et al*, 1991). These results suggest that acrosin could have a proteolytic activity on the zona pellucida.

Acrosin is initially synthesized as a

preprotein, and is transported into the acrosome of the mammalian sperm as proacrosin, an enzymatically inactive form. The affinity of acrosin to artificial and natural membranes (Brown and Hartree, 1976; Parrish *et al*, 1978; Straus *et al*, 1981) and the capacity of phospholipid vesicles to promote autoactivation of the zymogen proacrosin (Parrish *et al*, 1978), indicate an association of the protease with the acrosomal membranes.

It has been described in several mammalian species that fucoidan prevents the conversion of the zymogen proacrosin into the acrosomal enzyme acrosin that occurs in the presence of intact and solubilized zona pellucida (Töpfer-Petersen *et al*, 1990). In addition to the protease activity, acrosin and its precursor molecule have the capacity to bind carbohydrate groups of zona pellucida glycoproteins, as well as synthetic neoglycoproteins with L-Fucose or D-Manose. At the same time, acrosin and a group of low mass proteins (14-17 kDa) are responsible for the fucose binding capacity of boar spermatozoa (Töpfer-Petersen *et al*, 1990), suggesting that acrosin participates in the complex events of sperm-egg interactions by means of its fucose-binding sites (Töpfer-Petersen *et al*, 1991).

Recent studies have shown that proteins and glycosaminoglycans of the intercellular matrix of the human oophorus are able to convert proacrosin into acrosin (Drahorád *et al*, 1991). It has been proposed that during sperm-egg interaction in mammals, proacrosin, released during the early stages of the acrosome reaction, would mediate secondary or consolidated binding of spermatozoa to the zona pellucida by virtue of its carbohydrate binding capacity (Jones *et al*, 1988). It has also been reported that proacrosin has properties analogous to those described for bindin, the sperm-egg adhesion protein found within the acrosomal vesicle of sea urchin spermatozoa (Jones, 1991). It has also been suggested that proacrosin binding to the zona pellucida may serve as recognition or primary sperm ligand, as well as to maintain the sperm on the zona pellucida once the acrosome reaction has occurred (Urch and Patel, 1991). Studies carried out on the boar have shown that the zona pellucida glycoproteins participate not only in the activation of proacrosin to acrosin, but also in its subsequent degradation (Eberspaecher *et al*, 1991).

Using immunochemical techniques, proacrosin/acrosin has been shown to be present in the intact acrosome of the spermatozoa in ram (Huneau *et al*, 1984), human (Tesarik *et al*, 1988; Barros *et al*, 1990, 1992; Capote *et al*, 1992; Escalier *et al*, 1991); boar (Catellani-Ceresa *et al*, 1983), rabbit (Valdivia *et al*, 1991), hamster (Barros *et al*,1990, 1993), guinea pig (Barros *et al*, 1990, 1993) and bull (De Los Reyes and Pérez, 1991). It has also been shown that human acrosin is able to digest hamster egg zonae pellucidae and that this digestion can be inhibited by the action of the monoclonal antibody anti-human acrosin ACRO-C2E5 (Elce *et al*, 1986).

Sperm penetration

After the acrosome reaction, bound spermatozoa detach from the acrosomal cap and start to cross the zona pellucida (Fig 1).

Since the first work that showed the existence of acrosomal enzymes in the mammalian spermatozoon (Srivastava *et al*, 1965a), acrosin has been involved in the process of sperm penetration through the zona pellucida. However, when rabbit eggs were rendered resistant to acrosin and trypsin digestion by treating them with wheat germ agglutinin, they were fertilized when placed back into the oviduct of another mated female rabbit (Bedford and Cross, 1978).

On the other hand, ample evidence supports the involvement of acrosin during sperm penetration through the zona pellucida. A variety of trypsin inhibitors block *in vitro* and *in vivo* fertilization (Stambaugh *et al*, 1969; Zaneveld *et al*, 1971; Miyamoto and Chang, 1973; Bhattacharyya *et al*, 1979; De Ioannes *et al*, 1990). In mice and hamsters, it has been shown that SBTI added before sperm-egg interaction significantly inhibits fertilization (Saling, 1981; De Ioannes *et al*, 1990). A close study of the oocyte surface with the scanning electron microscope showed that neither the acrosome reaction nor the initial

binding and localized lytic activity were inhibited as evidenced by the presence of small sperm tracks (Fig 4). A significant inhibition was obtained when SBTI (0.1 mM) was added up to 30 minutes after cumulus-free hamster oocytes were inseminated with capacitated spermatozoa (Fig 5). Addition of the inhibitor, 45 and 60 minutes after insemination, had no effect on the fertilization rate. A significant inhibitory effect was also found when the number of spermatozoa penetrating each oocyte was considered. While 70% of control eggs had two spermatozoa, 4, 9, 18 and 33% of eggs transferred to SBTI 15, 30, 45 and 60 minutes, respectively, contained 2 spermatozoa (Fig 6). These results are at variance with those obtained in the mouse where no inhibition was reported when SBTI was added after *in vitro* insemination (Saling, 1981). The source of variation could have been the experimental procedure employed on each work.

In golden hamster spermatozoa, using anti human proacrosin/acrosin antibodies, it has been shown that as a result of the acrosome reaction, most proacrosin/acrosin is lost from the sperm head while significant proacrosin/acrosin remains associated to the acrosomal cap. The loss of proacrosin/acrosin was concomitant with the loss of the sperm ability to cross the zona pellucida. This evidence supports the idea that hamster spermatozoa preincubated for long periods of time cannot penetrate the zona pellucida, due to a loss of acrosin from the acrosomal region (Barros *et al*, 1992). This is also consistent with the inhibitory effect of ACRO-C2E5 anti-human proacrosin/acrosin monoclonal antibody on hamster *in vitro* fertilization (De Ioannes *et al*, 1990). Studies in human, guinea pig, rabbit and bull spermatozoa have 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 (Barros *et al*, 1992; Valdivia *et al*, 1991; De Los Reyes and Pérez, 1991). In guinea pig spermatozoa incubated for 7 hours, only 7% of the spermatozoa had reacted acrosome and no proacrosin/acrosin over the inner acrosomal surface; 17% of spermatozoa with reacted acrosomes showed proacrosin/acrosin over the inner acrosomal surface (Barros *et al*, 1992). This is in agreement with the findings that acrosome-reacted guinea pig spermatozoa can bind and penetrate the guinea pig zona pellucida (Huang *et al*, 1981; Huang and Yanagimachi, 1985). This evidence or these findings would support the hypothesis that proacrosin may serve as recognition and/or primary ligand for a sperm receptor. It could also maintain the sperm on the zona pellucida, after the acrosome reaction (Jones and Williams, 1990; Urch and Patel 1991). Proacrosin/acrosin left on the surface of the inner acrosomal surface could also be involved in the sperm passage through the zona pellucida.

There is additional evidence for the role played by acrosin on the sperm passage through the zona pellucida: Kuzan *et al* (1984) showed that rabbit spermatozoa recovered from the perivitelline space can fertilize other unfertilized rabbit eggs at rates of 23%. Twenty-four percent of perivitelline rabbit spermatozoa had proacrosin/acrosin, as evidenced by the silver enhanced immunogold technique (Barros *et al*, 1993). On the other hand, none of 197 perivitelline hamster spermatozoa recovered from 23 oocytes hamster eggs had proacrosin/acrosin as shown by the same technique (Yunes *et al*, 1992; Barros *et al*, 1993). This evidence supports the hypothesis that acrosin would be involved in the sperm passage through the zona pellucida.

It has been suggested that a controlled digestion of ZP3 and ZP2 by acrosin would allow the exposure of oligosaccharides present in ZP2 to maintain a tight association with the inner surface of the sperm after the acrosome reaction (O'Rand and Fisher, 1987; De Ioannes *et al*, 1990). Cycles of binding, activation of acrosin and digestion would allow the sperm to progress through the zona pellucida. Thus, acrosin is believed to have two physiological functions: limited proteolysis of the glycoprotein matrix of the egg zona pellucida, and recognition and binding of the zona pellucida at the initial stages of fertilization (Baba, 1993).

More recently it has been reported that acrosin is also involved in the development of the fusibility of the sperm plasma

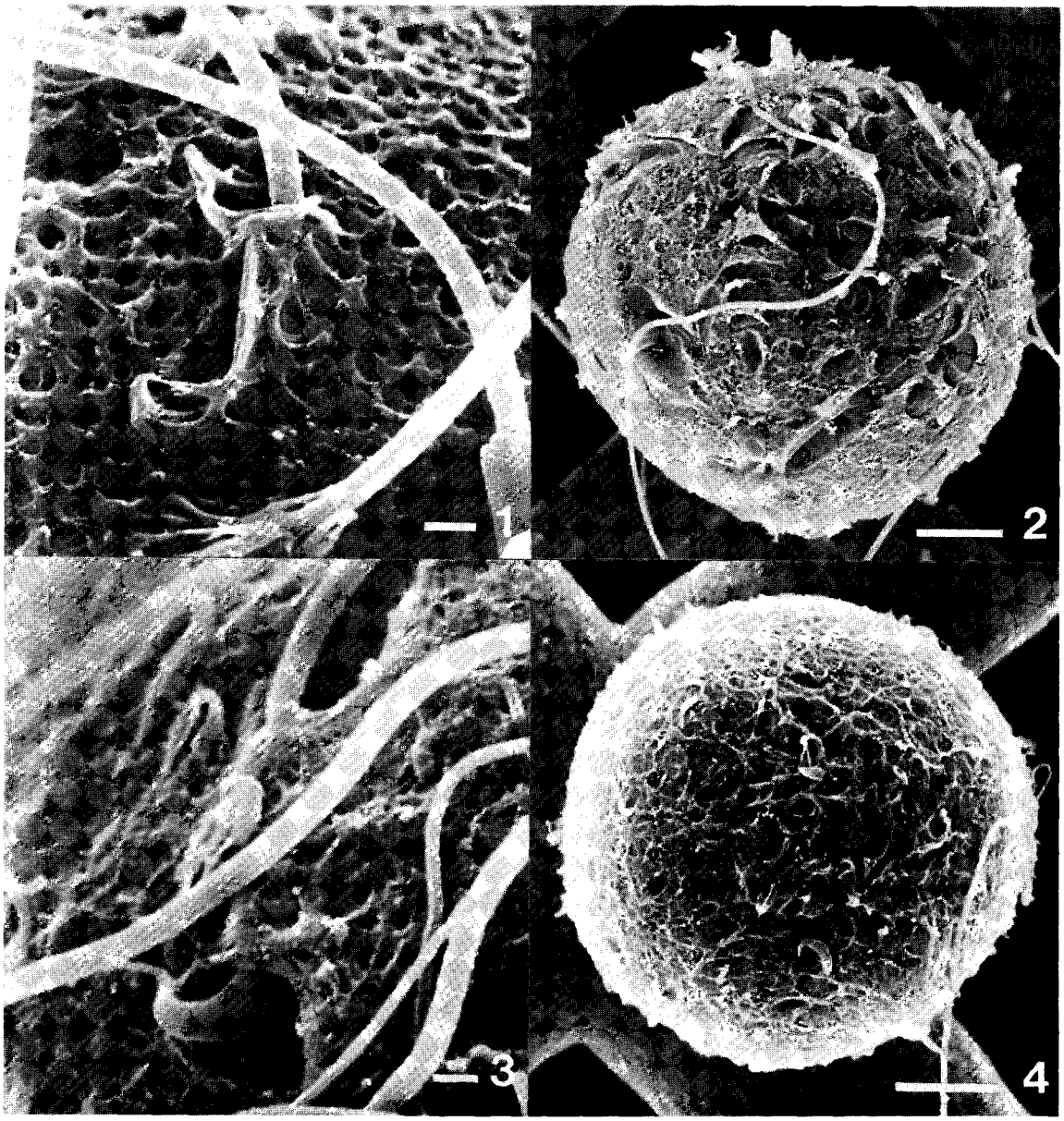

Fig 1. Scanning electron microscope micrograph of hamster egg inseminated *in vitro* with capacitated hamster spermatozoa. The sperm has started its passage through the zona pellucida and the head is already in the thickness of this egg coat. Bar equals 1 μm.

Fig 2. Scanning electron microscope micrograph of hamster egg inseminated *in vitro* with long-preincubated hamster spermatozoa. On the surface of the zona pellucida it is possible to observe many sperm tracks made by spermatozoa that failed to cross the zona pellucida. Bar equals 1 μm.

Fig 3. Scanning electron microscope micrograph of hamster egg inseminated *in vitro* with long-preincubated hamster spermatozoa. On the surface of the zona pellucida it is possible to observe a spermatozoon that is crossing tangentially to the zona pellucida. Bar equals 1 μm.

Fig 4. Scanning electron microscope micrograph of hamster egg inseminated *in vitro* with SBTI (0.1 mM) pre-treated spermatozoa. No spermatozoa can be seen at the zona surface. Only small sperm tracks and acrosomal caps of spermatozoa initially bound to the zona surface that failed to penetrate due to the action of SBTI may be observed. Bar equals 1 μm.

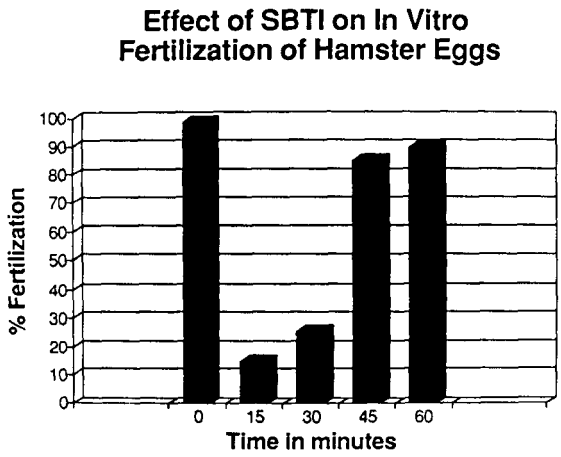

Fig 5. Percentage of *in vitro* fertilization of hamster eggs when the gamete mixture was transferred to 0.1 mM SBTI at 15, 30, 45 and 60 minutes after insemination with capacitated spermatozoa.

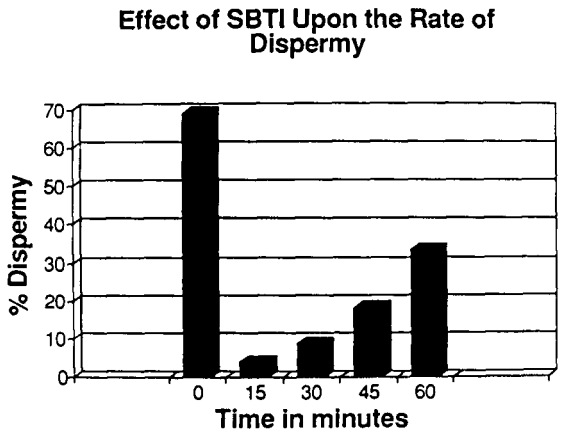

Fig 6. Percentage of dispermic *in vitro* fertilization of hamster when the gamete mixture was transferred to 0.1 mM SBTI at 15, 30, 45 and 60 minutes after insemination with capacitated spermatozoa.

membrane. When the acrosome reaction occurs in the presence of trypsin inhibitors, the gamete membrane fusion fails to occur. But, while trypsin inhibitors prevent acrosome reacted spermatozoa to fuse with the oocyte, the fusion of acrosome intact spermatozoa is not hindered by acrosin or trypsin alone (Takano *et al*, 1993). Therefore, the acrosome reaction would also be a prerequisite for the development of fusibility, thus supporting an early hypothesis proposed by Barros and Berrfos (1977), *i.e.*, that the acrosome reaction -besides its function of releasing acrosomal enzymes- would render the post acrosomal membrane able to fuse with the oocyte.

CRUSTACEA

Crustacean decapods constitute a particular model of gamete interactions because their spermatozoa are non motile spermatozoa. Spermatozoa of the crustacean Decapod *Rhynchocinetes typus* recovered from the vas deferens have the shape of a thumb tack with a semispherical body of 30 μm of diameter and a rigid spike of 53 μm long with a longitudinal external striation (Fig 7). When the spermatozoon is placed into sea water it undergoes an important change in shape and the semispherical body changes acquiring the shape of an inverted umbrella (Dupré and Barros, 1983).

The mature egg of *R. typus* measures 600 μm, and is surrounded by three egg coats; the outer one measures 0.5 μm, the middle one 2 μm, and the innermost 4.2 μm, which has a fenestrated appearance (Barros *et al*, 1986).

Gamete association in *R. typus* occurs between the tip of the rigid spike and the outer surface of the egg and is probably mediated by a bindin-like substance. At the time of gamete interaction, the tip of the spike appears at the ultrastructural level similar to that of the spermatozoon immediately after its release from the vas deferens; that is, there is no evidence of an exocytotic event that could account for the release of an acrosomal content. The spermatozoon is able to cross the egg coats by means of the tip of the rigid spike which seems to exert a lytic activity upon them (Barros *et al*, 1986), forming a channel through the egg coats (Fig 8). *R. typus* sperm extracts have a trypsin-like activity which may be involved in fertilization since the trypsin inhibitors, SBTI, PMSF and pAB, significantly inhibit *in vitro* fertilization of mature eggs (Rfos, 1993). This enzymatic activity might be involved in the observed digestion of the egg surface at the site of sperm-egg interaction.

The participation of sperm proteases in fertilization seems to be of general occurrence in invertebrate gamete interaction. Sperm extracts of several marine invertebrates have an enzymatic activity capable of dissolving the egg coats (Dan, 1967). Sperm proteases have been recognized in ascideans, molluscs, sea

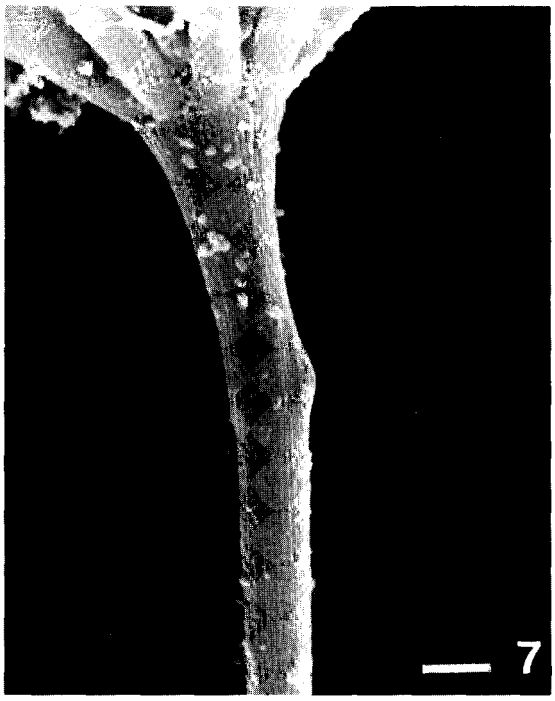

Fig 7. Scanning electron microscope micrograph of *R. typus* sperm showing the longitudinal external striations of the spike. Bar equals 1 μm.

Fig 8. Scanning electron microscope micrograph of *R. typus* sperm penetrating the egg coats. The hole made by the fertilizing spermatozoon is visible around the spike. Bar equals 1 μm.

urchins and starfish. There is evidence that those enzymatic activities may involve trypsin (Hoshi *et al*, 1981; Heller and Raftery, 1973; Hoshi *et al*, 1979; Green and Summers, 1980; Yamada and Aketa, 1981; Matsumura and Aketa, 1991; Sousa *et al*, 1992), chymotrypsin (Pinto *et al*, 1990; Hoshi *et al*, 1979; Yamada and Aketa, 1981; Matsumura and Aketa, 1991) and aryl sulfatase-like enzyme (Hoshi and Moriya, 1980). Sea urchin sperm has an acrosin-like activity evidenced biochemically (Levine *et al*, 1978). Ultrastructurally, it is localized in the acrosome using SBTI tagged to ferritin (Green and Summers, 1979, 1980). Similarly, in crude extracts of ascidean spermatozoa there is an enzyme that closely resembles mammalian acrosin, at least in its enzymatic properties, including optimum pH, substrate specificity, susceptibility to inhibitors, response to $CaCl_2$, and molecular weight (Sawada *et al*, 1982). These sperm enzymes have been involved in fertilization since specific inhibitors reduce the *in vitro* fertilization rates (Green and Summers, 1982; Sawada *et al*, 1982).

CONCLUSIONS

According to the evidence discussed here, sperm penetration through the egg coats seems to be aided by enzymatic activity of proteases present in spermatozoa of mammals and of invertebrates.

In **mammals**, the involvement of proacrosin/acrosin on sperm penetration is supported by:

- Inhibition of hamster sperm penetration through the zona pellucida when SBTI was added before or after *in vitro* insemination with capacitated spermatozoa.
- Inhibition of *in vitro* fertilization in the hamster by antiacrosin antibodies.
- Loss of hamster sperm ability to cross the zona pellucida when no detectable proacrosin/acrosin could be found on the inner acrosomal surface of live acrosome reacted spermatozoa.
- Lack of detectable proacrosin/acrosin on perivitelline hamster spermatozoa.

- Presence of proacrosin/acrosin on perivitelline rabbit spermatozoa that could account for their ability to fertilize other rabbit eggs.

The involvement of trypsin-like protease in *R. typus* sperm penetration is supported by:

- The presence of a digestion area at the point of sperm-egg interaction.
- Presence of trypsin-like activity of sperm extracts that can be inhibited by SBTI and pAB.
- The significant inhibition of *in vitro* fertilization of mature eggs when the spermatozoa were preincubated with SBTI, PMSF, and pAB.

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