

INDUCTION OF THE ACROSOME REACTION OF GOLDEN HAMSTER SPERMATOZOA WITH BLOOD SERUM COLLECTED AT DIFFERENT STAGES OF THE OESTROUS CYCLE

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(Received 1st January 1971, accepted 16th June 1971)

Summary. Blood serum collected from female hamsters on each day of the oestrous cycle was assayed for its ability to induce the acrosome reaction. The maximum activity was found in sera obtained from females around the time of ovulation, and the minimum on Day 2. Ovariectomy did not affect the activity of the serum but oestrogen treatment significantly increased it. Blood serum from progesterone-treated and pregnant females significantly depressed the incidence of the acrosome reaction. Progesterone counteracted the effect of oestrogen with regard to the incidence of the acrosome reaction. The counteracting effect was maximal when both hormones were administered at the same time.

The possible rôle played by the hormones in this phenomenon is discussed.

INTRODUCTION

Barros & Austin (1967) showed that oocytes recovered from preovulatory follicles could be fertilized *in vitro* with epididymal spermatozoa in the absence of any other secretion from the female tract. They also showed that a high incidence of the sperm acrosome reaction could be induced if epididymal spermatozoa were incubated in the presence of the contents of preovulatory follicles. The inference was then made that female tract secretions were not necessary for the occurrence of sperm capacitation.

Cow (Yanagimachi, 1969b; Gwatkin & Andersen, 1969), mouse and rat (Yanagimachi, 1969a) follicular fluids were found to induce the acrosome reaction of hamster spermatozoa *in vitro*.

Furthermore, Yanagimachi (1969b) found that there are at least two fractions in bovine follicular fluid which are important for the process of capacitation; one fraction is dialysable and heat stable at 90° C and has sperm-activating properties, and a second fraction is non-dialysable and heat labile at 90° C and is responsible for the induction of the acrosome reaction. The same two fractions seem to be present in blood serum. The large molecular weight fraction appears to be albumin or to consist of substances closely associated with albumins (Yanagimachi, 1970).

Hamster, rat, rabbit, human (Barros & Garavagno, 1970; Yanagimachi, 1970), bull and guinea-pig (Yanagimachi, 1970) blood sera preheated at 56° C can readily induce the acrosome reaction of hamster spermatozoa *in vitro*. Barros & Garavagno (1970) suggested that the agent that capacitates spermatozoa and is present in the female genital tract secretions, including the follicular fluid, might be synthesized in a place other than the genital tract and subsequently brought there by the blood stream. The results presented by Barros & Garavagno (1970) seem also to suggest a sex dependence of the abilities of blood serum with regard to the induction of capacitation and the acrosome reaction, since female blood serum appeared to have better acrosome-reaction properties than male blood serum.

The relationship between capacitation of rabbit spermatozoa in the female genital tract and the reproductive phase of the female was demonstrated by Chang (1958). He showed that capacitation could be obtained in the uterus of immature and ovariectomized female rabbits, with or without gonadotrophin or oestrogen treatment. He also showed that capacitation was inhibited in the pseudopregnant or progesterone-dominated uterus. Chang's results were later confirmed by Hamner, Jones & Sojka (1968). Soupart (1967), however, showed that human chorionic gonadotrophins enhance rabbit sperm capacitation with a clear dose-response relationship. Similar results were obtained by Wettemann & Hafs (1970). The gonadotrophin action is mediated through the ovary, since in ovariectomized females capacitation is almost completely abolished. Oestrogen injections into ovariectomized females restore the sperm-capacitating properties of the uterus (Soupart, 1967).

The purpose of this study was to investigate the effect of reproductive phase and of oestrogen and progesterone treatment on the acrosome-reaction-inducing properties of female hamster blood serum.

MATERIAL AND METHODS

Recovery of spermatozoa

Spermatozoa were obtained from the cauda epididymidis of mature, 5-month-old male hamsters. The cauda was blotted on a piece of filter paper to rid it of blood. Small incisions were made on the epididymis and then it was placed under mineral oil. A 0.15 M-sodium chloride solution, buffered with 0.1 M-tris at pH 7.6, was used to suspend spermatozoa. After 15 min, the epididymis was removed and, later, a sample of spermatozoa was taken in order to determine their concentration.

Recovery of blood serum

Human blood was drawn from the cubital vein, and hamster blood was obtained by heart puncture or, under ether anaesthesia, from the abdominal aorta. The blood was transferred to chilled siliconized tubes, and left to clot at room temperature for almost 2 hr. The clotted blood was centrifuged for 10 min at 1500 rev/min on a clinical centrifuge. The clear serum thus obtained was placed under mineral oil in vials, and heated in a water bath for 1 hr at 56° C. The heated serum was stored at 5° C until use.

Acrosome reaction assay of blood serum

The acrosome-reaction-inducing property of blood serum was assayed by incubating, under mineral oil, 1×10^4 spermatozoa/ μ l of serum. Preparations were made in disposable tissue culture Petri dishes (Falcon 3001), and incubated at 37.5° C. Samples of spermatozoa were taken after 5 hr incubation and examined under the phase-contrast microscope. Over 100 actively motile spermatozoa were counted and the incidence of spermatozoa with acrosomes which had undergone the reaction was recorded.

Assay of the method

Since our interest was to study the possible variation of the incidence of the acrosome reaction during the reproductive phase of the female hamster, it seemed necessary to determine the reliability of the method used in order to make sure that we would not have variations other than those under study. In preliminary studies, we compared the incidence of the acrosome reaction produced by an individual blood serum with that produced by pooled sera. We also compared the incidence of the acrosome reaction observed when spermatozoa were obtained from one male with that found when pooled spermatozoa from several males were used. Table 1 shows that the results obtained in the four experiments

TABLE 1
MEAN PERCENTAGE ACROSOME REACTIONS WITH MALE HUMAN BLOOD
SERA UNDER DIFFERENT COMBINATIONS

Blood serum	Spermatozoa	No. of experiments	Mean %	S.E.
Individual (10)	Individual (10)	10	82.9	2.6
	Pool of ten males	10	76.1	2.6
Pool of ten males	Individual (10)	10	78.5	2.7
	Pool of ten males	10	89.3	1.4

Numbers in parentheses indicate the numbers of individuals used.

are not significantly different. We also found no significant differences when blood serum was stored under mineral oil at 5° C for several days. In the light of these results and to economize on animals, spermatozoa were obtained in each experiment from one male and individual blood serum was used.

Effect of reproductive phase on the ability of blood serum to induce the acrosome reaction

Females kept under controlled light conditions (lights on from 06.00 until 21.00 hours) were used to provide blood during the different reproductive phases.

Normal cycling females. A group of mature female hamsters whose oestrous cycle was determined according to Orsini's (1961) method was used in this series of experiments. Only females showing at least four regular cycles were used as blood donors. Sixteen females were bled at 10.00 hours on Days 1, 3 and 4, and thirteen females on Day 2 of the oestrous cycle, and the individual sera were assayed for the acrosome reaction. The day on which the white viscous discharge appeared in the vagina (Orsini, 1961) was considered as Day 1.

Pregnant females. A group of fifteen pregnant females was bled on the 11th day of pregnancy. Day 1 was taken as the day when spermatozoa were found in the vagina.

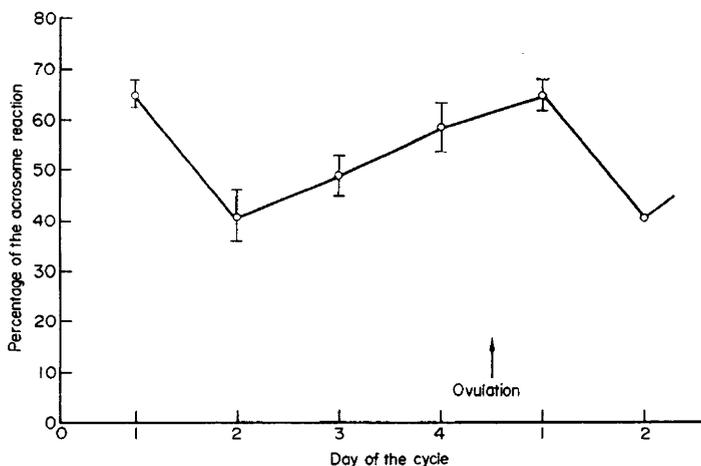
Ovariectomized females. A group of adult females with regular oestrous cycles was ovariectomized under Avertin anaesthesia and was then allowed to recover for 1 to 2½ months. After this time, the females were allotted to four groups as follows: *Group I:* A group of forty-one females was ovariectomized. After full recovery they were bled and the serum assayed with hamster spermatozoa for the incidence of the acrosome reaction. In this group, as in the others, a careful examination was made to ensure that ovariectomy had been complete. *Group II:* A group of twenty-three ovariectomized females was injected daily with 5 mg progesterone (Primolut, Berlimed Labs.) and was bled 24 hr after the last injection. Four females received one injection, four females received two injections and fifteen females received five injections of progesterone. *Group III:* A group of ten ovariectomized females was injected daily with 1 µg oestradiol benzoate for 9 consecutive days. They were bled 24 hr after the last injection. *Group IV:* A group of twelve females was injected daily with 1 µg oestradiol and 5 mg progesterone for 5 days. They were bled 24 hr after the last injection.

An additional ten females were primed with a daily injection of 1 µg oestradiol benzoate for 9 days. During an additional period of 5 days, they received oestrogen and progesterone.

RESULTS

Effect of the reproductive phase on the ability of blood serum to induce the acrosome reaction

Normal cycling females. Blood serum from sixty-one females was used in this series of experiments. The results (Text-fig. 1 and Table 2) show a variation in



TEXT-FIG. 1. Mean percentage acrosome reactions with female hamster blood serum on the 4 days of the oestrous cycle. The vertical bars represent \pm S.E.

the mean percentage of acrosome reactions through the oestrous cycle. The mean percentage of acrosome reactions obtained with blood serum of females

on Day 1 was $65.0 \pm \text{S.E. } 3.1$; with blood serum from females on Day 2 it was 41.0 ± 5.1 ; with blood serum from females on Day 3 it was 49.0 ± 3.6 ; and with blood serum from females on Day 4 it was 57.0 ± 4.9 . The results also show (Table 3) significant differences between Day 1 and Day 2 ($P < 0.001$); between Day 1 and Day 3 ($P < 0.01$); and between Day 2 and Day 4 ($P < 0.02$).

TABLE 2
MEAN PERCENTAGE ACROSOME REACTIONS OF HAMSTER SPERMATOZOA INCUBATED IN FEMALE BLOOD SERUM FOR THE 4 DAYS OF THE OESTROUS CYCLE

Day of cycle	No. of animals used	No. of experiments	Mean %	S.E.
Day 1	16	16	65.0	3.1
Day 2	13	13	41.0	5.1
Day 3	16	16	49.0	3.6
Day 4	16	16	57.0	4.9

TABLE 3
SIGNIFICANCE AND PROBABILITY OBTAINED BY STUDENT'S TEST WITH REGARD TO THE ACTIVITY OF SERA OBTAINED ON EACH OF THE 4 DAYS OF THE OESTROUS CYCLE IN THE INDUCTION OF THE ACROSOME REACTION (SEE TABLE 2)

Days of cycle	t	P
1 to 2	4.13	0.001
1 to 3	3.33	0.01
1 to 4	1.60	0.2
2 to 3	1.36	0.2
2 to 4	2.30	0.02
3 to 4	1.30	0.2

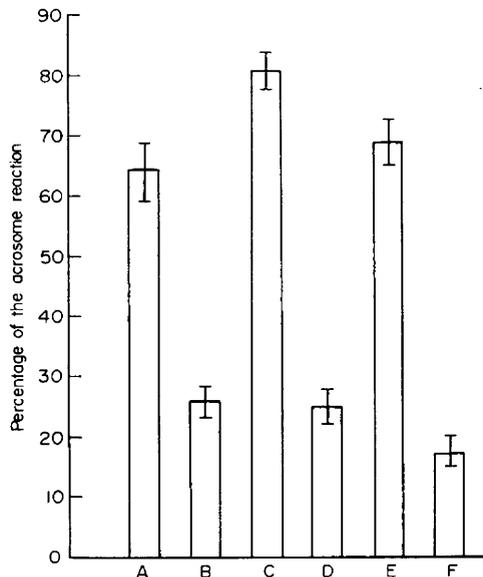
t, Significance; *P*, probability.

Pregnant females. The results show (Text-fig. 2, Table 4) that during pregnancy the ability of blood serum to induce the acrosome reaction is considerably depressed as compared with the serum of non-pregnant adult females. The mean percentage of the acrosome reactions in twenty-two experiments was 25.0 ± 3.2 .

Ovariectomized females. Blood sera from ovariectomized females kept under different experimental conditions were assayed for the incidence of the acrosome reaction.

In the series of experiments involving Group I, the uterine horns were completely atrophic, showing that ovariectomy was successful. The incidence of acrosome reactions was not affected when blood serum from ovariectomized females was used since the mean percentage of reactions was 64.5 ± 4.8 , which is not significantly different from 65.0 obtained with normal females on Day 1 (see Table 5).

In the series of experiments involving Group II, the uterine horns of females receiving one or two injections of progesterone were still as atrophic, as those of ovariectomized females receiving no progesterone treatment. However, after 5 days of treatment, the uterine horns showed a considerable weight increase characteristic of progesterone treatment.



TEXT-FIG. 2. Mean percentage acrosome reactions of hamster spermatozoa with female blood serum under different conditions. (A) Ovariectomized females; (B) ovariectomized and progesterone-treated (5 mg for 5 days); (C) ovariectomized and oestrogen-treated (1 μ g for 9 days); (D) pregnant females; (E) ovariectomized and primed with oestrogen for 9 days, and then treated with both oestrogen and progesterone; (F) ovariectomized and treated with oestrogen and progesterone for 5 days. The vertical bars represent \pm S.E.

TABLE 4

MEAN PERCENTAGE ACROSOME REACTIONS OF SPERMATOZOA INCUBATED WITH BLOOD SERA FROM FEMALES UNDER DIFFERENT CONDITIONS

Type of animals used	Days of treatment		No. of animals used	No. of experiments	Mean %	S.E.
	Oestradiol (1 μ g daily)	Progesterone (5 mg daily)				
(a) Ovariectomized*	—	—	41	41	64.5	4.8
(b) Ovariectomized	—	1	4	4	63.0	5.2
(c) Ovariectomized	—	2	4	4	60.0	3.0
(d) Ovariectomized	—	5	15	18	26.5	2.5
(e) Ovariectomized	9	—	10	12	81.5	3.1
(f) Ovariectomized	5	5	12	26	17.9	2.2
(g) Ovariectomized†	5	5	10	24	69.0	4.0
(h) Pregnant (11 days)	—	—	15	22	25.0	3.2

* This group of females were injected with 0.2 ml of the hormone vehicle. The results obtained with the serum of ovariectomized females injected with oil and those receiving no oil injections were not significantly different so that they have been combined in this table.

† Females were primed with oestrogen for 9 days.

The ability of blood serum to induce the acrosome reaction was not significantly modified after one or two injections, but the incidence of reactions was considerably depressed after 5 days' treatment (see Table 4). The mean percentage of acrosome reactions obtained with this group of females was 26.5 ± 2.5 which is significantly different ($P < 0.001$) from the percentage obtained with blood serum of ovariectomized females receiving no treatment.

TABLE 5
SIGNIFICANCE AND PROBABILITY OBTAINED
BY STUDENT'S TEST WITH REGARD TO THE
ACTIVITY OF SERA OBTAINED FROM FEMALES
UNDER DIFFERENT CONDITIONS

<i>Animals compared (see Table 4)</i>	t	P
a-d	7.10	0.001
a-e	3.20	0.001
a-g	0.72	0.4
a-f	8.90	0.001
f-g	11.30	0.001
d-f	2.66	0.01

t, Significance; P, probability.

The uterine horns of the females in Group III showed an increase in weight characteristic of oestrogen treatment. Consistently, their blood serum elicited the acrosome reaction at a higher incidence than that of untreated females (see Tables 4 and 5, Text-fig. 2). The mean percentage of 81.5 ± 3.1 found in this group of females was significantly different from that for untreated ovariectomized females ($P < 0.001$).

In the series of experiments involving Group IV, we studied the combined effect of progesterone and oestradiol benzoate. In the first series, ten females were primed with $1 \mu\text{g}$ oestradiol benzoate for 9 consecutive days and on the 10th to the 14th day, in addition to $1 \mu\text{g}$ oestradiol benzoate, they were injected with 5 mg progesterone. The results (see Tables 4 and 5) show that the mean percentage of acrosome reactions was 69.0 ± 4.0 . This percentage was not found to be significantly different from that observed with ovariectomized females receiving no treatment ($P < 0.4$). Different results were obtained in a second series of experiments when progesterone and oestradiol benzoate treatment was begun without pretreatment with oestradiol benzoate. Blood serum from twelve females treated for 5 days with 5 mg of progesterone and $1 \mu\text{g}$ oestradiol benzoate gave a mean percentage of acrosome reactions of 17.9 ± 2.2 . This percentage is significantly different from the previous one ($P < 0.001$).

DISCUSSION

We have confirmed the results of Barros & Garavagno (1970) and of Yanagimachi (1970) with regard to the ability of blood serum to induce the acrosome reaction of hamster spermatozoa.

The new findings in the present work are related to the dependence of this activity on the reproductive phase of the female hamster. The cyclic changes observed in the ability of blood serum to induce the acrosome reaction through the oestrous cycle would indicate a close correlation between the female sex hormones and the action of the active agent(s) present in the serum. This cyclic activity might be in agreement with the changes in the levels of progesterone and oestrogens during the cycle.

The lowest incidence of the acrosome reaction appeared on Day 2 of the cycle, which correlates with the finding of Lukaszewska & Greenwald (1970) of a maximum blood progesterone level on Day 2. On the other hand, the highest percentage of the acrosome reaction was found to correspond with the day of ovulation. This latter fact would be expected since it would be necessary to have the highest activity at the time when fertilization normally occurs.

It was interesting to find that after ovariectomy, the activity of blood serum was similar to that found in normal females on Day 1. We are aware of the fact that the hamster and the rabbit might be different biological models with regard to sperm capacitation but a few comparisons seem appropriate. Chang (1958) found that rabbit spermatozoa, incubated in the uterus of an oestrous doe, could fertilize 63% of the eggs, while those incubated in the uterus of an ovariectomized female could fertilize 37% of the eggs. Chang's results would agree with our's in the sense that ovariectomy does not inhibit capacitation. However, Soupart (1967) found that rabbit spermatozoa, incubated in the uterus of an ovariectomized doe, yielded only 4.2% of fertilized eggs. Moreover, he found a dose-response relationship of capacitation with increasing doses of oestradiol benzoate injected into ovariectomized females. The maximum activity corresponded to a dose of 1 $\mu\text{g}/\text{kg}$ of body weight. At higher doses, the capacitation process was slowly depressed. In spite of the fact that we did not find a depression in the ability of blood serum to induce the acrosome reaction after ovariectomy, we did find that the acrosome reaction was enhanced after oestrogen treatment. The mean percentage of 81.5% of acrosome reactions in ovariectomized females treated with oestradiol benzoate was significantly different from the percentage with ovariectomized females receiving no oestrogen treatment ($P < 0.001$).

The present work has also shown that progesterone has a powerful inhibitory effect on the ability of blood serum to induce the acrosome reaction. The percentage of acrosome reactions with ovariectomized females treated with progesterone was not significantly different from that obtained with blood serum from pregnant females. These results would agree with those of Chang (1958) and Hamner *et al.* (1968), who reported that rabbit spermatozoa do not gain the capacity to fertilize eggs when they are incubated in the uterus of a pseudopregnant female.

This suggestion is perhaps further supported by the fact that many oral contraceptives containing progestational compounds exert an inhibitory effect on fertilization by inhibition of sperm capacitation (Chang, 1967, 1969).

Our results also show that there is an interaction between the effects of oestrogen and progesterone which affects the ability of blood serum to induce the acrosome reaction. Thus, serum obtained from oestrogen-treated females

which are later treated with progesterone can induce the acrosome reaction to a degree which is similar to that of blood serum obtained from ovariectomized females with no oestrogen treatment, and also similar to that found in normal cycling females on Day 1. The effect of blood serum from oestrogen-primed females is counteracted by progesterone. The counteracting effect of progesterone was more pronounced when both hormones were administered to ovariectomized females which had not been primed with oestrogen. In these circumstances, the mean percentage was 17.9 which is significantly different from 26.5% found for ovariectomized females treated with progesterone ($P < 0.01$). A similar inhibitory effect on capacitation was found by Soupart (1970) in oestrogen-primed rabbit females treated with high doses of 20α -hydroxypregn-4-en-3-one. It would seem that oestrogen treatment augments the effect of progesterone (Martin, Finn & Carter, 1970).

The question of what rôle the hormones play in controlling the ability of blood serum to induce the acrosome reaction is one that cannot be answered at the present time, since we do not yet know the nature of the active agent involved in this process. Yanagimachi (1969b) showed that the active agent(s) of bovine follicular fluid is (are) present in the macromolecular fraction. The active agent of blood serum also appears to be present in the macromolecular fraction (Yanagimachi, 1970; Barros & Garavagno, unpublished observation). This fact does not rule out the possibility that the active agent(s) might be molecules of low molecular weight and bound to the proteins of blood serum. Both oestrogen and progesterone are bound to serum proteins (Williams, 1964). On the other hand, Gwatkin & Williams (1970) studied the effect *in vitro* of several steroids on hamster sperm capacitation. They found that the progestagen, chlormadinone acetate, was the most effective agent to inhibit capacitation as judged by fertilization *in vitro*, but sperm motility was greatly reduced. Capacitation was also inhibited with 17β -oestradiol but at a dose of $50 \mu\text{g/ml}$, which is much higher than the physiological one. At lower doses ($2 \mu\text{g/ml}$), they found an incidence of capacitation similar to that of the control group (28%).

Our results would not lend support to the idea of a direct effect of oestrogen and progesterone on the acrosome reaction, because of the high incidence of acrosome reactions found with the blood serum of ovariectomized females and with the blood serum of males (see Table 1), unless we accept that the amounts of oestrogen secreted by the testis (Williams, 1964) and adrenal glands (Zarrow, Jochim & McCarthy, 1964) would be enough for the induction of the acrosome reaction.

The same argument could be advanced for the possible controlling action of oestrogen and progesterone on the synthesis of the active agent(s).

A more likely possibility is that both hormones could control the action of the active agents. It is known that the incidence of the acrosome reaction could be modified not only with regard to the hormone administered, but also with regard to the interrelationship between the hormones. This interrelationship makes it conceivable that the active agents may be present in the female tract at all times, but may become active only at the time of fertilization.

ACKNOWLEDGMENTS

We are indebted to Professor C. R. Austin for reading this manuscript. Grateful acknowledgments are due to Mr Ronald Michels for suggestions which improved the English presentation for this manuscript.

This work was supported by Grants: 6856 from The Rockefeller Foundation; 190/68 from the Comision Nacional de Investigacion Cientifica y Tecnologica and 9/69 from the Catholic University of Chile.

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