Comparison of Capacitation and Fertilizing Ability of BALB/c and ICR Mouse Epididymal Spermatozoa: Analysis by In Vitro Fertilization with Cumulus-intact and Zona-free Mouse Eggs

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Abstract: *The* in vitro *capacitation and fertilizing ability of epididymal spermatozoa of BALB/c and ICR mice were compared. The capacitation rate of spermatozoa from BALB/c was significantly (P<0.05) lower than that of those from ICR mice when examined by chlortetracycline (CTC) fluorescence assay after 1 and 2 h of preincubation. An* in vitro *fertilization technique has revealed that the time taken for sperm penetration into intact and zona-free eggs was longer for BALB/c than for ICR spermatozoa (P<0.01). Fertilization rates for spermatozoa from BALB/c mice were significantly (P<0.01) lower than for spermatozoa from ICR regardless of the type of eggs (BALB/c, ICR). These results suggest that the low fertilizing ability of epididymal spermatozoa from BALB/c mice is associated with a lower efficiency of capacitation* in vitro.
Key words: *Capacitation, In vitro fertilization, ICR, BALB/c, Mice*

Mammalian spermatozoa must undergo physiological changes in the female reproductive tract, called capacitation, before they are capable of fertilization. Capacitation can be accomplished *in vitro* by using appropriate media in various mammalian species [1]. The reproductive characteristics of the inbred BALB/c mouse, which is a common research model in immunology, include a high proportion of abnormal sperm heads [2, 3] and low *in vitro* fertilization (IVF) rates [4–6]. In a previous study [7], we observed that epididymal spermatozoa from BALB/c mice had a low ability to fertilize cumulus-intact or cumulus-free eggs after 6 h insemination without preincubation (9–10% and 0–4% fertilization rates, respectively). In contrast, spermatozoa from ICR mice fertilized the eggs without preincubation, if the co-incubation time of eggs and spermatozoa was more than 2 h [8].

To examine the differences in the fertilizing ability of epididymal spermatozoa from BALB/c and ICR mice, spermatozoa were compared by means of *in vitro* fertilization techniques with cumulus-intact and zona-free mouse eggs. In the case of zona-free eggs, Hoechst 33342, which was previously reported as an efficient DNA-specific fluorochrome of sperm-egg fusion [9, 10], was used to ascertain the early events in fertilization.

Materials and Methods

Preparation of eggs

Inbred BALB/cA and outbred Jcl:ICR mice were commercially purchased from CLEA Japan Inc. (Tokyo, Japan). Female mice older than 2 months were superovulated by an i.p. injection of 5 I.U. PMSG, followed 48 h later by an i.p. injection of 5 I.U. hCG. Fifteen to 17 h after the hCG injection, the females were killed by cervical dislocation for egg collection. Cumulus-intact eggs were collected from the swollen ampulla of the oviducts and were transferred to 400 μl drops of TYH [8] containing 4.0 mg/ml BSA (fatty acid-free; Yagai Co., Tokyo, Japan). To prepare zona-free eggs, cumulus cells were removed by treatment with 0.05% hyaluronidase (Sigma Chemical Co., St. Louis, MO) and 3 protease inhibitors (50 μM benzamidine, 50 μM PMSF and 0.25 mg/ml aprotinin; Sigma) in TYH; the eggs were then washed 3

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times in fresh TYH. The eggs were placed in an acidic Tyrode's solution (pH 2.5) for 20–30 sec to completely dissolve the *zona pellucida*, then rapidly washed 3 times in TYH and transferred to 200 μl drops of TYH.

Preparation of spermatozoa

BALB/c and ICR males, more than 2 months old, were killed by cervical dislocation. Cauda epididymides were excised from two mice, punctured with a 23 G needle and submerged in 200 μl drops of TYH. The spermatozoa were allowed to disperse for 5–10 min and were preincubated for 2 h at a concentration of 0.6–2 × 10^7 cells/ml under 5% CO_2 in air at 37°C. When zona-free eggs were inseminated, small aliquots of the sperm suspension were preincubated for 60 min at the original concentration and then diluted to 200 μl drops of fresh TYH to 10^6 cells/ml and preincubated for an additional 60 min.

Chlortetracycline fluorescence assay

Fluorescence assay was performed with the method originally developed by Ward and Storey [11] and slightly modified by Xian *et al.* [12]. Chlortetracycline (CTC; C-4881, Sigma) at a 1 mM concentration was dissolved in a chilled buffer of 20 mM Tris, 130 mM NaCl, and 5 mM cysteine. The solution was kept at 4°C and shielded from light. The sperm suspension (20 μl) was mixed with an equal volume of the CTC solution. After 10 sec, 5 μl of 1% glutaraldehyde in 1 M tris buffer was added to the suspension, and one drop (5 μl) was put on a glass slide and covered with a coverslip. The slides were examined under a microscope equipped with epifluorescence optics (Nikon, Tokyo, Japan) with a 380–420-nm filter and a DM 430 dichroic mirror. Aliquots of the sperm suspensions from BALB/c and ICR mice were removed after 5 min, 1 h and 2 h of preincubation. A minimum of 200 spermatozoa with a normal or quasi-normal head [3] was scored for each aliquot.

Grossly misshapen (collapsed and triangular) heads [3] were omitted from scoring because it was difficult to observe the acrosome and postacrosomal areas. Spermatozoa were classified as uncapacitated (F-pattern), capacitated (B-pattern), and acrosome reacted (AR).

Hoechst-33342 staining of eggs

Zona-free eggs were stained by using a slight modification of the method described by Takahashi *et al.* [10]. A stock solution of Hoechst-33342 (1 μg/ml; H-33342; Sigma) was prepared in modified PBS containing 1.0 mg/ml of polyvinylalcohol (cold water soluble, Sigma); the solution was stored in the dark at −20°C prior to use. Zona-free eggs were incubated in TYH containing H-33342 for 30 min at 37°C. After preloading, the eggs were washed 3 times in TYH and preincubated in the same medium for 30 min.

In vitro fertilization

In vitro fertilization was conducted as described by Choi *et al.* [13]. Cumulus-intact eggs were transferred to 400 μl drops of TYH. Seven to 10 μl of sperm suspension, with or without preincubation, was introduced to 400 μl of TYH medium containing cumulus-intact mouse eggs. The final concentration of spermatozoa was 2–5 × 10^5 cell/ml. Zona-free eggs preloaded with H-33342 and preincubated in TYH were transferred to 200 μl drops of TYH for insemination with preincubated spermatozoa. The final sperm concentration was 1 × 10^4 cell/ml.

Assessment of fertilization

After an insemination period of 6 h, cumulus-intact eggs were washed and whole-mounted on a glass slide. The eggs were fixed with 2.5% glutaraldehyde for 2 h at room temperature and stained with 0.25% acetolacmoid [14]. Eggs possessing an enlarged sperm head and/or a male pronucleus with a sperm tail were considered fertilized. In zona-free eggs, coincubation of eggs with sperm for 5, 10, 30, 60 and 90 min was stopped by adding 10 μl of 2.5% glutaraldehyde in PBS (pH 7.4) to the co-incubation medium. Eggs were mounted on glass slides under a coverslip and examined under an epifluorescence microscope.

Statistical analysis

Each experiment was done in 3 to 5 replicates. Capacitation rates of spermatozoa were compared by means of an arcsin transformation followed by one-way analysis of variance. Fertilization rates of mouse eggs from BALB/c and ICR were compared by chi-square test.

Results

Capacitation rates of spermatozoa for BALB/c and ICR strains of mice

Capacitation rates of sperm from BALB/c and ICR strains of mice, estimated by CTC test and calculated by proportions of the B pattern, are shown in Fig. 1. Immediately after collection, the capacitation rate of epididymal spermatozoa from BALB/c mice was little different ($P>0.05$) from that of ICR spermatozoa (29.3 vs. 23.0%), but the capacitation rates of spermatozoa from BALB/c mice were lower ($P<0.05$) than from ICR mice after 1 h (50.8 vs. 60.3%) and 2 h (49.1 vs. 64.3%)

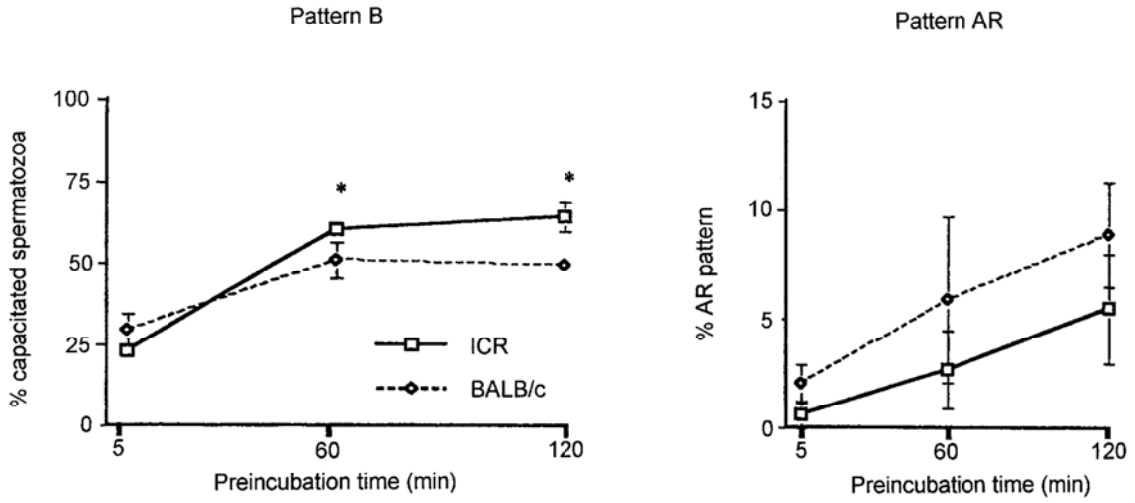

Fig. 1. Comparison of the percentages of capacitated (pattern B) and acrosome reacted (AR) spermatozoa in ICR and BALB/c mouse strains. *$P<0.05$.

Table 1. *In vitro* fertilization between BALB/c and ICR mouse gametes without sperm preincubation[A]

Sperm Donor	Egg donor	No. of eggs examined	No. (%) of eggs fertilized Total	polyspermic
ICR	ICR	58	46 (79)[a]	2 (3)
ICR	BALB/c	53	48 (91)[a]	3 (6)
BALB/c	ICR	46	8 (17)[b]	0 (0)
BALB/c	BALB/c	59	11 (19)[b]	0 (0)

[A]Superovulated eggs and non-preincubated spermatozoa were used. Coincubation time of sperm and eggs was 6 h. Experiment was conducted in 4 replicates. a vs. b; $P<0.01$.

of preincubation. Proportions of AR pattern increased steadily during preincubation in both strains. BALB/c sperm showed a higher proportion of AR pattern at any time during preincubation, but the difference was not significant. The motility of spermatozoa before and after incubation was 70 ± 10% in both strains.

Cross fertilization between BALB/c and ICR strains of mice

In vitro fertilization rates of cumulus-intact eggs with epididymal spermatozoa that were not preincubated are shown in Table 1. Fertilization rates for ICR spermatozoa were higher ($P<0.01$) than for BALB/c spermatozoa without relation to egg donors when the sperm were not preincubated. The interstrain fertilization between ICR spermatozoa and BALB/c eggs resulted in a high fertilization rate (91%), comparable to the rate within ICR gametes (79%). After 2 h preincubation, the fertilization rate for BALB/c sperm and BALB/c eggs was significantly higher than that for BALB/c sperm and ICR eggs (46% vs. 22%). These fertilization rates were, however, still lower ($P<0.01$) than those of ICR spermatozoa and cumulus-intact eggs of either strain (95–98%) (Table 2).

Time course of spermatozoa penetration of the cumulus-intact eggs

The time required for fertilization was observed by using spermatozoa preincubated for 2 h and cumulus-intact eggs of the same strain (Fig. 2). Rates of penetration of eggs by sperm from BALB/c mice were lower ($P<0.01$) than from ICR at each time point. In BALB/c, the penetration rate was increased from 2 to 4 h post-insemination (26.2% to 64.2%, $P<0.01$). At 4 h post-insemination, 30% of the penetrated eggs were found to have a male pronucleus. It was at 6 h after insemination that most (95%) of the penetrated eggs

Table 2. *In vitro* fertilization between BALB/c and ICR mouse gametes after sperm preincubation[A]

Sperm donor	Egg donor	No. of eggs examined	No. (%) of eggs fertilized Total	polyspermic
ICR	ICR	58	55 (95)[a]	16 (28)
ICR	BALB/c	51	50 (98)[a]	11 (22)
BALB/c	ICR	63	14 (22)[b]	1 (2)
BALB/c	BALB/c	56	26 (46)[c]	0 (0)

[A]Superovulated eggs and spermatozoa preincubated for 2 h were used. Co-incubation time for sperm and eggs was 6 h. Experiment was conducted in 3 replicates. [a–c]; Different superscripts show significant differences ($P<0.01$).

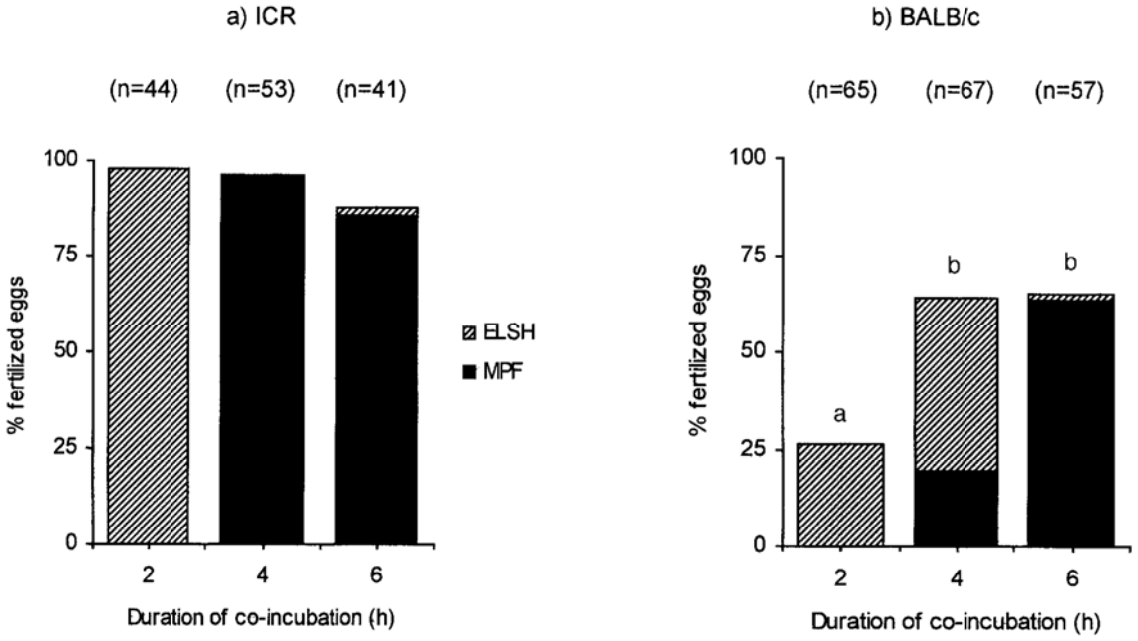

Fig. 2. Time course of sperm penetration in ICR and BALB/c mouse strains. Spermatozoa from each strain of mice were preincubated for 2 h. ELSH: enlarged sperm head; MPF: male pronucleus formation. Number of eggs examined is indicated in parentheses. a vs. b: $P<0.01$.

contained a pronucleus. In contrast, the penetration rate for sperm from ICR mice reached 98% at 2 h, and all penetrated eggs formed a male pronucleus by 4 h after insemination.

Time course of sperm-egg fusion in ICR and BALB/c mouse strain

Eggs freed from zonae with acidic Tyrode's solution and preloaded with 1 μg/ ml H-33342 were inseminated at the rate of 10 sperm/μl. Data were obtained from 5 independent experiments (total number of eggs examined ranged from 40 to 51). For ICR gametes (Fig. 3a), sperm-egg fusion was observed by 5 min, increased ($P<0.01$) between 5 and 10 min, and reached 91.8% at 30 min. An enlarged sperm head was first observed at 30 min post-insemination, and 88.6% of fused eggs had an enlarged sperm head after 60 min of co-incubation. For BALB/c mice (Fig. 3b), sperm-egg fusion was first observed at 30 min and reached 72.5% at 90 min. Only 25% of eggs contained an enlarged sperm head after 90 min of co-incubation with BALB/c sperm. The average numbers of spermatozoa attached to eggs at 5, 20, 30 and 60 min were 2.1, 3.5, 6.9 and 9.4 in ICR, and 1.1, 3.1, 5.9 and 9.6 in BALB/c, respectively.

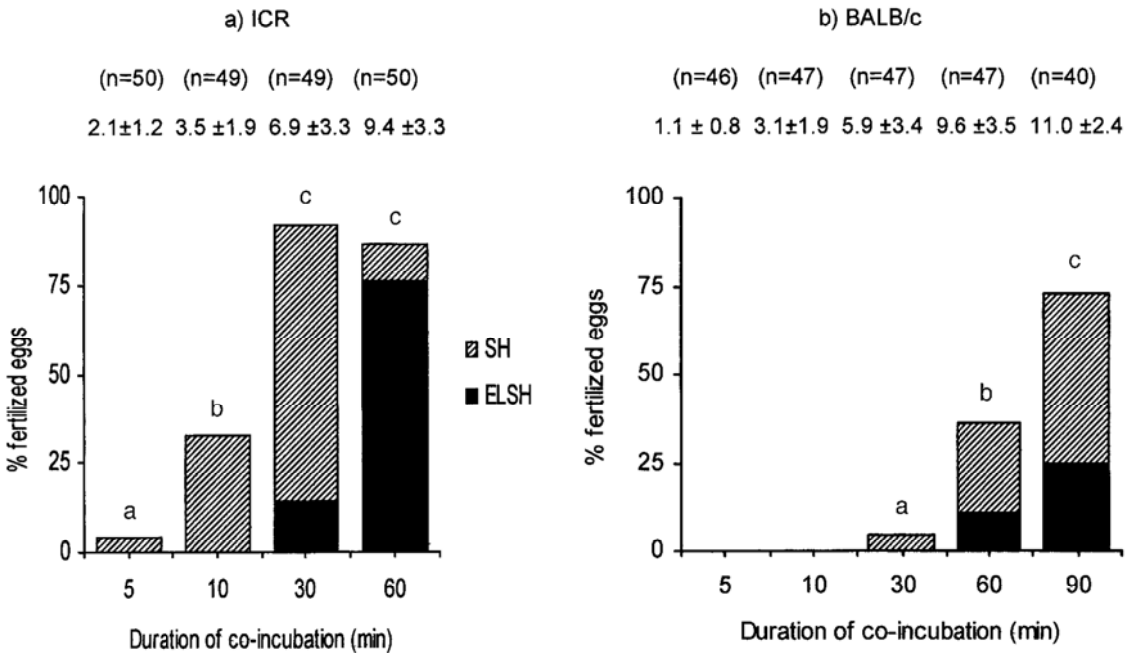

Fig. 3. Time course of sperm-egg fusion in ICR and BALB/c mouse strains. Spermatozoa were preincubated for 2 h. SH: unswollen sperm head fused with egg cytoplasm; ELSH: enlarged sperm head. Number of eggs examined is indicated in parentheses. Number of bound sperm per egg (mean ± SD) is shown above the graph. $^{a-c}$; Different superscripts indicate significantly different ($P<0.01$).

Discussion

In the present experiment, the ability of spermatozoa from the BALB/c strain of mice to fertilize eggs *in vitro* was reduced when compared to spermatozoa from ICR mice. Sperm penetration of the zona pellucida and sperm-egg fusion was slower for sperm from BALB/c than for sperm from ICR mice. The reduced fertilizing ability of BALB/c sperm may be a result of lower capacitation rates for epididymal spermatozoa from the BALB/c mouse *in vitro*.

Investigators have suggested that chlortetracycline may bind to a Ca^{2+}-affinity substance [15] or an anionic polypeptide which is secreted by the epididymis and has a sperm decapacitating activity [16, 17]. In the present study, *in vitro* capacitation rates of epididymal spermatozoa from ICR mice at different incubation time points in medium supplemented with BSA (0.4%) were consistent with rates reported by Ward and Story [11]. After 1 and 2 h of preincubation, however, the capacitation rates of BALB/c spermatozoa did not increase to the same degree as rates for ICR spermatozoa. This may be partly due to the high incidence of deformed sperm head in the BALB/c preparation, although the difference between normal and quasi-normal sperm heads was not examined in this study. The present results suggest low efficiency of *in vitro* capacitation in the BALB/c sperm population, but further analysis is necessary to elucidate the relationship between the high incidence of abnormal sperm heads and low efficiency of capacitation in this particular strain.

High fertilization rates of cumulus-intact mouse eggs have been obtained for ICR mice by using spermatozoa without preincubation if sperm and eggs were coincubated for at least 2 h [8]. On the other hand, BALB/c spermatozoa immediately after collection did not show high fertilizing ability with cumulus-intact mouse eggs. After preincubation of spermatozoa for 2 h, BALB/c spermatozoa fertilized BALB/c and ICR eggs, but the maximal fertilization rate was significantly lower than that of ICR sperm (46% vs. 98%). BALB/c eggs have always given a slightly higher fertilization rate than ICR ones (Tables 1 and 2), but the reason for this difference is not yet clear. It may be possible that high fertilizability of BALB/c eggs might compensate for low fertilizing ability of BALB/c spermatozoa.

The time required for BALB/c sperm to penetrate BALB/c cumulus-intact eggs was prolonged when compared to ICR sperm and cumuls-intact eggs (Fig. 2). These results suggest that *in vitro* capacitation of BALB/

c spermatozoa is slower or is not sufficiently achieved, not only during preincubation but also during co-incubation with cumulus-enclosed eggs.

Sperm-egg fusion normally occurs after an acrosome-reacted spermatozoon passes through the *zona pellucida*. The acrosome reaction may be spontaneously induced during preincubation *in vitro*, and spontaneously acrosome-reacted spermatozoa might penetrate zona-free eggs [1]. Nevertheless, in the present series of experiments, the acrosome reaction rate of ICR spermatozoa preincubated for 2 h was rather lower than that of BALB/c spermatozoa, but the time course of sperm-egg fusion proceeded much faster in the ICR than in the BALB/c strain. Sperm from BALB/c mice had no sperm-egg fusion until 30 min after co-incubation. In contrast, sperm-egg fusion started within 5 min and reached 92% at 30 min for sperm from ICR mice. These differences between AR and sperm-egg fusion rates suggest that most of the spontaneously acrosome reacted sperm in BALC/c might not be able to fuse with zona-free eggs. On the other hand the number of spermatozoa attached to the plasma membrane at each time point was similar for both strains. Therefore the ability of BALB/c and ICR spermatozoa to bind to zona-free eggs appeared similar, but fusion ability may be different.

The present series of experiments demonstrated that the reduced ability of BALB/c spermatozoa to penetrate cumulus-intact eggs is related to the delay in sperm-egg fusion. This distinct strain difference in sperm fertilizing ability may be useful for molecular analysis of sperm-egg fusion mechanisms.

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