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Sperm Binding Characteristics of the Murine Zona Pellucida

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ABSTRACT

Experimental conditions were established for an investigation of the sperm binding characteristics of the mouse zona pellucida. Fresh epididymal sperm binding to zonae of unfertilized eggs was a time-dependent process, reflecting the occurrence of sperm capacitation under *in vitro* incubation conditions. In contrast, capacitated sperm binding in large numbers to zonae of unfertilized ova occurred independently of temporal considerations. A dramatic difference was apparent in the binding of capacitated sperm to zonae of unfertilized eggs (>100 per zona) and to *in vivo* produced 2-cell embryos (0–5 per zona), providing a basis for assessing the occurrence of a zona reaction. Under comparable conditions, capacitated sperm bound in large numbers to zonae of *in vitro* produced 2-cell embryos. Possible reasons for a subnormal or inadequate zona reaction in eggs inseminated *in vitro* have been discussed.

INTRODUCTION

Sperm binding to the mammalian zona pellucida represents the initial step in sperm penetration of the mature, fertilizable ovum. The zona, comprised principally of glycoprotein (Austin, 1965), contains species specific sperm receptor sites that, in the hamster, are susceptible to modification by exposure to trypsin (Hartmann and Gwatkin, 1971).

In those mammalian eggs which undergo a zona reaction, the sperm binding properties of the zonae are presumed to alter substantially following fertilization. Indeed, dramatic differences in hamster zonae sperm binding characteristics have been demonstrated *in vitro* when zonae of unfertilized eggs were contrasted with those of penetrated eggs recovered from the oviducts of artificially inseminated animals or those of unfertilized eggs pretreated with cortical granule secretory product preparations (Barros and Yanagimachi, 1971; Gwatkin et al., 1973). The zona reaction *per se* is thought to result from the interaction of zonae with egg cortical granule material released in response to sperm penetration (Austin and Braden, 1956; Barros and Yanagimachi, 1971; Vacquier et al., 1972, 1973; Gwatkin et al., 1973). Changes in the solubility properties of zonae have also been associated with fertilization or the occurrence of a zona reaction in the rabbit, mouse and rat (Chang and Hunt, 1956; Gwatkin, 1964; Gould et al., 1971; Inoue and Wolf, 1974b), but not in the hamster (Chang and Hunt, 1956; Inoue and Wolf, 1975).

Interactions between fresh and capacitated sperm and isolated zonae or zonae surrounding unfertilized cumulus-free eggs from the hamster have been studied in some detail (Hartmann et al., 1972; Hartmann and Hutchinson, 1974a, b). In the present study, we have defined a zona sperm binding assay system for gametes of the mouse. Interactions between fresh and *in vitro* capacitated epididymal sperm and zonae surrounding unfertilized mouse eggs or *in vitro* and *in vivo* produced 2-cell embryos have been examined.

MATERIALS AND METHODS

The procedures employed in the recovery and handling of unfertilized eggs from superovulated Swiss mice have been described previously (Inoue and Wolf, 1974a, b). To disperse cumulus cells, egg clots were transferred to culture medium, pH 7.4–7.5, containing hyaluronidase (0.1 percent, Sigma Type I). After 5–10 min, denuded eggs were removed, washed three times and stored in culture medium until egg collection was completed. For sperm binding assays, completely denuded (cumulus-free) eggs were selected

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directly from hyaluronidase treated ova or were produced by manual removal (washing with a small bore micropipette) of the remaining corona cells from hyaluronidase treated ova. All subsequent manipulations were conducted at 37 C under oil (Silicon, Dow Corning 200 Fluid). *In vivo* fertilized 2-cell embryos were recovered from superovulated females as described previously (Inoue and Wolf, 1974b). To obtain *in vitro* fertilized 2-cell embryos, eggs in cumulus were transferred to a drop of medium (120–150 μl) under oil in a microdish and inseminated with 10 μl of a "capacitated" epididymal sperm suspension. Inseminated eggs in microdishes were transferred to French square bottles (4 oz.) and were incubated (26 h) at 37 C in the presence of 5 percent CO_2 in air (Mintz, 1967). "Capacitated" sperm suspensions were prepared by mincing 2 caudae epididymides in 0.4 ml of culture medium and incubating the suspension for 1–3 h prior to insemination (Toyoda et al., 1971a). Sperm concentrations varied from $1-3\times10^7$ cells/ml. Sperm were washed, where indicated, by centrifuging a suspension containing sperm from 2 pairs of epididymides in 0.8 ml for 5 min at 1400 × g. Collected sperm were resuspended in 0.8 ml, and the process was then repeated to give a minimum dilution of soluble epididymal secretions of 1 to 50. The culture medium employed in these studies was a modified Krebs-Ringer bicarbonate medium (Brinster, 1965) which contained the following (final concentrations): 1 mM sodium pyruvate, 25 mM sodium lactate, 5.56 mM glucose, phenol red 0.001 percent and 20 mg/ml BSA (Fraction V-Sigma). The medium was adjusted to pH 7.4 immediately before sterilization by Millipore filtration.

Trypsin (2× crystallized; Nutritional Biochem Co.) was employed in assessing the sensitivity of zonae sperm binding sites in unfertilized ova. Tryptic activity in culture medium was only slightly depressed by high BSA concentrations, as determined in control experiments monitoring the hydrolysis of Benzoyl-DL-arginine-β-naphthylamide-HCl (Meizel, 1972).

For measurements of the sperm binding properties of zonae, unfertilized eggs and 2-cell embryos (10 each) were transferred to the same plastic culture dish containing 0.2 ml of culture medium under silicon oil and inseminated with 20 μl of a 2–3 h preincubated capacitated sperm suspension. After a 5 min incubation, unless indicated otherwise, eggs were transferred to 3.7 percent formalin-saline solution containing 0.1 percent PVP-0.01 M phosphate at pH 7.2. Unbound or loosely bound sperm were removed by washing (gently sucking eggs in and out of a 150–200 μ diameter micropipette). Washed eggs were mounted on a slide, and the number of sperm adherent to zonae was scored. Formalin pretreated sperm did not bind to either the zonae of unfertilized or fertilized eggs in control experiments. In contrast, limited zonae binding (1–20 sperm/egg) could be demonstrated between aged sperm (standard epididymal sperm suspension held 24 h in culture medium at room temperature) and zonae of unfertilized ova in the absence of formalin exposure. To score sperm penetration of *in vitro* fertilized 2-cell ova, eggs were freed of sperm by pipetting, mounted, fixed and stained with aceto lacmoid and examined under the microscope (Toyoda and Chang, 1974).

RESULTS

Zona Sperm Binding Assay

Unfertilized hyaluronidase-treated mouse eggs (10 ova/assay) exposed to capacitated sperm become covered by large numbers of sperm bound by the head to their zonae (>100 per egg) (Fig. 1). The number of sperm that remained bound to zonae following removal from the insemination dish was dependent upon the experimental treatment employed, i.e., the number of washings, the diameter of the micropipette and the force used in expelling embryos from the micropipette during washing. However, even under vigorous washing conditions, some sperm remained adherent to unfertilized zonae. In marked contrast, zonae of 2-cell embryos recovered from natural matings retained limited numbers of capacitated sperm whose subsequent removal is readily accomplished by washing several times with a 150–200 μ diameter micropipette (Fig. 1). Since we were interested in characterizing fertilization-associated changes in the sperm binding properties of mouse zonae, the sperm binding assay developed utilized unfertilized ova and 2-cell embryos as internal controls. Washing of eggs or embryos following insemination was defined as the transfer of eggs and adherent sperm (with a 150–200 μ diameter micropipette) through culture medium until zonae of 2-cell embryos retained less than 10 sperm/zona.

The use of a spermicidal agent in zona sperm binding assays was desirable to prevent gamete interaction during washing and scoring and to allow precise control of incubation times. Accordingly, sperm binding to zonae of unfertilized ova or of 2-cell embryos was examined in the presence and absence of formalin (Vacquier and Payne, 1973). In triplicate experiments, 120 2-cell embryos were incubated for 5 min with capacitated sperm. Gamete interaction was then limited by formalin addition or by washing in medium alone. Formalin treated embryos retained 0–7 sperm/zonae following 3 washings, while embryos treated in medium alone retained 0–5 sperm/zonae. Zonae of unfertilized ova bound greater than 100 sperm each independent of the washing procedure employed.

Sensitivity of Zonae Sperm Binding Sites to Tryptic Attack

The sensitivity of sperm binding sites on zonae of unfertilized ova to trypsin was exa-

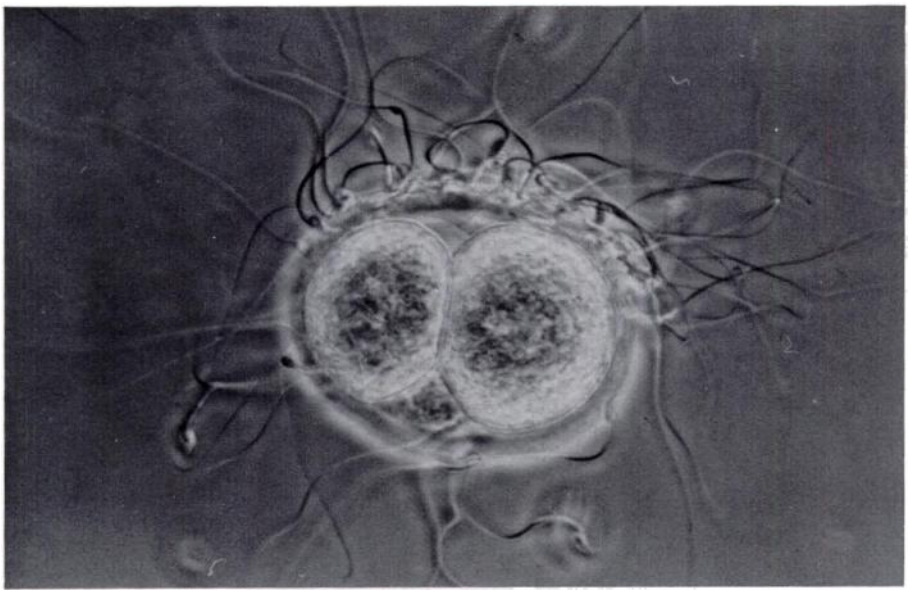

FIG. 1. Capacitated sperm binding to zonae of unfertilized eggs and *in vivo* produced 2-cell embryos. Eggs were inseminated for 5 min with 2 h preincubated sperm, washed in formalin-saline, mounted and photographed. Phase contrast X150.

mined. Hyaluronidase treated, unfertilized ova subjected to trypsin at concentrations as high as 500 μg/ml in culture medium for 30 min at 37° retained the ability to bind >100 sperm/zona. Experiments were conducted in the presence of impure preparations of serum albumin (Fraction V) as well as in crystalline bovine serum albumin. The details of a typical experiment follow. Cumulus-free unfertilized ova (60) were exposed to 200 μg/ml trypsin-5 mg/ml crystalline bovine serum albumin in culture medium for 30 min. Ova were washed 3X in culture medium, and lots of 10 were transferred to drops of culture medium, each containing 10 2-cell embryos. Capacitated epididymal sperm were added, and the mixtures were incubated for 5 min before sperm-egg interaction was terminated by transferring eggs and embryos to formalin. After washing, trypsin treated eggs retained >100 sperm per zona, identical to unfertilized egg controls and in marked contrast to zonae of 2-cell embryos. Identical results were realized in duplicate experiments employing different sperm preparations, indicating that sperm binding sites on zonae of unfertilized mouse ova are insensitive to tryptic attack.

Gamete Contact Time and Sperm Binding to Zonae of Unfertilized and In Vivo Fertilized 2-cell Embryos

The temporal dependency of sperm binding to zonae of unfertilized and 2-cell embryos was examined. Eggs or embryos were incubated for 5, 10, 30 or 60 min with capacitated sperm, washed and scored for sperm binding. Unfertilized eggs retained >100 sperm bound per zona, and 2-cell embryos retained 0–5 sperm per zona, at all times tested. Similar results were obtained in three separate experiments conducted with the gametes of different animals, and it can be concluded that sperm binding to these zonae types is insensitive to gamete interaction time.

Fresh Sperm Binding to Zonae

Fresh epididymal sperm do not bind in large numbers to zonae of unfertilized eggs or to zonae of 2-cell embryos following 5 min of gamete interaction (Fig. 2). However, binding of fresh sperm to zonae of unfertilized ova is a time dependent process. Thus, limited sperm binding was apparent following a 10 min incubation; and by 30 min, zonae of unfertilized ova bound approximately the same number of sperm (>100/zona), as was observed throughout the incubation with capacitated sperm. Plausible explanations for the temporal dependency observed for fresh sperm binding to zonae of unfertilized ova included the occurrence of sperm capacitation *in vitro* or a time dependent modification of zonae by sperm or by epididymal components. To dis-

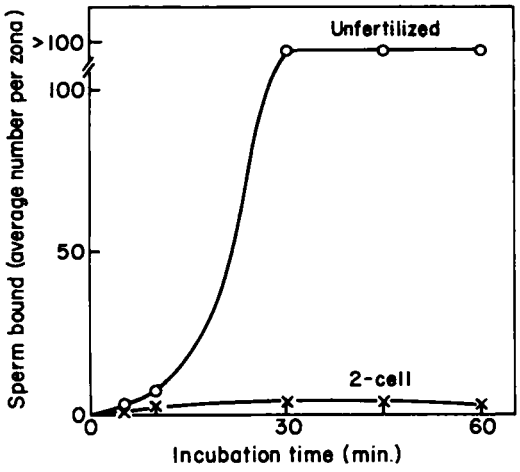

FIG. 2. Fresh epididymal sperm binding to mouse zonae as a function of incubation time. Fresh epididymal sperm were incubated for the times indicated with unfertilized eggs or *in vivo* produced 2-cell embryos (10 eggs or embryos per time point). Loosely bound sperm were removed by washing in formalin-saline before eggs were mounted for scoring.

tinguish between these possibilities, a sperm binding time course was conducted with fresh eggs and preincubated sperm and contrasted with the experiment presented in Figure 2 involving direct incubation of sperm and eggs. Identical results were obtained in the two experiments, indicating that the temporal dependency observed in fresh epididymal sperm binding is attributable to capacitation *in vitro*.

Sperm Binding to In Vitro Produced 2-Cell Ova

The sperm binding properties of zona pellucida surrounding *in vivo* produced 2-cell embryos were markedly different from those of embryos resulting from fertilization *in vitro*. Results obtained with fresh and capacitated sperm preparations are summarized in Table 1. Fresh sperm did not bind in large numbers to zonae from unfertilized eggs or either *in vivo* or *in vitro* produced 2-cell embryos. However, the binding of capacitated sperm to zonae of *in vitro* produced 2-cell embryos was nearly equal to that of unfertilized zonae (Fig. 3). This behavior was in marked contrast to the limited sperm binding capacity observed with zonae from *in vivo* produced 2-cell embryos under identical conditions. Similar results were obtained for 3 different experiments involving 40–60 eggs of each type. All of the *in vitro produced embryos exa*mined contained a fertilizing sperm tail, and some of these embryos were penetrated by only one sperm. The former observation discounts the possibility of parthenogenetic activation, while the latter is important in considering the contribution of multiple sperm penetration to a subnormal zona reaction in embryos fertilized *in vitro*.

To examine the possibility that epididymal components were contributing to the subnormal changes in zonae sperm binding properties, 2× washed sperm were prepared and employed during *in vitro* fertilization. However, zonae of 2-cell embryos resulting from *in vitro* insemination with washed sperm also displayed sperm binding characteristics (30–100 sperm/zona) intermediate between zonae of unfertilized ova and those of 2-cell embryos recovered from natural matings.

DISCUSSION

In defining a suitable sperm binding assay for investigating fertilization-associated changes in the mouse zona pellucida, attention was focused on the use of internal controls, i.e., unfertilized eggs and 2-cell embryos recovered from natural matings. Consequently, a rigorous definition of attached versus bound sperm, comparable to that reported for the hamster

TABLE 1. Sperm binding properties of the murine zona pellucida.[a]

Epididymal sperm added	Sperm bound per zona		
	Unfertilized eggs	2-cell embryos produced	
		In vivo	*In vitro*
Fresh, uncapaciated	0-5	0	0-5
In vitro capaciated	>100	0-10	30∿100

[a]Eggs were inseminated for 5 min with epididymal sperm, washed 3 times in 3.7% formalin-saline, mounted on a slide and scored.

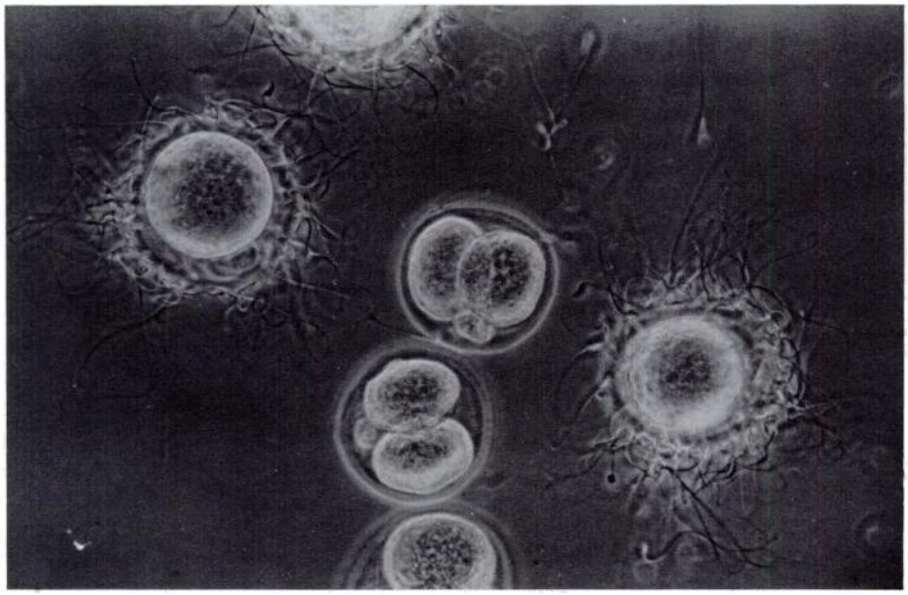

FIG. 3. Capacitated sperm binding to the zona of an *in vitro* produced 2-cell embryo. Embryos were inseminated for 5 min with 2 hr preincubated epididymal sperm, washed in formalin-saline and mounted. Zonae from *in vitro* produced 2-cell embryos bound nearly as many sperm as those of unfertilized eggs. Phase contrast ×300.

(Hartmann et al., 1972), is unavailable in the mouse. We do, however, presume that interactions between homologous gametes of the type characterized in this work carry physiologic implications. Unfertilized eggs, completely free of cumulus, were employed here in sperm binding studies, since sperm often attach to cells remaining on zonae, thereby invalidating quantitative measurements. Temporally, the sperm binding characteristics of naked zonae are obviously different from those of zonae surrounded by an intact cumulus. Moreover, the cumulus-free egg often shows an altered fertilizability (summarized by Fukuda et al., 1972). If the cumulus plays a role in limiting sperm access to the zona surface or, conversely, is instrumental in initiating sperm binding to the zona, the physiologic significance of sperm binding to the naked zona may be limited. However, under the high sperm concentrations normally employed for *in vitro* fertilization of hamster and mouse ova, premature cumulus dispersion occurs; and these ova often display abnormally high penetration levels (Yanagimachi, 1969; Iwamatsu and Chang, 1970; Inoue and Wolf, unpublished observations). Cumulus presence does not, therefore, appear critical to successful sperm-egg interaction.

Sperm binding sites on the unfertilized mouse zona are resistant to tryptic hydrolysis relative to those of hamster zonae (Hartmann and Gwatkin, 1971). This finding is consistent with the dramatic differences documented in the solubility properties of whole zonae from the two species. Thus, at least an order of magnitude difference exists in their susceptibility to solubilization by tryptic attack, the mouse being the more resistant (Chang and Hunt, 1956; Gwatkin, 1964). This observation, in conjunction with additional zonae solubility studies conducted in mercaptoethanol, low pH and periodate containing solutions, serves to document the species specificity inherent in zonae molecular architecture (Inoue and Wolf, 1975).

Capacitation can be defined as those changes in the sperm prerequisite to its penetration of the egg, specifically of the egg membranes including the zona pellucida (Pavlok and McLaren, 1972). In the mouse, fresh, noncapacitated epididymal sperm do not bind to zonae of cumulus-free unfertilized eggs. However, they quickly acquire this ability upon incubation in culture medium. A 30 min incubation was sufficient to transform a fresh epididymal sperm suspension to one that displayed an affinity for zonae comparable to that

of a 2 h preincubated "capacitated" sperm suspension. Previous studies (Toyoda et al., 1971a, b) support the conclusion that capacitation of mouse sperm occurs rapidly in culture. Based on these observations, sperm binding to the zona pellucida of unfertilized eggs represents a relatively simple, rapid method for detecting the presence of capacitated sperm. In the hamster, fresh, "uncapacitated" epididymal sperm bind to the zona of the unfertilized ovum immediately upon insemination while capacitated sperm binding is time dependent. Thus, species specificity is apparently of paramount importance when the sperm binding characteristics of hamster and mouse zonae are contrasted. The rather complex pre-penetration interactions between sperm and eggs of the hamster have been studied in detail (Hartmann et al., 1972; Hartmann and Hutchison, 1974a, b).

In the present study, a striking difference was documented in the sperm binding characteristics of zonae from cumulus-free unfertilized ova and those from *in vivo* produced 2-cell embryos. These findings support the existence of a zona reaction in which zona sperm binding sites are unavailable in the fertilized egg. The mouse is known to undergo a moderately effective zona reaction *in vivo* (Braden et al., 1954), and changes in zona solubility properties in both *in vitro* and *in vivo* produced 2-cell embryos relative to unfertilized eggs have been defined (Inoue and Wolf, 1974b). The temporal sequence of fertilization-associated changes in the mouse zona will be the subject of a separate report.

Zonae surrounding *in vitro* produced 2-cell embryos displayed somewhat reduced sperm binding properties when compared with unfertilized eggs but in marked contrast to zona of *in vivo* fertilized embryos. Epididymal fluid did not appear to contribute to this phenomena despite the earlier report that epididymal extracts alter penetration and fertilization of mouse eggs *in vitro* (Iwamatsu and Chang, 1971). Explanations for this phenomena may derive from the occurrence of a subnormal zona reaction or from zona modification following completion of the reaction. Subnormal changes in zona susceptibility to dissolution by pronase have been recorded for parthenogenetic mouse embryos with the suggestion that such changes can be attributed to inadequate release of cortical granule contents (Mintz and Gearhart, 1973). Fertilizing sperm tails were detected in all *in vitro* inseminated eggs examined in this study, and ultrastructural investigations confirm the complete dehiscence of cortical granules from *in vitro* inseminated eggs (Nicosia, Inoue and Wolf, unpublished results). The high incidence of polyspermy observed with *in vitro* inseminated hamster eggs has also been associated with subnormal zona changes. In this case, loss of cortical granule secretory product through multiple sperm slits produced in the zona of such eggs represents a plausible molecular mechanism accounting for the subnormal reaction (Barros and Yanagimachi, 1972). However, in the mouse, at least some *in vitro* produced 2-cell embryos were penetrated by a single sperm yet displayed zona sperm binding properties intermediate between the unfertilized egg and the *in vivo* produced 2-cell embryo, i.e., identical with those of other *in vitro* produced 2-cell embryos. The loss of cortical granule material from heavily penetrated eggs does not, therefore, appear to provide a plausible explanation for the zona sperm binding properties of *in vitro* fertilized mouse embryos. Experimentation designed to clarify the underlying differences in the zonae of *in vivo* and *in vitro* produced 2-cell embryos is currently in progress.

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