Nature of the rabbit acrosome reaction-inducing activity of follicular fluid

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Summary. Follicular fluid samples from rabbits, cats, pigs, women and cows had acrosome reaction-inducing activity (ARIA) on rabbit spermatozoa as determined by differential staining after incubation with these fluids. Activity was retained after dialysis and at least 50% was found to be labile when heated to 56°C for as little as 20 min. The induction of the acrosome reaction by bovine follicular fluid showed a dependence on the concentration of follicular fluid and spermatozoa and on calcium ions, and had a pH optimum of approximately 8. Enzyme treatments showed that proteases destroyed the ARIA and this activity was completely blocked by treatment of bovine follicular fluid with goat anti-bovine antiserum. Electron microscope observations indicated the similarity of the reactions observed to that occurring *in vivo*. It is concluded that the rabbit acrosome reaction-inducing activity of bovine follicular fluid is a serum component(s), probably a protein(s) of high molecular weight.

Introduction

The sperm acrosome, thought to be a differentiated lysosome (Fawcett, 1958), apparently functions during the fertilization process by releasing its contents of hydrolytic enzymes and thus facilitating sperm penetration through investments which surround the ovum. The process by which the enzymes may be released is the result of fusion and vesiculation of the plasma membrane specifically covering the acrosome and the outer acrosomal membrane. This process has been termed the acrosome reaction and occurs universally in mammalian spermatozoa (Barros, Bedford, Franklin & Austin, 1967). Ovarian follicular fluid has been shown to induce a functional acrosome reaction in hamster (Yanagimachi, 1969) and mouse (Iwamatsu & Chang, 1969), and to facilitate fertilization of rabbit ova *in vitro* (Rosado, Hicks, Reyes & Blanco, 1974; Oliphant, 1976).

The work presented in this paper attempts to provide data on the acrosomal changes induced in rabbit spermatozoa by follicular fluid, thus defining the rabbit system for further study and further characterizing the component(s) of follicular fluid affecting the sperm acrosome.

Materials and Methods

Follicular fluid was obtained from bovine or porcine ovaries collected from an abattoir. Ovaries were placed on ice immediately upon removal from the animal. Within 2½ to 3½ h fluid was aspirated from the follicles, pooled, divided into 0.5 ml aliquots, and stored frozen at –20°C until use. Human follicular fluid was collected by aspiration of follicles at the time of surgical procedures. Rabbit and cat follicular fluids were obtained from normal ovaries of animals used in this laboratory.

Rabbit spermatozoa were collected from New Zealand White bucks of proven fertility by use of an artificial vagina. The sperm concentration was determined with a haemocytometer and the ejaculates diluted to the required concentrations with CB medium (Cross & Brinster, 1970). Spermatozoa exposed for 5 min to an environment of 380 mosmol by treatment with a modified Krebs–Ringer–bicarbonate medium adjusted with NaCl: this was the High Ionic Strength (HIS) medium (Brackett & Oliphant, 1975). A volume of 25 µl of a suspension of pretreated rabbit spermatozoa was then transferred to the mixture for induction of the acrosome reaction. The reaction mixture, maintained at 37°C under paraffin oil, consisted of 50 µl CB medium equilibrated with 5% CO₂ in air and 50 µl of an

acrosome reaction-inducing agent. For control treatments, 50 μ l medium was used instead of inducing agent. These conditions were altered only during determination of the concentration dependence of the acrosome reaction-inducing activity in follicular fluid when appropriate volumes (to a total of 125 μ l) of medium and follicular fluid were used to obtain the desired follicular fluid concentration. When necessary for pH dependence assays, the standard medium was altered by addition of HCl or NaOH. Follicular fluid was dialysed against the adjusted medium for 12–18 h before use. The pH in the small volume of the reaction mixture was tested with litmus paper before and after the experiments to show that the buffering capacity was sufficient in each case to maintain the required pH during the reaction times.

For each assay, the time course of the reaction was followed by taking 25 μ l samples at 0, 60, and 120 min of incubation. The samples were fixed and stained by the method of Wells & Awa (1970) and 50–100 spermatozoa were counted for each sample to evaluate the completion of the acrosome reaction. Only spermatozoa from which acrosomes were completely removed were considered as reacted. For electron microscopy, samples were fixed in 3 % glutaraldehyde and post-fixed in osmium tetroxide. Sections were dehydrated in graded acetone, embedded in epon, sectioned and stained with uranyl acetate.

Follicular fluid treatments

Each of the enzymes (purest commercially available) used for treatment of follicular fluid was dissolved in CB medium at a concentration of 5 mg protein/ml. Activities of these enzymes were: pronase, 45,000 PUK units/g; trypsin, 2×10^6 BAEE units/g; α -amylase, 5000 SKB units/g; β -amylase, 785,000 maltose units/g; lysozyme, 175×10^5 units/g; lipase, 3000 Wilson units/g. Enzyme solutions were substituted for the CB medium to test the direct effects of these enzymes on spermatozoa. Follicular fluid was incubated with these solutions for 10 min before introduction of spermatozoa. Follicular fluid was also pretreated with trypsin which was bound to agarose (Miles Laboratory) to give 38.4 BAEE trypsin units/200 μ l. Preliminary experiments indicated that little if any trypsin was solubilized from the agarose gel. For equilibrium, the bound enzyme (200 μ l) was suspended in 0.5 ml follicular fluid for 5 min and recovered by centrifugation (10,000 g, 15 min). The bound trypsin was then suspended in 0.5 ml of the follicular fluid to be tested. After reaction for 10 min at 4°C and with continuous swirling the follicular fluid was separated from the bound enzyme by centrifugation (10,000 g, 15 min).

The effect of Ca^{++} on the acrosome reaction was determined. Follicular fluid was dialysed against 10^{-3} M-EDTA, pH 7.0, with an effective dilution of $1:2 \times 10^4$ and then half of the sample was dialysed (effective dilution $1:2 \times 10^4$) against CB medium (normally containing Ca^{++}) and the other half was dialysed against CB medium without Ca^{++} . The spermatozoa in the assays were initially washed in phosphate-buffered saline to remove calcium from the ejaculate, and Ca^{++} -free CB was used for both the HIS medium treatment and dilution of follicular fluid.

Attempts were also made to inhibit the acrosome reaction immunologically. Goat anti-bovine antiserum (Cappel Labs) was heated at 56°C for 30 min and dialysed against CB medium (without BSA). This antiserum preparation (50 μ l) was used instead of the CB medium in the assay mixture. Antiserum was allowed to react with follicular fluid for 30 min at 37°C before introduction of spermatozoa.

Results and Discussion

Species variation, dialysis and heating

The acrosome reaction of rabbit spermatozoa preincubated in HIS medium was readily induced in human, cow or pig follicular fluid, and less so in cat and rabbit follicular fluids (Table 1). Only 2–3 % of spermatozoa incubated in control CB medium lost their acrosomal caps, presumably through autolysis. These results are consistent with previous reports that bovine follicular fluid induces the acrosome reaction in hamster (Yanagimachi, 1969) and mouse (Iwamatsu & Chang, 1969) and that human follicular fluid induces the acrosome reaction of mouse, rat and human spermatozoa

Table 1. Induction of the rabbit acrosome reaction (% completion) by follicular fluids of various species

Species	Untreated	Dialysed	Heated 30 min at 56°C
Man	38 ± 7.4	30 ± 7.5	15 ± 1.0*
Cow	32 ± 9.9	33 ± 8	16 ± 2.6*
Pig	30 ± 3.3	34 ± 7	16 ± 3.1*
Cat	17 ± 4.2	—	—
Rabbit	12 ± 4.6	—	—

Values are mean ± S.D. for 6 determinations. The 1×10^7 spermatozoa/ml were incubated in 40% follicular fluid for 120 min.

* Statistically different from untreated follicular fluid values at least at $P = 0.01$ (χ^2 analysis).

(Mukerjee & Lippes, 1972). However, this activity is not confined to follicular fluid because the serum of many species effects these changes in hamster spermatozoa (Yanagimachi, 1970; Talbot, Franklin & Fussell, 1974).

No significant activity was lost when the follicular fluids were dialysed (Table 1), indicating and confirming (Yanagimachi, 1969) the macromolecular nature of the effective component(s) (mol. wt > 10,000). For the hamster, Yanagimachi (1969) noted the requirement for a small dialysable component for optimal results but such does not appear to be necessary for the rabbit.

Heating caused a 50–60% loss of activity (Table 1). When bovine follicular fluid was heated for 0, 5, 15, 30 and 60 min and used to induce the acrosome reaction, 50% of the activity (36% of spermatozoa reacted at 0 min) was lost after 5 min (18%) and there was only 64% loss after 60 min (13%). Heating followed by dialysis produced results similar to those of heating alone. It seems possible that two forms of activity were present, one being due to a very labile material and the other being relatively heat stable.

Follicular fluids have been reported as having a toxic effect on spermatozoa (Yanagimachi, 1969; Mukerjee & Lippes, 1972) and this has been overcome in some investigations by heating follicular fluid for 20–30 min at 56°C. Except for the experiments described above, we did not heat the follicular fluid samples used. Approximately 30% of the samples caused immobilization of spermatozoa (often because the spermatozoa were trapped in the coagulated follicular fluid), while in the remaining samples spermatozoa were viable for the duration of the experiment and motility ranged from 50 to 75%. Any samples in which < 50% of the spermatozoa were motile were excluded from the results.

Concentration of follicular fluid and spermatozoa

The extent of induction of the rabbit acrosome reaction was directly dependent on the bovine follicular fluid concentration (Table 2) as found by Yanagimachi (1969) for hamster spermatozoa. When the follicular fluid concentration of the assay mixture was maintained at 40% and the sperm concentration was varied, a dependence on sperm concentration was observed Text-fig. 1. If the regression line in Text-fig. 1 is extrapolated to low sperm concentrations, as would be the case under

Table 2. Dependence of induction of the rabbit acrosome reaction (% of completely reacted spermatozoa) on bovine follicular fluid concentration*

Time	Follicular fluid conc. (%)			
	0	20	40	80
60 min	2 ± 1.1	10 ± 2.8	28 ± 5.7	41 ± 11
120 min	2 ± 2.1	21 ± 3.8	42 ± 4.1	67 ± 1.5

*The final incubation mixture had a sperm concentration of 1×10^7 /ml and was incubated at 37°C under oil. Values are mean ± S.D. for 6 determinations.

physiological conditions, about 75% of the spermatozoa would undergo the acrosome reaction in 2 h. This is in general agreement with the findings of Talbot *et al.* (1974) for the hamster acrosome reaction induced by sera at various sperm concentrations.

Text-fig. 1. Dependence of induction of the rabbit acrosome reaction on sperm concentrations. After sperm pretreatment incubations were in a final concentration of 40% bovine follicular fluid at 37°C under oil. Each point is the average of 2 determinations and represents spermatozoa from 5 different bucks.

pH

When the effect on the acrosome reaction of pH values 5, 6, 7, 8, 9 and 10 was examined the response (mean $\pm$ S.D., 3 determinations, 2 h incubation) was 0 ± 2.1 , 10.5 ± 1.5 , 20 ± 4.1 , 29 ± 3.8 , 11 ± 3.2 and 3 ± 2.3 % reacted spermatozoa, respectively. The optimum response of pH 8 would presumably be similar to that in the oviduct, the pH of which is about 7.8 (Hamner & Williams, 1965). The activity of the acrosome reaction induced was irreversibly lost at pH 5 but not totally destroyed at pH 10. Activity in the control samples was 5–10% at pH 9 and 10 and < 2% at pH 8 or lower.

Hydrolytic enzymes

Treatment of follicular fluid with various hydrolytic enzymes (Table 3) indicated that proteases destroyed the activity of the acrosome reaction inducer but other enzymes had no significant effect. When spermatozoa were treated with the enzymes alone they were similar in appearance and motility to untreated controls. Deleterious effects resulted from pronase treatment, however: there was some separation of heads from tails and decreased motility, but the acrosomes still appeared intact.

To eliminate the possibility that the effect of the proteolytic enzymes was on the spermatozoa rather than on the follicular fluid, follicular fluid was pretreated with Agarose-bound trypsin. Most of the acrosome reaction-inducing activity was destroyed by the trypsin treatment: control untreated

Table 3. The effect of enzymatic treatment of bovine follicular fluid on the rabbit acrosome reaction (% complete)

Enzyme	Treatment of sperm.	Treatment of follicular fluid
α -Amylase	4 ± 2.8	35 ± 1.6
β -Amylase	3 ± 1.6	33 ± 3.0
Lysozymes	0 ± 0	30 ± 3.0
Lipase	1 ± 1	30 ± 1.0
Pronase	0 ± 0	0 ± 0
Trypsin	0 ± 0	0 ± 0
Control medium	2 ± 1.0	35 ± 7.5

Values are mean $\pm$ S.D. of 3 experiments.

Fig. 1. Thin section of ejaculated rabbit spermatozoa after incubation for 30 min in CB medium (no HIS-medium pretreatment). The nucleus (N) is surrounded over the anterior aspect by the acrosome (AR) which is enclosed by the inner (IAM) and outer (OAM) acrosomal membranes. The plasma membrane (PM) is continuous over the head. $\times 9800$.

Fig. 2. Tangential section of a spermatozoa preincubated for 5 min in HIS medium followed by a 30-min incubation in CB medium. The acrosome and its surrounding membranes remain intact. Arrows indicate the equatorial segment. $\times 890$.

Fig. 3. Tangential section of a spermatozoa pretreated in HIS medium and incubated for 30 min in the presence of bovine follicular fluid. Fusion has occurred between the outer acrosomal and plasma membranes with loss of the acrosomal substance. The inner acrosomal membrane (IAM) remains intact. V, vesicles. $\times 17,100$.

Fig. 4. Terminal stage of the acrosome reaction. The acrosomal cap has been shed and the contents released. All that remains is the inner acrosomal membrane with some adherent electron-dense material. Arrows indicate remnants of acrosomal material. $\times 12,100$.

follicular fluid gave $41 \pm 4.1\%$ (S.D., 6 determinations) induction while trypsin-treated follicular fluid gave $0.5 \pm 2.1\%$ induction. However, the surface of the spermatozoa could still have been affected by the trypsin in the experiments in which direct exposure occurred, and it is possible that trypsin-like enzyme within the spermatozoa could play a role in the acrosome reaction as suggested by Meizel & Lui (1976). The finding does, however, suggest that the acrosome reaction-inducing activity can be destroyed by proteolytic enzymes and that a protein is the effective follicular fluid component.

Calcium ion

Dialysis of follicular fluid against EDTA followed by dialysis against CB medium without calcium resulted in a decreased activity from $27 \pm 2.5\%$ to $5 \pm 1\%$ acrosome reactions, i.e. a loss of $>80\%$ of the reaction-inducing activity. When the EDTA-dialysed follicular fluid was redialysed against normal CB medium (containing calcium), $25 \pm 2\%$ acrosome reactions were induced in the same time, indicating that there was little or no loss of activity.

This calcium dependence is consistent with that reported by Yanagimachi & Usui (1974) for induction of the hamster acrosome reaction. Na^+ , K^+ and Mg^{++} are not involved because these are still present in the medium. In contrast to findings with the hamster, however, the rabbit acrosome reaction is not induced by calcium alone, as demonstrated by the negligible loss of acrosomes in control medium which contained 1.71 mM-CaCl_2 .

Antiserum

When antiserum directed against bovine serum components was used to pretreat bovine follicular fluid, induction of the acrosome reaction was completely inhibited, the % of acrosome-reacted spermatozoa being 2 ± 1.5 (S.D., 6 determinations) compared with 28 ± 3.8 for follicular fluid (40%) alone. The antiserum alone had little effect on the spermatozoa ($3 \pm 1.8\%$ acrosome reaction) within the 120-min incubation period. These results indicate that the factors in follicular fluid which induce the acrosome reaction are also present in serum. This is consistent with the results of others who showed that 'heat detoxified' serum will induce the hamster acrosome reaction (Yanagimachi, 1970; Talbot *et al.*, 1974). Albumin has been implicated as the protein factor necessary in the hamster acrosome reaction (Yanagimachi, 1970), but this is not the sole factor required for the rabbit reaction. Our experiments do not exclude a role for albumin but concentrations of 4–30 mg albumin/ml alone in a balanced salt medium do not induce the rabbit sperm acrosome reaction.

Ultrastructural observations

After incubation in control media (Pl. 1, Fig. 1), capacitation in HIS and incubation for 30 min in control medium (Pl. 1, Fig. 2), the sperm membranes were intact and ultrastructurally unchanged. After capacitation in HIS medium and incubation in follicular fluid (Pl. 1, Figs 3 and 4), the spermatozoa showed vesicle formation between the outer acrosome and the plasma membrane, the inner acrosomal membrane remaining intact, the apparent release of the contents of the acrosome and the ultimate loss of the outer acrosome membrane and adjacent plasma membrane.

These observations, like those relating to successful fertilization *in vitro* (Rosado *et al.*, 1974; Oliphant, 1976), suggest that the changes in the rabbit acrosome induced by follicular fluid *in vitro* are similar to those of the acrosome reaction *in vivo*.

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