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Improved In Vitro Fertilization and Development by Use of Modified Human Tubal Fluid and Applicability of Pronucleate Embryos for Cryopreservation by Rapid Freezing in Inbred Mice

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We examined in vitro fertilizability and development of 10 inbred mouse strains (C57BL/6J, C57BL/10, C57BL/10.D2/newSn, C57BL/10-Thy1.1, C57BL/10.Br/Sn, C3H/He, RFM/Ms, STS/A, BALB/c-*nu* and C.B-17/Icr), and the viability of frozen-thawed in vitro fertilized (IVF) embryos after embryo transfer (ET). In seven strains, fertilizability was significantly greater in modified human tubal fluid (mHTF) compared with modified Krebs-Ringer's bicarbonate solution (TYH medium). The TYH medium supported almost no fertilization in four strains. More than 80% of IVF embryos developed to the blastocyst stage by 120 h in potassium-enhanced simplex optimization medium (KSOM). Reciprocal fertilization between C57BL/6J and BALB/c-*nu* gametes in TYH medium yielded poor fertilization of BALB/c-*nu* due to spermatozoal deficiencies. Increased concentrations of bovine serum albumin and spermatozoa during capacitation and Percoll washing did not drastically affect fertilization. The mHTF, but not TYH medium, supported BALB/c-*nu* spermatozoa penetration into the zona pellucida irrespective of capacitation media. In vitro fertilized embryos frozen-thawed rapidly were transferred to surrogate mothers at the two-cell stage. Compared with that of unfrozen controls, rapid freezing had no significant effect on fetus development except in C57BL/10.D2/newSn mice. These results suggest that mHTF medium is superior with respect to IVF of inbred mice, and that KSOM adequately supports in vitro fertilized embryo development in inbred mice. The data also indicate that rapid freezing of pronucleate embryos following IVF is suitable for cryopreservation and embryo banking of inbred mice and for the production of genetically modified mice.

Embryo and gamete manipulation in vitro provides a powerful and essential tool not only for handling and maintaining laboratory animals, but also for clinical applications and domestic animal reproduction. In vitro fertilization (IVF) has enabled the simultaneous harvest of zygotes and embryos for use in animal husbandry and cryopreservation. Importantly, improved in vitro embryo culture techniques have increased the efficiency of producing genetically modified animals and have enhanced the survival of frozen-thawed embryos following embryo transfer (ET).

Embryo and gamete manipulation has been most successful in mice, producing enormous numbers of mutant, transgenic, and knockout mice and facilitating embryo banking of genetically modified mice. With regard to cryopreservation, these techniques also have reduced the cost and labor associated with animal husbandry and breeding. Furthermore, they have helped prevent genotypic mutation and contamination, augmented the stocking of animals in case of environmental accidents or infection, and facilitated safety during animal transport (24, 25, 30, 32).

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Cryopreservation of in vitro fertilized embryos is preferred over in vivo fertilization, especially in genetically modified strains, in terms of efficiency, cost, and labor because only a few males are required to obtain a large number of zygotes at a time. However, the sensitivity of embryos and gametes to manipulations such as IVF (3, 6, 9, 16, 17, 27), embryo culture (4, 12, 23, 35), and cryopreservation (5, 34), varies among genetically distinct mice. Furthermore, limited and varied outcomes of these three techniques are often encountered in some inbred mice. For example, low success rates for IVF and development have been reported in BALB/c and C3H strains (3, 11, 29, 31, 32), both of which are frequently used to produce congenic strains. Thus, fertilization and developmental competence need to be assessed on a strain-by-strain basis. Poor fertilization success has been explained by deficiencies in spermatozoa (hereafter referred to as sperm) capacitation, resulting in the inability of sperm to penetrate the zona pellucida (15, 31). In addition, as with other mammalian species, retarded (or blocked) development in vitro (the "two-cell block" [2]) has been reported at the two-cell stage of outbred and some inbred mouse strains. Because poor IVF and development is detrimental to subsequent embryo manipulations such as cryopreservation, the development of high-percentage (at least 50%) IVF and development techniques is a prerequisite to successful embryo manipulations.

Table 1. Composition of TYH, mHTF, KSOM, and PB1 media

Component	TYH (m*M*)	mHTF (m*M*)	KSOM[a] (m*M*)	PB1 (m*M*)
NaCl	119.3	101.6	95.0	137.0
KCl	4.7	4.70	2.5	0.27
$CaCl_2$	1.71	5.14	1.71	0.11
KH_2PO_4	1.2	0.40	0.35	0.15
Na_2HPO_4	-	-	-	0.81
$MgSO_4$	1.2	0.20	0.20	-
$MgCl_2$	-	-	-	0.049
$NaHCO_3$	25.1	25.0	25.0	
Glucose	5.56	2.78	0.2	5.56
Lactate (Na^+ salt)	-	18.36	10.0	-
EDTA (disodium salt)[b]	-	-	0.01	-
Pyruvate (Na^+ salt)	1	0.34	0.20	0.33
Glutamine	-	-	1	-
BSA (Fraction V)[c]	4 mg/ml	4 mg/ml	1 mg/ml	3 mg/ml
Streptomycin[d]	0.05 mg/ml	0.05 mg/ml	0.05 mg/ml	0.05 mg/ml
Penicillin G (K^+ salt)[e]	100 IU/ml	100 IU/ml	100 IU/ml	100 IU/ml

[a]KSOM also contains 10 µl of MEM essential amino acid solution/ml and 5 µl of non-essential amino acid solution/ml.
[b]Wako Pure Chemical Industries, Ltd. (Osaka, Japan) 343-01861.
[c]Bovine serum albumin, Nakalai Tesque (Kyoto, Japan) 012-02.
[d]Meiji Seika (Tokyo, Japan).
[e]Sigma Chemical Co. (St. Louis, Mo.) P-4687.
TYH = modified Krebs-Ringer's bicarbonate solution modified by Toyoda, Yokoyama and Hoshi (33); mHTF = modified human tubal fluid (25); KSOM = potassium-enhanced simplex optimization medium (21).

In the study reported here, we examined fertilization and developmental competence in vitro as well as the viability of frozen-thawed embryos after ET in 10 major inbred strains (C57BL/6J, C57BL/10, C57BL/10.D2/newSn, C57BL/10-Thy1.1, C57BL/10.Br/Sn, C3H/He, RFM/Ms, STS/A, BALB/c-*nu* and C.B-17/Icr). We compared two IVF media: the modified Krebs-Ringer's bicarbonate solution, TYH medium (33), and human tubal fluid (HTF) (28) modified (mHTF) by Nakagata for mouse IVF (25). We determined the developmental competence of IVF embryos by culturing them in KSOM formulated by Lawitts and Bigger (21) and modified by Ho and co-workers (13) to overcome the two-cell block of outbred mouse embryos. We also attempted to elucidate the cause of low fertilization success in BALB/c-*nu* in TYH medium by varying the conditions of sperm capacitation and insemination. Finally, we observed that the viability of pronucleate IVF embryos frozen and thawed by the procedure of rapid freezing described by Nakao and co-workers (26) is comparable to that of unfrozen embryos for most strains. Our study will provide useful data for various in vitro manipulations of gametes and embryos.

Materials and Methods

Animals. Ten inbred mouse strains (C57BL/6J, C57BL/10, C57BL/10.D2/newSn, C57BL/10-Thy1.1, C57BL/10.Br/Sn, C3H/He, RFM/Ms, STS/A, BALB/c-*nu* and C.B-17/Icr) were obtained from the specific-pathogen-free (SPF) breeding facility of the National Institute of Radiological Sciences, Japan, where mice were bred by sib mating and maintained under conditions described previously (22). Mice used in this study were transferred from the SPF facility to a conventional animal facility, then were maintained for at least a week (females) or 3 to 4 weeks (males) before use. For BALB/c-*nu* mice, males were always BALB/c-*nu*/+ and females were always BALB/c-*nu*/*nu*. For ET, MCH:ICR mice (CLEA JAPAN, Inc., Tokyo, Japan) were used as recipients and 4 mice were housed in each cage. Mice were housed in sterilized metal cages (30 × 17 × 10.5 cm) with autoclaved bedding that was changed once a week. A maximum of five female inbred mice were housed per cage, and males were housed individually. Mice were allowed ad libitum access to MB-1 food pellets (Funahashi Farm Co., Ltd., Chiba, Japan) and chlorinated water that was changed twice weekly. The animal room was maintained at 24 ± 2°C and 55 ± 10% humidity under a light/dark regimen of 12 h on and 12 h off (lights on at 7 a.m.). For ova and sperm recovery, the oviducts and the distal portions of the cauda epididymides were removed from animals after sacrifice by cervical dislocation, and ET was performed on animals under anesthesia induced with avertin (14).

Animal treatment and experimental procedures were in strict accordance with Guidelines for Handling of Laboratory Animals for Biomedical Research compiled by the Safety and Ethical Handling Regulations Committee for Laboratory Animal Experiments of the National Institute of Radiological Sciences, Japan.

Medium preparation. All salts were purchased from Nacalai Tesque Inc. (Kyoto, Japan), unless indicated otherwise. Media (Table 1) lacking pyruvate, bovine serum albumin (BSA), glutamine, and amino acids were stored at 4°C for no longer than 1 week. Pyruvate (P-4562, Sigma Chemical Co., St. Louis, Mo.), glutamine (G-5763, Sigma Chemical Co.), BSA (012-02, Nacalai Tesque Inc.), minimal essential amino acid solution (MEM; 11130-051 GIBCO BRL, Rockville, Md.) and non-essential amino acid solution (11140-050, GIBCO BRL) were added as necessary immediately before equilibration. Media used for IVF (TYH and mHTF) were equilibrated overnight under 5% CO_2 in air at 37°C and saturated humidity, and KSOM was equilibrated for 3 h under 5% CO_2, 5% O_2, and 90% N_2 at 37°C and saturated humidity.

Sperm preparation, in vitro fertilization and embryo culture. Ova and sperm were harvested as described (20). Briefly, females 8 to 12 weeks of age were injected intraperitoneally with 5 IU of equine chorionic gonadotropin (eCG; Serotropin; Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG; Gonatropin; Teikoku Hormone Mfg. Co., Ltd.) 46-48 h apart. The females were sacrificed 16 h after hCG injection, and cumulus-oocyte complexes (COCs) were collected from the oviducts into mineral oil (M-8410, Sigma Chemical Co.). To compare fertilization in TYH medium versus mHTF, COCs from individual animals were divided evenly and directly trans-

ferred to insemination drops by use of a watchmaker's forceps. Sperm were collected from the distal cauda epididymides under mineral oil and were capacitated in either TYH medium or mHTF at 1 to 2×10^6 sperm/ml (approx. 1:500 dilution) for 1.5 to 2 h under 5% CO_2 in air at 37°C. Capacitated sperm suspensions were diluted 1:10 to a final concentration of 1 to 2×10^5 sperm/ml. After a 5-h sperm:ova coincubation, ova were rinsed vigorously to remove adherent but non-penetrating sperm, and ova were transferred to 50 µl of KSOM supplemented with half the strength of MEM amino acids (13, 21) for culture under 5% CO_2, 5% O_2, and 90% N_2 at 37°C. Fertilization was defined as ova that had developed to the two-cell stage by 24 h postinsemination (PI). In vitro development to the blastocyst stage was examined at 96 and 120 h PI. Blastocysts were scored for expansion and hatching. Hatching blastocysts included embryos in the process of hatching and completely hatched embryos.

Capacitation of sperm and fertilization of BALB/c-*nu* mice in vitro. The poor fertilization success of BALB/c-*nu* mice in TYH medium was studied in more detail. To determine whether the deficiency was associated with the sperm or ova, IVF was performed, using TYH medium, by reciprocal exchange of gametes between BALB/c-*nu* and C57BL/6J mice. Next, we examined the effect of BSA concentration (4 to 20 mg/ml) during capacitation and sperm:ova coincubation as well as the effect of sperm concentration (1 to 2×10^6 and 1 to 2×10^7 sperm/ml) during capacitation in BALB/c-*nu* mice. Then, we studied how immediate postcollection washing of sperm with Percoll (Pharmacia, Piscataway, N.J.) affected fertilization because there is evidence that washing can remove decapacitation factors from the surface of sperm (8). Sperm were dispersed in TYH medium for 10 min at 37°C and were gently placed at the top of the 30 and 80% Percoll layers in a conical tube (No. 2095, Becton Dickinson, Franklin Lakes, N.J.). The suspension was centrifuged for 10 min at 750 ×*g* at room temperature, and the sperm pellet was recovered and capacitated in TYH for 2 h before insemination. The effect of Percoll washing also was examined in C.B-17/Icr mice because this strain has the same ancestor as that of the BALB/c-*nu* strain.

Lastly, we compared the effect of TYH medium and mHTF on sperm capacitation and sperm:ova coincubation. In vitro fertilization was performed as described above. In this series of experiments, ova at 5 h PI were washed and fixed in 2% formaldehyde and 2% glutaraldehyde (19). Fixed ova were mounted on glass slides and overlaid with coverslips supported by a paraffin wax-Vaseline mixture (3:1). Ova were stained with aceto-orcein and were examined for zona penetration and male pronuclear formation by use of Nomarski interference microscopy. Zona penetration was defined as an ovum with a sperm head within the zona pellucida. Ova were scored as fertilized if they had a 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 study.

Viability of inbred IVF embryos cryopreserved by rapid freezing. Sperm capacitation and sperm/ova coincubation were performed in mHTF as described previously. Ova containing the second polar body and pronuclei at 5 h PI were transferred to PB1 solution (Table 1) warmed to 37°C and were divided into two groups: one for freezing and another for unfrozen controls that were immediately transferred to 50-µl drops of KSOM. The procedure of rapid freezing and thawing has been described elsewhere (26). After storing for 0.5 to 1 h in liquid nitrogen, the cryotubes were warmed at room temperature for 1 min followed by addition of 900 µl of warm (37°C) PB1 solution containing 0.3*M* sucrose (304-04, Nacalai Tesque Inc.). Ova were transferred to 60-mm Petri dishes (No. 1007, Becton Dickinson) containing warm PB1 solution. After incubation for 10 min, the ova were rinsed three times in PB1 solution before culture in KSOM.

Female recipient mice were housed in a cage with vasectomized male ICR mice (CLEA JAPAN) for mating in advance. The embryos were examined for cleavage at 24 h PI and two-cell embryos were transferred to the oviducts of pseudopregnant recipient mice on the day of vaginal plug formation (day 1 of pseudopregnancy). The number of embryos transferred to each recipient ranged from 7 to 20, depending on the number of embryos obtained and survival after thawing. Implantation and fetus development were examined by necropsy on day 19 of pregnancy.

Experimental design and statistical analyses. To control the variance in data derived from individual animals, the COCs or fertilized ova from individual females were assigned equally to all treatment groups. All data were recorded as percentages, then were subjected to arcsin (Tukey-Freeman) transformation (38). Transformed data were analyzed by analysis of variance with a random block design, using the SAS program, except for the study of the effect of BSA concentration that was analyzed by the χ^2-test. Males were defined as blocks because motility and viability (and most likely fertilizability) varied among male donors (19). When necessary, multiple comparisons were made using the least significant difference test and 5% significance level.

Results

In vitro fertilization and development of inbred mice. Fertilization was defined as development to the two-cell stage by 24 h PI. The percentage of fertilized ova from C57/BL6J, STS/A and RFM/Ms strains did not differ significantly between TYH medium and mHTF (Table 2). Fertilization was > 80% in C57/BL6J and RFM/Ms strains in both media. The mHTF yielded significantly higher fertilization percentages than did TYH medium for C57BL/10, C57BL/10.D2/newSn, C57BL/10-Thy1.1, C57BL/10.Br/Sn, C3H/He, BALB/c-*nu* and C.B-17/Icr mice ($P < 0.05$, Table 2). Differences in fertilization success between the two media were prominent in C57BL/10-Thy1.1, C57BL/10.Br/Sn, BALB/c-*nu*, and C.B-17/Icr stains (Table 2). Fertilization of strain STS/A was not medium dependent, but rather varied with the sources of ova and sperm because sperm from one male or ova from one female yielded fertilization percentages ranging from 0 to 100% in both media (data not shown). Using KSOM, high percentages of development to the blastocyst stage at 96 h PI (> 78%) and the expanded blastocyst stage at 120 h PI (> 75%) were observed for all inbred strains. There were only marginally significant differences in development of C57BL/10, C3H/He and RFM/Ms embryos between the two media used for IVF (Table 2). Development to the hatching blastocyst stage ranged from 27 to 87% depending on strain, and there was a tendency toward lower hatching in C57BL derivative strains compared with others.

In vitro fertilization of BALB/c-*nu* mice. In IVF between C57BL/6J and BALB/c-*nu* gametes, significantly lower fertilization was observed for sperm from BALB/c-*nu* mice ($P < 0.05$, Fig.

Table 2. Comparison of in vitro fertilization in TYH medium and mHTF and subsequent development in vitro of various inbred mice[a]

Strain	No. of males	No. of females	IVF medium	Total no. of ova	No. of 2-cell embryos at 24 h PI (% ± SEM)[b]	No. (% ± SEM)[c] of embryos at 96 h PI		No. (% ± SEM)[c] of embryos at 120 h PI		
						Blastocysts	Expanded blastocysts	Blastocysts	Expanded blastocysts	Hatching blastocysts
C57BL/6J	5	10	TYH	90	85 (93 ± 4)	68 (78 ± 4)	54 (64 ± 4)	73 (85 ± 5)	65 (75 ± 6)	43 (51 ± 9)
			mHTF	98	96 (98 ± 1)	80 (84 ± 7)	73 (77 ± 8)	87 (92 ± 4)	77 (81 ± 5)	53 (55 ± 5)
C57BL/10	6	12	TYH	94	55 (58* ± 8)	47 (88 ± 4)	42 (81 ± 6)	54 (99 ± 1)	53 (97* ± 2)	18 (27 ± 6)
			mHTF	96	94 (98* ± 2)	79 (85 ± 4)	63 (66 ± 5)	87 (92 ± 3)	76 (79* ± 3)	34 (35 ± 6)
C57BL/10-Thy1.1	5	10	TYH	85	18 (20* ± 7)	15 (ND)	13 (ND)	16 (ND)	16 (ND)	5 (ND)
			mHTF	104	101 (86* ± 2)	89 (87 ± 3)	73 (71 ± 5)	95 (93 ± 3)	90 (89 ± 3)	46 (46 ± 5)
C57BL/10.BR/Sn	5	10	TYH	88	1 (1* ± 0)	1 (ND)	1 (ND)	1 (ND)	1 (ND)	1 (ND)
			mHTF	72	70 (98* ± 1)	61 (90 ± 5)	47 (67 ± 9)	64 (93 ± 3)	60 (85 ± 6)	29 (40 ± 10)
C57BL/10.D2/newSn	9	17	TYH	201	103 (53* ± 6)	95 (94 ± 2)	82 (82 ± 4)	96 (96 ± 2)	94 (94 ± 2)	74 (75 ± 5)
			mHTF	192	133 (70* ± 6)	113 (84 ± 4)	103 (77 ± 5)	121 (95 ± 3)	118 (91 ± 3)	93 (71 ± 7)
C3H/He	8	16	TYH	286	123 (46* ± 5)	103 (83* ± 4)	92 (73* ± 5)	111 (91* ± 3)	105 (84* ± 4)	97 (77* ± 4)
			mHTF	260	254 (97* ± 1)	232 (91* ± 3)	212 (83* ± 4)	248 (97* ± 2)	237 (93* ± 4)	221 (87* ± 3)
STS/A	13	25	TYH	434	250 (54 ± 7)	236 (94 ± 2)	221 (88 ± 4)	244 (97 ± 2)	237 (95 ± 2)	215 (84 ± 5)
			mHTF	443	260 (59 ± 6)	236 (88 ± 4)	201 (75 ± 4)	242 (90 ± 4)	231 (86 ± 5)	208 (76 ± 5)
RFM/Ms	7	14	TYH	348	280 (82 ± 4)	222 (76* ± 3)	156 (55* ± 4)	264 (92* ± 3)	255 (89 ± 4)	210 (74 ± 4)
			mHTF	361	328 (89 ± 3)	264 (90* ± 2)	199 (70* ± 3)	324 (99* ± 1)	313 (95 ± 1)	241 (77 ± 4)
BALB/c-*nu*	16	32	TYH	326	18 (6* ± 1)	9 (ND)	5 (ND)	13 (ND)	13 (ND)	5 (ND)
			mHTF	337	297 (86* ± 3)	268 (89 ± 3)	225 (71 ± 4)	273 (90 ± 2)	247 (82 ± 3)	215 (69 ± 6)
C.B.-17/Icr	6	12	TYH	196	21 (11* ± 4)	17 (ND)	16 (ND)	20 (ND)	17 (ND)	13 (ND)
			mHTF	220	201 (93* ± 3)	193 (95 ± 3)	170 (83 ± 5)	187 (93 ± 2)	173 (86 ± 3)	156 (77 ± 4)

[a]Capacitation and insemination were performed in TYH medium or mHTF, and at 5 h postinsemination (PI) the eggs were washed and transferred to KSOM with amino acids.
[b]Percentage of the total embryos inseminated. Percentage data were transformed by arcsin transformation, and were analyzed by analysis of variance (ANOVA). Multiple comparisons were made using the least significant difference test.
[c]Percentage of 2-cell embryos at 24 h PI. Percentage data were transformed by arcsin transformation, and were analyzed by ANOVA. Multiple comparisons were made using the least significant difference test.
ND = not determined because a low number of embryos was obtained.
*Significant difference in individual strains between the two in vitro fertilization (IVF) media ($P < 0.05$).

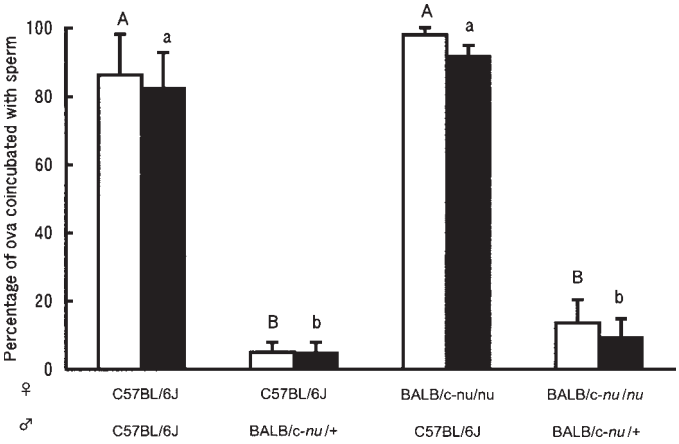

Figure 1. Penetration through the zona pellucida (open bars) and fertilization (filled bars) in modified Krebs-Ringer's bicarbonate solution (TYH medium) between BALB/c-*nu* and C57BL/6J gametes in three replicate experiments. Ova were fixed and stained at 5 h post-insemination (PI) to examine penetration through the zona pellucida and fertilization under Nomarski interference microscopy. Error bars indicate SEM. Percentage data were transformed by arcsin transformation and were analyzed by analysis of variance (ANOVA), and multiple comparisons were made using the least significant difference test. Within each fertilization process (noted with uppercase letters [penetration] and lowercase letters [fertilized]), data that are significantly different ($P < 0.05$) are labeled with different letters.

1), indicating that BALB/c-*nu* sperm in TYH medium was deficient in this regard. Fertilization was not significantly affected by BSA concentration (Table 3) or sperm concentration during capacitation (Table 4). Washing sperm with Percoll (Table 5) significantly improved fertilization from 10% to 20% in BALB/c-*nu* mice

Table 3. Effect of BSA concentration on in vitro fertilization of inbred BALB/c-*nu* mice[a]

BSA concentration (mg/ml)	No. of males tested	No. of ova coincubated with sperm	Fertilized ova (%)
4	2	39	4 (10.3)
10	2	35	2 (5.7)
20	2	41	4 (9.8)

[a]Sperm were capacitated and coincubated with eggs under the various concentrations of BSA. After 5 h of sperm:ova coincubation, ova were fixed and stained for examination. The data were analyzed by the χ^2-test.

Table 4. Effect of sperm concentration during capacitation on in vitro fertilization of inbred BALB/c-*nu* mice[a]

Concentration (sperm/ml)	No. of males tested	No. of ova co-incubated with sperm	Fertilized ova	Mean % ± SEM[b]
$1\text{-}2 \times 10^6$	5	53	7	15.9 ± 7.2
$1\text{-}2 \times 10^7$	5	55	3	6.4 ± 3.8

[a]Sperm were capacitated at various concentrations for 1.5 to 2 h, then were inseminated. Ova were fixed and stained for examination at 5 h PI. A total of five replicates, each having 7 to 16 ova, were performed depending on the number of ova collected in each experiment. Percentage data were transformed by arcsin transformation, and were analyzed by ANOVA.
[b]Calculated from percentage data. For ANOVA, percentage data were transformed by arcsin transformation.

($P < 0.05$), and from 6.4% to 18.4% in C.B-17/Icr mice ($P < 0.05$), although these improved percentages were still low compared with the results of IVF using mHTF (Table 2).

The effect of medium on capacitation and sperm:ova coincubation was compared at 5 h PI in BALB/c-*nu* mice. Zona penetration and fertilization of ova coincubated in TYH medium was significantly lower than with ova inseminated in mHTF ir-

Table 5. Effect of washing sperm with Percoll on in vitro fertilization of inbred BALB/c-*nu* and C.B.-17 strains[a]

Strain	No. of males tested	Percoll wash	No. of ova coincubated with sperm	No. of fertilized ova	Mean % ± SEM[b]
BALB/c-*nu*	7	–	92	10	7.7 ± 3.4
	7	+	98	14	20.0* ± 6.6
C.B.-17	13	–	160	13	6.4 ± 2.4
	13	+	160	27	18.4* ± 5.9

[a]Sperm were dispersed in TYH medium briefly, then were centrifuged in Percoll. Recovered sperm were capacitated and coincubated with ova. At 5 h PI, ova were fixed and stained for examination. Seven replicates with 5 to 23 ova in each replicate for BALB/c and 13 replicates with 5 to 23 ova in each replicate for C.B.-17 were performed depending on the number of ova collected in each experiment.
[b]Calculated from percentage data. For ANOVA, percentage data were transformed by arcsin transformation.
*Values are significantly different ($P < 0.05$).

respective of the capacitation medium ($P < 0.05$), indicating that TYH medium supports capacitation but not penetration through the zona pellucida and vitellus (Fig. 2).

Viability of inbred IVF embryos cryopreserved by rapid freezing. The development of cryopreserved embryos to the two-cell stage did not differ significantly from that of unfrozen controls except in C57BL/10 and C.B-17/Icr mice ($P < 0.05$; Table 6). The percentage of implantation sites did not differ significantly between treatment groups for any of the strains (Table 6). The C57BL/10.D2/newSn was the only strain to show significantly less fetal development on day 19 compared with that for controls ($P < 0.05$). For the other strains, there were no significant differences in fetal development between treatment groups (Table 6), although there was a tendency toward reduced development in frozen-thawed embryos compared with unfrozen controls.

Discussion

We have documented that mHTF is superior to TYH medium for IVF of inbred mouse strains. For all strains except STS/A, we obtained > 80% fertilization, a level that is sufficient for applications involving in vitro embryo manipulations. We also observed that, in all strains tested, KSOM supported a high degree of embryo development without the two-cell block observed in some inbred and outbred mice. Furthermore, fetal development of pronucleate IVF embryos frozen-thawed rapidly was comparable to that of unfrozen control embryos except in C57BL/10.D2/newSn, indicating that pronucleate-stage embryos are usable for cryopreservation.

In vitro fertilizability in mice varies depending on genotype and the conditions used for capacitation and fertilization (3, 6, 9, 16, 17, 27). The inbred strains that yielded low IVF results included BALB/c (3, 31, 32), C3H (32) and 129 (31), which are among the most frequently used inbred strains for human disease models or congenic gene modification. Thus, establishing a series of IVF and cryopreservation procedures is important for the advancement of laboratory animal science. Given a rate of approximately 50% for in vivo fertilization of superovulated inbred mice (C57BL/6J: [18], BALB/c: [29]), IVF success rate > 50%, and ideally, > 80% will be required for practical use of IVF.

Four types of media have been used for mouse IVF, namely modified Krebs-Ringer's bicarbonate solution (15, 33), modified Tyrode's solution (9, 36), tissue culture medium with Earl's balanced salt solution supplemented with amino acids and vitamins (13), and mHTF, a medium based on human tubal fluid (28) as modified by Nakagata (25). In most instances, these media have been evaluated for only a few mouse strains. Here, we examined TYH medium and mHTF, and found that the former is only useful for IVF in a limited number of inbred strains (C57BL/6J and RFM/Ms), whereas mHTF yielded a high percentage of fertilization in most inbred strains except STS/A. The results for STS/A mice were quite paradoxical in that, irrespective of IVF medium, fertilization rate ranged from zero to 100% depending on the particular animals from which sperm and ova were harvested. The factors affecting IVF in this strain are yet to be determined.

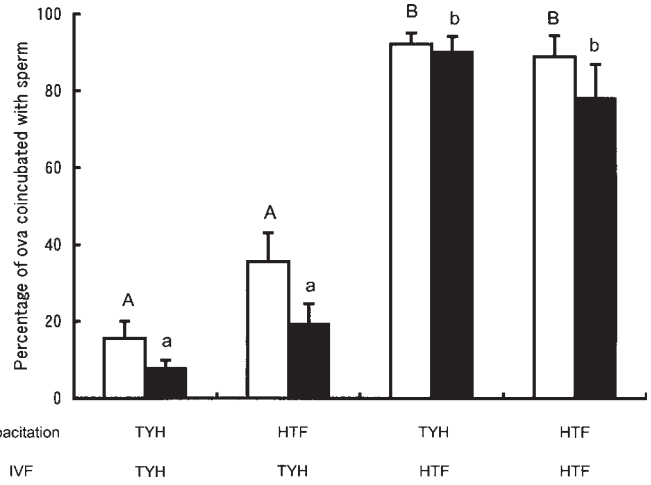

Figure 2. Penetration though the zona pellucida (open bars) and fertilization (filled bars) of BALB/c-*nu* mice capacitated and inseminated in either TYH medium or modified human tubal fluid (mHTF) in three replicate experiments. Ova were fixed and stained at 5 h PI to examine penetration through the zona pellucida and fertilization under Nomarski interference microscopy.

Developmental competence in vitro varies depending on genotype and culture conditions (4, 12, 23, 35). Although we found substantial effects of the IVF medium on in vitro development of C3H/He, C57BL/10 and RFM/Ms embryos, we consider the differences marginal because more than 90% of the embryos reached the blastocyst stage by 120 h PI, indicating that the two-cell block did not occur in KSOM in any of the strains examined. One of the known genetic factors involved in in vitro development is haplotype of the H-2 complex. Previous work suggested that embryos having the H-2^k haplotype develop more slowly than do those with the H-2^b haplotype (12). In our study, there was no correlation between H-2 haplotype and in vitro development because the development of C57BL/10.Br/Sn and C3H/He embryos (both H-2^k) was comparable to that of other strains (Table 2). These results suggest that the previously documented effect of the H-2 haplotype on in vitro development may reflect anomalies of the culture environment.

The cause of poor fertilization in the BALB/c strain in TYH medium was addressed by reciprocal insemination between BALB/c-*nu* and C57BL/6J gametes. Deficient fertilization always resulted when BALB/c-*nu* sperm were used irrespective of the ovum source, indicating the inability of sperm to capacitate or penetrate the zona pellucida rather than deficiency in the ova (e.g., zona hardening) (37). These results are consistent with work by Choi and co-workers (3) and Sztein and co-workers (31), that BALB/c sperm exhibit low fertilizability during reciprocal insemination between hybrid and BALB/c strains. Factors that affect IVF include BSA concentration (37), sperm concen-

Table 6. Survival, two-cell development, and fetal development after embryo transfer (ET) of frozen-and-thawed embryos[a]

Strain	Treatment	Total no. of embryos (n[b])	No. (% ± SEM)[c]		No. of two-cell embryos transferred	No. (% ± SEM)[d]	
			Embryos with normal morphology after thawing	Two-cell stage at 24 h PI		Implantation site	Fetus
C57BL/6J	Control	179 (8)		166 (93 ± 4)	160	124 (77 ± 5)	84 (52 ± 4)
	Frozen	178 (8)	169 (95 ± 3)	159 (94 ± 3)	159	119 (74 ± 8)	72 (45 ± 6)
C57BL/10	Control	100 (7)		79 (79* ± 4)	76	57 (76 ± 6)	47 (63 ± 5)
	Frozen	99 (7)	92 (93 ± 2)	58 (58* ± 6)	53	47 (89 ± 3)	37 (72 ± 4)
C57BL/10-Thy1.1	Control	83 (6)		70 (85 ± 5)	70	52 (73 ± 15)	36 (50 ± 11)
	Frozen	83 (6)	82 (97 ± 2)	67 (81 ± 4)	67	45 (70 ± 14)	32 (49 ± 11)
C57BL/10.BR/Sn	Control	104 (6)		102 (98 ± 1)	102	75 (73 ± 6)	50 (72 ± 6)
	Frozen	105 (6)	100 (95 ± 3)	95 (91 ± 3)	95	67 (70 ± 12)	46 (63 ± 11)
C57BL/10. D2/newSn	Control	76 (6)		64 (85 ± 5)	108	57 (89 ± 4)	44 (69* ± 3)
	Frozen	76 (6)	69 (90 ± 3)	56 (74 ± 5)	56	43 (79 ± 7)	26 (45* ± 8)
C3H/He	Control	119 (9)		108 (90 ± 3)	108	94 (87 ± 5)	68 (62 ± 4)
	Frozen	119 (9)	102 (85 ± 4)	84 (70 ± 8)	84	73 (92 ± 7)	44 (53 ± 9)
STS/A	Control	61 (6)		59 (97 ± 2)	59	52 (88 ± 5)	26 (44 ± 11)
	Frozen	67 (6)	65 (97 ± 2)	56 (83 ± 7)	56	48 (88 ± 6)	17 (32 ± 6)
RFM/Ms	Control	128 (9)		112 (87 ± 4)	110	86 (79 ± 10)	68 (64 ± 10)
	Frozen	129 (9)	122 (94 ± 2)	102 (79 ± 5)	84	73 (74 ± 7)	44 (45 ± 7)
BALB/c-*nu*	Control	109 (9)		103 (94 ± 2)	103	87 (85 ± 6)	46 (45 ± 5)
	Frozen	109 (9)	105 (97 ± 2)	91 (82 ± 6)	91	60 (76 ± 5)	24 (27 ± 6)
C.B.-17/Icr	Control	116 (8)		113 (97* ± 1)	113	90 (80 ± 2)	45 (40 ± 7)
	Frozen	116 (8)	111 (96 ± 4)	101 (87* ± 3)	101	66 (64 ± 8)	34 (34 ± 7)

[a]Fertilization was performed in mHTF medium, and, at 5 h PI, the ova were cultured either in KSOM with amino acids (control) or frozen and subsequently thawed and cultured in KSOM with amino acids (frozen).
[b]Number of males tested that are equivalent with number of replicates.
[c]Percentage of total embryos tested. Percentage data were transformed by arcsin transformation and were analyzed by ANOVA. Multiple comparisons were made using the least significant difference test.
[d]Percentage of total transferred embryos. Percentage data were transformed by arcsin transformation and were analyzed by ANOVA. Multiple comparisons were made using the least significant difference test.
*Significantly different from control ($P < 0.05$).

tration during capacitation and insemination (6, 16), and the extent of removal of decapacitation factors from the epididymal sperm surface (8). We addressed each of these issues. Although washing sperm with Percoll increased fertilization to some extent (7 to 20%) in BALB/c-*nu* and its derivative strain C.B-17/Icr, none of the aforementioned factors were sufficient to overcome the fertilization deficiency. It should be noted that non-motile sperm were layered between the 30% and 80% Percoll layers, and that, for our experiments, we harvested only motile sperm that sediment in the layer below 80% Percoll. Since non-motile sperm do not contribute to fertilization (1), this simple method for separating motile from non-motile sperm in cryopreserved samples may enhance IVF success.

Fertilization deficiency of BALB/c-*nu* in TYH medium was further studied by changing the medium for capacitation and sperm:ova coincubation. Although both TYH medium and mHTF were adequate for sperm capacitation, mHTF was far superior during sperm:ova coincubation. This result indicates that sperm penetration of the zona pellucida is not supported in TYH medium and that capacitation and zona penetration have different requirements. The notable differences in components between TYH medium and mHTF are the higher concentrations of calcium and lactate and lower concentration of potassium, magnesium and glucose in mHTF (Table 1).Since lactate, potassium, magnesium, and glucose concentrations were within the range sufficient for mouse IVF (7, 10, 27), poor zona penetration in TYH medium most likely reflects the relatively low calcium concentration. Calcium is critical for early embryo development in many mammalian species, contributing to sperm capacitation, the acrosome reaction, sperm-zona and sperm-ooplasm interactions, block to polyspermy, and consequent cleavage (37). It is also possible, as reported by Fraser and Herod (7), that ionic balance such as Na^+/K^+ affects sperm capacitation and zona penetration during IVF. Our IVF data suggest the possibility that differences in sensitivity to calcium or ionic balance exist among inbred and outbred strains. Defining the medium components that control fertilization may lead not only to formulation of a standard IVF medium for all mouse strains, but also to a medium that can be used throughout IVF and early embryo development. Formulation of such medium would help reduce the stress that ova encounter when forced to adjust to a changing environment. Toward this goal, we are currently using BALB/c gametes as a sensitive model for determining the medium components that affect capacitation and sperm-ova interactions.

The viability of cryopreserved embryos (rapid or slow freezing) varies depending on genetic background and the developmental stage at which the embryos are frozen (5, 24, 30, 34). Inbred strains such as BALB/c are more sensitive than outbred strains, and freezing at early embryo stages is less successful than at later stages (11, 24, 30). Results of our study indicated a tendency toward inferior fetal development in frozen and thawed embryos compared with unfrozen controls, although the differences were not statistically significant, except for C57BL/10.D2/newSn embryos. The lack of statistical significance may be due to the large variations within groups. Our fetal developmental data are comparable to those of other studies in which inbred and hybrid two-cell embryos were rapidly frozen (26), indicating that the pronucleate stage of IVF embryos is useful in cryopreservation of inbred mice.

The procedures that span IVF and rapid freezing are ame-

nable not only to inbred mice, but also to mice carrying gene modifications. In particular, the procedures may be applied to mice produced by microinjection of gene-modified embryonic stem cells. The mice used in such studies have been derived principally from 129 strains, and must be crossed with predetermined inbred strains for 8 to 12 generations to be useful for biomedical studies. Breeding by use of IVF and embryo culture is advantageous over natural mating by its elimination of uncontrolled factors of reproduction such as irregular estrus cycle and cannibalism of newborns. Thus, use of IVF results in synchronous production of large numbers of descendant generations in predetermined intervals without delay. In addition, cryopreserved IVF embryos constitute a reliable source of animals for transgenic experiments (25) because they lend flexibility to experimental scheduling as well as preparation of pronucleate-stage embryos, recipients, and DNA samples. Our data establish a basis for in vitro preparation of competent embryos in numbers sufficient for studies involving embryo manipulations.

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