Effects of Female Reproductive Tract Secretions on Rabbit Sperm III. Acrosomal Enzyme Changes Related to Capacitation (37361)

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(Introduced by B. C. Wexler)
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The release of hyaluronidase by rabbit sperm incubated in uterine fluid is related to the presence of a specific macromolecular component (1, 2). Uterine fluid which does not contain this component will block the release of sperm hyaluronidase. This macromolecular component is readily found in post-ovulatory uterine fluid and may be present in pre-ovulatory uterine fluid, but in greatly reduced concentration. Although our evidence indicated that hyaluronidase was being released by the sperm (1, 2) we wished to further confirm this observation by measuring changes in the sperm acrosomal content following incubation in uterine fluid.

Purified hyaluronidase has no effect on the zona pellucida of rabbit ova. Instead, sperm apparently contain an acrosomal proteinase which is capable of removing the zona pellucida from rabbit ova (3). Although proteolytic enzyme can be chemically extracted from rabbit sperm (4), it is not known whether the zona-penetrating enzyme is released by the sperm or whether it functions in a membrane-bound form (5). In this work we have investigated the changes in proteolytic activity in rabbit sperm incubations as well as changes in acrosomal proteolytic content. Thus, the work described in this report is a continuation of our studies on the relationship of rabbit uterine fluid, sperm acrosomal enzymes, and the process of capacitation (1, 2).

Methods and Materials. As we have described previously (1, 2), uterine fluid was collected from ligated uteri, either before or after ovulation. Rabbit sperm, collected by means of an artificial vagina, were incubated in aliquots of the pooled uterine fluid following removal of the seminal plasma.

At various time intervals during the sperm incubation, aliquots were removed and the sperm separated from the uterine fluid by centrifugation at 55,000*g*. This uterine fluid supernatant was assayed for hyaluronidase activity (6) and proteolytic activity (7). The sperm pellet was resuspended in a 10 m*M* barbituric acid solution to provide a mild disruption of the outer acrosomal membrane (4). This suspension was allowed to stand at room temperature for 10–15 min after which the sperm were removed by centrifugation at 55,000*g*. The final supernatant ("acrosome extract") was assayed for hyaluronidase activity and proteolytic activity.

Incubated sperm were bioassayed for capacitation (8). Ovum donors received an ovulating dose of human chorionic gonadotropin (HCG)[3] 12–14 hr prior to sperm insemination. Ova were then recovered within 24 hr and examined for evidence of fertilization by examination of stained as well as fresh ova. Criteria for fertilization were the presence of a male pronucleus and/or a sperm tail in the vitellus, or, in the case of cleaved ova, the presence of chromatin in each blastomere.

The results of the chemical assays were analyzed by either the analysis of variance or the analysis of covariance as applicable (9).

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[3] HCG was kindly provided by Dr. John T. Jewell, Ayerst Labs., New York.

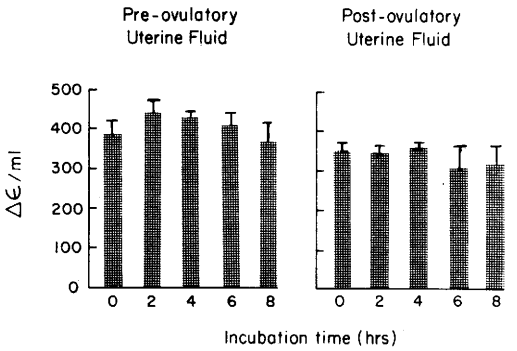

FIG. 1. Supernatant proteolytic activity measured in aliquots from sperm incubations in pre-ovulatory and post-ovulatory uterine fluid. Proteolytic activity is measured as the change in extinction coefficient ($\Delta \epsilon$) of hemoglobin and calculated per ml of incubation medium. Each bar represents the mean of at least four experiments using different sperm pools and uterine fluid pools. Vertical lines represent the standard error. There is no significant difference in proteolytic activity at any of the various intervals.

Results. The proteolytic activity of the supernatant was measured during sperm incubation in uterine fluid collected prior to ovulation, as well as in uterine fluid collected within four hours after ovulation. There was no significant change in the amount of proteolytic activity during the course of the sperm incubation (Fig. 1).

Acrosomal proteolytic activity was extracted from aliquots of the incubated sperm at varying intervals of time. There was no significant change in proteolytic activity of the acrosome extracts as a result of incubation in post-ovulatory uterine fluid (Fig. 2). However, there was a significant decrease in acrosome extract proteolytic activity after 6 hr incubation in pre-ovulatory uterine fluid. A similar decrease occurred in acrosome extracts from sperm incubated in Tyrode's solution. In this latter incubation, there appeared to be a correlation between the decrease in proteolytic activity and a decrease in the numbers of motile sperm.

Hyaluronidase was measured in the acrosome extracts of sperm incubated in pre-ovulatory uterine fluid and in post-ovulatory uterine fluid (Fig. 3). There was no signifi-

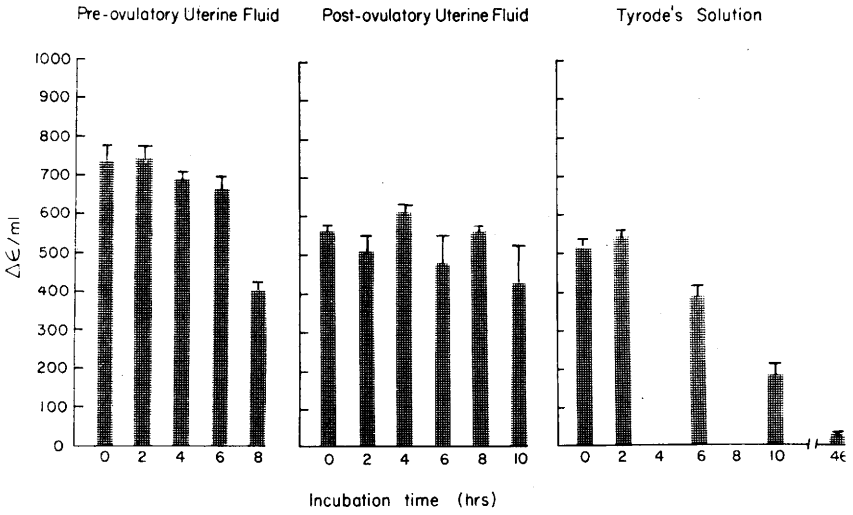

FIG. 2. Proteolytic activity measured in the acrosomal extract from aliquots of sperm incubated in pre-ovulatory or post-ovulatory uterine fluid, or in Tyrode's solution. Activity is measured in terms of the change in the extinction coefficient ($\Delta \epsilon$) of hemoglobin per ml of incubation medium. Bars represent the mean of at least four experiments and vertical lines represent the standard error. There is no significant difference in the proteolytic activity in sperm incubated in post-ovulatory uterine fluid. Sperm incubated in either pre-ovulatory uterine fluid or Tyrode's solution show a significant decrease in acrosomal proteolytic activity with increasing length of incubation ($p < 0.01$).

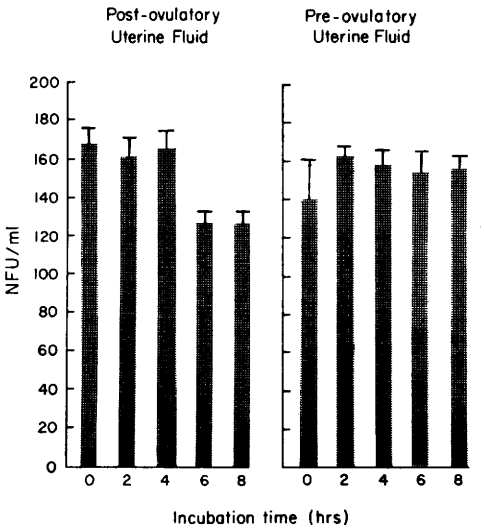

FIG. 3. Hyaluronidase activity measured in acrosomal extracts from aliquots of sperm incubated in either pre-ovulatory uterine fluid or post-ovulatory uterine fluid. Enzyme activity is given in terms of National Formulary Units (NFU) per ml of incubation medium. Bars represent the mean of at least four different experiments with the vertical line designating the standard error. There is a significant decrease in acrosomal hyaluronidase activity after 4 hr incubation in post-ovulatory uterine fluid ($p < 0.001$); there is no significant difference in enzyme activity in sperm incubated in pre-ovulatory uterine fluid.

cant change in hyaluronidase in the acrosome extracts from the sperm incubated in pre-ovulatory uterine fluid. However, after 4–6 hr incubation in post-ovulatory uterine fluid, there was a significant decrease in hyaluronidase activity. It has been observed that the number of motile sperm is not greatly reduced as a result of incubation for this length of time in this medium (1, 2).

Rabbit sperm were incubated sequentially in pre-ovulatory uterine fluid, then in post-ovulatory uterine fluid. The proteolytic activity of the incubation supernatant as well as that of the acrosome extract did not show any significant change during the sequential incubation (Fig. 4). The hyaluronidase activity of the acrosome extracts shows a significant decrease following transfers of the sperm to the post-ovulatory uterine fluid. Unfortunately, the supernatant hyaluronidase activity was below the sensitivity of the assay during the second part of the incubation in all of our experiments.

Sperm were incubated in a post-ovulatory uterine fluid pool which had been previously tested for the capacity to release acrosomal hyaluronidase after 6 hr incubation. Using the bioassay techniques for capacitation (8), incubated sperm were transferred to the oviducts of 10 ovum donor does after either 4, 5, 6, or 7 hr incubation. Ovulation had occurred in these donor does 2–4 hours prior to the sperm insemination. Only those sperm which had been incubated for 5.0–5.5 hr *in vitro*, fertilized any ova (8 of 9 ova recovered from 3 does). Sperm incubated *in vitro* for shorter or longer periods of time did not fertilize any ova. *In utero* incubated control sperm (14 hr incubation), inseminated in the contralateral oviducts of the donor does, fertilized 26 of 31 ova. Sperm incubated in Tyrode's solution, or *in utero* for 6 hours or less did not fertilize any ova in this bioassay system.

Discussion. The measurement of proteolytic activity in the sperm incubations and sperm extracts by our assay system indicates that no active release of any proteolytic enzyme occurs. These results are consistent with evidence indicating that the acrosomal proteinase responsible for sperm penetration of the zona pellucida functions as a membrane-bound enzyme, rather than as a soluble enzyme released by the sperm (4, 5, 11). One question we have been unable to answer is why there should be a decrease in sperm extract proteolytic activity following extended incubation in Tyrode's solution or pre-ovulatory uterine fluid. This decrease in activity may be related to increased formation of proteinase–proteinase inhibitor complexes (12, 13). However, this decrease in proteolytic activity also coincided with increasing numbers of inactive sperm and might be related to cell death.

The measurements of hyaluronidase activity in the sperm extracts strengthens our previous assumption that the increase in hyaluronidase activity was a result of release from the sperm (1, 2). We also find it of interest that measurable levels of hyaluronidase activity remain in the sperm acrosomes

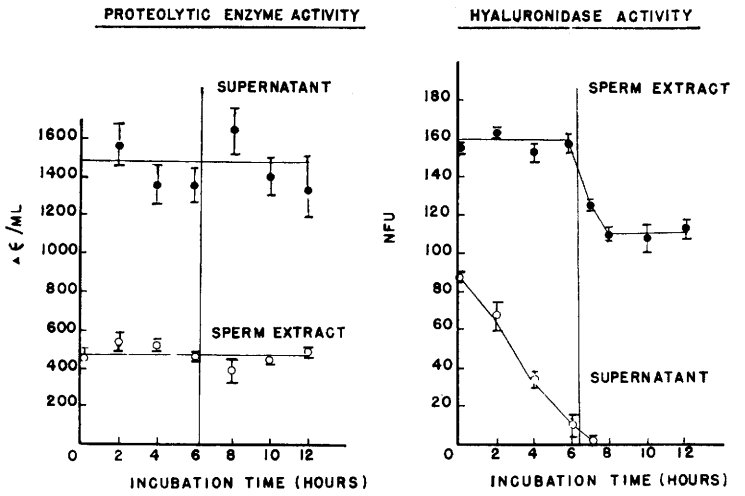

FIG. 4. Enzyme measurements during the sequential incubation; first in pre-ovulatory uterine fluid and then in post-ovulatory uterine fluid. Bars represent the mean of four measurements plus or minus the standard error. The vertical line at six hours represents the time of transfer of the sperm between the two incubation media. There is no significant difference in the proteolytic activity of the supernatant or of the acrosome extracts at the indicated time intervals. The change in hyaluronidase activity is significant in both the supernatant and the acrosome extracts ($p < 0.001$).

even after 12 hr of incubation in pre- and post-ovulatory uterine fluid. This may indicate that hyaluronidase has a further function beyond dissolution of the matrix which binds the cells of the cumulus oophorus.

Our results further confirm that a relationship does exist between the release of hyaluronidase and the process of capacitation. The fact that sperm incubated beyond the time of acrosomal hyaluronidase release were not capable of fertilizing ova indicates that there is an infertile post-capacitation stage in rabbit sperm. This may or may not be related to loss of the outer acrosomal membrane. However, it does correlate with other evidence that once capacitated, the fertile life span of the sperm is considerably shortened (5). We have also observed that sperm incubated in pre-ovulatory uterine fluid are not capable of fertilizing ova unless the period of incubation exceeds ten hours (unpublished results). Thus, it appears from these results and those of our earlier studies (1, 2) that the length of time required for capacitation of rabbit sperm is related to the concentration of a macromolecular component of uterine fluid. The concentration of this component in uterine fluid increases following ovulation. As capacitation of some mammalian sperm occurs in follicular fluid (14), sera (15), and chemically defined media (16), it is quite conceivable that this uterine fluid component could be found in tubal fluid and/or follicular fluid. We believe that measurement of hyaluronidase release in sperm incubations *in vitro* may serve as a method of studying the process of capacitation of rabbit sperm while obviating the necessity for an *in vivo* bioassay step or the more difficult test of *in vitro* ovum fertilization.

Summary. Rabbit sperm incubated in uterine fluid *in vitro* did not show any decrease in acrosomal proteolytic activity. There is a measureable decrease in acrosomal hyaluronidase activity following incubation in postovulatory uterine fluid, but not during incubation in pre-ovulatory uterine fluid. Rabbit sperm incubated in post-ovulatory uterine fluid for 5 hr were capable of fertilizing ova and were considered to be capacitated. Sperm incubated past the time of hyaluronidase release were not capable of fertilizing ova. It is believed that the release of

hyaluronidase by rabbit sperm incubated in uterine fluid is related to the completion of the process of capacitation. Thus, the release of hyaluronidase can be used, under the proper conditions, as an end point for the study of *in vitro* capacitation of rabbit sperm.

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