Sperm Capacitation by Uterine Fluid or Beta-Amylase in vitro

Abstract. *Rabbit sperm developed the capacity to fertilize ova when incubated* in utero *or in vitro in fluids from estrous uteri. Incubation of sperm with beta-amylase in phosphate-buffered Locke's solution also resulted in capacitation. The data suggest that sperm capacitation involves enzymatic alteration of carbohydrate-containing seminal macromolecules which coat sperm and inhibit fertilization.*

Chang (*1*) and Austin (*2*) independently demonstrated that rabbit sperm normally require a period of residence in the female reproductive tract to develop the capacity to fertilize ova. Subsequent evidence suggested that this sperm capacitation was prerequisite to fertilization in many mammals (*3*).

Conventionally, sperm capacitation has been demonstrated by incubation of sperm in the uterus or oviducts of an estrous female. Sperm capacitation did not occur in blood serum (*4*) or when sperm were placed in a dialysis bag in the uterus (*5*). However, one report presented evidence suggesting that sperm capacitation may occur in such seemingly unrelated sites as the anterior chamber of the eye, the isolated bladder of the male or female, and the glandularis vesicularis of the male rabbit (*5*).

The object of this research was to find means to capacitate sperm in vitro. For this purpose, uteri in estrous rabbits were ligated either at the cervix or at both extremities to accumulate uterine fluids (*6*). Sperm were injected through a midventral incision into uteri which had been ligated for about 2 weeks. The sperm were aspirated with accumulated uterine fluid after 6 or 10 hours of *in utero* incubation, adjusted to a concentration of about 25×10^6 per milliliter, and about 1.25×10^6 sperm were inseminated to a depth of about 2 cm in each oviduct of does, at 0 to 2 hours after the time of ovulation. In these experiments, injection of luteinizing hormone (2.0 to 2.5 mg Armour PLH) was used to induce ovulation, which occurred 10.0 to 10.5 hours later. Ova were flushed from the oviducts 22 to 48 hours after ovulation and examined for evidence of fertilization. Tubal inseminations with sperm which had been washed with saline but not incubated *in utero* (negative control) were infertile; 78 ova were recovered and none were cleaved (Table 1). Tubal inseminations with similar sperm about 10 hours before ovulation (positive control) resulted in 22 ova cleaved of 23 recovered.

When sperm were incubated in ligated uteri for 6 or 10 hours and tubally inseminated into does about 0 to 2 hours after ovulation, a total of 149 ova were recovered, of which 38 were cleaved (Table 1). Inseminations performed immediately after ovulation with sperm that had been incubated *in utero* for 6 hours were more fertile than those performed about 2 hours after ovulation (30 percent and 7 percent cleavage, respectively). Ten hours of *in utero* incubation yielded higher fertility than 6 hours (39 percent and 7 percent cleavage, respectively) when inseminations were performed about 1.5 to 2.0 hours after ovulation. These observations suggested that partial capacitation occurred *in utero* and that capacitation was completed in the oviducts of the tubally inseminated does.

In all of these experiments, recovered ova were examined by bright-field, dark-field, and phase-contrast microscopy. Subsequently, ova were stained to reveal unclear details and, in each series of experiments, some tubally inseminated does were examined at midpregnancy for viable fetuses. These procedures were adopted to distinguish fragmented ova or parthenogenically cleaved ova from normally fertilized ova. For example, seven of ten does that had been tubally inseminated after ovulation with sperm incubated *in utero* for 6 hours possessed 21 viable fetuses at 14 and at 27 days of gestation (Table 1). These data demonstrated that sperm capacitation occurred in uteri which had been ligated for as long as 2 weeks.

Table 1. Capacitation of sperm *in utero*.

Sperm incubation		Time of insemination*	No. of ova	No. of ova cleaved
Environment	Hr			
Washed	0	0.0	23	22
Washed	0	10.0	49	0
Washed	0	12.0	29	0
In utero†	6	12.5	41	3
In utero	10	12.0	28	11
In utero	6	10.5	80	24
In utero	6	10.5	21 fetuses	

* Tubal inseminations; hours after injection of luteinizing hormone (LH). Ovulation occurred about 10.0 to 10.5 hours after LH injection. † Washed sperm injected into ligated uteri.

Subsequently, fluids were aspirated from ligated uteri to find whether they could capacitate sperm in vitro. Incubation of sperm for 6 or 8 hours in phosphate-buffered (0.016*M*; *p*H, 7.40) Locke's solution, which served as a negative control, resulted in very low fertility; 116 ova were recovered, of which only five were cleaved (Table 2). When sperm were incubated for 6 hours in uterine fluid in vitro and used for tubal inseminations shortly after ovulation, 95 ova were recovered, of which 40 were cleaved. These 95 ova were from four experiments: 39 were from an experiment in which the *p*H of uterine fluid was maintained at 7.4 with CO_2 to approximate *in utero* conditions (*3*), 25 were from an experiment with freshly collected uterine fluid, 10 were from an experiment in which the uterine fluid was frozen before it was used to incubate sperm, and 21 were from an experiment in which seminal plasma was added to uterine fluid with the sperm during incubation. Because no significant fertility differences were apparent among these treatments, the data were combined in Table 2. Only two ova were cleaved of 32 recovered after tubal inseminations about 2 hours after ovulation with sperm which had been incubated in uterine fluid for 8 hours, which indicated that the period of incubation in uterine fluid did not completely capacitate sperm.

Uterine fluid was dialyzed in a vacuum ultrafilter (LKB). Sperm incubated for 6 hours in the dialysate failed to fertilize any of 51 ova, but sperm incubated for 6 hours in the macromolecular portion of uterine fluid fertilized 8 of 48 ova recovered (Table 2).

Chang (*7*) reported that sperm which had been capacitated *in utero* could be decapacitated by addition of 5 percent seminal plasma to the sperm before tubal insemination. Consequently, we incubated (presumably capacitated) sperm in uterine fluid and apparently decapacitated them by addition of seminal plasma before tubal inseminations (Table 2).

Recently, Williams *et al.* (*8*) reported that beta-amylase inactivated the seminal plasma decapacitation fac-

Table 2. Capacitation of sperm in Locke's solution (phosphate-buffered) and in uterine fluid (UF) in vitro.

Sperm incubation		Time of insemination*	No. of ova	No. of ova cleaved
Environment	Hr			
Locke's	6	10.5	22	3
Locke's	6	12.0	33	2
Locke's	8	10.5	32	0
Locke's	8	12.0	29	0
UF	6	10.5	95	40
UF	6	12.0	47	1
UF	8	10.5	29	15
UF	8	12.0	32	2
UF, dialy.†	6	10.5	51	0
UF, macro.†	6	10.5	48	8
UF, decap‡	7	10.5	17	0

* Tubal inseminations; hours after injection of LH. † Dialysate (dialy.) and macromolecular (macro.) portions of uterine fluid. ‡ Sperm incubated in uterine fluid and decapacitated with seminal plasma.

tor. Previous research from our laboratory had demonstrated a rabbit uterine fluid component which electrophoretically and immunochemically resembled beta-globulin (*9*). These observations suggested to us that reactant(s) in uterine fluid similar to amylase may be normally involved in sperm capacitation. Consequently, we assayed (*10*) for amylase activity and found that 1 ml of rabbit uterine fluid liberated an average of 0.15 mg of maltose equivalents from starch. The comparable average for blood serum was 0.93.

Preliminary data (Table 3) revealed that sperm incubated in beta-amylase (1.0 or 0.1 mg/100 ml of phosphate-buffered Locke's solution) apparently were capacitated. A total of 114 ova were recovered, of which 40 were cleaved following tubal inseminations, about 0 to 1.5 hours after ovulation, with sperm which had been incubated for 8, 10, or 12 hours in beta-amylase. The control data for these experiments are those resulting from sperm incubations with phosphate-buffered Locke's solution (Table 2). Eight does were similarly inseminated with sperm incubated with beta-amylase, and two of them possessed fetuses at 12 days of gestation. The fertility data (Table 3) from sperm incubations with beta-amylase supported the view that partial capacitation resulted from the beta-amylase and that capacitation was completed in the oviducts of the tubally inseminated does. However, the possibility that longer periods of incubation with beta-amylase might completely capacitate sperm could not be excluded on the basis of these data.

Table 3. Capacitation of sperm with beta-amylase.

Sperm incubation		Time of insemination†	No. of ova	No. of ova cleaved
β-amylase* (mg%)	Hr			
1.0	8	10.5	11	3
0.1	8	10.5	13	5
.1	8	12.0	18	4
.1	10	10.5	18	8
.1	10	12.0	17	5
.1	12	10.5	21	10
.1	12	12.0	16	5
		Total	114	40

* Beta-amylase in phosphate-buffered Locke's solution. † Tubal inseminations; hours after injection of LH.

Sperm were incubated at 38° to 39°C in all of these experiments. Motility was always observed before and after treatment of the sperm. Incubation *in utero* or in uterine fluid in vitro always resulted in a reduction of the percentage of motile sperm, from 60 to 70 percent initially to 20 to 50 percent at the end of the incubation periods. Of washed sperm and sperm incubated in buffered Locke's solution, with or without beta-amylase, at least 50 percent were motile at the time of tubal inseminations. Thus, the differences in fertility apparent in Tables 1, 2, and 3 were not attributable to differences in sperm motility.

Bedford and Chang (*11*) observed that the seminal plasma decapacitation factor was a macromolecule and proposed a hypothesis which was recently modified (*12*) to suggest that capacitation may involve destruction or removal of seminal plasma factors on sperm. Weil and Rodenburg (*13*) demonstrated that rabbit sperm are coated with seminal plasma antigens, and Hunter and Hafs (*14*) demonstrated that some seminal antigens firmly coat bull sperm before the secretions of the major accessory sex glands come in contact with the sperm. These observations are compatible with data which indicated that epididymal as well as washed ejaculated sperm must be capacitated to fertilize ova (*1*).

To our knowledge, the data reported in this paper provide the first demonstration of in vitro capacitation of sperm. Furthermore, the data suggest that sperm capacitation involves enzymatic alteration of carbohydrate-containing macromolecules from seminal plasma which coat sperm and inhibit fertilization.

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Herbicide Metabolism: N-Glycoside of Amiben Isolated from Soybean Plants

Abstract. *The* N-*glycoside of the herbicide 3-amino-2,5-dichlorobenzoic acid (amiben) was synthesized in the laboratory. The compound was chromatographically identical with a conjugate of amiben isolated from soybean plants,* Glycine Max *(L.) Merr. The seeds of a tolerant plant, soybean, and of a susceptible one, barley* (Hordeum vulgare *L.), were incubated in amiben labeled with carbon-14, and the seedlings were extracted. Essentially all the radioactivity in soybean was present as a compound corresponding to* N-*glycosyl amiben. Only a small amount of this conjugate was present in barley; most of the radioactivity was present as free amiben or as other unidentified metabolites.*

Conjugates of the herbicide 3-amino-2,5-dichlorobenzoic acid (amiben) have been isolated from plants. Their structure was not reported, but acidic or alkaline hydrolysis of conjugates from tomato plants (*1*) and from soybean plants (*2*) released free amiben. I now report on a conjugate of amiben in soybean plants, amiben-X, and on its possible physiological significance.

Soybean plants were grown in sand culture in pots in the greenhouse. When the plants were 10 to 12 days old, amiben (1 mg per plant) was added