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Acrosomal status and motility of guinea pig spermatozoa during *in vitro* penetration of the cumulus oophorus

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Summary

Previous studies have suggested that both acrosome-intact and acrosome-reacted guinea pig sperm are capable of binding to the zona pellucida of cumulus-free oocytes, but the acrosomal status of guinea pig sperm during penetration of the cumulus has not been reported. We made video recordings of the interaction between capacitated guinea pig sperm and cumulus-invested guinea pig oocytes. The videotapes were analysed to identify sperm with hyperactivated motility and to classify the acrosomal status of sperm during penetration of the cumulus and after binding to the zona pellucida. The resolution of the video recordings was not sufficient to recognise sperm with swollen acrosomes. However, sperm that had completed the acrosome reaction were easily identified. Acrosome-reacted sperm were found adherent to the outer boundary of the cumulus, but were never observed to penetrate the cumulus. The percentage of acrosome-intact, hyperactivated sperm was higher in the cumulus oophorus than in culture medium, suggesting that changes in motility were elicited in response to contact with the cumulus. Fully acrosome-reacted sperm were found adherent to the zona pellucida, and solubilised guinea pig zona pellucida was capable of inducing acrosome reactions in capacitated guinea pig sperm. Acrosome-intact sperm were also observed on the zona, but they were not tightly bound and did not have hyperactivated motility, suggesting that these sperm were not functionally capacitated. Our observations demonstrate that guinea pig sperm penetrate the cumulus matrix in an acrosome-intact state. Although we did not observe sperm undergoing the acrosome reaction, our observations and experimental data suggest that the acrosome reaction of guinea pig sperm is completed on or near the surface of the zona pellucida.

Key words: Acrosome reaction, Cumulus, Guinea pig, Sperm, Zona pellucida

Introduction

The cumulus complex which surrounds the mammalian oocyte presents a formidable barrier to fertilisation. Several factors may assist the sperm in penetrating the extracellular matrix (ECM) of the cumulus cell layers including enzymes carried by the sperm. PH-20, the sperm hyaluronidase, is capable of dispersing the cumulus ECM and is located on the outer surface of the sperm plasma membrane (Phelps & Myles, 1987; Lin *et al*., 1993; Jones *et al*., 1996; Li *et al*., 1997). Numerous enzymes including a soluble form of PH-20 are released when sperm undergo the acrosome reaction and could aid in sperm penetration if the acrosome reaction took place in the cumulus. Most previous observations on sperm acrosomal status during cumulus penetration have been made using sperm and oocytes from the golden hamster. It has been observed that hamster sperm can pass through the cumulus layer and bind to the zona pellucida in an acrosome-intact state (Cherr *et al*., 1986). Alterations in acrosomal morphology have been observed during

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sperm penetration of the hamster cumulus (Yanagimachi & Phillips, 1984), but it is likely that these changes are manifestations of acrosomal swelling (Yudin *et al*., 1989, 1998) rather than the membrane fusion events of a true acrosome reaction. In most species, sperm that acrosome react outside the cumulus layer become adherent to the cumulus and cannot penetrate to the zona (Cummins & Yanagimachi, 1986; Saling, 1989). Acrosome-reacted rabbit sperm have been shown to penetrate both the cumulus and zona pellucida (Kuzan *et al*., 1984), but because these sperm were collected from the perivitelline space of fertilised oocytes, they had lost any remnant of the vesiculated acrosomal cap, which may be responsible for sperm adhesion to granulosa cells (Drobnis & Katz, 1991). In the mouse model, denuded oocytes are commonly studied and only acrosome-intact sperm are thought to be capable of binding to the zona pellucida (Saling & Storey, 1979; Florman & Storey, 1982).

The guinea pig has been suspected to be an exception to the generally accepted model that sperm first bind to the zona pellucida and then undergo the membrane fusion events of the acrosome reaction on the zona. It has been shown that acrosome-reacted guinea pig sperm are able to initiate binding to the zona pellucida. In fact, early studies of sperm–zona binding in this species suggested that acrosome-intact sperm were unable to bind to the zona (Huang *et al*., 1981), although later studies demonstrated zona binding of acrosome-intact sperm (Myles *et al*., 1987). In most species, PH-20 is located on the surface of the entire sperm head, but in the guinea pig it is localised to the posterior head region of the acrosome-intact sperm (Myles & Primakoff, 1984). The fact that acrosome-reacted guinea pig sperm can initiate zona binding raises the possibility that guinea pig sperm could acrosome react before or during penetration of the cumulus cell layer, thereby utilising enzymes of the inner acrosomal membrane (Lin *et al*., 1993) or the acrosomal contents (Talbot, 1985) for digestion of the ECM. This fact also suggests that fertilisation in the guinea pig may be fundamentally different from fertilisation in the other mammalian species which have been studied in detail.

The aim of this study was to document the acrosomal status of guinea pig sperm during penetration of the cumulus matrix and after binding to the zona pellucida, when cumulus-enclosed guinea pig oocytes were incubated with capacitated guinea pig sperm *in vitro*. We hypothesised that if only acrosome-reacted sperm were found penetrating the cumulus, it would indicate that acrosome-reacted guinea pig sperm might initiate binding to the zona pellucida *in vivo*. If, on the other hand, acrosome-intact guinea pig sperm initiate binding with the zona pellucida *in vivo*, then acrosome-intact sperm should be found to penetrate the cumulus. In order to observe guinea pig sperm in the cumulus, it was necessary to develop methods for obtaining large numbers of mature oocytes with expanded cumulus masses from the ovaries of young female guinea pigs and for the capacitation of guinea pig sperm with a low rate of spontaneous acrosome reactions and agglutination.

Materials and methods

Animals

Hartley guinea pigs were purchased from Charles River and Kingston Breeding Labs. Males were retired breeders and larger than 700 g. Females were prepuberal to peripubertal and ranged in weight from 200 to 350 g.

Equipment

Phase-contrast micrographs of oocytes were taken with a Leitz Laborlux microscope equipped with a Nikon AFX-DXII automatic 35 mm camera. Gamete interactions were videotaped using an Olympus BH2-S microscope with a ×10 or ×20 positive phase-contrast objective, a ×6.7 photo-ocular, an MTV-3 adapter, a COHU Solid State camera, a Panasonic AG-6300 video recorder and BT-S1300n colour video monitor, a FOR-A video time generator and a Motion Analysis stage warmer. Observations of sperm motility and percentage acrosome reactions in cumulus-free medium following treatment with various substances were made using the ×20 negative phase contrast objective of a Nikon Labophot-2 microscope (with no photo-ocular), a Sony CCD-IRIS HyperHAD black and white video camera, a Sony DA Pro 4 Head video recorder, a Philips RGB video monitor and an LEC Instruments LEC916 microscope stage warmer. Photomicrographs were made from the videotaped images with a Sony SVO-1450 videotape player. Images were grabbed as still frames using a Video Designer PC system software/hardware capture board developed by Grass Valley Group (Grass Valley, CA). This equipment runs on a Pentium PC computer. The images were exported as TIFF graphic images files at 200 DPI. The images were cleaned and cropped using Adobe Photoshop (San Jose, CA) and printed on a Fujix Pictrography 3000 digital colour printer.

Reagents

Human chorionic gonadotropin (hCG), bovine testicular hyaluronidase and Sigmacote surface treatment, as well as D-glucose, sodium lactate, sodium pyruvate, streptomycin sulphate, potassium penicillin G, NaHCO3, and bovine serum albumin (BSA) used in

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media preparation were purchased from Sigma Chemical Co. NaCl and KCl were purchased from Fisher Scientific Co. $MgCl_2.6H_2O$ was purchased from J. T. Baker Chemical Co.

Media

Basic medium was a modified Tyrode's solution containing 635.2 mg NaCl, 20 mg KCl, 10 mg $MgCl_2.6H_2O$, 210 mg $NaHCO_3$, 300 mg BSA, 100 mg D-glucose, 112 mg sodium lactate, 11 mg sodium pyruvate, 50 mg streptomycin sulphate, and 10^7 IU potassium penicillin per 100 ml (Fleming & Yanagimachi, 1981). The pH was adjusted to 7.6 with HCl. Capacitation medium consisted of basic medium plus an anti-WH-30 monoclonal antibody. This antibody binds to the sperm surface antigen WH-30, which is involved in cell–cell adhesion (Flaherty *et al.*, 1993), and was included to reduce sperm agglutination. Cumulus penetration assays were carried out in basic medium + 2 mM $CaCl_2$.

Recovery of oocytes

Twenty-four hours prior to each experiment female guinea pigs were injected subcutaneously with 250 IU hCG. Females were killed in a CO_2 chamber and the ovaries were removed and placed in a small Petri dish containing basic medium + $CaCl_2$. Follicles were ruptured with forceps and the oocyte–cumulus complexes (OCCs) were transferred by glass pipette to a glass well containing basic medium + $CaCl_2$.

Recovery and capacitation of spermatozoa

Male guinea pigs were killed in a CO_2 chamber. One cauda epididymidis was removed aseptically and placed in a glass well containing approximately 0.5 ml basic medium. The epididymis was cut into several pieces and sperm were allowed to swim out. The percentage motility of the sperm suspension was estimated by placing a drop of the suspension on a slide with a cover slip and counting the number of motile sperm per total sperm seen in six different microscope fields at a magnification of ×100. The sperm concentration was adjusted to 10^6/ml, and 100 µl of this suspension were added to a 1.5 ml microtube containing 1 ml of capacitation medium. The contents of the tube were covered with mineral oil and incubated for 4 h at 37 °C under 5% CO_2.

Cumulus penetration assay

Three or four OCCs were placed in approximately 50 µl basic medium + $CaCl_2$ in a glass well. A 5 µl aliquot of capacitated sperm suspension (approx. 10^5 sperm/ml, or 500 sperm) was added to the well. After 2–3 min of incubation, the OCCs were gently washed twice through fresh basic medium + $CaCl_2$. They were then placed on a glass slide and covered with a cover slip supported by 300 µm glass beads and the edges of the cover slip sealed with mineral oil equilibrated with basic medium at 37 °C under 5% CO_2. The slide was observed at 37 °C with positive phase-contrast microscopy at ×100 and ×200 for 40–60 min and a videotape record of sperm–cumulus interaction was made. The interaction of individual gametes on the slide was not videotaped continuously, rather each OCC with which sperm interacted was observed discontinuously, allowing periodic monitoring of each site of activity on the slide. The aim of this method of recording gamete interaction was to maximise the opportunity for observing the sequential steps of fertilisation.

Both overall and frame-by-frame visual analyses of the videotape records were made at a later date. The overall analysis involved the classification of recorded sperm as acrosome-intact or acrosome-reacted and the tallying of the numbers of sperm of each class found within the cumulus matrix and at the surface of the zona pellucida. We classified acrosome-intact sperm as those which clearly retained the acrosomal cap and acrosomal contents, and acrosome-reacted sperm as those which had no evidence of the acrosomal cap. Our video recordings were capable of identifying changes in the appearance of the acrosomal cap during early stages of the acrosome reaction. However, no such changes were observed in sperm recorded either in the cumulus or on the zona pellucida. We were not able to distinguish sperm with swollen acrosomes in the cumulus matrix or vesiculated acrosomal caps (acrosomal shrouds) of sperm bound to the zona pellucida. Motility patterns of recorded sperm were classified as nonhyperactivated or hyperactivated based upon characteristics of flagellar bending and sperm trajectory. Sperm exhibiting low-amplitude, high-frequency flagellar bending and a progressive trajectory were classified as non-hyperactivated, while sperm exhibiting high-amplitude flagellar bending and a non-progressive, convoluted trajectory were classified as hyperactivated. Sperm within the cumulus were classified on the basis of flagellar bending patterns alone. A sperm was considered to have penetrated the zona once the entire head was within the zona, while fertilisation was defined as the fusion of the sperm head with the egg plasma membrane such that the sperm head was no longer distinguishable but the tail of the fertilising sperm was still seen extending through the zona pellucida.

A frame-by-frame analysis was performed to describe the motility patterns exhibited by the sperm within the cumulus matrix, and at the zona pellucida. Tracings of sperm trajectories and flagellar bending

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were made by placing an acetate sheet over the video monitor screen. The scale of these tracings was established by recording a stage micrometer at the same magnification as that at which the sperm were viewed. In addition, the linear swimming velocities of sperm, which were recorded both within the cumulus and outside the cumulus, were calculated manually by placing an acetate sheet on which a grid had been drawn over the video monitor screen. The squares of this grid represented an area 10 μm × 10 μm for videotaped images recorded at a magnification of ×200, or 20 μm × 20 μm for images recorded at ×100. This scale was established by viewing a stage micrometer at ×200. The velocities of five sperm which were recorded swimming in the cumulus matrix as well as outside the cumulus were determined in each location by advancing the videotape frame by frame and measuring the distance travelled over time using the acetate grid and the time display provided by the video time generator.

Solubilisation of zona pellucida

OCCs were placed in a minimal volume of basic medium in a glass well. They were then treated with bovine testicular hyaluronidase at a concentration of 1 mg/ml for 30 min to remove the cumulus matrix. The cumulus-free oocytes were washed three times through fresh basic medium, then three times through phosphate-buffered saline (PBS), and placed in a minimal volume of PBS in a glass well. The oocytes were treated with 0.1 M sodium acetate in 0.9% saline (pH 2.8) at a volume of 1 μl per oocyte for 15 min to remove the zonae. The solubilised zonae were recovered by centrifugation in a Fisher Scientific microcentrifuge (Model 59A) over a 5000 MW ultrafilter (Whatman Chemical Co.) at 4650 rpm, followed by dilution back to a concentration of one zona per microlitre with basic medium + $CaCl_2$, then frozen at –20 °C for later use.

Study of sperm in cumulus-free medium

For this series of experiments, sperm from each cauda epididymidis of the male were capacitated separately. Capacitated sperm were diluted to 10^4 to 10^5 sperm/ml with either basic medium or basic medium containing calcium (final concentration: 2 mM $CaCl_2$), or calcium and solubilised zona pellucida (final concentration: 2 mM $CaCl_2$, 0.5 ZP/μl). Thus, for each treatment, with the exception of the solubilised zona, each animal contributed two samples for analysis. Only one sample per animal was treated with solubilised zona due to the limited availability of this material. After a 10 min incubation period at 37 °C, 20 μl of the diluted sperm suspension were placed on a Sigmacote-coated slide and covered by a similarly coated cover slip. Each slide was viewed at ×200 with a negative phase-contrast objective, and 100 sperm per slide were scored for acrosomal status and for hyperactivated motility. For treatments in which two samples were scored per animal, the results were averaged to obtain a single mean value for each parameter measured. Sperm were also videotaped for frame-by-frame analysis, which was performed as described above.

The capability of solubilised zona pellucida to induce acrosome reactions was also tested in a separate set of experiments. An aliquot of capacitated sperm suspension was added to 10 μl of either solubilised zona suspension or basic medium + $CaCl_2$ in a small Petri dish such that the final concentration of sperm in each drop approximated 10^4 sperm/ml. In these experiments, the concentration of solubilised zona pellucida was increased to 1 ZP/μl and the incubation time was extended to 30 min. The drops were covered with a layer of mineral oil and incubated at 37 °C under 5% CO_2. Following incubation, the contents of each drop were fixed with 10 μl 4% paraformaldehyde which was added under the oil layer. The percentage acrosome-reacted sperm per 100 sperm was calculated in each drop.

Statistical analysis

A one-sided paired *t*-test was used to compare manually calculated mean swimming velocities of acrosome-intact sperm in cumulus-free medium versus cumulus matrix. A simple one-sided *t*-test was used to compare percentage acrosome reactions in untreated sperm versus sperm treated with solubilised zonae.

Results and discussion

Gamete recovery and preparation

Superovulation of females

In other species such as the hamster and mouse multiple OCCs may be recovered following superovulation with gonadotropins. However, the guinea pig is remarkably unresponsive to such manipulation of the reproductive cycle (Donovan & Lockhart, 1972). Obtaining spontaneously ovulated oocytes from the female guinea pig is complicated by the 15–17 day oestrous cycle and the fact that at least two cycles must be observed to establish the normal cycle length of each animal. Guinea pigs have a short oestrus period (mean duration 8 h) which does not occur at a predictable time in the photoperiod, and there is a 10 h delay between the onset of heat and ovulation. Although ovulation can be induced a few days prior to oestrus by administration of luteinising hormone

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(Dempsey *et al*., 1936), mature females ovulate only three or four oocytes per cycle (Phoenix, 1970).

Other investigators have retrieved immature ovarian oocytes from guinea pigs and used an overnight incubation in medium containing gonadotropins to stimulate oocyte maturation (Fiedler & Seifert, 1992). In preliminary experiments we utilised this approach, but we were not convinced that the ECMs of such OCCs were physiologically normal because the cumulus matrices did not appear fully expanded and treatment with hyaluronidase did not result in cumulus dispersion. Therefore, we developed a protocol for *in vivo* maturation of ovarian follicles based on the work of Reed & Hounslow (1971). Prepubertal female guinea pigs were treated with hCG and OCCs were recovered from the follicles. Because these animals had no pre-existing cycle which could affect the response to exogenous gonadotropins they could be treated at any time. Approximately 10 mature oocytes with expanded cumulus matrices were recovered per female (Figs. 1, 2). Preliminary experiments demonstrated that these OCCs were physiologically mature as evidenced by the presence of polar bodies, the ability of hyaluronidase to disperse the cumulus matrix, and fertilisation by guinea pig sperm *in vitro* (data not shown). The ovaries of immature females produced more OCCs than those of mature females (data not shown).

Sperm capacitation

Capacitated guinea pig sperm are relatively unstable in that spontaneous acrosome reactions occur with high frequency. Also, capacitated sperm tend to agglutinate *in vitro* forming aggregates of the sperm stacks,

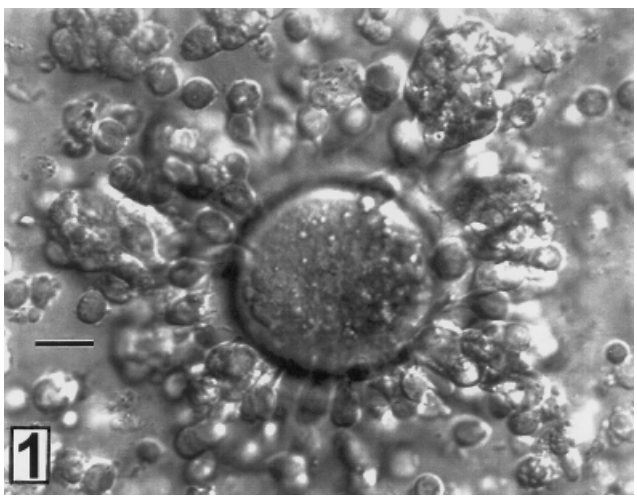

Figure 1 Phase-contrast photomicrograph of a guinea pig oocyte–cumulus complex obtained from an ovarian follicle 24 h after stimulation with 250 IU of hCG. The granulosa cells form a corona radiata around the zona pellucida. Scale bar represents 25 µm.

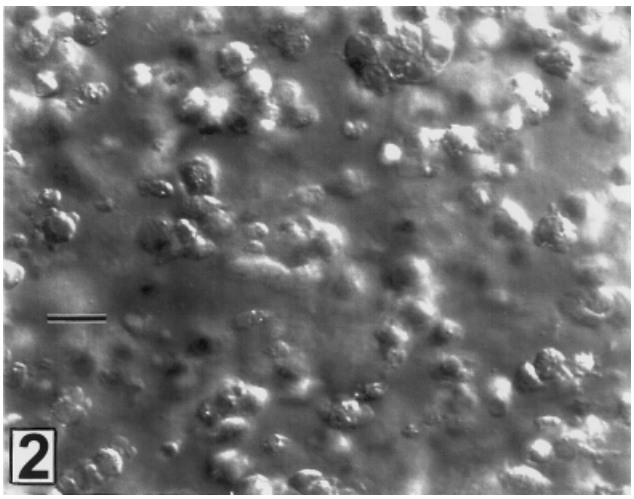

Figure 2 Higher-magnification phase-contrast photomicrograph of the expanded cumulus oophorus of the guinea pig oocyte. Scale bar represents 20 µm.

or rouleaux, which occur normally *in vivo* (Flaherty & Olson, 1991). Because of the presence of these sperm aggregates, we were unable to make observations of individual sperm cells. To overcome this problem, we carried out preliminary experiments in which sperm were incubated in basic medium with and without the anti-WH-30 antibody over a range of times from 1 h to 24 h. The anti-WH-30 antibody was used because the WH-30 surface molecule is involved in cell–cell adhesion, and the anti-WH-30 antibody has been shown to dissociate rouleaux (Flaherty *et al*., 1993). When sperm were incubated for 4 h in a calcium-free modified Tyrode's medium which included the anti-WH-30 antibody, at least 75% motility was maintained and less than 5% of sperm had spontaneous acrosome reactions. There was little or no clumping or rouleaux formation. Incubation of guinea pig sperm in Ca^{2+}-free medium has been shown to block the occurrence of acrosome reactions without affecting the rate of capacitation (Yanagimachi & Usui, 1974). When resuspended in Ca^{2+}-containing medium, the sperm in our experiments responded to soluble and intact zona pellucida with acrosome reactions, suggesting that the presence of the WH-30 antibody on the sperm surface did not affect capacitation.

Sperm interaction with the oocyte–cumulus complex

Sperm penetration of the cumulus was recorded in seven replicated experiments in which a total of 18 OCCs was observed. Twenty-seven acrosome-intact sperm were videorecorded during penetration through the cumulus matrix. No acrosome-reacted sperm were ever observed within the cumulus, and no acrosome-intact sperm were observed to undergo the acrosome reaction during cumulus penetration.

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Ten acrosome-reacted sperm were found adhered to cumulus cells at the edges of the cumulus matrix. These results are consistent with previous reports that acrosome-reacted sperm of other species are unable to enter the cumulus matrix (Florman & Storey, 1982; Yanagimachi & Phillips, 1984). This inability may be due to a change in sperm surface properties resulting from the acrosome reaction which leaves the sperm head more likely to adhere to the cumulus cells and ECM (Cherr *et al*., 1986).

The sequence of acrosomal changes which take place in the cumulus prior to sperm–zona interaction has not been widely studied. In the mouse, the acrosome reaction is thought to be induced as a consequence of sperm–zona binding (Florman & Storey, 1982), but the experiments on which this model is based have used cumulus-free oocytes. Cherr *et al.* (1986) found very few acrosome-reacted hamster sperm within the cumulus matrix, and most sperm reached the zona with the acrosome still intact. Nevertheless, there is evidence that changes in acrosomal morphology of hamster sperm may be initiated in the cumulus at some distance from the zona pellucida. Cummins & Yanagimachi (1986) found that virtually all hamster sperm which were able to penetrate the cumulus matrix exhibited some degree of acrosomal swelling indicative of imminent acrosome reaction, although it was noted that these sperm were not acrosome-reacted. On the other hand, sperm which had already undergone the acrosome reaction outside the cumulus were unable to enter the matrix. In addition, *in vivo* studies of hamster sperm have shown that most of the sperm found within the cumulus matrix have modified acrosomal caps consistent with acrosomal swelling (Yanagimachi & Phillips, 1984).

The significance of acrosomal swelling is not entirely clear. Such morphological changes have been interpreted as an initial step of the acrosome reaction of hamster sperm (Cummins & Yanagimachi, 1986). Experiments with macaque sperm suggest that acrosomal swelling is a preliminary event which takes place in sperm before the acrosome reaction is initiated, rather than being an initial step in the reaction itself (Yudin *et al*, 1998). This interpretation is based on the results of treatments with antibodies to the PH-20 sperm surface protein, which aggregate the protein and induce acrosomal swelling without causing acrosome reactions (Yudin *et al*., 1998). We were not able to distinguish guinea pig sperm with modified acrosomal caps on our videotapes; therefore we cannot determine whether acrosomal swelling was initiated within the cumulus prior to sperm contact with the zona pellucida. It is interesting to note that the percentage of acrosome-intact sperm within the cumulus which exhibited hyperactivated motility was greater than that seen in acrosome-intact sperm populations which were not exposed to OCCs. This result may be due to an increase in internal sperm calcium during penetration of the cumulus (Cherr *et al*., 1999), an event which is closely related to acrosomal swelling (Yudin *et al*., 1998).

Altogether, 28 acrosome-intact and 12 acrosome-reacted sperm were observed bound to the zonae pellucidae of 18 oocytes. Both acrosome-intact sperm and acrosome-reacted sperm were observed on the zona within 5 min of sperm–cumulus co-incubation, which was the earliest time of observation. Sperm were never observed to undergo the acrosome reaction either in the cumulus or on the zona, suggesting that all sperm capable of acrosome-reacting reached the zona within the 5 min co-incubation period. Because the acrosome reaction was not observed directly, we cannot define the precise location at which it occurred. However, the inability of acrosome-reacted guinea pig sperm to penetrate the cumulus strongly suggests that the membrane fusion events of the acrosome reaction were completed on or near the zona pellucida.

Acrosome-reacted sperm were tightly bound to the zona (Figs. 3, 4), and of 12 acrosome-reacted sperm observed on the zona, none was ever seen moving over the zona surface or detaching from the zona during the 40 min observation period. These sperm may have initiated binding to the zona pellucida before or after the acrosome reaction. Acrosome-intact sperm, on the other hand, appeared to bind to the zona relatively loosely; they were observed to swivel their heads around a point on the zona (Figs. 5, 6) and to slide along the zona surface. Of the 28 acrosome-intact sperm bound to the zona, 82% were observed to detach after binding, some as briefly as 1 min after binding and some as long as 10 min after binding. These acrosome-intact sperm may not have been functionally

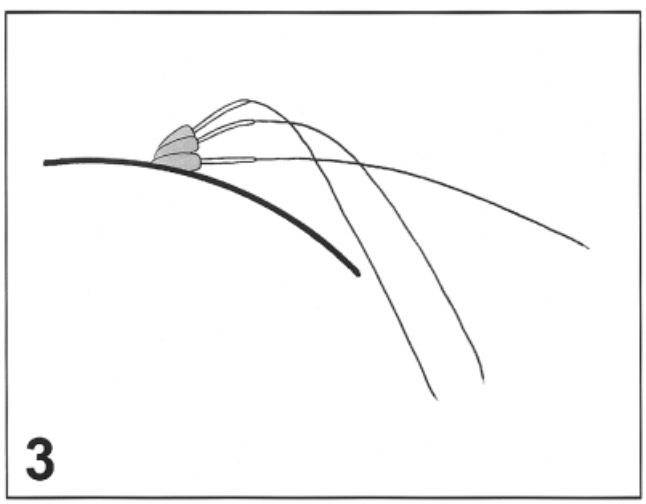

Figure 3 Illustration of the motility pattern of an acrosome-reacted sperm soon after initial binding to the zona pellucida. Superimposed tracings were made from consecutive frames of videotape (frame interval 0.03–0.04 s).

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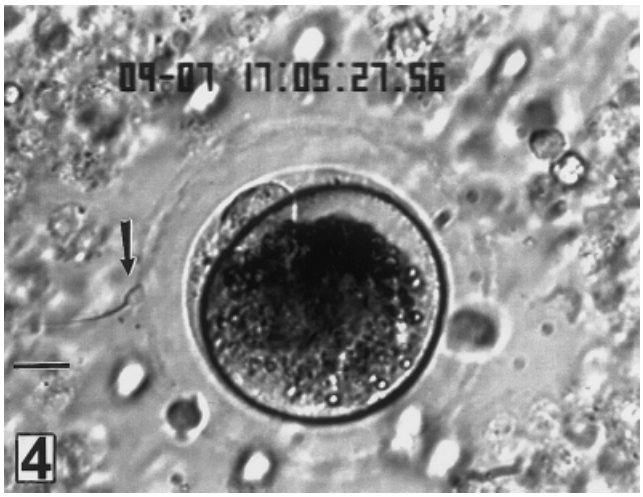

Figure 4 Photomicrograph taken from the videotape of an acrosome-reacted sperm (arrow) bound to the zona pellucida. Scale bar represents 20 µm.

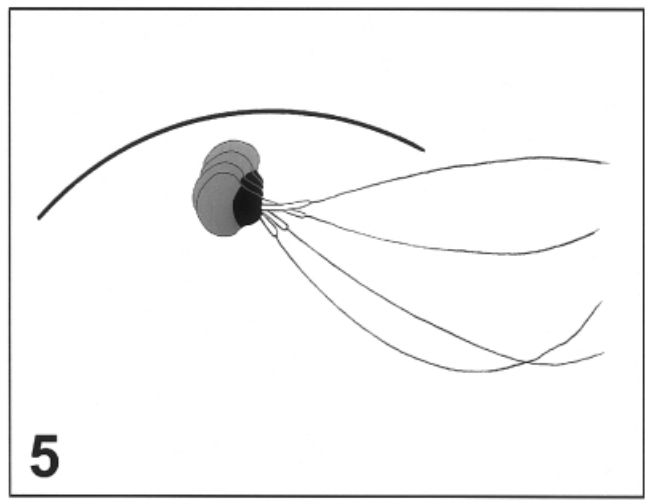

Figure 5 Illustration of the motility pattern of an acrosome-intact sperm soon after initial binding to the zona pellucida. Superimposed tracings were made from consecutive frames of videotape (frame interval 0.03–0.04 s).

capacitated, and thus could not initiate tight zona binding or respond to zona binding with an acrosome reaction. This interpretation is supported by the absence of hyperactivated motility in this group of sperm (Fig. 5). It is possible that if sperm capacitation conditions had been optimal, then all sperm bound to the zona would have been acrosome-reacted.

There is good evidence that the vesiculated acrosomal cap or 'shroud' anchors the acrosome-reacted sperm to the zona pellucida in hamsters (Yanagimachi & Phillips, 1984; Cherr *et al*., 1986), rabbits (Esaguy *et al*., 1988) and monkeys (VandeVoort *et al*., 1997), and there is circumstantial evidence that a similar sequence of events also occurs in cattle (Crozet, 1984), sheep (Crozet & Dumont, 1984), goats (Crozet *et al*., 1987) and

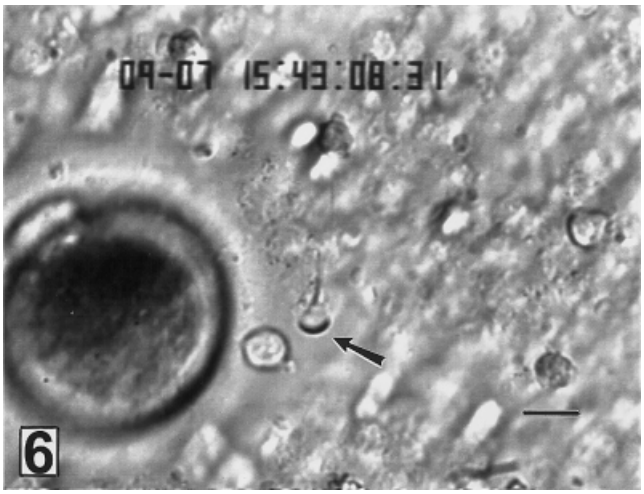

Figure 6 Photomicrograph taken from the videotape of an acrosome-intact sperm (arrow) soon after attachment to the zona pellucida. Scale bar represents 20 µm.

humans (Tesarik *et al*., 1988). It is also clear that under experimental conditions *in vitro* fully acrosome-reacted sperm can bind without a shroud to the zona in guinea pigs (Huang et al., 1981; Myles *et al*., 1987), rabbits (Kuzan *et al*., 1984), humans (Morales *et al*., 1989) and monkeys (VandeVoort *et al*., 1997). The resolution of our videorecordings was not sufficient to enable us to determine whether the acrosome-reacted guinea pig sperm observed in the present experiments were bound to the zona by their inner acrosomal membrane or by an acrosomal shroud.

Penetration of the zona pellucida by acrosome-reacted sperm was recorded nine times in five of the seven penetration assays. The time of zona penetration after addition of sperm to cumulus ranged from 17 min to 107 min. These penetration times may be overestimates, because zona penetration may have occurred earlier and not been detected. Fertilisation was recorded in a single experiment after 75 min of gamete co-incubation.

Movement characteristics of guinea pig sperm

Capacitated, acrosome-intact sperm swimming free in culture media were either progressively motile with linear trajectories and propelled by symmetrical flagellar beats of low amplitude and high frequency (Fig. 7), or they exhibited non-progressive hyperactivated motility (Fig. 8). Acrosome-reacted sperm in culture media were always hyperactivated. These sperm were propelled rapidly in a highly convoluted pattern with flagellar beats of high amplitude and low frequency (Fig. 9). Experiments in other species have shown that sperm motility changes to a pattern defined as hyperactivation during capacitation (Yanagimachi, 1970), and hyperactivated motility is also observed following

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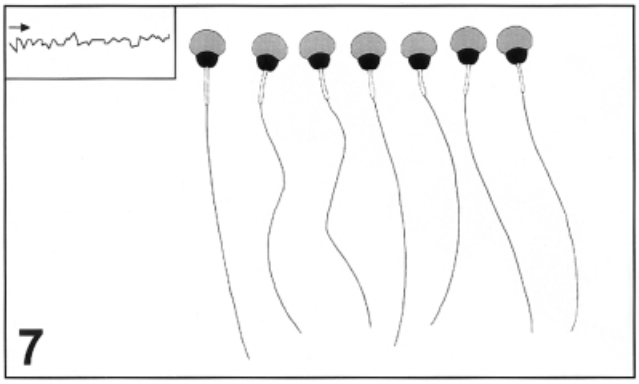

Figure 7 Illustration of an acrosome-intact sperm swimming in medium. Tracings were made from consecutive videotape frames to show the flagellar beat pattern (0.03–0.04 s/frame). Sperm heads are aligned along a horizontal axis for presentation. The insert shows the sperm swimming trajectory over a 1.5 s interval.

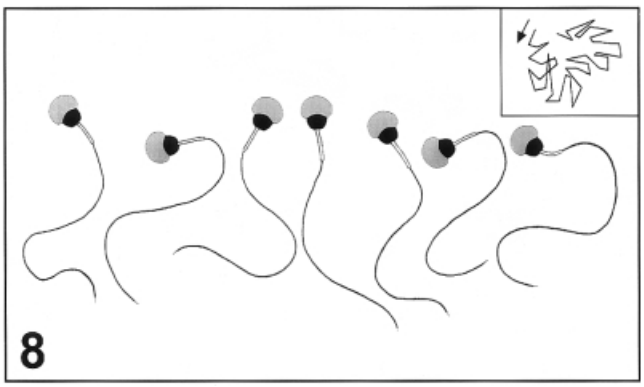

Figure 8 Illustration of an acrosome-intact hyperactivated sperm swimming in medium. Tracings were made from consecutive videotape frames to show the flagellar beat pattern (0.03–0.04 s/frame) over a 0.25 s interval. Sperm heads are aligned along a horizontal axis for presentation. The insert shows the sperm swimming trajectory over a 1.5 s interval.

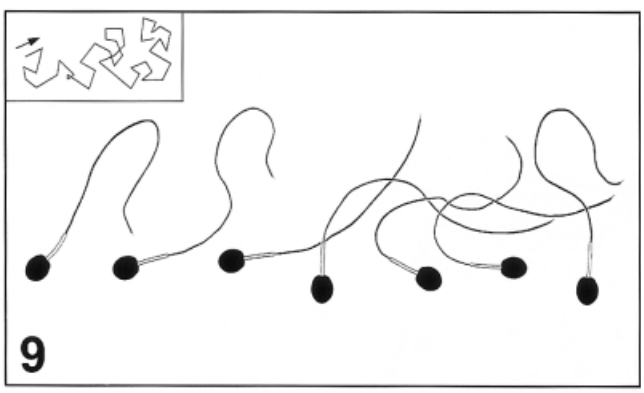

Figure 9 Illustration of an acrosome-reacted hyperactivated sperm swimming in medium. Tracings were made from consecutive videotape frames to show the flagellar beat pattern (0.03–0.04 s/frame) over a 0.25 s interval. Sperm heads are aligned along a horizontal axis for presentation. The insert shows the sperm swimming trajectory over a 1.5 s interval.

the acrosome reaction (Suarez *et al.*, 1984). Hyperactivated motility is thought to be instrumental in sperm transport to the site of fertilisation (Katz & Yanagimachi, 1980) and in penetration of the cumulus and zona pellucida (Suarez *et al.*, 1991).

The trajectories of non-hyperactivated sperm within the cumulus were less linear than those seen outside the matrix, probably as a result of movement around granulosa cells. Five sperm were recorded both in the cumulus and after their exit from the cumulus. Within the cumulus matrix, the manually determined mean linear swimming velocity of nonhyperactivated acrosome intact sperm was 39.32 ± 25.76 μm/s, which was significantly less than the mean velocity of 112.76 ± 17.10 μm/s observed for the same sperm after their exit from the cumulus layer ($n = 5$, $\alpha = 0.05$, $p < 0.005$).

Hyperactivated, acrosome-intact sperm swimming in the cumulus matrix exhibited intervals of progressive motility interspersed with episodes of extreme flagellar bending during which the head moved slowly in the direction of the flagellum and then straightened out quickly, leading with the convex edge of the acrosome (Figs. 10, 11). After such a manoeuvre, the flagellum appeared to follow a path cleared by the motion of the head. These movement characteristics are similar to the hatchet-like movements reported for hyperactivated hamster sperm during cumulus penetration (Drobnis *et al.*, 1988). The motility pattern of hyperactivated guinea pig sperm in the cumulus differed from that seen in the hamster in that the concave face of the guinea pig sperm head moved towards the tail during the initial slower deviation from the midline, and with the more rapid straightening of the tail the convex face of the sperm head was pushed through the ECM. In the hamster, such slow bends occur in the opposite direction (Drobnis *et al.*, 1988). This difference may be significant in light of the fact that the sperm surface hyaluronidase PH-20 has been localised to the posterior head region of the sperm surface of acrosome-intact guinea pig sperm (Myles & Primakoff, 1984). Therefore, a motility pattern in which the posterior head precedes the rest of the sperm head through the ECM would be consistent with the proposed function of this sperm-surface enzyme in cumulus penetration.

The observation of two motility patterns for acrosome-intact sperm in OCCs suggests the existence of two subpopulations of sperm. One population of sperm may have hyperactivated motility as a result of increased intracellular calcium. Macaque sperm in the cumulus ECM have motility patterns consistent with hyperactivation and also have increased intracellular calcium (Cherr *et al.*, 1999). The components of the ECM which elicit this response include hyaluronic acid, and macaque sperm must be capacitated in order to respond to hyaluronic acid with an increase in intracellular calcium (Cherr *et al.*, 1999). These observations

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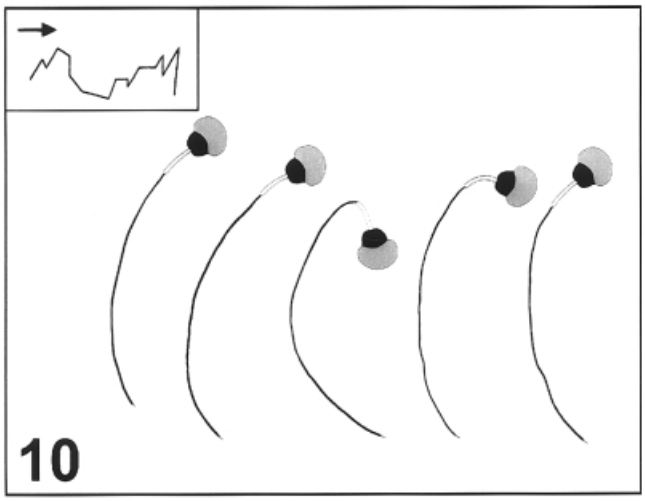

Figure 10 Illustration of an acrosome-intact hyperactivated sperm swimming in the cumulus oophorus. Tracings were made from consecutive videotape frames to show the flagellar beat pattern (0.03–0.04 s/frame) over a 0.18 s interval. Sperm heads are aligned along a horizontal axis for presentation. The insert shows the sperm swimming trajectory over a 0.6 s interval.

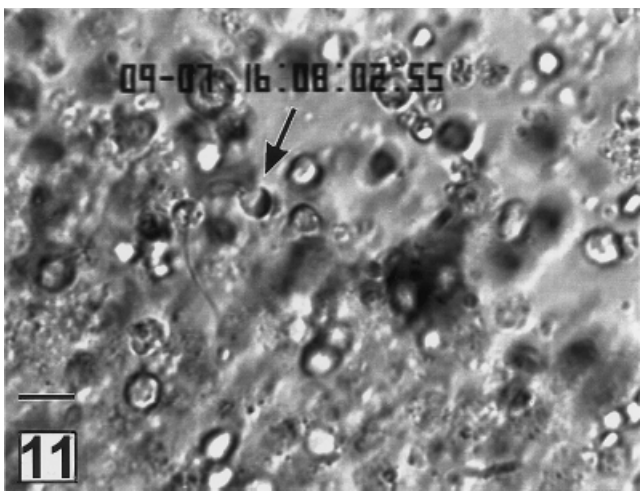

Figure 11 Photomicrograph taken from the videotape of an acrosome-intact sperm (arrow) during penetration of the cumulus oophorus. Scale bar represents 25 µm.

in macaques support the interpretation that the guinea pig sperm which were not hyperactivated in the cumulus or on the zona (Figs. 5, 6) may not have been fully capacitated and, as a consequence, were unable to bind tightly to the zona or to acrosome-react on the zona surface (see above). Acrosome-reacted sperm that were bound to the zona always had flagellar bending patterns that were consistent with hyperactivation, i.e. relatively low-frequency, high-amplitude beats (Figs. 3, 4). The hyperactivated motility pattern seen in acrosome-reacted guinea pig sperm which were bound to the zona was translated into strong thrusts which appeared to force the sperm head into the zona material. It is likely that this shearing force, rather than lytic enzymes, is primarily responsible for sperm penetration through the zona (Bedford, 1998). The flagella of zona-bound, acrosome-reacted sperm were observed bending in two-dimensional planes in areas adjacent to the zona and which were free of cumulus matrix. The motility pattern of acrosome-reacted guinea pig sperm, when swimming freely and when bound to the zona pellucida, is similar to that reported for hyperactivated hamster sperm (Suarez *et al.*, 1984).

Response of guinea pig sperm to calcium and solubilised zona pellucida

Capacitated sperm which were not exposed to calcium exhibited few or no acrosome reactions, and none of the acrosome-intact sperm had hyperactivated motility (Table 1). After a 10 min incubation in cumulus-free medium which contained calcium, a small percentage of the acrosome-intact sperm were hyperactivated, and the percentage of acrosome reactions increased to 16%. These percentages were higher when sperm were incubated briefly with solubilised zona pellucida in the presence of calcium (Table 1). Since solubilised zona pellucida also induced acrosome reactions, this evidence of hyperactivation may reflect an initial stage of the acrosome reaction.

In three replicate experiments, using three different males, sperm were exposed to a higher concentration of solubilised zona pellucida (1 ZP/µl) during a longer incubation period (30 min). Solubilised zona pellucida induced the acrosome reaction in 40.3 ± 7.57% of the treated sperm, significantly more than the 25.0 ± 5.29% background level of acrosome reactions of untreated sperm in calcium-supplemented medium (n = 3, α = 0.1, $p < 0.025$). These data are consistent with previous

Table 1 Acrosomal status and motility of capacitated guinea pig sperm following 10 min of incubation in different media

Treatment[a]	Percent acrosome-reacted	Percentage hyperactivated[b]
–Ca2+	0.33 ± 0.58	0.00
+Ca2+	16.00 ± 13.23	3.33 ± 0.76
+ZP	33.67 ± 11.50	8.67 ± 4.16

Data are mean values ± SD (n = 3).
[a]Calcium-free medium ($-Ca^{2+}$) was capacitation medium. Calcium-plus medium ($+Ca^{2+}$) was capacitation medium with 2 mM Ca Cl_2. Solubilised zona pellucida medium (+ZP) was calcium-plus medium with 0.05 ZP/µl.
[b]Percentage of hyperactivated sperm among the acrosome-intact sperm. All acrosome-reacted sperm were hyperactivated.

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studies which have identified zona glycoproteins as factors capable of stimulating the acrosome reaction (Bleil & Wassarman, 1983; Meizel, 1985; Meizel & Turner, 1986).

The capability of the zona pellucida to induce the acrosome reaction in homologous sperm was first demonstrated in the mouse (Bleil & Wassarman, 1983) and has since been confirmed in a number of other species (Yanagimachi, 1994). Taken together, our observations and experimental results suggest that the events of fertilisation in the guinea pig are similar to those proposed for other rodent species. Although our observations were limited by the resolution of the video camera, they are consistent with those made in the hamster model, which suggest that hamster sperm undergo acrosomal swelling during cumulus penetration but the acrosome reaction is not completed until sperm binding to the zona pellucida takes place (Yanagimachi & Phillips, 1984; Cherr *et al*., 1986). Most significantly, our observations clearly show that acrosome-reacted guinea pig sperm, like hamster sperm, are not capable of penetrating the cumulus investment of the oocyte, and they suggest that guinea pig sperm *in vivo* are unlikely to reach the zona pellucida in a fully acrosome-reacted state. Thus, sperm–oocyte interaction in the guinea pig does not appear to be exceptional and, as a consequence, the results of experiments performed in the guinea pig may have wider relevance to mammalian fertilisation than was previously supposed.

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