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Effects of Testis Hyaluronidase and Seminal Fluids on the Fertilizing Capacity of Rabbit Spermatozoa.

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An enzyme, extracted from mammalian testis, which increases the permeability of skin and other tissues containing hyaluronic acid is known as hyaluronidase.[1-4] The cumulus cells surrounding the tubal ova of the rabbit were shown to be dispersed in the presence of fairly large numbers of sperms by a heat labile enzyme,[5,6] now identified as hyaluronidase.[7-10] On the basis of these observations, the participation of hyaluronidase in fertilization,[7-10] the capacity of hyaluronidase to increase the fertilizing power of sperms,[11] and the clinical use of hyaluronidase for sterility[12] have been reported. Rowlands' report[11] on increased fertilizing capacity in rabbits was based on the use of heated seminal fluid which contains hyaluronidase. In the following experiments we have examined the effects of purified testis hyaluronidase on fertilization *in vivo*.

Methods. Adult non-pregnant doe rabbits were superovulated according to Pincus.[13] They were inseminated with a minimal effective number of spermatozoa (number of sperms needed to fertilize only a small number of ova) suspended in different fluids just before the intravenous injection for the induction of ovulation. The rabbits were sacrificed 25 to 30 hours later and the ova were flushed out from the oviducts. The fertilized and unfertilized ova were counted.

The semen of a single male rabbit was collected with an artificial vagina[14] for insemination in order to control the variation of sperm quality between different individuals. The interval between each collection of sperm was 3 to 4 days in order to keep the sperm quality constant. The general method of insemination was carried out according to Walton.[15,16] The semen just after collection was diluted with saline (0.9% NaCl) about 1 part to 1,000. Then the sperm concentration was immediately counted by means of the hemocytometer technique. Saline or sperm was added if the concentration of sperms was too high or too low. Then 0.5 ml of this suspension was added to one of the following: (1) 0.5 ml of saline containing a known amount of hyaluronidase, (2) 0.5 ml of supernatant fluid of normal semen after heating, (3) 0.5 ml of semen from a vasectomized male, (4) 0.5 ml of saline serving as a parallel control. These mixtures (all 1 ml in volume) were introduced into the vagina of each rabbit. Usually, 6 rabbits were inseminated at a time. The time interval between collection of semen and the first insemination was about 10 minutes, and that between the first and last insemination was about 20-30 minutes.

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The testis hyaluronidase used was obtained from bull testicles (Schering Corporation). The seminal hyaluronidase was prepared according to Rowlands;[11] *i.e.*, normal semen was heated at 50° C for 8 to 11 minutes to kill sperms, and then kept on ice for 5 minutes and centrifuged. The supernatant fluid was used. After insemination, the heated seminal supernatant fluid and the vasectomized male semen were assayed by the viscosimetric method[17] and expressed as mg of testis hyaluronidase. The number of spermatozoa in the suspension was counted again 4 to 6 times and the average number of sperms inseminated was calculated.

Results. The complete data of these experiments are presented in Table I. It is evident that the fertilizing capacity of spermatozoa was not affected when testis hyaluronidase (1 to 0.65 mg per ml) was added. The average percentage of fertilized ova for 10 experimental does was 30 and that of 10 parallel control does was 27. The total number of fertilized ova in the experimental group was 70 out of 203 (34%), while that in the control group was 48 out of 198 (24%).

The average percentage of fertilized ova (60%) in the 13 experimental does inseminated with supernatant fluid of normal semen which contained hyaluronidase (1.05 to 0.2 mg per ml) was higher than that of 13 parallel control does (38%). But there is no statistical significance of the difference ($t=1.56$, $P<0.2>0.1$). However, the total number of ova fertilized in the experimental group (62%) is higher than that of the parallel control group (39%).

The average percentage of fertilized ova in those 10 does inseminated with sperms suspended in saline and the semen of vasectomized bucks which contains no hyaluronidase was 63, while that of 9 parallel control does was only 15. The difference is statistically significant ($t=4.66$, $P<0.01$). The total number of ova fertilized in the experimental group (65%) is higher as compared with that in the parallel control group (21%).

It is quite clear from these data that the extra hyaluronidase added to the sperm suspension does not influence the fertilizing capacity of spermatozoa. On the other hand, seminal fluid with or without hyaluronidase does increase the fertilizing capacity of rabbit spermatozoa.

Discussion. It is a common thought that seminal fluid is not important for the fertilizing capacity of spermatozoa because the epididymal spermatozoa,[15,16,18] spermatozoa separated from seminal fluid by centrifugation,[19] and semen in a very diluted form[20,21] are able to insure fertilization. The present investigation, however, reveals clearly that the importance of seminal fluid shows up when the number of spermatozoa is decreased to a minimum. Thus, any disturbances of accessory glands may affect the fertility of a male though clinical data on this point are still scarce.[22]

The great variation in the percentage of fertilized ova per doe (Table I) under strictly controlled experimental conditions leads one to reject those positive conclusions based upon only a few clinical cases or based on some experimental studies without strict control of variations of sperm quality in different individuals and in different time intervals of collection in the study of such an intrinsic process as fertilization.

Although the dispersal of cumulus cells surrounding the ovum by sperms or by hyaluronidase *in vitro* is unquestionable, the role of hyaluronidase in the complicated process of fertilization *in vivo* is still uncertain. Even if hyaluronidase *per se* plays an important role in fertilization, the hyaluronidase of spermatozoa is quite adequate to perform its function without further addition of hyaluronidase.

Summary. Thirty-three doe rabbits were inseminated with a minimal effective number of spermatozoa suspended in saline containing

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TABLE I. Effect of Hyaluronidase and of Seminal Fluid on the Fertilizing Capacity of Rabbit Spermatozoa.

Experimental series	Exp. No.	No. of sperms inseminated × 1000	Experimental group (E) Does No.	Conc. of hyaluronidase mg/ml	Experimental group (E) No. ova Fertilized	Unfertilized	Total	% fertilization	Control group (C) Does No.	Control group (C) No. ova Fertilized	Unfertilized	Total	% fertilization	% difference in fertilization (E-C)
Testis Hyaluroni-	2	228	410	1	2	13	15	13	411	18	3	21	86	—17
			413		20	19	39	51	414	2	11	13	15	
idase added	3	226	416	”	0	2	2	0	417	0	23	23	0	—15
			419		0	28	28	0	420	7	18	25	28	
	4	342	363	”	13	0	13	100	365	0	27	27	0	27
			371		6	31	37	16	378	4	6	10	40	
	6	50	421	.65	23	6	29	79	423	2	11	13	15	48
			424		0	13	13	0	426	1	29	30	3	
	8	116	337	”	0	11	11	0	434	7	13	20	35	—17
			436		6	10	16	38	437	7	9	16	44	
Avg % of fertilization per doe								30					27	3
Total No. of ova					70	133	203	34		48	150	198	24	10
Supernatant fluid	5	274	394	.625	20	0	20	100	393	23	0	23	100	— 2
			397		31	3	34	91	400	12	1	13	92	
of heated nor-	6	50	422	.20	0	18	18	0	423	2	11	13	15	1
			425		3	16	19	16	426	1	29	30	3	
mal semen added	7	111	472	.325	19	11	30	63	428	14	2	16	88	— 3
			431		5	5	10	50	432	0	6	6	0	
	8	116	433	.75	12	0	12	100	434	7	13	20	35	50
			435		5	2	7	71	437	7	9	16	44	
	9	96	444	.56	10	0	10	100	445	2	12	14	14	34
			446		1	12	13	8	447	1	7	8	13	
	10	130	456	.5	16	7	23	70	458	3	24	27	11	59
	11	173	460	1.05	10	6	16	63	461	1	18	19	5	— 3
			463		1	1	2	50	464	18	8	26	69	
Avg % of fertilization per doe								60					38	22
Total No. of ova					133	81	214	62		91	140	231	39	23
Semen from	10	130	454	.05†	16	0	16	100	455	0	11	11	0	78
			457		22	6	28	79	458	3	24	27	11	
vasectomized	11	173	459	0	11	8	19	58	461	1	18	19	5	16
			462		7	5	12	58	464	18	8	26	69	
bucks added	12	150	467	0	4	4	8	50	466	1	12	13	8	49
			469		24	8	32	75	468	1	26	27	4	
									470	13	19	32	41	
			508	0	19	8	27	70	510	0	16	16	0	53
	17	125	511		6	17	23	26	513	0	9	9	0	
			509*	0	10	3	13	77						
			512*		6	9	15	40						
Avg % of fertilization per doe								63					15	48
Total No. of ova					125	68	193	65		37	143	180	21	44

*Semen of vasectomized male heated at 50°C for 20 minutes was used. † Very few dead sperms present.

purified testis hyaluronidase, or saline and supernatant fluid of heated normal semen containing hyaluronidase, or in saline and semen of vasectomized buck containing no hyaluronidase. Thirty-two does were inseminated at the same time with the same number of sperms collected from the same rabbit but suspended in saline, serving as parallel controls. It was found that it was the seminal fluid, not hyaluronidase, which really increased the fertilizing capacity of spermatozoa.

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Range of Antibiotic Activity of Protoanemonin.*†

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Extracts of buttercups and *Anemone pulsatilla* exert an inhibitory action on the growth of a number of pathogenic bacteria.[1,2] The active principle extracted from *A. pulsatilla* proved to be protoanemonin.[3] The present studies deal with the titration of the antibiotic activity of protoanemonin when tested against a variety of bacteria and fungi, as well as a few representatives among the viruses and protozoa.

Methods of testing the bacteria. The protoanemonin used in these experiments was extracted from dried ground *A. pulsatilla* or was prepared synthetically, as described previously.[3] The stock was a 1-100 dilution by volume in sterile distilled water from which further dilutions in the test media were prepared.

The *in vitro* susceptibility of the bacteria, with the exception of the Mycobacteria, was determined by inoculating 0.5 cc of a 10^{-3} dilution of a 24- or 48-hour culture of the test organisms into 4.5 cc of media containing decreasing concentrations of the antibiotic. The media chosen, as indicated in the tables, were those which provided favorable growth conditions for the bacteria under investigation. In some cases the organisms were tested when grown in each of 2 media. After a period of incubation at 37°C, sufficient for optimum growth of the control tubes, containing no protoanemonin, the cultures were examined for the presence of visible turbidity or other evidence of growth, such as the production of a pellicle, pigment or gas. The maximum dilution of protoanemonin capable of preventing the appearance of growth in the period of time specified is recorded in the tables.

Testing of the Mycobacteria required modifications in technique. A loopful of pellicle 1 cm in diameter from a 25-day-old culture was used to inoculate 100 cc volumes of

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