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Inhibition of Mammalian Fertilization *in Vitro* by Membrane Vesicles from Seminal Plasma (38033)

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Before mammalian spermatozoa express their fertilizing ability, they change in the female tract in some incompletely understood manner, termed capacitation [1–4]. Capacitated rabbit sperm recovered from the uterus of a doe several hours after mating are reversibly decapacitated when exposed to seminal plasma [5]. It was postulated that rabbit seminal plasma contains a macromolecular inhibitor of fertilization when ultracentrifugation was found to remove the inhibitory effect in a few hours [6–8]. Recently, two rapidly sedimenting fractions, consisting of distinct classes of vesicles, have been isolated from the seminal plasma of rabbit, bull and man [9]. The less dense, slower sedimenting vesicle fraction in rabbit seminal plasma inhibited fertilization following oviductal insemination of treated capacitated spermatozoa [10]. The sedimentation rate (ca 55S) of this fraction, moreover, conformed with that anticipated for the proposed inhibitor, which was reported to have a clearance time of approximately 3 hr in a 'Spinco' 40 rotor operated at 105,000*g* [6]. At the concentrations employed in this study [10], the denser, faster sedimenting vesicle fraction had no significant influence on the fertilizing ability of rabbit spermatozoa.

It has been implicitly assumed that a single factor causes decapacitation [11] and as rabbit epididymal fluid is attributed with decapacitation activity, it was theorized the factor present in seminal plasma may also occur in the epididymis [12]. This view suggested an obvious explanation for the need to capacitate epididymal sperm [13–15]. However, rabbit epididymal fluid was found to be relatively deficient in the vesicle fraction (Fr II) shown to cause decapacitation, whereas Fr I vesicles were abundant [9]. This suggested the former may not be the only factor capable of decapacitation and that possibly the latter is also active. Furthermore, since spermatozoa from at least ten mammalian species require capacitation [11] and as seminal plasma from several mammalian species is known to inhibit the fertilization of rabbit eggs [8], seminal plasma might be expected to inhibit fertilization in species other than the rabbit.

In the present investigation an endeavor was made to resolve the following questions: (a) Can seminal plasma fractions inhibit fertilization in other mammalian species in addition to the rabbit? For this purpose their influence on the fertilization of rat eggs has been studied. (b) Do both vesicle fractions inhibit fertilization? (c) Does inhibition of fertilization directly involve the gametes? This should permit its expression under defined *in vitro* conditions, such as those employed recently to capacitate rat spermatozoa [16–17].

Materials and Methods. Seminal plasma was obtained from fertile bucks of mixed strain with an artificial vagina. The fresh semen was centrifuged at 1000*g* for 30 min to sediment spermatozoa. The seminal plasma was aspirated, and frozen at −30°C until required. Fractionation of the seminal plasma was performed by sedimentation on a discontinuous sucrose density gradient with 20%, 40% and 60% (w/v) sucrose zones in Krebs-Ringer phosphate buffer. Centrifugation was performed at 25,000 rpm for 8 hr in an SW25.1 rotor using a

'Spinco' model L ultracentrifuge operated at about 4°C. Fractions of 1 ml were collected after puncturing a hole in the base of the cellulose nitrate tube containing the sucrose density gradient preparation. The ultraviolet absorbance by these fractions was measured at 270 nm. Fractions in each region of peak absorbance were pooled and dialyzed with Visking tubing against buffer. Fraction I and II are arrested by 60% and 40% sucrose zones, respectively [9]. Fraction III was recovered from the top of the gradient. These fractions were kept at 4°C and used within a few days of preparation. Protein concentrations have been determined with the method of Lowry [18].

Spermatozoa obtained from the cauda epididymis of mature Sprague-Dawley rats, CD strain, were suspended in 0.4 ml medium [16] containing 0.4% (w/v) bovine serum albumin and covered with mineral oil pre-equilibrated with 5% CO_2 in air [19]. Eggs were obtained 2–3 hr after ovulation from superovulated immature rats (23–24 days). They were released from an excised oviduct by means of a probe into 0.5 ml sperm suspension ($0.5–1 \times 10^{-6}$ spermatozoa/ml) which contained 10–30 μl seminal plasma fractions. After 8 hr incubation at 37°C, the eggs were mounted on a glass slide and stained with 0.25% lacmoid and subsequently examined for evidence of penetration or fertilization.

Following incubation spermatozoa were withdrawn from the medium with a microcapillary pipette, deposited on a cleaned glass coverslip, and allowed to air dry for a few minutes. The coverslips were then immersed for 1 hr in 1% glutaraldehyde and 0.1 *M* cacodylate buffer, pH 7.4, and they were then left overnight in 3% glutaraldehyde and cacodylate buffer. The fixed sperm cells were dehydrated in a graded ethanol series that was completed with 95% ethanol. After dusting with gold under vacuum, the sperm cell preparations were examined under a scanning electron microscope (AMR 900).

Data obtained concerning the frequency of egg penetration by spermatozoa, fertilization and polyspermy have been analyzed with the Chi-square test adjusted for data discontinuity by Yates' correction. Statistical significance was assessed in contingency tables with zero entries after analyzing the more probable arrangement having an entry of 5. In each test, this procedure permitted significance ($P < 0.05$) to be established.

Results. It can be seen in Table I that

TABLE I. Effect of Whole and Fractionated Rabbit Seminal Plasma on the Fertilization of Rat Eggs *in Vitro*.

Additive (μg protein/ml)	No. of eggs examined	Condition of eggs: Zona pellucida penetrated	Undergoing fertilization	Polyspermy (% fert. eggs)
		Number (%)		
Nil	81	49 (60)	43 (53)	1 (2)
10–30 μl Dialysate	87	50 (58) n.s.	48 (55) n.s.	4 (8) n.s.
Seminal plasma (266–525)	37	[a]	0*	0*
Fr I (83–155)	34	0*	0*	0*
Fr II (43–85)	72	0*	0*	0*
Fr III (38–175)	40	[a]	38 (95) *	27 (71) *

[a] Dissolution of the zona pellucida occurred under this treatment.

* Significant ($P < 0.05$) Chi-square difference from Control. n.s. not significant.

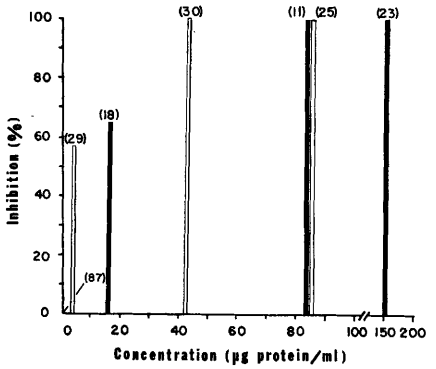

FIG. 1. Extent of inhibition in the fertilization of rat eggs *in vitro* by rabbit seminal plasma fractions I and II. A solid bar indicates Fr I and an open bar denotes Fr II. When dialysate from seminal plasma fractions was added to the incubation medium, no inhibition of fertilization was noted. The number of eggs examined is shown in parenthesis.

the presence of 266–525 µg protein/ml rabbit seminal plasma in the incubation medium blocked fertilization completely. Whereas, 38–175 µg protein/ml Fr III significantly enhanced fertilization (95 vs 53%) and the occurrence of polyspermy (71 vs 2%) compared with control values. Addition of vesicle containing fractions I and II, however, entirely blocked sperm penetration and fertilization at concentrations of 83–155 and 43–85 µg protein/ml, respectively. No significant change occurred in the rates of penetration and fertilization following addition of 10–30 µl dialysate obtained following the dialysis of seminal plasma fractions.

Figure 1 shows that the inhibition of *in vitro* fertilization by Fr I and II displayed a dependence on concentration over the range employed. At a concentration of 5 µg protein/ml Fr II caused a 57% inhibition in the fertilization rate and 17 µg protein/ml Fr I induced a 65% decrease. With concentrations above 43 µg protein/ml Fr II and 83 µg protein/ml Fr I fertilization was entirely blocked.

While the zona pellucida was removed from eggs exposed to seminal plasma and Fr III, it was retained in the other experimental groups. When Fr III was heated for 5 min at 65°C this property was lost. Nearly all Fr III treated eggs were fertilized and they frequently possessed more than one fertilizing spermatozoa. In the control group fertilized eggs were ordinarily monospermic (Table I). Spermatozoa failed to penetrate the vitelline membrane when seminal plasma was added to the incubation medium, although numerous sperm became attached to these eggs (Fig. 2).

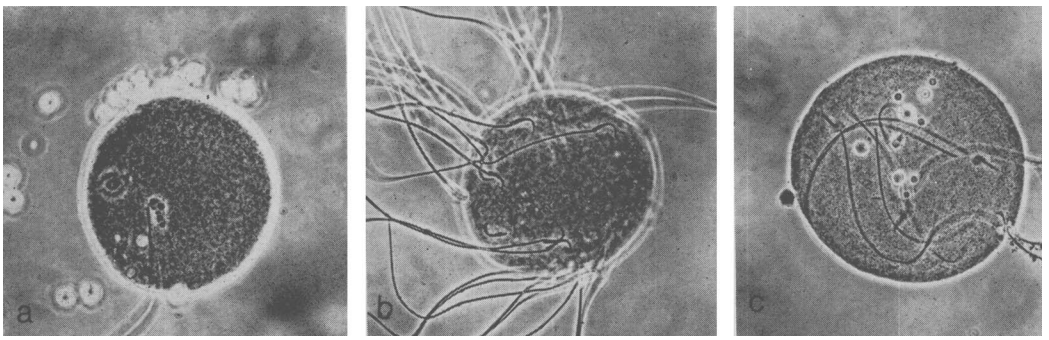

FIG. 2. Rat eggs eight hours after insemination with epididymal spermatozoa *in vitro* that were stained with lacmoid and photographed under phase contrast, mag. × 250. (a) A monospermic egg showing formation of male pronucleus. The zona pellucida was removed during fixation. (b) A zona-free unfertilized egg exposed to seminal plasma. None of the sperm cells attached to this egg pentrated the vitellus. (c) A zona-free polyspermic egg that was incubated with Fr III. Seven spermatozoa have penetrated the vitelline membrane as shown by enlarged sperm heads and the presence of pronuclei.

When spermatozoa from these experimental groups were scanned under an electron microscope it was observed that exposure to seminal plasma and seminal plasma vesicle fractions prevented loss of the plasma membrane over the acrosomal region of the sperm head (Fig. 3). Examination of sperm cells from medium to which no seminal plasma fractions had been added, revealed membrane breakdown and removal in this region.

Discussion. These observations demonstrate that the fertilization of rat eggs under defined *in vitro* conditions was inhibited by rabbit seminal plasma fractions I and II, which have been shown to consist of small vesicles [9]. Suppressed fertilization in does inseminated with uterine capacitated spermatozoa exposed to Fr II was attributed to decapacitation [10]. It may be noted that addition of 38–175 μg protein/ml nonsedimenting components of rabbit seminal plasma (Fr III) caused removal of the zona pellucida from treated eggs and elevated the fertilization rate. While 266–525 μg protein/ml whole seminal plasma blocked fertilization although it also caused dissolution of the zona. Consequently, an action on the zona pellucida by seminal plasma vesicles is unlikely to be responsible for the inhibition of fertilization with seminal plasma. Impairment of the capacitation process is however consistent with the effects of vesicle containing fractions on sperm cell plasma membranes seen in Fig. 3. Vesiculation and loss of the plasma membrane and outer acrosomal membrane was not observed in treated sperm cells. These events are believed to be necessary before fertilization by mammalian spermatozoa [20, 21].

When eggs from immature rats were pretreated with 200 μg/ml chymotrypsin [22] to remove the zona pellucida, 14.5% of these zona-free eggs became penetrated by epididymal spermatozoa during 4–8 hr *in vitro*. Whereas, untreated eggs were not penetrated at all. It was deduced that the vitelline membrane does not impede fertilization by uncapacitated spermatozoa and, as a corollary, that capacitation of spermatozoa only facilitates penetration of the zona pellucida. The results of the present experiments do not support this contention. For as noted, zona-free eggs that had been exposed to seminal plasma were not penetrated by epididymal spermatozoa. It is

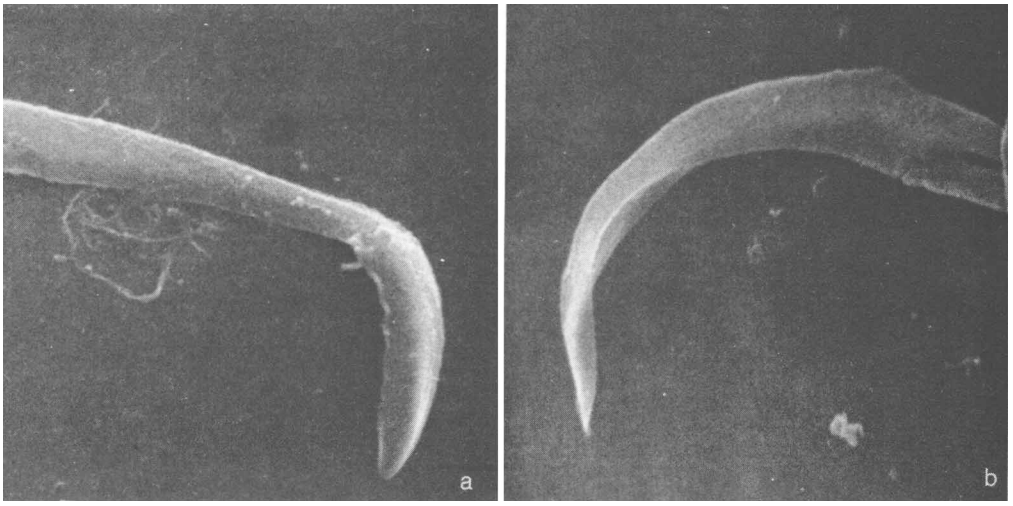

FIG. 3. Rat epididymal spermatozoan heads seen under a scanning electron microscope following incubation *in vitro* for 8 hr (mag. × 7100). (a) A sperm cell from medium to which no seminal plasma fractions were added; this medium allowed fertilization. The plasma membrane over the acrosome region has been largely removed. (b) The plasma membrane is intact in this sperm cell that was incubated in a medium containing 83 μg protein/ml Fr I. Fertilization was completely inhibited in this medium.

difficult to discount the possibility that some degree of capacitation was achieved by the chymotrypsin treated spermatozoa [22]; parenthetically, the fertilization rate (30%) approximately doubled when uterine (presumably already capacitated) spermatozoa were incubated with these zona-free eggs.

Since epididymal fluid has a relatively high concentration of Fr I vesicles [9], addition of epididymal sperm in excessive numbers to the incubation medium would be detrimental to fertilization [17] because of the presence of these vesicles. Moreover, their presence in epididymal fluid could account for the need to capacitate epididymal sperm of the rat and various other species [13–15] and the decapacitation of rabbit spermatozoa by this fluid [12]. Evidently, the inability of 48–160 μg protein/ml Fr I to decapacitate rabbit sperm that were inseminated into the oviducts of does after ovulation [10] resulted from insufficiently high concentrations for this *in vivo* assay, rather than a lack of decapacitation activity. Fraction II was more inhibitory than Fr I in both *in vitro* and *in vivo* systems.

In the *in vitro* assay used in this work, 5 μg protein/ml Fr II inhibited fertilization by 57% (Fig. 1). A previous study [10], which involved the *in vivo* assay [5], showed 3.2–7.2 μg protein/ml Fr II was ineffective and that concentrations above 20 μg protein/ml were needed to decapacitate rabbit spermatozoa. More efficient capacitation *in vivo* could possibly account for this difference in sensitivity between the *in vitro* and *in vivo* assay systems. The nature of the biochemical changes produced in sperm cells by these seminal plasma vesicles is an intriguing problem that is presently under investigation.

Summary. Vesicle fractions I and II, isolated from rabbit seminal plasma following ultracentrifugation, inhibited fertilization of rat eggs *in vitro*. Fertilization was reduced by 65% when the medium contained 17 μg protein/ml Fr I and by 57% when it had 5 μg protein/ml Fr. II. It was completely prevented with 83–155 and 43–85 μg protein/ml Fr I and II, respectively. Addition of 266–525 μg protein/ml whole seminal plasma or 38–175 μg protein/ml nonsedimenting seminal plasma components (Fr III) caused dissolution of the zona pellucida by constituents that were heat labile. Fertilization was inhibited by seminal plasma and promoted by Fr III. Electron micrographs of spermatozoa following incubation indicated that vesicle containing fractions from rabbit seminal plasma prevent breakdown of the plasma membrane covering the acrosome region of the head. The results are interpreted as suggesting that both classes of seminal plasma vesicles block fertilization by inhibiting sperm capacitation under defined *in vitro* conditions.

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