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A Demonstration of Cat Seminal Plasma Antifertility Activity (37757)

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About 15 years ago, Chang (1) made the significant observation that capacitated rabbit sperm lose their ability to fertilize eggs *in vivo* after they are incubated with rabbit, bull, or human seminal plasma. These decapacitated sperm regain their fertilizing ability after further female tract incubation. These observations were the basis for the hypothesis that capacitation involves the removal of functional inhibitors from spermatozoa (2). One method to support such a hypothesis is to determine if the seminal plasmas of other mammalian species whose sperm require capacitation contain these inhibitors of fertilization. This has been shown to be the case for the rabbit, boar, bull, stallion (3), monkey, human, and perhaps for the dog (4). This work, then is directed toward expanding this list by proving that cat seminal plasma contains antifertility activity.

Materials and Methods. Cats (*Felis catus, L.*) were obtained from the University of Virginia Vivarium breeding colony. Rabbits were New Zealand whites, obtained from licensed dealers.

Four basic experiments were performed. First, cat seminal plasma (CSP) was assessed for its ability to inhibit rabbit sperm from fertilizing rabbit eggs using a modification of Chang's assay for decapacitation factor (1) and Brown and Hamner's *in vivo* assay for capacitation (5). Rabbit sperm were collected by artificial vagina from four to six bucks of proven fertility. After a dilution with physiological saline to a concentration of approximately 5–10 $\times$ 10^6 sperm/ml, 0.1 ml of this sperm suspension was injected into the uterine cornua of an estrous doe after surgical exposure of her uterus. Sixteen hours after deposition of the sperm, they were recovered by flushing the uterus with 37° Krebs–Ringer phosphate bicarbonate buffer (KRPB) containing 0.31% bicarbonate and 0.30% crystalline bovine sperm albumin (Pentex– A grade) at 7.8. The population of capacitated sperm was divided into two equal parts and centrifuged at approximately 500*g* for 4 min. Both supernatants were discarded. The control sperm pellet was resuspended in 0.20 ml 37° KRPB while the experimental pellet was resuspended in 0.20 ml 37° cat seminal plasma (CSP) collected previously by artificial vagina from 10 healthy toms, centrifuged at 700*g* for 15 min, and the supernatant stored at —20° until use. Both control and experimental suspensions were allowed to incubate for 20 min at 37°. Both were then recentrifuged at 500*g* for 4 min, and both sperm pellets resuspended in 0.20 ml KRPB. One-tenth milliliter of the control sperm suspension was inseminated through the infundibulum into the right oviduct of one egg-donor doe and into the left oviduct of another. Experimental sperm suspensions were introduced into the contralateral oviducts. Both egg-donor rabbits had been given an ovulation-inducing intravenous injection of 50 IU of human chorionic gonadotropin (Follutein-E.R. Squibb and Sons, Inc.) 12.75 hr earlier. Thus, viable eggs near the end of their fertilizability were present in the oviducts when the sperm were deposited. Egg donor rabbits were killed 30 hr later, their oviducts flushed with physiological saline, and the eggs examined for evidence of fertilization (usually the presence of four blastomeres within the zona). As a viability check, CSP-incubated sperm were deposited in the oviducts of egg donors 4 hr prior to ovulation to determine if CSP-treated sperm

could be recapacitated.

The second and third experiments utilized this same procedure. In the second experiment, CSP was diluted to ½, ⅓, ⅕, and its original concentration with KRPB. It was then tested for antifertility activity. In the third experiment, CSP was heated to either 60 or 100° for 10 min before its antifertility activity was assessed.

The fourth experiment was a preliminary attempt to identify one of the possible factors involved in CSP antifertility activity. It is known that rabbit seminal plasma contains two trypsin inhibitors, at least one of which must be removed from sperm acrosin, an acrosomal proteinase, as a part of capacitation (6). This may activate the acrosin, allowing the sperm to penetrate the zona pellucida (7, 8). Based on this, CSP was examined for trypsin inhibitor activity. CSP (0.01 ml) was preincubated for 5 min with bovine pancreatic trypsin (Calbiochem; B grade) before its activity was assayed by a standard BAEE method (9). Two-tenths milliliter of trypsin solution (0.01 mg/ml) in 0.001 *N* HCl was added to 3.0 ml of 0.1 *M* borate buffer, pH 8.0, containing 0.05 *M* $CaCl_2$. CSP (0.01 ml) was also added at this time. The trypsin/CSP solution was allowed to incubate for 5 min whereupon 0.2 ml of a solution containing 1.7 mg/ml *N*-benzoyl-L-arginine ethyl ester (BAEE; Sigma) in borate buffer was added to the mixture. After a 30-sec equilibration, the change in absorbance at 253 nm was monitored for 5 min. One milliunit of trypsin activity was defined as causing a change of 0.001/min in the optical density of the 3.4-ml assay. One inhibitor milliunit (Imu) is defined as causing a decrease in optical density change of 0.001/min in the same assay system.

TABLE I. Effect of Cat Seminal Plasma on Fertilizing Capacity of Capacitated Rabbit Sperm.

	Sperm treatment			
	Krebs–Ringer phosphate		Seminal plasma[a]	
Expt no.	No. ova	% Fertilized	No. ova	% Fertilized
1	4	25	1	0
2	12	17	4	0
3	4	50	2	0
4	9	44	7	0
5	3	33	4	0
5	32	31	18	0

[a] Sperm were treated with cat seminal plasma for 20 min prior to their insemination into the oviduct of egg donors.

TABLE II. Effect of Dilution on Antifertility Activity of Cat Seminal Plasma (CSP).

Sperm treatment	No. ova	No. fert.	% Fert. (range)	No. expt
Control (KRP)	32	10	31 (17–50)	5
Whole CSP	18	0	0 (—)	
Control	33	21	64 (25–86)	5
1/2 CSP	25	11	44 (0–67)	
Control	50	25	50 (18–73)	5
1/3 CSP	22	8	36 (0–57)	
Control	32	16	50 (11–100)	5
1/5 CSP	29	14	48 (0–75)	
Control	11	4	36 (0–100)	5
1/10 CSP	14	5	36 (0–100)	

CSP was also heated to 60° or 100° for 10 min and the effect of this treatment on CSP trypsin-inhibiting activity was assessed by the above procedure.

Chi square with Yate's correction was used to determine significance of the fertilization results. Linearity of the progressive decrease in CSP antifertility activity upon dilution was determined by the least-squares method on a Wang Series 370 electronic calculator.

Results. Table I shows the effects of exposing capacitated rabbit sperm to CSP prior to their insemination into egg donors. In five experiments, control sperm fertilized a total of 10 of 32 ova, while CSP-incubated sperm fertilized none of 18 ($P < 0.01$). When CSP was progressively diluted from full strength

TABLE III. Effect of Heating on Antifertility Activity of Cat Seminal Plasma.

Sperm treatment[a]	No. ova	No. fert.	% Fert. (range)	No. expt
Control (KRP)	32	10	31 (17–50)	5
CSP unheated	18	0	0 (—)	
Control (KRP)	26	19	73 (33–100)	5
CSP 60°	29	3	10 (0–20)	
Control (KRP)	39	26	67 (29–90)	5
CSP 100°	50	14	28 (11–46)	

[a] Cat seminal plasma (CSP) heated 10 min.

to 1/10 strength, the antifertility effect became progressively less in a linear fashion (correlation coefficient > 0.95). That is, the percentage of the control fertilization rate shown by the treated sperm increased linearly (slope $= 1.12$) as the percentage volume of CSP in the sperm incubation media decreased (Table II). Heating CSP to 60° for 10 min had a negative effect on its antifertility activity (Table III), causing an increase of the fertilization rate of CSP-treated sperm from 0 to 10%. Boiling the CSP for 10 min further reduced, but did not destroy its antifertility activity. Both 60° and 100° heated CSP-incubated rabbit sperm fertilized significantly fewer ova than their respective control sperm ($P < 0.005$ in both cases).

CSP (0.010 ml) contains trypsin inhibitor activity at a level of approximately 13.4 inhibitor milliunits, *i.e.* 1340 Imu/ml. Upon heating CSP to 60° for 10 min, the value drops to 1090 Imu/ml. Boiling CSP for 10 min further reduced trypsin inhibitor activity to 223 Imu/ml.

CSP did not affect rabbit sperm viability, since the sperm could be "recapacitated" and would fertilize eggs at a rate not significantly different from similarly treated control sperm fertilization rates if insemination took place prior to ovulation of the egg donors.

Discussion. The capacitation process may involve the removal of seminal plasma acrosomal enzyme inhibitor(s) from spermatozoa (2, 6). Since these inhibitors have been shown to be both bound to sperm and free in the seminal plasma (2, 4, 6, 8, 10, 11), one would expect that those species requiring capacitation would show antifertility factors in their seminal plasmas. As stated in the introduction, this has been shown for the rabbit, boar, bull, stallion, primate, and dog. Recently, it has been shown (12) that cat sperm also require capacitation. Therefore, the real significance of these preliminary observations is not that they demonstrate antifertility activity in CSP *per se*, but that they extend the applicability of this capacitation scheme to another species.

Originally, the antifertility activity in rabbit seminal plasma was assigned the name "decapacitation factor," D.F. (1). Unfortunately, this nomenclature led to much confusion due to species differences and due to the fact that D.F. activity may not be the result of single molecule. (See Ref. 10 for a review of this problem). In the rabbit, for example, D.F. is associated with a several hundred thousand molecular weight proteinaceous material (13). D.F. itself may be a smaller polypeptide (14) that is just one of several seminal plasma inhibitors of the sperm acrosomal enzymes which participate in fertilization (6, 14–16). Therefore, antifertility activity of seminal plasma may be the functional result of the redeposition of its several acrosomal enzyme inhibitors upon capacitated sperm.

CSP exhibits an analogous situation, insofar as these experiments have shown it to contain both antifertility and trypsin inhibitor activities. And, since rabbit and human seminal plasma both contain at least one inhibitor which inhibits both bovine pancreatic trypsin and acrosin (17, 18), it could be assumed that CSP contains an inhibitor for cat sperm acrosin.

Summary. CSP has a reversible, partially heat-labile, antifertility effect on rabbit spermatozoa. In an *in vivo* fertilization assay for capacitation, CSP-treated sperm fertilize significantly fewer ova than do control sperm ($P < 0.005$). Heating the CSP to 60° for 10

min destroys some of the activity, while heating it to 100° for 10 min further attenuates it. In both cases, CSP-treated sperm still fertilize fewer ova than do control sperm ($P = 0.005$). CSP also contains partially heat-labile trypsin inhibitor activity which may be partially responsible for the antifertility activity.

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