Kinetics of In Vitro Fertilization and Development of an Inbred Mouse Strain: A Study Comparing RFM/Ms with C57BL/6J

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Abstract: *In vitro fertilizability and subsequent developmental competence of inbred RFM/Ms mice, which have high incidence of myeloid leukemia by radiation, was studied. When response of RFM/Ms females to various amount of eCG (1.25–10.0 i.u.) was examined, superovulation (>20 ovulated ova) occurred in animals injected with ≥5.0 i.u. eCG. The kinetics of in vitro fertilization and embryo development were examined and compared among RFM/Ms, C57BL/6J zygotes and their hybrid zygotes. The fertilization kinetics were significantly slower in RFM/Ms zygotes than in C57BL/6J zygotes. Crossbreeding experiments between C57BL/6J and RFM/Ms showed that the speed and incidence of fertilization were affected by paternal and maternal factors, respectively. The in vitro development to the blastocyst stage of RFM/Ms zygotes was significantly slower than that of C57BL/6J zygotes and their hybrid zygotes at 96 h post-insemination (PI), but not at 120 h PI. The nuclear number of RFM/Ms embryos was significantly lower than that of C57BL/6J and their hybrid embryos throughout the culture period (72, 96 and 120 h PI). Conversely, hatching at 120 h PI in C57BL/6J embryos was much slower than that of the other groups of embryos. This study showed that gametes and zygotes of RFM/Ms strain can be fertilized and developed well in vitro and that maternal and paternal factors and their interaction have complicated effects on fertilization and developmental kinetics in vitro.*

Key words: *Inbred mice, RFM/Ms, C57BL/6J, In vitro fertilization, In vitro development*

In the past few decades, the development of techniques such as *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) for many mammalian species has significantly contributed to clinical intervention in human and endangered species infertility, as well as the animal science of domesticated and laboratory animals [1]. Of these mammals, mice are most frequently used as models for reproductive physiological studies. Although many mouse strains have been bred for specific purposes of research, their reproductive profiles vary among strains [2]. In vitro handling, such as IVF and IVC is not necessarily possible in all inbred strains of mice [3–6]. The feasibility of IVF and IVC of a particular mouse strain has to be determined on a strain by strain basis in most cases.

We report the ability to manipulate gametes and embryos from an inbred RFM/Ms mouse strain that has often been used in the study of leukemia [7, 8]. In spite of the importance of this strain in radiation biology and immunology due to its high incidence of myeloid leukemia after radiation exposure [9, 10], the reproductive profile and gamete/zygote handling *in vitro* have not been studied. Therefore, we examined this strain's ovulatory response to hormone stimulation and the fertilization and developmental competence of RFM/Ms gametes and zygotes in IVF and IVC including comparison with C57BL/6J gametes and embryos. As we observed differences in the kinetics of fertilization and embryo development, an attempt was made to investigate how paternal and maternal factors affect fertilization and developmental kinetics *in vitro* by producing hybrid embryos.

Materials and Methods

Animals

Inbred RFM/Ms and C57BL/6J mice have been maintained by sib mating over 80 generations in the specific pathogen free animal facility of the National

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Table 1. Composition (mM) of TYH, kSOM and FHM

	TYH	kSOM	FHM
Phenol red	–	–	0.01 mg/ml
NaCl	119.3	95	95
KCl	4.7	2.5	2.5
$CaCl_2$	1.71	1.71	1.71
KH_2PO_4	1.2	0.35	0.35
$MgSO_4 \cdot 7H_2O$	1.2	0.2	0.2
$NaHCO_3$	25.1	25	4
Glucose	5.56	0.2	0.2
HEPES	–	–	20
EDTA-2Na[1]	–	0.01	0.01
Na-Pyruvate[2]	1	0.2	0.2
Glutamine[3]	–	1	1
BSA (Fraction V)[4]	4 mg/ml	1 mg/ml	1 mg/ml
Streptomycin[5]	0.05 mg/ml	0.05 mg/ml	0.05 mg/ml
Penicillin G (K-salt)[6]	100 U/ml	100 IU/ml	100 IU/ml

[1]Dojindo, 343-01861. [2]Sigma, P-4562. [3]Nacalai Tesque, 012-02. [4]Sigma, G-5763. [5]Meiji Seika. [6]Sigma, P-4687.

Institute of Radiological Sciences, thus the precise indication of these strains is RFM/MsNrs and C57BL/6JNrs. In this manuscript, we omit institutional code for convenience. Animals were kept on a 12 h light:12 h dark schedule (lights on at 07:00 am), with food (MB-1, Funahashi Farm Co., Ltd., Chiba, Japan) and water provided ad libitum. Mature males (≥10 weeks) were housed individually and were mated with mature females (≥8 weeks) or sacrificed for sperm collection. This study was conducted after authorization by the Safety and Ethical Handling Regulations Committee for Laboratory Animal Experiments at the National Institute of Radiological Sciences, Japan.

Culture media

All salts were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless mentioned elsewhere. Manipulation of gametes and zygotes in air was performed in prewarmed flushing and handling medium (FHM, Table 1) [11] supplemented with half-strengths of both MEM essential amino acid solution (10 μl/ml, GIBCO BRL #11130-051, Rockville, MD, USA) and non-essential amino acid solution (5 μl/ml, GIBCO BRL #11140-050). Glutamine (Sigma G-5763), pyruvate (Sigma P-4562), BSA (Nacalai Tesque 012-02) and the amino acid solutions were added to FHM on the day of the experiment and pH was adjusted to 7.4. Modified KRB [12], called TYH (Table 1), was used for sperm preincubation and IVF. Pyruvate was added immediately after overnight equilibration of the medium. In vitro fertilized zygotes were cultured in kSOM (Table 1) supplemented with half-strengths of both MEM essential amino acid solution (10 μl/ml) and MEM non-essential amino acid solution (5 μl/ml) [13]. Glutamine, pyruvate, BSA and the amino acid solutions were added immediately before equilibration of the medium.

Exp 1. Ovulatory response of RFM/Ms to eCG

In this experiment, the ovulatory response of RFM/Ms female mice to various amonunts (0–10 i.u.) of eCG was examined. For natural ovulation, male and female mice were kept overnight in the same cage and the next morning only females with vaginal plugs were used for ova collection. To examine the ovulatory response to eCG, female animals were injected intraperitoneally (IP) with either 1.25, 2.5, 5.0, 7.5 or 10.0 i.u. of eCG (Serotropin®, Teikoku Hormone Mfg. Co., Ltd., Japan), followed by an IP injection of 5.0 i.u. hCG (Gonatropin®, Teikoku Hormone) 46–48 h after eCG injection. Animals were sacrificed by cervical dislocation the day after hCG injection. Both oviducts were dissected, gently blotted on sterile filter paper to remove blood, and immersed in mineral oil (Sigma, M-8410). The cumulus-oocyte complexes (COCs) were liberated from the oviduct into a 100 μl drop of FHM and the cumulus cells were removed by pipetting with 1 mg/ml bovine testicular hyaluronidase (Sigma, H-3506). Ova with the first polar body and normal morphology were counted as ovulated ova.

Experiment 2. Comparison of fertilization kinetics in vitro

Two experiments were performed. First, fertilization

kinetics were determined for RFM/Ms and C57BL/6J mice. Fertilization was scored at 3, 5 and 7 h post-insemination (PI). The second experiment compared the fertilization kinetics *in vitro* among RFM/Ms, C57BL/6J and their hybrids (both ♀ RFM/Ms × ♂ C57BL/6J and ♀ C57BL/6J × ♂ RFM/Ms) using 2 × 2 factorial design. Fertilization was scored at 3 and 5 h PI. Males were sacrificed by cervical dislocation and epididymides were dissected. The distal cauda epididymal contents were expressed into mineral oil and sperm were capacitated in TYH for 1.5–2 h at a concentration of approximately 1 × 10^6 sperm/ml under 5% CO_2 in air at 37°C with saturated humidity. Females superovulated with 5 i.u. eCG were sacrificed 17 h post hCG injection. The COCs were collected in mineral oil equilibrated at 5% CO_2 in air and then transferred into TYH. The COCs were inseminated with capacitated sperm at a concentration of 1.0–2.0 × 10^5 sperm/ml. At a specific time point after insemination, ova were rinsed vigorously to remove adherent but non-penetrating sperm and then fixed [14]. Fixed ova were mounted on glass slides and overlaid with coverslips supported by 3:1 paraffin wax-Vaseline mixture. Ova were stained with aceto-orcein, and examined under a Nomarski interference microscope for sperm penetration and male pronuclear (MPN) formation. Ova were scored as penetrated if they had decondensed sperm head(s) in the vitellus or two or more pronuclei. Ova with no sperm heads that had resumed second meiosis or ova that had only one pronucleus were scored as parthenogenotes and were excluded from the data.

Exp 3. Comparison of developmental kinetics in vitro

Developmental kinetics were compared among RFM/Ms, C57BL/6J, and their hybrids (♀ RFM/Ms × ♂ C57BL/6J and ♀ C57BL/6J × ♂ RFM/Ms) using 2 × 2 factorial design. Ova were collected and fertilized as described in exp. 2. Ova were rinsed at 7 h PI, and those with the second polar body were selected for embryo culture in kSOM under 5% CO_2, 5% O_2 and 90% N_2 at 37°C. Embryo development to the blastocyst stage was scored at 72, 96, 120 h PI and that to the hatching blastocyst (HB) stage at 96 h, and 120 h PI. Embryos in this experiment were defined as zygotes undergoing at least the first cleavage division. For the determination of nuclear number, one-third of the embryos were fixed in 1% formalin at each time point, stained with Hoechst 33342 (Sigma, B-2261) and observed by fluorescence microscopy [15].

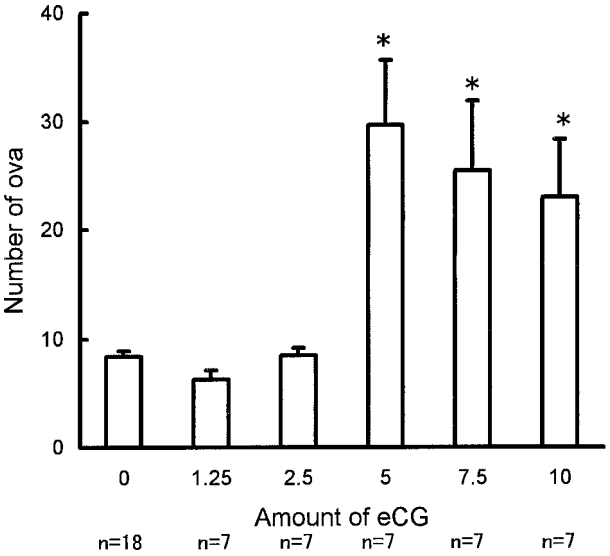

Fig. 1. Ovulatory response of RFM/Ms female mice to various amounts of eCG. Control animals (0 i.u.) were animals with vaginal plugs after an overnight stay with a male. Others were injected with 1.25, 2.5, 5.0, 7.5 or 10.0 i.u. eCG followed by hCG after 46–48 h intervals. *: Significantly different from control (0 i.u.) at $P<0.05$.

Experimental design and statistical analysis

All experiments were repeated at least four times. In order to control the variance derived from individual animals, the COCs from individual females were equally assigned to all the treatment groups in experiments 2 and 3 (controlled pooling) [16]. Percentage data were transformed by arcsin transformation (Tukey-Freeman transformation) [17], and nuclear numbers, by log transformation. Analysis was performed by means of the GLM procedure of the analysis of variance (ANOVA) using the SAS program, and multiple comparisons were made using the least significant difference (LSD) test.

Results

Exp 1. Ovulatory response of RFM/Ms to eCG

Under natural ovulatory conditions, 8.4 ± 0.5 ova were ovulated (Fig. 1). Injection of ≥5.0 i.u. eCG induced ovulation of a significantly larger number of ova ($P<0.05$), with the largest number (29.7 ± 6.0) observed in animals injected with 5 i.u. eCG (Fig. 1).

Exp 2. Comparison of fertilization kinetics in vitro

Percent penetration and MPN formation reached a plateau by 3 h and 5 h PI in C57BL/6J, respectively. In

Table 2. *In vitro* fertilization of C57BL/6J and RFM/Ms mice[1]

Post-insemination (h)	Strain	No. ova inseminated	%[2] ± S.E.M. of ova	
			penetrated	with MPN[3]
3	C57BL/6J	54	$92^{a} \pm 7$	$39^{a} \pm 14$
3	RFM/Ms	212	$30^{b} \pm 3$	$4^{b} \pm 2$
5	C57BL/6J	61	83 ± 4	$80^{a} \pm 4$
5	RFM/Ms	217	77 ± 5	$19^{b} \pm 5$
7	C57BL/6J	62	88 ± 4	78 ± 7
7	RFM/Ms	206	82 ± 4	66 ± 7

[1]Total of four replicates of experiment. [2]Denoted as percentages of total ova inseminated. [3]Male pronucleus. [a, b]At each time point, values with different superscripted letters in the same column are significantly different (P<0.05).

Table 3. Fertilization kinetics *in vitro* of inbred and crossbred ova of C57BL/6J and RFM/Ms[1]

Post-insemination (h)	Gamete		No. ova inseminated	%[2] ± S.E.M. of ova	
	Male	Female		penetrated	with MPN[3]
3	C57BL/6J	C57BL/6J	145	$97^{a} \pm 2$	$49^{a} \pm 7$
	C57BL/6J	RFM/Ms	168	$81^{b} \pm 5$	$10^{b} \pm 4$
	RFM/Ms	C57BL/6J	148	$28^{c} \pm 6$	$4^{bc} \pm 2$
	RFM/Ms	RFM/Ms	183	$16^{c} \pm 3$	$0^{c} \pm 0$
5	C57BL/6J	C57BL/6J	152	$88^{a} \pm 6$	$86^{a} \pm 6$
	C57BL/6J	RFM/Ms	185	$76^{b} \pm 5$	$67^{b} \pm 5$
	RFM/Ms	C57BL/6J	149	$79^{ab} \pm 3$	$27^{c} \pm 5$
	RFM/Ms	RFM/Ms	184	$66^{b} \pm 6$	$15^{c} \pm 4$

[1]Total of four replicates of experiment. [2]Denoted as percentage of total ova inseminated. [3]Male pronucleus. [a, b, c]At each time point, values with different superscripted letters in the same column are significantly different (P<0.05).

RFM/Ms, percent penetration and MPN formation reached a plateau by 5 h and 7 h, respectively (Table 2), indicating slow penetration and MPN formation in RFM/Ms.

The results of hybrid fertilization between RFM/Ms and C57BL/6J are shown in Table 3. When C57BL/6J male gametes were used, penetration reached a plateau by 3 h PI, while it took 5 h to reach a similar level of penetration when RFM/Ms male gametes were used (Table 3). The main effects of both male and female strains, but not interaction, on penetration were significant at both 3 and 5 h PI (P<0.001, Table 4). Male pronuclear formation in C57BL/6J homologous fertilization at 3 h PI (49%) was significantly higher than those in the other groups (≤10%, P<0.05, Table 3). At 5 h PI, percentage MPN formation was significantly low when RFM/Ms males were used compared with ova fertilized with C57BL/6J sperm (P<0.05, Table 3). The main effects of both male and female strains on MPN were significant at all time points (P<0.001, Table 4).

Table 4. P-values of male and female strain effects and interaction in crossbreeding experiment

Post-insemination	Factor	P value	
		penetrated	with MPN
3	Male	<0.001	<0.001
	Female	<0.001	<0.001
	Interaction	0.356	<0.001
5	Male	0.021	<0.001
	Female	0.001	<0.001
	Interaction	0.772	0.463

Exp 3. Comparison of developmental kinetics in vitro

No zygotes developed to the blastocyst stage at 72 h PI. At 96 h PI, blastocyst development from RFM/Ms zygotes (55%) was significantly lower than that from the other group of zygotes (≥85%, P<0.05), however, the difference was eliminated by 120 h PI (Table 5). Both paternal (P<0.001) and maternal (P<0.013) main effects and interaction (P<0.025) were significant at 96 h of blastocyst development (Table 6). Development to the

Table 5. *In vitro* development of RFM/Ms, C57BL/6J and their hybrid embryos fertilized *in vitro*[1]

Gamete		n	%[2] ± S.E.M. of embryos developed to blastocyst by		%[2] ± S.E.M. of embryos developed to hatching blastocyst by	
Male	Female		96 h PI	120 h PI	96 h PI	120 h PI
C57BL/6J	C57BL/6J	96	89[a] ± 6	92 ± 6	30 ± 7	35[a] ± 5
C57BL/6J	RFM/Ms	82	90[a] ± 4	99 ± 1	47 ± 11	81[b] ± 8
RFM/Ms	C57BL/6J	86	85[a] ± 3	95 ± 2	40 ± 7	49[a] ± 4
RFM/Ms	RFM/Ms	55	55[b] ± 6	87 ± 6	12 ± 6	50[a] ± 13

[1]Total of four replicates of experiment. [2]Denoted as percentage of total ova fertilized. [a, b]At each time point, values with different superscripted letters in the same column are significantly different (P<0.05).

Table 6. P-value of male and female effects and their interaction in development experiment

Factor	96 h blastocysts	120 h blastocysts	96 h hatching blastocysts	120 h hatching blastocysts
Male	<0.001	0.231	0.235	0.230
Female	0.013	0.665	0.425	0.011
Interaction	0.025	0.106	0.021	0.012

Table 7. Nuclear numbers of RFM/Ms, C57BL/6J and their hybrid embryos

Gamete		Mean nuclear number ± S.E.M (n) at		
Male	Female	72 h PI	96 h PI	120 h PI
C57BL/6J	C57BL/6J	19.3[ab] ± 1.5 (19)	70.0[a] ± 4.1 (20)	112.1[a] ± 8.8 (20)
C57BL/6J	RFM/Ms	21.7[a] ± 1.6 (19)	63.6[a] ± 4.8 (18)	115.6[a] ± 7.0 (20)
RFM/Ms	C57BL/6J	17.8[b] ± 1.1 (19)	66.8[a] ± 5.2 (17)	110.2[a] ± 7.9 (17)
RFM/Ms	RFM/Ms	16.2[b] ± 0.9 (17)	44.6[b] ± 4.0 (13)	87.2[b] ± 4.7 (14)

[a, b]At each time point, values with different superscripted letters in the same column are significantly different (P<0.05).

Table 8. P-value of male and female effects and their interaction in terms of nuclear number

Factor	72 h	96 h	120 h
Male	0.009	0.008	0.019
Female	0.770	<0.001	0.192
Interaction	0.155	0.062	0.072

hatching blastocyst (HB) stage was not significantly different among the groups at 96 h; however, at120 h PI, the highest percentage (81%) HB was found in ♀ RFM/Ms × ♂ C57BL/6J embryos (P<0.05, Table 5). A major increase in HB during the 96 and 120 h PI periods was only observed in ♀RFM/Ms × ♂C57BL/6J embryos (Table 5). Maternal main effect (P=0.011) and interaction (P=0.012) were significant for HB at 120 h PI (Table 6). Nuclear numbers in RFM/Ms embryos were the smallest at 96 and 120 h PI (Table 7). The main effect of male on nuclear number was significant throughout the culture period (Table 8).

Discussion

The inbred RFM/Ms strain of mice exhibits a high incidence of both myeloid and lymphoid leukemia induced by X-irradiation and chemical carcinogens [8, 9, 18], and has been used for decades to study the developmental mechanisms of leukemia [7, 10]. In spite of it being an excellent candidate model to elucidate the mechanisms of leukemia by irradiation, its use has been limited due to a high incidence of fetal loss during pregnancy [19] and cannibalism of newborns by mothers. Thus, basic data on gametes and embryo manipulation *in vitro* will facilitate the maintenance of this inbred strain.

When RFM/Ms female mice were injected with ≥5 i.u. eCG, superovulation of more than 20 normal ova was obtained. This number of ovulations is quite high when

compared with other strains of mice examined elsewhere [2]. Then, we examined *in vitro* fertilization and subsequent *in vitro* development of RFM/Ms mice under conventional conditions and indicated that RFM/Ms can be successfully fertilized in TYH (82%, Table 2) and develop in kSOM to the blastocyst stage (87%, Table 5). These results suggested the promise of IVF and IVC, and even cryopreservation for the effective production and maintenance of RFM/Ms mice. However, when the *in vitro* kinetics of fertilization and development were compared with C57BL/6J, RFM/Ms ova showed significantly slower fertilization kinetics as indicated by penetration and MPN formation. Sperm motility is unlikely to influence slow fertilization of RFM/Ms mice as RFM/Ms sperm had better motility (>70%) compared with C57BL/6J mice (60–70%) [personal observation]. Other studies [3, 6, 20–23] have shown the involvement of genetic factors in not only fertilization competence but also fertilization kinetics *in vitro* as well. The observation of differences in fertilization kinetics of the two strains used in this study and other strains [3, 6, 20-23] prompted us to study the maternal and paternal effects on fertilization and subsequent developmental kinetics *in vitro* by conducting crossbreeding experiments of these two inbred strains of mice.

Gametes from inbred BALB/c mice have very poor competence to fertilize *in vitro* [6]. The BALB/c strain has a very high incidence of abnormal sperm morphology and a significantly low capacitation competence in TYH [4, 6], which suggest that the poor IVF competence of the BALB/c strain is due to poor sperm competence to capacitate *in vitro*. Other examples of poor fertilization *in vitro* have been shown in a mouse strain, B10.BR-Y^{del}, that has a partial deletion in the Y chromosome [23–25], and in the KE mouse strain [22, 26]. In these strains, male-related factors were shown to be the major contributor to fertilization competence. Our crossbreeding study revealed the influence of both female and male genetic factors on fertilization competence, or penetration and MPN formation. When C57BL/6J males were used, penetration reached a plateau by 3 h, which was significantly faster than when RFM/Ms males were used (Tables 3 and 4). A similar tendency was found in terms of MPN formation. These results suggest the effect of male factors on the speed of penetration and MPN formation. On the other hand, when the female strain was C57BL/6J, higher levels of penetration and MPN formation were found than when the RFM/Ms strain was used as the ovum source, indicating that female factors affect the total incidence of fertilization *in vitro*. Thus, our data indicate the influence of both male and female factors on fertilization and that the nature of paternal and maternal effects are different. Unlike the others reports [3, 6, 21], maternal influence on fertilization kinetics was detected because of the examination of fertilization kinetics in a controlled manner.

Differences in developmental speed among mouse strains were indicated by McLaren and Bowman [27] and Whitten and Dagg [28]. McLaren and Bowman [27] indicated that developmental speed was not affected by strain and concluded that maternal and paternal factors did not affect developmental speed, but that the nuclear number was affected by the maternal factor. In contrast, Whitten and Dagg [28] showed that by using BALB/c and 129 inbred and their hybrid embryos, paternal factors were the major contributor to the strain differences in the speed of the third cleavage division and blastocoel formation. Although we found genetic differences in *in vitro* embryo development, our study using RFM/Ms and C57BL/6J inbred strains showed results different from those cited above. Morphological observation revealed the significance of main effects of both male and female strains and their interaction (Table 6), but nuclear number data indicated the major contribution of the male factor (Table 8). Development to the hatching blastocyst stage revealed further the different contributions of male and female from the other variables examined, that is, hatching was under the strong influence of female and male $\times$ female interaction (Tables 5 and 6).

Although previously reported studies and ours cannot be compared due to the different conditions employed, not only fertilization and developmental kinetics, but also the maternal and paternal contribution to the kinetics is likely to vary with strain. The differences in the contribution of male and female factors to various developmental stages may reflect differences in genomic control of various stages of development. It is of interest to know how maternal and paternal factors control the early developmental program in mammalian species.

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