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Studies on Sperm Capacitation. V.[1] Characterization of Decapacitation Factor from Guinea-pig Spermatozoa

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Fr. I from guinea-pig spermatozoa which had DF activity against rabbit spermatozoa showed inhibitory effect on respiration and motility of guinea-pig spermatozoa. Measurement of DF activity was attempted in mouse *in vitro* fertilization system and the potent inhibition of sperm penetration into ova by Fr. I was confirmed, while the addition of anti-Fr.I-immunoglobulin (anti-Fr.I-Ig.) caused disappearance of fertilization inhibitory activity of Fr.I. The DF activity of Fr.I was partially destroyed during incubation at 60° for 2 hours and completely destroyed at 80° for 2 hours. Alkaline and acid treatment of Fr.I caused no effect on the DF activity. The Fr.I did not decrease its DF activity by the digestion with β-amylase. From the enzymatic point of view the inhibition of corona radiata penetrating enzyme by Fr.I was noted.

Since Chang[3] had found the reversible function of capacitation, Bedford and Chang,[4] and more recently Weinmann and co-workers[5] have demonstrated the presence of decapacitation factor (DF) in seminal plasma. In our previous paper,[6] it was shown that the macromolecular substance (Fr. I) was released from the sperm during incubation and the Fr. I had decapacitation activity against rabbit capacitated spermatozoa. The fact that the sperm had the DF seemed to be important to study the capacitation phenomenon.

The purpose of this study was to investigate the effect of Fr. I and anti-Fr. I-immunoglobulin (anti-Fr. I-Ig.) on the respiration and motility of guinea-pig spermatozoa and the enzyme inhibitory activity of Fr. I on some acrosomal enzyme *i.e.* hyaluronidase, trypsin and corona radiata penetrating enzyme (CPE). Moreover, in order to establish the available DF assay, the effects of Fr. I and anti-Fr. I-Ig. on the mouse *in vitro* fertilization were studied. Stability of Fr. I against temperature, pH and proteolytic or glycolytic enzymes was investigated by this method.

Experimental

Fr.I was obtained from guinea-pig spermatozoa and anti-Fr.I-Ig. was prepared from immunized rabbit as described in our previous report.[6]

Measurement of Sperm Respiration——Epididymal spermatozoa were obtained from male guinea-pig weighing about 500 g and suspended in Ca-free KRP buffer (pH 7.4) containing penicillin (50IU/ml) and streptomycin (50 μg/ml) at 37°. Spermatozoa were washed twice carefully to avoid mechanical damages.

Oxygen uptake was measured using Warburg apparatus. Fructose was added into the apparatus as substrate (2.5 mg/apparatus). Fr.I (5 mg) or anti-Fr. I-Ig. (5 mg) was put into the chamber. The sperm number was counted for each experimental flask and all values were expressed as μl of O_2 uptake per 10^8 sperm cells.

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Measurement of Sperm Motility——Fr.I (2.5 mg/ml) or anti-Fr. I-Ig. (2.5 mg/ml) was added into the sperm suspension and as a control bovine serum γ-globulin (2.5 mg/ml) was used. Sperm motility was measured according to the method of Aonuma, *et al.*[7]

Mouse *in Vitro* Fertilization——Mouse *in vitro* fertilization was performed by the method of Aonuma, *et al.*[8] using modified KRB buffer as medium. In order to induce capacitation, mouse spermatozoa were preincubated and inseminated into culture medium containing mouse ova and incubated under mineral oil in an atmosphere of 5% CO_2–air at 37° for 4 hours. Under phase contrast microscopy ova were observed about the sperm penetration or male and female pronuclei production.

To determine the effect of anti-Fr. I-Ig. on DF activity in Fr. I, capacitated spermatozoa and ova were incubated with Fr. I preincubated with anti-Fr. I-Ig. for 1 hour. The γ-globulin fraction (fractionated with 20—50% saturation of ammonium sulfate) of normal rabbit serm was used as a control sample against anti-Fr. I-Ig..

Heat, Acid and Alkaline Treatment of Fr. I——The effect of temperature of DF activity in Fr. I was demonstrated by treating Fr. I (1 mg/ml, 0.05 M phosphate buffer, pH 7.4) at 20, 40, 60 and 80° respectively for 2 hours.

Acid and alkaline treatment of Fr. I (1 mg) was done at pH 2.0 (1 ml of 0.05 M glycine–HCl buffer) and pH 10.0 (1 ml of 0.05 M glycine–NaOH buffer) respectively at 4° for 48 hours.

Digestion of Fr. I by Pronase-P, Trypsin and β-Amylase——Enzymatic digestion of Fr. I was performed by using each enzyme at the weight ratio of 1/20 per Fr. I in the following medium;

1) Pronase-P (Kaken Kagaku); modified KRB buffer pH 8.0 without bovine serum albumin
2) Trypsin (Sigma Chem.); modified KRB buffer pH 8.0 without bovine serum albumin
3) β-Amylase (Sigma Chem.); 0.05 M citrate buffer pH 4.8

Fr. I treated with each enzyme was diluted to 25 μg/ml by modified KRB buffer, followed by the estimation of the fertilization inhibitory activity.

Inhibitory Activity of Fr. I Against Hyaluronidase, Trypsin-like Enzyme (TLE) and Corona Radiata Penetrating Enzyme (CPE)——After incubation of trypsin or hyaluronidase with Fr. I in various ratio for 2 hours, the trypsin or hyaluronidase activity was measured using N-benzoyl-DL-arginine-*p*-nitroanilide or hyaluronic acid as substrate.

Inhibitory activity of Fr. I against CPE was assayed by the modified method of Zanaveld and Williams.[9] CPE was prepared from rabbit spermatozoa according to the modified method of Hartree and Srivastava.[10] Ova obtained from the oviduct of female rabbit treated with HCG 13 hours before, were incubated with CPE (50 μg) treated with Fr. I (500 μg) for 24 hours under mineral oil in an atmosphere of 5% CO_2–air. CPE activity was expressed according to the next index;

(+) the corona cells were completely dispersed
(±) some corona cells were dispersed
(−) only the cumulus cells were removed

Result

Effect of Fr. I and Anti-Fr. I-Ig. on Guinea-pig Sperm Respiration and Motility

Fr. I showed a inhibition on sperm respiration (Fig. 1). A great suppression of sperm motility by Fr. I was likewise observed and its motility was almost disappeared after 4 hours incubation (Fig. 2). It seems that the effect of Fr. I on respiration and motility was corelated each other. But the addition of anti-Fr. I-Ig. was promotive to respiration and suppressive to motility.

Effect of Fr. I on the Mouse *in Vitro* Fertilization

As shown in Table I, in control, 47% of ova were at the male and female pronuclei forming stage. But the spermatozoa treated with Fr. I were not able to pass through the zona pellucida. And Fr. I was not affected by treatment with normal γ-globulin (200 μg), but lost its activity by incubation with anti-Fr. I-Ig. more than 100 μg. Anti-Fr. I-Ig. had no effect on mouse *in vitro* fertilization.

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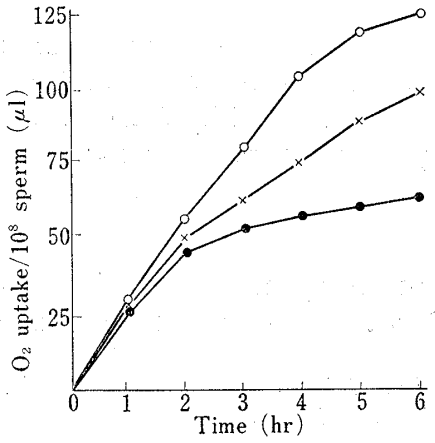

Fig. 1. Effects of Fr. I and Anti-Fr. I-Ig. on Guinea-pig Sperm Respiration

Respiration was determined with Warburg Apparatus in Ca-free KPP buffer at 37°.
—×—: control (bovine serum γ-globulin, 5 mg/vessel)
—●—: Fr. I (5 mg/vessel)
—○—: anti-Fr. I-Ig. (5 mg/vessel)
vessel volume; 2 ml

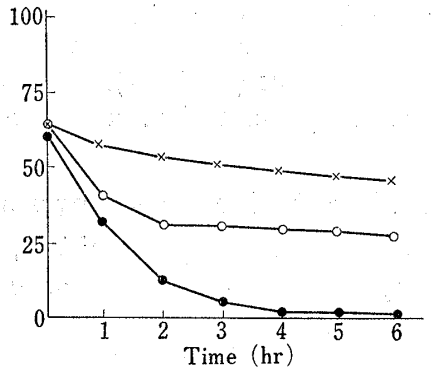

Fig. 2. Effects of Fr. I and Anti-Fr. I-Ig. on Guinea-pig Sperm Motility

Epididymal spermatozoa were incubated in Tyrode's buffer at 37°
sperm concentration; 7—9×10^6/ml
substrate; fructose (2.5 mg/ml)
—×—: control (bovine serum γ-globulin, 5 mg/ml)
—●—: Fr. I (5 mg/ml)
—○—: anti-Fr. I-Ig. (5 mg/ml)

TABLE I. Effects of Fr. I and Anti-Fr. I-Ig. on Mouse Fertilization *in Vitro*

Treatment	No. of ova examined	No. of ova fertilized[a)]	% fertile
Control	64	30	47
Fr. I[b)]	67	0	0
Fr. I+anti-Fr. I-Ig. (50 μg)	58	2	3
Fr. I+anti-Fr. I-Ig. (100 μg)	54	21	39
Fr. I+anti-Fr. I-Ig. (200 μg)	46	19	41
Fr. I+normal serum γ-globulin[c)] (200 μg)	36	0	0
Anti-Fr. I-Ig. (200 μg)	55	22	40

Mouse spermatozoa (2.2×10^5) and ova were incubated in 0.4 ml of modified KRB buffer (pH 7.4) under mineral oil in 5% CO_2-air for 4 hours at 37°.
a) ova with male and female pronuclei
b) Fr. I; 10 μg/tube
c) fractionated from normal rabbit serum

TABLE II. Effects of Temperature and pH on DF Activity of Fr. I in Mouse Fertilization *in Vitro*

Pretreatment of Fr. I	Ova fertilized / Ova examined	% fertile
Control (—Fr. I)	21/35	60
20° for 2 hr	1/26	4
40° for 2 hr	0/36	0
60° for 2 hr	8/34	23
80° for 2 hr	18/35	51
pH 2.0 for 48 hr	4/56	7
pH 10.0 for 48 hr	4/46	9

Mouse spermatozoa and ova were incubated in modified KRB buffer (pH 8.0) under mineral oil in 5% CO_2-air for 4 hours at 37°.

Stability of DF Activity in Fr. I

DF activity of Fr. I was partially destroyed by incubation at 60° for 2 hours and was completely destroyed after incubation at 80° for 2 hours. Alkaline and acid treatment of Fr. I caused no effect (Table II). DF activity was not inactivated by β-amylase. With or without Fr. I, trypsin and pronase caused inhibitory effect on mouse *in vitro* fertilization (Table III).

Effect of Fr. I on the Acrosomal Enzymes

Fr. I did not inhibit hyaluronidase, an enzyme dispersing cumulus cells (Table IV), and not affect trypsin which dissolves zona pellucida (Table V). Meanwhile, as shown in Table VI,

Table III. Effects of Pronase-P, Trypsin and β-Amylase on DF Activity of Fr. I in Mouse Fertilization *in Vitro*

Pretreatment of Fr. I	Ova fertilized	% fertile
Control (−Fr. I)	30/45	67
Control (+Fr. I)	2/70	3
Pronase-P (−Fr. I)	11/59	19
Pronase-P (+Fr. I)	21/67	31
Trypsin (−Fr. I)	0/56	0
Trypsin (+Fr. I)	6/73	9
β-Amylase (−Fr. I)	39/64	61
β-Amylase (+Fr. I)	18/35	51

Mouse spermatozoa and ova were incubated in modified KRB buffer under mineral oil in 5% CO_2-air for 4 hours at 37°. Fr. I was pretreated with each enzyme (Fr. I: enzyme=20: 1) for 6 hours at 37°. Treated Fr. I (10 μg) was assayed in mouse fertilization.

Table IV. Effect of Fr. I on Hyaluronidase Activity

Addition to hyaluronidase	Hyaluronidase activity (mU)
None	26
Fr. I (10 μg)	26
Fr. I (50 μg)	26
Fr. I (100 μg)	26
Fr. I (200 μg)	27

Hyaluronidase (50 μg) was incubated with or without Fr. I for 2 hours at 37° before assay.

One unit of hyaluronidase activity was defined as the amount of N-acetylglucosamine (1 μmole) liberated from hyaluronic acid.

Table V. Effect of Fr. I on Trypsin Activity

Addition to trypsin	Trypsin activity (mU)
None	38
Fr. I (10 μg)	33
Fr. I (25 μg)	34
Fr. I (50 μg)	37

Trypsin (10 μg) was incubated with or without Fr. I for 2 hours at 37° before assay.

One unit of trypsin activity was defined as the amount of *p*-nitroaniline (1 μmole) liberated from N-Benzoyl-DL-arginine-*p*-nitroanilide.

Table VI. Effect of Fr. I on CPE Activity

Treatment of ova	Denudation index (+)	Denudation index (±)	Denudation index (−)	No. of ova
None	2	2	6	10
CPE (50 μg)	10	1	0	11
CPE (50 μg)+Fr. I (500 μg)	1	1	11	13

Denudation index;
(+) the corona cells were completely dispersed
(±) some corona cells were removed
(−) only the cumulus cells were removed
CPE was incubated with or without Fr. I for 2 hours at 37° before treatment with ova.
CPE: corona radiata penetrating enzyme from rabbit spermatozoa

most of ova were denuded by treatment with CPE, but by addition of Fr. I only the cumulus cells were removed and corona cells were remained in most ova. It seemed that the released substance from guinea-pig spermatozoa, Fr. I, had the inhibitory activity against CPE.

Discussion

Chernoff[11] reported that spermatozoa treated with DF pellet from rabbit seminal plasma by the method of Bedford and Chang[12] lose fertilizing capacity, and decrease their motility, but possess their viability. As shown in our results, DF active substance released from guinea-pig spermatozoa also decreased sperm motility and its respiration. In our previous report, it was described that Fr. I from guinea-pig spermatozoa possesed DF activity to rabbit capacitated spermatozoa and in the present results it was demonstrated that Fr. I also inhibited mouse *in vitro* fertilization. To purify the DF, mouse *in vitro* fertilization system is seemed to be an effective means by its convenience. Recently, using this method, we purified the DF from Fr. I, and noted that the DF is a small molecular glycoprotein.[13]

Previously, we reported that the ^{131}I-anti-Fr. I-Ig. attached more to the epididymal spermatozoa than to the sperm incubated in estrous uterus. So disappearance of DF activity of Fr. I treated with anti-Fr. I-Ig. (Table I) indicate that the sperm capacitation is the disappearance of DF on (in) spermatozoa.

Zanaveld and Williams reported that the DF from rabbit seminal plasma did not inhibit hyaluronidase or TLE, but showed the inhibition against CPE.[9] As shown in this paper, the DF from guinea-pig spermatozoa showed the same enzyme inhibitory effect. And the characters of DF from seminal plasma[14] is similar to those from sperm described here. These two DF seem to at least include the same substance.

It is clear that β-amylase failed to degenerate Fr. I, but trypsin and pronase-P caused inhibition of mouse *in vitro* fertilization by themselves alone. It seems like that pronase-P and trypsin degenerate the surface of sperm or (and) ova which may take an important role in fertilization.

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