Influence of genetic factors on the fertilization of mouse ova *in vitro*

Elżbieta Kaleta

Department of Genetics and Evolution, Institute of Zoology, Jagellonian University, Krupnicza 50,
30-060 Kraków, Poland

Summary. In-vitro fertilization was studied in F₁ hybrids, 4 inbred and outbred strains of mice. Experiments were performed with intact ova (Series I) and denuded ova (Series II). In Series I the highest percentages of fertilization were obtained with gametes of F₁ hybrids (86.7%) and of the CBA strain (87.5%). The KP, KE, and particularly the C57 strains, which *in vivo* give less than 100% fertilization, also gave low indices *in vitro* (42.9, 26.0 and 8.6% respectively). Testing gametes of these inbred strains with F₁ gametes showed that spermatozoa and ova are responsible for the low percentages of fertilization. The rate of fertilization was mainly dependent on the genotype of the spermatozoon. In Series II, high percentages of fertilization (97–100%) were regularly obtained in all groups, indicating that differences between strains pertained mainly to binding of spermatozoa with the zona pellucida. The incidence of polyspermy, which did not exceed 5% in Series I and reached 58% in Series II, was dependent on the genotype of both gametes.

Introduction

Inbred strains of mice show different indices of fertilization *in vivo*, e.g. the percentage and rate of fertilization, occurrence of polyspermy and penetration of supplementary spermatozoa (Braden, 1958; Krzanowska, 1960, 1970; McLaren & Bowman, 1973). These differences indicate that genetic factors play a significant role in the functioning of gametes. Under natural conditions, however, analysis is complicated by a number of additional factors: disturbances of synchronization between ovulation and copulation may result in sterile copulations or in a lower percentage of fertilization (Krzanowska, 1960; Kaleta, Antos & Nowicka, 1976) and the environment of the female tract may affect the transport of spermatozoa (Nicol & McLaren, 1974), the viability of the gametes and their interaction.

These complications can be avoided by using fertilization *in vitro* in medium of known composition. This method has been successfully used for several years, but most experiments have been carried out with highly viable gametes from F₁ hybrids or outbred mice which give high percentages of fertilization *in vitro*. There is little information about differences between inbred strains of mice (Iwamatsu & Chang, 1971; Pavlok, 1971), which generally show lower indices of fertilization *in vitro*.

Because mouse inbred strains with known parameters of fertility were available, the present study, comparing the effectiveness of fertilization *in vivo* and *in vitro* under various experimental conditions, was an analysis of the malfunctions of spermatozoa and ova which result in a reduction of the percentage and rate of fertilization in some inbred strains.

Materials and Methods

The mice used were 2–4 months old and from the inbred strains KP, KE (Krzanowska, 1965), CBA/Kw and C57BL/Kw (referred to hereafter as CBA and C57, respectively), their F₁ hybrids, and from an outbred stock. When describing F₁ hybrids, the strain of the female is given first.

Superovulation in females was induced by injecting 8–10 i.u. PMSG and, 48–50 h later, 8–10 i.u.

HCG. Ova were removed from the oviducts 14–18 h after the HCG injection. Fertilization *in vitro* was performed in two series. In the first series, intact ova, as isolated from the oviducts, were used, and in the second, the ova were denuded enzymatically of cumulus cells and zona pellucida. As it was impossible to test all genetic groups in every experiment, fertilization of gametes from the inbred strains was performed in parallel with fertilization of the F_1 hybrid gametes which served as a control. If fertilization of the F_1 gametes dropped below 60%, the results of this experiment were discarded.

Series I

Ova were placed into a drop (about 0.4 ml) of culture medium prepared as described by Toyoda, Yokoyama & Hosi (1971), but freeze-dried bovine serum albumin (Fraction V) inactivated for 90 min at 56–57°C was used instead of crystalline albumin. The number of ova per drop ranged from 10 to 30. Spermatozoa were expressed from the vasa deferentia of male mice to another drop of culture medium (about 0.4 ml), after gentle pressure of the cauda epididymidis. The spermatozoa were allowed to disperse for 10–15 min at 37°C. After thorough mixing, about 0.02 ml sperm suspension was transferred to the drop containing ova, giving a final concentration of 100–600 spermatozoa/ml. After incubation at 37.5°C for 7 h in an atmosphere of 5% CO_2 in air, the ova were washed with 0.9% NaCl and inspected under the microscope, fresh and then after fixation in absolute alcohol:glacial acetic acid (3:1 v/v).

Ova at metaphase of the second meiotic division, with chromosomes arranged in the equatorial plane of the spindle, were classified as unfertilized, regardless of the possible presence of spermatozoa under the zona pellucida. In fertilized ova, three stages were distinguished: (1) those at anaphase and containing a sperm head in the cytoplasm; (2) those with the second polar body and a sperm head in the cytoplasm; and (3) those with the second polar body and two or more pronuclei. Ova identified as old, i.e. with a distinctly detached zona pellucida and scattered chromosomes, or as activated, i.e. with the second polar body and only one pronucleus, were excluded from analysis.

In experiments in which the numbers of supplementary spermatozoa were estimated, ova were incubated for 8–9 h. By this time nearly all fertilized ova had formed pronuclei which were visible without fixation (during which the zona pellucida dissolves), and the presence of supplementary spermatozoa in the perivitelline space could easily be detected in fertilized or unfertilized ova.

Series II

Ova were isolated in a drop of culture medium of pH 7.6 (without bovine serum albumin), containing testicular hyaluronidase (0.025%). The cumulus cells dispersed within 10 min, and old ova which had fragmented or had a detached zona pellucida were removed. The ova were then washed in culture fluid and transferred to the culture fluid containing α -chymotrypsin (9000 ATEE units/mg: Koch-Light) at a concentration of 0.003%. Depending on the genotype, the time required to dissolve the zona pellucida varied, but prolonged treatment with enzyme solution considerably reduced the percentage of fertilized ova. The zona pellucida was therefore removed mechanically from ova of the C57 and KE strains after incubation with α -chymotrypsin for 15 min. After two washings in culture fluid, denuded ova were incubated under the same conditions as in Series I, except that the concentrations of spermatozoa were reduced by 50%.

Statistical differences were determined by a 2×2 or $R \times 2$ test of independence (Snedecor, 1955).

Results

Series I: % and rate of fertilization of intact ova *in vitro*

The results obtained (Table 1) show that the effectiveness of fertilization was clearly dependent on the genotype. Gametes of F_1 hybrids showed high percentages of fertilized ova and fertilization rates, and relatively low coefficients of variation. There were no significant differences according to the cross and the results for reciprocal hybrids were therefore combined. The highest percentage of

Table 1. Percentage and rate of fertilization *in vitro* of ova surrounded by the zona pellucida and cumulus cells

Genotype		No. of eggs tested (no. of exps)	No. of fertilized ova:				Ova with pronuclei/ no. fertilized (%)	Poly-spermic eggs (%)
Ova	Spermatozoa		At ana-phase	With 2nd polar body	With pro-nuclei	Fertilized ova (%)		
F ₁ (KE × CBA) F ₁ (CBA × KE)	F ₁ (KE × CBA) F ₁ (CBA × KE)	438 (22)	1	64	315	86.7	82.8	2.3
F ₁ (CBA × C57) F ₁ (C57 × CBA)	F ₁ (CBA × C57) F ₁ (C57 × CBA)	298 (22)	4	85	142	77.5	61.5	1.3
F ₁ (C57 × KE)	F ₁ (C57 × KE)	191 (8)	2	84	55	73.8	39.0	—
Outbred	F ₁	264 (22)	7	57	142	78.1	68.9	1.9
CBA	CBA	241 (14)	10	37	164	87.5	77.7	4.3
CBA	F ₁	229 (12)	1	44	153	86.5	77.3	3.0
F ₁	CBA	228 (13)	2	21	181	89.5	88.7**	3.9
Outbred	CBA	297 (14)	1	27	229	86.5	89.1**	0.7
KP	KP	256 (10)	1	54	55	42.9	50.0	—
KP	F ₁	249 (13)	3	29	111	57.4**	77.6**	0.7
F ₁	KP	314 (15)	3	6	23	10.1**	71.8**	—
Outbred	KP	231 (13)	5	17	16	16.4**	42.1	—
KE	KE	200 (15)	2	11	39	26.0	75.0	1.9
KE	F ₁	151 (13)	1	9	34	29.1	77.3	2.2
F ₁	KE	304 (17)	2	31	131	53.9**	79.8	—
Outbred	KE	228 (15)	3	19	47	30.3	68.1	1.4
C57	C57	231 (23)	1	9	10	8.6	50.0	—
C57	F ₁	197 (17)	2	9	43	27.4**	79.6*	3.7
F ₁	C57	194 (13)	5	24	13	21.6**	30.9	2.4
Outbred	C57	261 (15)	1	12	9	8.4	40.9	—
CBA	C57	319 (14)	9	23	15	14.7	31.9	—
C57	CBA	164 (14)	1	12	43	34.2	76.8	1.8

F₁ refers to F₁ hybrid gametes from the reciprocal crosses between CBA and C57 or CBA and KE strains—both groups being used in the same proportions for each testing.

Difference between the intrastrain value and that for the cross significant at ** $P < 0.005$; * $P < 0.05$.

fertilization was obtained with F₁ hybrids from crosses between the KE and CBA strains, while the other groups of F₁ hybrids gave significantly lower results ($P < 0.005$).

The CBA mice and their hybrids had high fertilization percentages and rates of fertilization. The other inbred strains gave significantly lower ($P < 0.005$) results, especially the C57 mice. In comparison with the F₁ hybrids and the inbred CBA mice, the inbred KP and C57 strains had the lowest rates of fertilization (50% of eggs with pronuclei).

To ascertain whether ova or spermatozoa were responsible for the low percentages of fertilization *in vitro*, gametes of the inbred strains were tested with gametes from F₁ hybrids, or from outbred females. The results for the CBA strain gave a percentage fertilization as high as that for the inbred or F₁ hybrid gametes. However, insemination of ova of KP, KE and C57 strains with F₁ spermatozoa and vice versa in most cases improved the percentages of fertilization indicating that the genotype of both gametes was a contributory factor. Comparison of the percentages of F₁ or outbred ova fertilized by spermatozoa from each of the four strains gave highly significant differences ($R \times 2$; $P < 0.005$), confirming the influence of sperm genotype on fertilization. A similar analysis made with F₁ spermatozoa and ova of inbred and outbred strains showed that the effectiveness of fertilization also depended on the genotype of the ova ($P < 0.005$).

The rate of fertilization was mainly dependent on the genotype of the spermatozoa. Spermatozoa of F_1 hybrids usually accelerated the rate of fertilization, especially of the KP and C57 ova. In all variants in which C57 spermatozoa were used, the percentage and rate of fertilization were reduced. This was particularly noticeable in an additional test in which fertilization of CBA ova with C57 spermatozoa gave significantly lower results than the opposite combination.

The percentages of polyspermic ova in relation to all fertilized ova were low and did not exceed 5%.

Supplementary spermatozoa. When the ova of outbred females were inseminated with CBA spermatozoa (209 ova) supplementary spermatozoa (1–2/ovum) were found in 43.6% of fertilized ova and in 30.3% of unfertilized ova (difference not significant). When KE spermatozoa were used (306 ova), supplementary spermatozoa (1–5/ovum or more) were found in 69.8% of fertilized ova, and in only 26.2% of unfertilized ova ($P < 0.005$). Statistical analysis showed that KE spermatozoa penetrated the zona pellucida of fertilized outbred ova in a significantly higher percentage of cases compared with CBA spermatozoa ($P < 0.005$). In unfertilized ova, KE and CBA spermatozoa were noted in the perivitelline space in approximately similar proportions (not significant).

Table 2. Fertilization *in vitro* of eggs without a zona pellucida

Genotype		No. of ova tested	No. of fertilized eggs				Polyspermic eggs (%)	No. of pronuclei/ polyspermic egg (range)
Ova	Spermatozoa		At anaphase	With 2nd polar body	With pronuclei	Fertilized eggs (%)		
F_1	F_1	114	—	—	114	100	54.3	3.4 (3–6)
CBA	CBA	141	—	—	141	100*	41.8	3.6 (3–7)
C57	C57	120	—	—	118	98.3	3.4	3.0 —
KP	KP	112	2	4	103	97.3	2.7	3.0 —
KE	KE	127	—	1	119	94.5*	2.5	3.0 —
CBA	C57	153	—	1	151	99.3	4.6	3.0 —
C57	CBA	144	—	—	144	100	58.3	3.8 (3–7)
Outbred	F_1	155	1	1	152	99.3	7.8	3.2 (3–5)
Outbred	CBA	132	—	1	128	97.7	18.6	3.2 (3–4)
Outbred	C57	125	—	2	116	94.4	3.4	3.0 —

$F_1 = \text{CBA} \times \text{C57}$.

* Significantly different, $P < 0.02$.

Series II: % and rate of fertilization of denuded ova in vitro

In all the experimental groups, very high (up to 100%) and similar percentages of fertilization were obtained (Table 2, Text-fig. 1).

The rate of fertilization was much faster than in Series I, and varied little. Nearly all ova contained pronuclei. Only the KP strain showed a tendency to slower fertilization (in comparison with the CBA strain, $P < 0.025$).

The occurrence of polyspermy was very variable, but much higher than in Series I, and was related to the genotype of both gametes. The CBA spermatozoa gave high indices of polyspermy, dependent on the genotype of the ova (C57, CBA and outbred: $R \times 2$, $P < 0.005$). However, the C57 spermatozoa, which in Series I always had a low percentage and rate of fertilization, regardless of the genotype of ova (outbred, C57 or CBA), gave low and similar percentages of polyspermy.

Outbred ova which were tested with F_1 , CBA and C57 spermatozoa showed generally low percentages of polyspermy depending on the genotype of the male gametes ($R \times 2$, $P < 0.005$).

Abnormalities of fertilization in vitro

In the culture medium employed, fertilization appeared normal and the stages were comparable with those observed after fertilization *in vivo*. In Series I only 12 of 5497 ova were activated; and in

Text-fig. 1. Comparison of the effectiveness of fertilization *in vivo* and *in vitro* of inbred strains of mice. Solid columns, *in vivo* (CBA, KE and C57 strains: Kaleta, Antos & Nowicka, 1976; KP strain, Krzanowska, 1960); hatched columns, *in vitro*, eggs with zona pellucida; open columns, *in vitro*, eggs without zona pellucida.

Series II, only 3 of 1326 ova were activated. In all cases, activation consisted of extrusion of the second polar body without penetration of a spermatozoon into the oocyte cytoplasm. Moreover, 8 of 2721 fertilized ova in Series I showed abnormal extrusion of the second polar body: instead of a single second polar body, two bodies separated from the cytoplasm, and the cytoplasm of the ova contained only the head of the spermatozoon, or an already formed male pronucleus. Female pronuclei were present in both polar bodies.

Discussion

In this study gametes of F_1 hybrid mice gave high percentages of fertilization comparable with the results of other authors (Toyoda, Yokoyama & Hosi, 1971; Hoppe & Pitts, 1973; Miyamoto & Chang, 1973; Hoppe & Whitten, 1974). Fertilization ran a normal course, abnormalities and spontaneous activations occurring only sporadically. Thus the method used seems to be adequate for the analysis of genetic differences in the effectiveness of fertilization. The results with intact ova confirmed the data obtained with material of the same genotype *in vivo* (Krzanowska, 1960, 1964, 1970; Kaleta, Antos & Nowicka, 1976), indicating that spermatozoa from different inbred strains exhibit different abilities to penetrate ova, and that the effectiveness of fertilization depends on the genotypes of both gametes. The relationships appeared very clearly *in vitro*, although the effectiveness of fertilization was generally lower.

Indices of fertilization *in vitro* were high only for those genotypes which attain 100% fertilization *in vivo* (F_1 hybrids and the CBA strain). Mice of the KP, and especially of the C57 and KE strains, have a low percentage and rate of fertilization *in vivo* (Text-fig. 1). However, when gametes of other strains are used (e.g. CBA strain: Krzanowska, 1970; F_1 : Kaleta, Antos & Nowicka, 1976) those of the KP, C57 and KE strains are capable of normal fertilization *in vivo* and an improved, but still low, rate *in vitro*. Tests of C57, KE and KP gametes with F_1 gametes showed that the ova and spermatozoa were responsible for the fertilization inefficiency, suggesting a common genetic control of the quality of ova and spermatozoa. The present results do, however, indicate that the rate of fertilization *in vitro* depends mostly on the genotype of spermatozoa.

The experiments of Series II with denuded ova were performed to test the assumption that the low percentages of fertilization in inbred strains were mainly due to disturbances of sperm capacitation or binding of spermatozoa with the receptors on the zona pellucida. Our results confirm those of Pavlok & McLaren (1972) that the completion of sperm capacitation is not necessary for penetration of ova deprived of the zona pellucida. In Series II very high percentages of fertilization were obtained

and the differences between inbred strains observed in Series I (Text-fig. 1) were abolished. The percentage of fertilization was significantly lower only in the KE strain. These data corroborate observations *in vivo* (Krzanowska, 1960, 1970; Kaleta, Antos & Nowicka, 1976), which show that many unfertilized ova of this strain contain spermatozoa in the perivitelline space. Thus some disorders of fertilization must appear in the process of binding of the spermatozoa with the vitelline membrane.

Inbred strains of mice differ in the number of supplementary spermatozoa reaching the perivitelline space of ova fertilized *in vivo*, and the genotype of both gametes is involved (Krzanowska, 1970), suggesting differences in the effectiveness of the block against polyspermy at the level of the zona pellucida (Krzanowska, 1972). Despite such differences, polyspermy *in vivo* is rare, indicating an efficient block at the vitelline membrane. These two blocks, although efficient *in vivo*, become inadequate *in vitro* because of the large numbers of capacitated spermatozoa surrounding the ovum, especially those without a zona pellucida (Hanada & Chang, 1972; Pavlok & McLaren, 1972).

In the present experiments the incidence of supplementary spermatozoa was tested only in the outbred ova fertilized with CBA or KE spermatozoa. Higher numbers of outbred $\times$ KE gametes confirm the differences obtained *in vivo* (Krzanowska, 1970).

In both series polyspermy was clearly influenced by the genotype of both gametes. In Series II there were differences in the index of polyspermy according to the genotype of the ova, suggesting differences in the effectiveness of the block at the vitelline membrane. Since outbred, C57 and KE ova fertilized *in vivo* often contain supplementary spermatozoa under the zona pellucida but do not develop polyspermy, a rapid and effective block on the vitelline membrane of these ova was to be expected. This was fully confirmed *in vitro* only for outbred ova, which showed little polyspermy in Series II even with very active F_1 and CBA spermatozoa. However, when C57 ova were fertilized with CBA spermatozoa, the block proved inadequate.

Ova of the CBA strain exhibit a low incidence of supplementary spermatozoa *in vivo* and it was expected that the block at the vitelline membrane would be less effective. When CBA spermatozoa were used in Series II, the percentage of polyspermy was much higher in CBA ova than in outbred ova and the low indices obtained when CBA ova were fertilized with C57 spermatozoa can be attributed to low fertilizing capacity of these spermatozoa.

This work was supported by the Polish Academy of Sciences within the project 09.3.1. I thank Dr Halina Krzanowska for critical review and discussion of the manuscript.

References

- BRADEN, A.W.H. (1958) Variations between strains of mice in phenomena associated with sperm penetration and fertilization. *J. Genet.* **56**, 37–47.
- HANADA, A. & CHANG, M.C. (1972) Penetration of zona-free eggs by spermatozoa of different species. *Biol. Reprod.* **6**, 300–309.
- HOPPE, P.C. & PITTS, S. (1973) Fertilization *in vitro* and development of mouse ova. *Biol. Reprod.* **8**, 420–426.
- HOPPE, P.C. & WHITTEN, W.K. (1974) Maturation of mouse sperm *in vitro*. *J. exp. Zool.* **188**, 133–136.
- IWAMATSU, T. & CHANG, M.C. (1971) Factors involved in the fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* **26**, 197–208.
- KALETA, E., ANTOS, K. & NOWICKA, L. (1976) Indices of *in vivo* fertilization in inbred strains of mice and F_1 hybrids. *Zwierzęta Laboratoryjne* **13**, 3–18.
- KRZANOWSKA, H. (1960) Studies on heterosis. II. Fertilization rate in inbred lines of mice and their crosses. *Folia biol., Kraków* **8**, 269–279.
- KRZANOWSKA, H. (1964) Time interval between copulation and fertilization in inbred lines of mice and their crosses. *Folia biol., Kraków* **12**, 231–244.
- KRZANOWSKA, H. (1965) New inbred strains of mice. *Mouse News Letter* **32**, 54–56.
- KRZANOWSKA, H. (1970) Relation between fertilization rate and penetration of eggs by supplementary spermatozoa in different mouse strains and crosses. *J. Reprod. Fert.* **22**, 199–204.
- KRZANOWSKA, H. (1972) Rapidity of removal *in vitro* of the cumulus oophorus and the zona pellucida in different strains of mice. *J. Reprod. Fert.* **31**, 7–14.
- MCLAREN, A. & BOWMAN, P. (1973) Genetic effects on the timing of early development in the mouse. *J. Embryol. exp. Morph.* **30**, 491–498.
- MIYAMOTO, H. & CHANG, M.C. (1973) The importance of serum albumin and metabolic intermediates for capacitation of spermatozoa and fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* **32**, 193–205.
- NICOL, A. & MCLAREN, A. (1974) An effect of the female

- genotype on sperm transport in mice. *J. Reprod. Fert.* 39, 421-424.
- PAVLOK, A. (1971) Study of some genetic factors participating in the fertilization of superovulated mouse ova. *Folia biol. Praha* 17, 50-53.
- PAVLOK, A. & McLAREN, A. (1972) The role of cumulus cells and the zona pellucida in fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* 29, 91-97.
- SNEDECOR, G.W. (1955) *Statistical Methods*. Iowa State College Press, Ames, Iowa.
- TOYODA, Y., YOKOYAMA, M. & HOSI, T. (1971) Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm. *Jap. J. Anim. Reprod.* 16, 147-151.

Received 26 January 1977