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In-vitro fertilization of hamster eggs in different media and the stimulating effect of heterologous and homologous spermatozoa

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Summary. Hamster eggs with follicular cells were fertilized by epididymal spermatozoa in two chemically defined media. The proportion of penetrated eggs was significantly higher in a medium for rabbit (16%) than in a medium for rat eggs (6%), but much lower than in Tyrode's solution containing follicular fluid or blood serum as reported by others. The optimal sperm concentration for sperm penetration ranged from 0·5 to 5 × 10^6/ml but penetration of denuded eggs failed in these media. When exposed to hamster spermatozoa in the rabbit medium containing living or dead spermatozoa of guinea-pig, rat, mouse or hamster, high proportions of denuded eggs (24–96%) and eggs with follicular cells (93%) were penetrated. By exposure of denuded hamster eggs to hamster spermatozoa in supernatant fluid of frozen–thawed guinea-pig spermatozoa, 97% of eggs were penetrated in 8 hr compared to 0% in the control group. Sperm capacitation was also efficiently induced by preincubation of hamster spermatozoa in the supernatant fluid. The fertilizing capacity of hamster spermatozoa was maintained for 12 hr during incubation with frozen–thawed guinea-pig spermatozoa when the concentration of hamster spermatozoa ranged between 10 and 20 × 10^6/ml. The beneficial factor of guinea-pig spermatozoa appeared to be from spermatozoa themselves, not from the vasal or epididymal fluids. The presence of follicular cells, blood serum, bovine serum albumin, or even polyvinylpyrrolidone in the media is essential for the capacitation and acrosome reaction of hamster spermatozoa. The components of guinea-pig spermatozoa appear to maintain the fertilizing capacity of hamster spermatozoa and stimulate the process of capacitation.

Introduction

Since the first achievement of in-vitro fertilization of hamster eggs by epididymal spermatozoa (Yanagimachi & Chang, 1963, 1964), in-vitro capacitation of spermatozoa and fertilization of hamster eggs in the presence of follicular fluid (Barros & Austin, 1967; Yanagimachi, 1969a, b; Gwatkin & Anderson, 1969) or of blood serum (Yanagimachi, 1970; Barros & Garavagno, 1970; Miyamoto & Chang, 1972, 1973) have been described. Sperm capacitation and fertilization of hamster eggs *in vitro* can be achieved in a chemically defined medium without addition of any biological fluids (Bavister, 1969), although the acrosome reaction was "only induced in the majority of spermatozoa following the addition of the products of ovulation" (Bavister, 1973).

In a study of effects of heterologous seminal plasma and sperm cells on fertilizing capacity of rabbit spermatozoa, Chang (1949) reported that the proportion of eggs fertilized was significantly increased by insemination of rabbits with minimal numbers of spermatozoa suspended in a fructose–Ringer solution containing dead human spermatozoa rather than fresh chicken egg yolk. A recent study by Bavister (1974) has shown that the motility and survival of hamster spermatozoa were considerably enhanced either by lowering the osmotic pressure of the medium, or by adding an extract of spermatozoa into the medium. The present experiments were conducted to investigate

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whether (a) hamster eggs can be penetrated in various chemically defined media, and (b) the presence of living or dead heterologous and homologous spermatozoa or extracts of them could enhance sperm capacitation and fertilization of hamster eggs *in vitro*. Since sperm concentration is an important factor for sperm capacitation and in-vitro fertilization in the rat (Niwa & Chang, 1974), mouse (Tsunoda & Chang, 1975) and hamster (Talbot *et al.*, 1974), the sperm concentration for each experiment was controlled in the present study. Because the cumulus components (Gwatkin *et al.*, 1972) or products of ovulation (Bavister, 1973) may play a role in the capacitation or acrosomal reaction of hamster spermatozoa, eggs with follicular cells in cumulus and denuded hamster eggs were tested in these experiments.

Materials and Methods

Culture media. The media used were that described by Toyoda & Chang (1974) for the rat and that described by Brackett (1970) for rabbit eggs, except that the rabbit medium contained 4 mg bovine serum albumin/ml instead of 3 mg/ml. The pH of the former was 7·4–7·5 in a 5% CO_2 incubator and that of the latter was adjusted to 7·8 by adding 1·5 N-NaOH. In later experiments, however, 21·58 mM-sodium lactate was added to the rabbit medium and designated as 'modified rabbit medium'. Its pH was not adjusted but was about 7·4–7·5 in a 5% CO_2 incubator. Tyrode's solution was prepared with conventional concentrations of salts and added glucose. These media were filtered through a 0·22 μm Millipore filter just before use. Usually 0·4 ml medium was placed in the centre of a plastic Petri dish (35 × 10 mm) covered with sterilized paraffin oil (Saybolt viscosity 125/135, Fisher Co.). The preparations were kept in a 5% CO_2 incubator overnight before use.

Preparation of hamster sperm suspensions. The tubules of an excised cauda epididymidis from mature male hamsters (weighing 100–200 g) were cut with a sharp knife and the emerging sperm mass was taken with a sterile glass needle and placed into 0·5–1 ml medium. After 5–10 min various amounts of the original suspension, depending on the sperm concentration, were taken with a glass pipette and placed into 0·4 ml medium for the introduction of eggs.

Preparation of heterologous sperm suspensions. Spermatozoa of mature guinea-pigs (weighing 675–1300 g) and rats (weighing 500–610 g) were obtained by squeezing the ductus deferens and cutting the cauda epididymidis, but those from mature mice (weighing 32–40 g) were obtained by cutting the tubules of the cauda epididymidis. The sperm mass was suspended in 1–2 ml medium covered with paraffin oil in a plastic dish. Care was taken to avoid contamination with blood. About 0·4 ml guinea-pig sperm suspensions covered with oil were kept in a CO_2 incubator for 16 hr as 'preincubated living sperm samples'. The same volume of sperm suspensions covered with oil was placed on a metal plate at 0°C for 20 min and then warmed to 37°C. This was repeated several times until all the spermatozoa were immotile. These were designated as 'temperature-shocked samples'. Similarly, sperm suspensions were placed on a block of solid CO_2 until completely frozen and then placed in the incubator for thawing ('frozen–thawed samples'). The preparations were kept in a 5% CO_2 incubator overnight before introduction of hamster spermatozoa and eggs.

For the study of the effects of supernatant fluid of frozen–thawed guinea-pig spermatozoa, the frozen–thawed samples were pooled in a sterile test tube, covered with oil and centrifuged at 900 *g* for 10 min at room temperature. The supernatant fluid was withdrawn and kept in a frozen condition before use.

For the study of the effect of epididymal fluid, living guinea-pig spermatozoa with ductal and epididymal fluids were suspended in modified rabbit medium ($\simeq 10 \times 10^6$ cells/ml), centrifuged for 20 min, and the supernatant fluid withdrawn. The centrifuged spermatozoa were resuspended, frozen, thawed, and centrifuged again to obtain sperm extract without contamination of tract fluids.

In the last experiment about 10–20 × 10^6 guinea-pig spermatozoa were suspended in 1 ml Tyrode's solution, in which they were frozen, thawed and centrifuged. The supernatant fluid was diluted with an equal volume of Tyrode's solution containing either 0·8% bovine serum albumin (BSA) or 0·8% polyvinylpyrrolidone (PVP, mol. wt 40,000: Sigma). A droplet of 0·4 ml covered with oil was equilibrated in 5% CO_2, then used for incubation of hamster spermatozoa and eggs.

Collection, insemination and examination of hamster eggs. Mature female hamsters weighing 96–165 g were induced to superovulate (Yanagimachi & Chang, 1964) by injection of PMSG (donated by NIAMDD) and killed 14–16 hr after injection of HCG. The oviducts were placed under oil in a Petri dish. The eggs in the cumulus were dissected out and then introduced into various sperm suspensions. For the removal of follicular cells surrounding the eggs or products of ovulation, the eggs in cumulus were placed into the medium without BSA but containing 0·1 % testicular hyaluronidase (Type 1, approximately 300 NF units/ml: Sigma). After dissolution of the cumulus and dispersal of follicular cells the denuded eggs were washed five times in the medium and then introduced to various sperm suspensions. These preparations were all incubated in an incubator saturated with 5% CO_2 in air at 37–37·5°C. At various times after incubation the eggs were picked up and mounted *in toto* on a slide. The eggs were fixed, stained and examined according to the method of Toyoda & Chang (1974). Eggs with spermatozoa in the perivitelline space and in the vitellus were considered as penetrated and those with an enlarged sperm head(s) or a male pronucleus with corresponding sperm tail were considered as undergoing fertilization. The motility of spermatozoa during and after incubation was noted, and the number of hamster and foreign spermatozoa was estimated by means of a haemocytometer. Statistical difference was determined by a χ^2 test.

Results

In-vitro penetration of hamster eggs with or without follicular cells in the rat or rabbit medium

Hamster eggs with or without follicular cells were exposed to various concentrations of hamster epididymal spermatozoa suspended in the rat or rabbit medium prepared 10 min to 1 hr previously (Table 1). The motility of spermatozoa decreased after 2–3 hr in both media. Their motility, however, was maintained for about 8 hr in the presence of eggs with follicular cells in the cumulus, but less than 8 hr in the presence of denuded eggs. The penentration rate of the eggs was very low in both media. An optimal sperm concentration appeared to be between 0·5 and 5×10^6/ml in both media. The rabbit medium, however, was better than the rat medium because the proportions of penetrated and fertilized eggs were significantly higher ($P < 0·001$). There was only one polyspermic egg (1.5%) out of 66 eggs undergoing fertilization in the rat medium, but 13 (11%) of 119 eggs were polyspermic in the rabbit medium. Sixteen (8·6%) of 185 eggs undergoing fertilization in both media were digynic.

Effect of heterologous and homologous spermatozoa on in-vitro penetration of hamster eggs

The motility of hamster spermatozoa was drastically improved when hamster epididymal spermatozoa were introduced in modified rabbit medium containing living guinea-pig spermatozoa preincubated for 16 hr, or temperature-shocked or frozen–thawed guinea-pig spermatozoa. Rat, mouse or hamster dead spermatozoa were also effective, but not as conspicuously as were guinea-pig spermatozoa.

The results in Table 2 show that penetration and fertilization rates were very high with rat and guinea-pig spermatozoa and higher ($P < 0·01$) than the control rates with mouse and frozen–thawed hamster spermatozoa. Among the 403 eggs undergoing fertilization in these experiments, 117 eggs (29%) were polyspermic and 96 eggs (23·8%) were polygynic.

Time of sperm penetration in the supernatant fluid of frozen–thawed guinea-pig spermatozoa

About 5×10^6 guinea-pig spermatozoa in 1 ml rabbit medium were frozen, thawed and centrifuged. Denuded hamster eggs were introduced to 0·4 ml supernatant fluid containing hamster spermatozoa, and examined at various times (Table 3). The supernatant fluid of guinea-pig spermatozoa effectively induced capacitation of hamster spermatozoa which were then able to penetrate and fertilize hamster eggs.

To test whether the effect was on the denuded eggs or on the spermatozoa, hamster epididymal spermatozoa were incubated in 0·4 ml supernatant fluid of frozen–thawed guinea-pig spermatozoa

Table 1. In-vitro fertilization of hamster eggs by epididymal spermatozoa in two chemically defined media

Conc. hamster sperm. ($\times 10^6$/ml)	Rat medium (pH 7·4–7·6)				Rabbit medium (pH 7·8)			
	No. of tests*	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)	No. of tests*	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
Eggs with follicular cells								
0·05–0·25	6	143	0	0	13 (6)	238	29 (12·2)	21 (8·8)
0·26–0·50	6	87	0	0	4 (2)	99	15 (15·2)	14 (14·1)
0·51–1·00	8 (3)	329	39 (11·9)	38 (11·6)	7 (3)	135	21 (15·6)	17 (12·6)
1·01–2·00	14 (5)	264	24 (9·1)	17 (6·4)	12 (7)	253	60 (23·7)	55 (21·7)
2·01–3·00	6 (2)	145	11 (7·6)	9 (6·2)	3	73	0	0
3·01–5·00	10 (3)	144	9 (6·3)	2 (1·4)	3 (2)	50	18 (36·0)	12 (24·0)
5·01–12·50	7	176	0	0	3	37	0	0
Total	57 (13)	1288	83 (6·4)**	66 (5·1)	45 (20)	885	143 (16·2)**	119 (13·5)
Denuded eggs								
1·00–13·00	7	315	0	0	13† (1)	256	3 (1)	2 (0·8)

Eggs were examined 8–12 hr after exposure to spermatozoa.

* Figure in parentheses indicates the number of tests in which at least more than one egg was penetrated.

† These tests were performed in modified rabbit medium which contained 21·58 mM-sodium lactate and the sperm concentration ranged from 0·4–15·20 $\times 10^6$/ml.

** Significant difference ($P < 0·001$) between these two media.

Table 2. Effects of heterologous and homologous spermatozoa on in-vitro fertilization of hamster eggs

Sperm. from	Treatment of hetero- or homologous sperm.	Sperm conc. ($\times 10^6$/ml)		Eggs	No. of tests	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
		Hetero- or homologous	Hamster					
Guinea-pig	Preincubated, living	3·67–4·92	0·77–1·05	With follicular cells	4	85	79 (92·9)	79 (92·9)
		3·67–20·10	0·77–2·90	Denuded	6	87	70 (80·5)	65 (74·7)
	Temperature-shocked	14·00–17·20	1·00–3·20	Denuded	2	38	33 (86·8)	30 (79·0)
	Frozen–thawed	5·00–40·00	0·30–1·18	Denuded	8	165	142 (86·1)	133 (80·6)
Rat	Frozen–thawed	20·00–22·50	0·40–0·80	Denuded	4	53	51 (96·2)	48 (90·6)
Mouse	Temperature-shocked	5·40–9·10	1·60–1·80	Denuded	2	37	12 (32·4)*	10 (27·0)
	Frozen–thawed	16·90–18·30	0·40–0·70	Denuded	3	38	9 (23·7)*	6 (15·8)
Hamster	Temperature-shocked	10·25–12·05	3·45	Denuded	4	76	0 (0)	0 (0)
	Frozen–thawed	20·00	0·75	Denuded	2	29	20 (69·0)*	15 (51·7)
Control (no hetero- or homologous spermatozoa)			0·40–4·50	With follicular cells	6	188	33 (17·6)	15 (8·0)
			0·40–1·00	Denuded	4	75	3 (4·0)*	2 (2·7)
			2·85–4·50	Denuded	5	105	0 (0)	0 (0)
			12·80–15·20	Denuded	4	76	0 (0)	0 (0)

Eggs were examined 8–10 hr after exposure to hamster spermatozoa suspended in a modified rabbit medium containing hetero- or homologous spermatozoa.
* Significant difference ($P < 0·01$) compared with the control.

Table 3. Time of sperm penetration during the incubation of denuded hamster eggs and epididymal spermatozoa in the supernatant fluid of frozen–thawed guinea-pig spermatozoa suspended in modified rabbit medium

Medium	Time of examination (hr after insemination)	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)	Morphology of eggs undergoing fertilization	
					With enlarged sperm head (%)	With male pronucleus (%)
Supernatant fluid	2	57	0 (0)	0 (0)	—	—
	4	58	5 (8·6)	2 (3·4)	2	0
	6	61	40 (65·6)	31 (50·8)	10 (32·3)	21 (67·8)
	8	58	56 (96·6)	54 (93·1)	10 (18·5)	44 (81·5)
Without supernatant fluid	2 to 8	112	0 (0)	0 (0)	—	—

Sperm concentration ranged from 1·85 to 3·5 × 10^6/ml. There were two or three tests at each time.

Table 4. Effect of preincubation of hamster epididymal spermatozoa in the supernatant fluid of frozen–thawed guinea-pig spermatozoa suspended in modified rabbit medium on in-vitro fertilization of denuded hamster eggs

Time of preincubation (hr)	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
1	59	0 (0)	0 (0)
2	55	7 (12·7)	0 (0)
4	61	23 (37·7)	21 (34·4)
6	63	48 (76·2)	25 (39·7)

Eggs were examined 1 hr after exposure to hamster spermatozoa at a concentration of 0·85–4·9 × 10^6 spermatozoa/ml. There were three tests at each time.

before exposure to denuded eggs. The results in Table 4 show that the effect was on the induction of sperm capacitation rather than on the eggs themselves. The concentration of the hamster spermatozoa also appeared to be involved because at a concentration of 0·85–1·23 × 10^6/ml penetration was observed in samples preincubated for 2–6 hr, but at a concentration of 3·7–4·9 × 10^6/ml penetration was observed only in the samples preincubated for 6 hr.

Maintenance of fertilizing capacity of hamster spermatozoa by incubation with frozen–thawed guinea-pig spermatozoa

Various numbers of hamster spermatozoa were suspended with frozen–thawed guinea-pig spermatozoa and preincubated for 12 hr. Denuded hamster eggs were then introduced to these suspensions and examined 7–8 hr later. The motility of hamster spermatozoa incubated for 12 hr in the absence of guinea-pig spermatozoa was very poor, but improved in the presence of guinea-pig spermatozoa. As shown in Table 5, the fertilizing capacity of hamster spermatozoa was completely lost after incubation for 12 hr in the absence of frozen–thawed guinea-pig spermatozoa, but was maintained for 12 hr in the presence of guinea-pig spermatozoa if the number of hamster spermatozoa was between 10 and 20 × 10^6/ml. Most of the eggs undergoing fertilization were monospermic; only two (4%) and four (8%) out of 48 eggs were either polyspermic or polygynic. Thus the fertilizing capacity of hamster spermatozoa is better maintained in the presence of guinea-pig spermatozoa but an optimal concentration of hamster spermatozoa is also important.

Table 5. The fertilizing capacity of hamster spermatozoa after preincubation in the presence of frozen–thawed guinea-pig spermatozoa for 12 hr

Sperm conc. ($\times 10^6$/ml)		No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
Guinea-pig	Hamster			
20·00	1·66–2·98	38	0 (0)	0 (0)
20·00	4·06–4·97	39	3 (7·7)	2 (5·1)
5·00	10·00	53	27 (50·9)	27 (50·9)
5·00	20·00	49	22 (44·9)	19 (38·8)
5·00	30·00	48	0 (0)	0 (0)
Control				
0	20·00	48	0 (0)	0 (0)
0	30·00	47	0 (0)	0 (0)

Denuded eggs exposed to spermatozoa were examined 7–8 hr later; two tests for each suspension.

Comparison of the effect of supernatant of guinea-pig spermatozoa with tract fluids and supernatant of frozen–thawed guinea-pig spermatozoa

Guinea-pig spermatozoa and fluids from the ductus deferens and epididymis were suspended in modified rabbit medium and centrifuged. The sperm pellet was resuspended in the rabbit medium, frozen, thawed, then centrifuged again. The number of spermatozoa in both preparations was adjusted to about 10×10^6/ml before centrifugation. Hamster epididymal spermatozoa were prepared in these supernatant fluids for the introduction of denuded hamster eggs. The results in Table 6 show that the beneficial factor is probably from the spermatozoa themselves rather than from the tract fluids.

Table 6. Effect of supernatant fluid of (a) guinea-pig spermatozoa with tract fluids and (b) of resuspended frozen–thawed guinea-pig spermatozoa on in-vitro fertilization of denuded hamster eggs

	No. of eggs examined	No. of eggs penetrated (%)	Mean no. of sperm. in:		No. of eggs undergoing fertilization (%)		
			Egg	Vitellus	Total	Mono-spermic	Poly-spermic
(a)	60	58 (97·7)	2·5	1·5	54 (90)	34	20
(b)	60	60 (100·0)	5·8	2·3	60 (100)	19	41

Eggs were examined 9–10 hr after exposure to spermatozoa; three tests for each experiment. Sperm concentration ranged from $0{\cdot}74–1{\cdot}43 \times 10^6$/ml.

Effect of BSA and PVP

The effect of follicular cells or BSA on the penetration rate was examined. Tyrode's solution was used as the basic medium for the preparation of hamster spermatozoa, and PVP was also used as a substitute for BSA. When hamster spermatozoa were suspended in these media (Table 7), their motility during incubation appeared to be less than that of spermatozoa in the rabbit medium, but motility was maintained and accelerated in the presence of supernatant fluid of guinea-pig spermatozoa and head-to-head agglutination was observed in all these media. The proportions of denuded eggs penetrated by spermatozoa were, in general, lower (43–54%) than when rabbit medium was used (96·6%, Table 3). In the presence of follicular cells even without BSA, 32 (60·4%) of 53 eggs were penetrated. In the absence of follicular cells, BSA, or PVP, practically no denuded eggs were penetrated (Table 7). The presence of the components from heterologous spermatozoa, therefore, appeared to stimulate capacitation.

Table 7. Effect of bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) on the penetration of hamster eggs with and without follicular cells in the presence of supernatant fluid of frozen–thawed guinea-pig spermatozoa in Tyrode's solution

Tyrode's solution	Eggs	No. of tests	No. of eggs examined	No. of eggs penetrated (%)	No. of eggs undergoing fertilization (%)
Without BSA	With follicular cells	4	53	32 (60·4)	21 (39·6)
Without BSA	Denuded	4	62	1 (1·6)	0 (0)
With 0·4% BSA	Denuded	4	59	32 (54·2)	13 (22·0)
With 0·4% PVP	Denuded	3	49	21 (42·9)	14 (28·6)

Half the volume of supernatant of 10–20 × 10^6 frozen–thawed guinea-pig spermatozoa/ml in Tyrode's solution containing 1·02–1·84 × 10^6 of hamster spermatozoa/ml was used. Eggs were examined 8–10 hr after incubation with spermatozoa.

Discussion

A variety of culture media with different chemical compositions has been used by different workers for in-vitro fertilization. Whether a particular medium for a particular species reflects the actual requirements for a given species or only reflects the experience or preference of different workers has always been in question. Since the procedures used by different workers were not the same, comparison of their results is difficult. In the present study, the results of the experiment to determine whether hamster eggs can be penetrated in a medium suitable for the rat (Toyoda & Chang, 1974) or for the rabbit (Brackett, 1970) showed that rabbit medium was superior to the rat medium but that the penetration rates were still quite low (12–36%, Table 1). Although hamster eggs can be fertilized in a modified Tyrode's solution containing BSA without any biological fluid (Bavister, 1969, 1973), high penetration rates occur in Tyrode's solution containing follicular fluids (Yanagimachi, 1969a, b), or blood serum (Yanagimachi, 1970; Miyamoto & Chang, 1972, 1973; Miyamoto *et al.*, 1974). It appears, therefore, that a particular medium is best for a given species and that a medium with biological fluid may be better than one without.

Capacitation has been used to denote only those physiological changes of spermatozoa before the occurrence of the acrosome reaction (Austin *et al.*, 1973; Bavister, 1973), but the acrosome reaction is induced in the majority of hamster spermatozoa only after addition of the products of ovulation (Bavister, 1973). The acrosome reaction was not examined in this study but the ability of spermatozoa to penetrate eggs was taken as the criterion of capacitation and acrosome reaction.

The role of cumulus components for the capacitation of hamster spermatozoa and penetration of hamster eggs has been reported by Gwatkin *et al.* (1972), but Miyamoto & Chang (1972) observed that there was no difference in the penetration rates (91 versus 87%) of hamster eggs with or without follicular cells incubated with epididymal spermatozoa in an equal volume of Tyrode's solution and heated hamster blood serum. Since only 4% of denuded hamster eggs were penetrated in the rabbit medium which contained BSA (Table 2), the heated hamster blood serum may play an important role for the capacitation and acrosome reaction of hamster spermatozoa in the absence of follicular cells. In the presence of follicular cells and cumulus components, however, capacitation and the acrosome reaction of hamster spermatozoa can be achieved in a medium without BSA (Table 7). The components in the blood serum or in the follicular cells that induce capacitation, and whether they act in the same way or in different processes during capacitation and acrosome reaction, however, await further investigation.

Stimulated by the work of Yanagimachi (1972), who meticulously described the motility, agglutination, capacitation and acrosome reaction of guinea-pig spermatozoa in culture, we thought that the head-to-head agglutination of hamster and guinea-pig spermatozoa incubated together may facilitate the acrosome reaction of hamster spermatozoa. By adding guinea-pig spermatozoa in a low concentration to a hamster sperm suspension the 'dilution effect' may be reduced. When hamster spermatozoa were introduced to guinea-pig sperm suspension or its supernatant fluid, not only the

motility of hamster spermatozoa was drastically improved but also very high proportions of denuded hamster eggs were penetrated. When hamster spermatozoa were suspended with frozen–thawed rat, mouse or hamster spermatozoa the proportions of penetrated eggs were also increased, although guinea-pig and rat spermatozoa appeared to be more potent than mouse and hamster spermatozoa. The failure of temperature-shocked hamster spermatozoa to increase the penetration rate may be due to a need of drastic treatment for the leakage of this factor from hamster spermatozoa. The factor not only has the ability to induce capacitation (Tables 3, 4 and 6) but also that of prolonging the fertilizing capacity of incubated spermatozoa (Table 5). In the presence of the factor but absence of BSA, penetration occurred in eggs with follicular cells but failed in denuded eggs. In the presence of the factor and BSA or PVP, penetration of denuded eggs was possible (Table 7), indicating that the presence of BSA or PVP is essential for the manifestation of the stimulatory effect of the sperm factor at least on sperm capacitation. The biochemical identification of the factor and its mode of action, however, require further study.

The presence of epididymal extract in the medium appeared to inhibit sperm penetration of mouse eggs *in vitro* (Iwamatsu & Chang, 1971), although the involvement of epididymal secretions and cumulus oophorus for the capacitation of mouse spermatozoa *in vitro* has been reported (Gwatkin *et al.*, 1974). Since the preparation of epididymal extract and of epididymal secretions were different, in the light of the present experiments the beneficial factor for sperm capacitation is not in the epididymal secretions but from the spermatozoa themselves (Table 6).

Hamster spermatozoa appeared to be inherently more fragile than mouse, rat and guinea-pig spermatozoa. Their motility was better and lasted longer when suspended in a medium containing BSA, blood serum, eggs with follicular cells, or foreign spermatozoa. The poor motility in a medium without these components cannot be explained simply as being due to the 'dilution effect' because the deterioration of their motility occurred in a wide range of sperm concentration ($1–13 \times 10^6$/ml). We have found, however, that this sperm factor not only can maintain the motility and the fertilizing capacity of spermatozoa, but also has the ability to induce capacitation.

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