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Rat Oocytes Fertilized in Modified Rat 1-Cell Embryo Culture Medium Containing a High Sodium Chloride Concentration and Bovine Serum Albumin Maintain Developmental Ability to the Blastocyst Stage

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ABSTRACT

A suitable chemically defined culture medium for 1-cell rat embryos (mR1ECM) was modified to obtain sperm penetration, and the developmental competence of oocytes fertilized in the medium was compared to that of oocytes fertilized in a traditional medium, modified Krebs-Ringer bicarbonate medium (mKRB). Sperm penetration was not observed when polyvinyl alcohol was replaced with BSA in mR1ECM (mR1ECM-BSA); the incidence was improved only when the osmolarity in mR1ECM-BSA was increased to that in mKRB (310 mOsm) by addition of NaCl. The proportion of oocytes penetrated in mR1ECM-BSA with NaCl increased (71.6 ± 6.9%), which was not different compared to that in mKRB (76.7 ± 13.7%). High incidences of sperm penetration (88.8 ± 4.1% to 93.1 ± 5.1%) were also observed when NaCl concentration in mR1ECM-BSA was increased from 76.7 mM to 100-130 mM. The incidence of embryos developing to the morula and blastocyst stages was higher when fertilized in mR1ECM-BSA containing 110-130 mM NaCl (91-94%) than in mKRB (70%). A total of 5 offspring were obtained after transfer of the morulae and blastocysts (69 embryos/7 females). These results demonstrate that a high developmental ability of rat embryos to the blastocyst stage is attained when the embryos have been fertilized in mR1ECM-BSA containing 110-130 mM NaCl and then cultured in mR1ECM.

INTRODUCTION

Modifying a chemically defined medium for hamster embryos (HECM-1) [1] has improved the early embryonic development of rat 1-cell embryos to the blastocyst stage [2, 3]. Currently, in a chemically defined medium supplemented with 20 amino acids, designated as modified rat 1-cell embryo culture medium (mR1ECM), 80-90% of 1-cell rat embryos can develop to the blastocyst stage [3]. Furthermore, cumulus-enclosed rat oocytes, fertilized during 10-h coculture with epididymal spermatozoa in modified Krebs-Ringer bicarbonate solution (mKRB) [4], developed in mR1ECM to the blastocyst stage [5]. Normal offspring have been produced after transfer of morulae/blastocysts fertilized and developed in vitro [6]. However, recent studies indicated that early development of 1-cell rat and hamster embryos in mR1ECM was reduced when the embryos were collected from oviducts before pronuclear formation or were produced by in vitro fertilization [6, 7]. Their development was maintained by preincubation of embryos in mKRB or by delaying the time of transfer from mKRB to mR1ECM [6]. Therefore, one or more factors in mKRB seem to be required during pronuclear formation to achieve successful early development of rat embryos. However, rat embryos fertilized in vitro show a complete developmental block at the 2- to 4-cell stage in mKRB [4]. Only 45-64% of embryos fertilized in vitro could develop to the blastocyst stage even when transferred from mKRB to mR1ECM at 5-30 h after in vitro insemination [6]. Therefore, although mKRB seems to contain critical factor(s) required for sperm penetration and pronuclear formation, mKRB may also contain factor(s) detrimental to early embryonic development. If sperm penetration and pronuclear formation can be achieved during coculture of cumulus-oocyte complexes with spermatozoa for 10 h in modified mR1ECM, the developmental ability of rat embryos fertilized in vitro may be improved. In the present studies, we compared mR1ECM, mKRB, and modified mR1ECM with respect to sperm penetration and developmental ability of rat embryos.

TABLE 1. Formulations of mKRB [4] and mR1ECM [6].

Ingredient	mKRB	mR1ECM
NaCl	94.6 mM	76.7 mM
KCl	4.78 mM	3.2 mM
$CaCl_2$	1.71 mM	2.0 mM
KH_2PO_4	1.19 mM	—
$MgSO_4$	1.19 mM	—
$MgCl_2$	—	0.5 mM
$NaHCO_3$	25.07 mM	25.0 mM
Sodium lactate	21.58 mM	10.0 mM
Sodium pyruvate	0.5 mM	0.5 mM
Glucose	5.56 mM	7.5 mM
BSA	4.0 mg/ml	—
Polyvinyl alcohol	—	1.0 mg/ml
Glutamine	—	0.1 mM
EAA[a]	—	2% (v/v)
NEAA[b]	—	1% (v/v)
Streptomycin sulfate	50 μg/ml	—
Potassium penicillin	75 μg/ml	—
Osmolarity	310 mOsM	246 mOsM

[a] Minimal essential medium (MEM) amino acid solution (GIBCO BRL).
[b] MEM nonessential amino acid solution (GIBCO BRL).

MATERIALS AND METHODS

Media

Basic media used for fertilization of oocytes were mKRB and mR1ECM [6]. The mKRB was basically the same as that developed and used by Toyoda and Chang [4] except that phenol red was omitted. The ingredients of mKRB and mR1ECM are shown in Table 1. Basically, mKRB and mR1ECM contain BSA and polyvinyl alcohol (PVA), respectively. In the present studies, mKRB with PVA (1.0 mg/ml) instead of BSA, and mR1ECM with BSA (4.0 mg/ml) instead of PVA, were also used and were named mKRB-PVA and mR1ECM-BSA, respectively. Culture medium for early development of rat embryos was

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mR1ECM. All fertilization and culture media (each 400 μl) were covered with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) and equilibrated in an atmosphere of 5% CO_2 in air at 37°C overnight. The pH of all media was 7.4 after equilibration.

Preparation of Sperm Suspension

According to a traditional procedure [4], a dense mass of spermatozoa from the epididymis of Wistar rats (10-12 mo old) was introduced into 400 μl of mKRB, which had been covered with paraffin oil and equilibrated in an atmosphere of 5% CO_2 in air at 37°C overnight. About 5 min after preparation, the sperm suspension (10-30 μl) was transferred into a fertilization medium (400 μl) to provide a final sperm concentration of 5-10 $\times$ 10^5 cells/ml. The diluted sperm suspension was preincubated in an atmosphere of 5% CO_2 in air at 37°C for 5-6 h.

Collection of Ovulated Oocytes and In Vitro Fertilization

Sexually mature female Wistar rats (2-3 mo old) were maintained under controlled lighting conditions (14L:10D; lights-on at 0600 h). For collection of ovulated oocytes, rats were killed between 0500 h and 0600 h on the day of estrus, which was identified by examination of vaginal smears. The oviducts were isolated and placed in a dish containing paraffin oil and the preincubated-diluted sperm suspension. The cumulus-oocyte complexes were dissected out of the oviducts, introduced into the sperm suspension, and cultured for 10 h in an atmosphere of 5% CO_2 in air at 37°C. After the coculture with spermatozoa, the oocytes were stripped of cumulus cells by pipetting with 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) in mR1ECM.

Assessment of Sperm Penetration

The denuded oocytes were mounted, fixed, dehydrated, stained with 0.25% lacmoid, and examined under a phase-contrast microscope as described previously [7]. Oocytes were designated as penetrated when they had at least one male pronucleus and corresponding sperm tail in the vitellus. Oocytes with spermatozoa in their perivitelline space were not considered penetrated.

Culture of Embryos Fertilized In Vitro and Assessment of Early Embryonic Development

Some of the denuded oocytes were washed three times with mR1ECM and observed for evidence of sperm penetration by means of an inverted phase-contrast microscope (Nikon Diaphot; Nikon Corp., Tokyo, Japan). Only penetrated zygotes were washed again three times with mR1ECM, transferred (10-20 zygotes) into 400 μl of the same medium, and cultured in an atmosphere of 5% CO_2 in air at 37°C. In vitro development of the zygotes was determined after 24, 72, 96, and 120 h after insemination by using an inverted phase-contrast microscope (Nikon Diaphot). Embryos showing compaction and blastocyst cavity formation were classified as morulae and blastocysts, respectively.

Transfer of Morulae and Blastocysts to Recipients

Embryos that had developed to the morula and blastocyst stages 99-101 h after insemination were transferred into the uteri of pseudopregnant female Wistar rats as described by Miyoshi et al. [3]. To induce pseudopregnancy, female rats were stimulated by insertion of a glass rod connected to an electric vibrator into the vagina between 1930 h and 2000 h on the day of proestrus (Day 0). Between 1000 h and 1200 h on Day 4, the females were anesthetized with an i.p. injection of tribromoethanol (Avertin, 0.012 ml/g body mass; Aldrich Chemical Co., Inc., Milwaukee, WI), and the uterus was exposed through a dorsal incision. The embryos were picked up with a mouth-controlled pipette with a curved tip 150-200 μm in diameter. The tip of the pipette was inserted into an opening made previously by inserting a 26-gauge needle through the uterine wall at the oviductal side, and embryos were transferred into each uterine horn. After transfer, the vaginal smears of the recipients were examined daily. Cyclicity was considered to have been reestablished when the day of proestrus was identified in the recipients by examination of vaginal smears. These nonpregnant recipients were immediately killed and examined for implantation sites.

Experiment 1

To determine the effect of BSA in mR1ECM on sperm penetration, cumulus-oocyte complexes were cocultured with spermatozoa in mKRB, mKRB-PVA, mR1ECM, and mR1ECM-BSA for 10 h.

Experiment 2

To determine the effect of phosphate, sodium lactate, and osmolarity in mR1ECM-BSA on sperm penetration, the concentration of KH_2PO_4 (0 mM) or sodium lactate (10 mM) or the osmolarity (246 mOsm) in mR1ECM-BSA was adjusted to that of mKRB (1.19 mM KH_2PO_4, 21.58 mM sodium lactate, and 310 mOsm), respectively. The osmolarity of mR1ECM was adjusted by increasing NaCl concentration (from 76.7 mM to 106.7 mM). After coculture of cumulus-oocyte complexes with spermatozoa in the above-mentioned modified mR1ECM-BSA for 10 h, the incidence of sperm penetration was compared to that in mKRB. Further, to examine the effect on sperm penetration of supplementation to adjust osmolarity, the osmolarity of mR1ECM-BSA was increased to the same level (310 mOsm) in mKRB by increasing NaCl concentration (106.7 mM) or by adding 60 mM sorbitol. After coculture of cumulus-oocyte complexes with spermatozoa in the modified mR1ECM-BSA for 10 h, the incidence of sperm penetration was compared to that in mR1ECM-BSA.

Experiment 3

To determine the optimal concentration of NaCl in mR1ECM-BSA for sperm penetration, cumulus-oocyte complexes were cocultured with spermatozoa in mR1ECM-BSA containing various concentrations (90-140 mM) of NaCl for 10 h.

Experiment 4

To compare the developmental competence of embryos fertilized in mR1ECM-BSA containing a high NaCl concentration with that of embryos fertilized in mKRB, zygotes fertilized in mKRB or mR1ECM-BSA containing various NaCl concentrations (100-130 mM) were cultured in mR1ECM. The incidence of embryos that had developed to the 2-cell, 4-cell, morula, and blastocyst stages was determined at 24, 72, 96, and 120 h after insemination, respectively. Furthermore, some of the embryos fertilized in mR1ECM-BSA containing 110 mM NaCl that had devel-

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TABLE 2. Effects of BSA in mKRB and mR1ECM on sperm penetration of rat oocytes inseminated with epididymal spermatozoa.

	Oocytes			
Media	No. examined[a]	% penetrated[b]	% with male and female pronuclei[c]	% polyspermic[c]
mKRB	76	88.2 ± 2.7	100 ± 0	36.3 ± 1.3
mKRB-PVA	77	0	—	—
mR1ECM	85	0	—	—
mR1ECM-BSA	79	0	—	—

[a] Oocytes were examined 10 h after insemination; experiments were replicated 4 times.
[b] Percentage (mean ± SEM) of oocytes examined.
[c] Percentage (mean ± SEM) of oocytes penetrated.

oped in mR1ECM to the morula and early blastocyst stages were transferred into the uterine horn of 7 recipients (9-13 embryos per recipient) 86-88.5 h after induction of pseudopregnancy as described previously [3]. The developmental competence of the embryos to term was also examined.

Statistical Analysis

Data from four replicate trials were expressed as means ± SEM. Statistical analyses for treatment comparisons were carried out by ANOVA and Duncan's multiple range test using the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arc sine transformation before statistical analysis. When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's multiple range test. A probability of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of BSA in mR1ECM (Experiment 1)

The incidence of oocytes penetrated during coculture of oocyte-cumulus complexes with spermatozoa in mKRB, mKRB-PVA, mR1ECM, and mR1ECM-BSA for 10 h is shown in Table 2. Sperm penetration was observed only in mKRB (88.2 ± 2.7%). Although no sperm penetration occurred in mR1ECM-BSA, this medium was used in the subsequent experiments because we found the presence of BSA to be of importance for sperm penetration in mKRB.

Effect of Phosphate, Sodium Lactate, and Osmolarity in mR1ECM-BSA (Experiment 2)

No penetrated oocytes were observed after coculture of oocyte-cumulus complexes with spermatozoa either in mR1ECM-BSA supplemented with 1.19 mM KH_2PO_4 or in mR1ECM-BSA with increased sodium lactate (21.58 mM). However, oocytes were penetrated (71.6 ± 6.9%) when the osmolarity of mR1ECM-BSA was increased to 310 mOsm by addition of NaCl (Table 3). The incidence was not different ($p > 0.05$) from that in mKRB (76.7 ± 13.7%). However, the incidence of sperm penetration was extremely low ($p < 0.01$; 7.0 ± 7.0%) when the osmolarity of mR1ECM-BSA was increased by adding 60 mM sorbitol (Table 4).

Optimal Concentration of NaCl in mR1ECM-BSA for Sperm Penetration (Experiment 3)

Table 5 shows the proportion of oocytes penetrated during coculture with spermatozoa in mR1ECM-BSA containing various concentrations (90-140 mM) of NaCl for 10 h. The incidence of oocytes penetrated was high, and no differences were noted ($p > 0.05$) among mR1ECM-BSA medium containing 100-130 mM NaCl (88.8 ± 4.1% to 93.1 ± 5.1%). Rates of total sperm and monospermic penetration in mR1ECM-BSA containing 100-130 mM NaCl were not different ($p > 0.05$) compared to those in mKRB (81.6 ± 3.8% and 24.0 ± 7.7%, respectively; Table 5). However, the incidence of sperm penetration was reduced (at least $p < 0.05$) in mR1ECM-BSA containing 90 and 140 mM NaCl (21.0 ± 5.1% and 58.8 ± 13.2%, respectively).

Developmental Ability of Embryos Fertilized in mR1ECM-BSA Containing a High NaCl Concentration (Experiment 4)

Early development of embryos fertilized in mR1ECM-BSA containing 100-130 mM NaCl or in mKRB is shown in Table 6. More than 80% of embryos fertilized in

TABLE 3. Effect of phosphate, lactate, and osmolarity in mR1ECM-BSA on sperm penetration of rat oocytes inseminated in vitro with epididymal spermatozoa.

	Oocytes			
Media	No. examined[a]	% penetrated[b]	% with male and female pronuclei[c]	% polyspermic[c]
mKRB	79	76.7 ± 13.7	94.5 ± 5.6	31.9 ± 10.9
mR1ECM-BSA	78	0	—	—
with phosphate[d]	72	0	—	—
with high lactate[e]	77	0	—	—
with high osmolarity[f]	74	71.6 ± 6.9	96.0 ± 2.4	26.6 ± 6.6

[a] Oocytes were examined 10 h after insemination; experiments were replicated 4 times.
[b] Percentage (mean ± SEM) of oocytes examined.
[c] Percentage (mean ± SEM) of oocytes penetrated.
[d] 1.19 mM KH_2PO_4 was added.
[e] Concentration of sodium lactate was increased from 10.0 mM to 21.58 mM.
[f] Osmolarity was adjusted to 310 mOsM by increasing NaCl concentration to 106.7 mM.

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TABLE 4. Sperm penetration of rat oocytes in mR1ECM-BSA with high osmolarity (310 mOsM) adjusted by adding NaCl or sorbitol.

Osmolarity (mOsM)	Adjusted method[a]	Oocytes			
		No. examined[b]	% penetrated[c]	% with male and female pronuclei[d]	% polyspermic[d]
246	—	52	0	—	—
310	NaCl	57	77.2 ± 8.3	96.7 ± 3.3	16.8 ± 8.3
310	Sorbitol	62	7.0 ± 7.0	0	0

[a] Osmolarity was adjusted to 310 mOsM by increasing NaCl concentration to 106.7 mM (NaCl) or by adding 60 mM sorbitol.
[b] Oocytes were examined 10 h after insemination; experiments were replicated 4 times.
[c] Percentage (mean ± SEM) of oocytes examined.
[d] Percentage (mean ± SEM) of oocytes penetrated.

mR1ECM-BSA containing 100-130 mM NaCl developed to the morula stage in mR1ECM within 96 h after insemination. The incidences of embryos developing to the morula and blastocyst stages were higher (at least $p < 0.05$) when oocytes were fertilized in mR1ECM-BSA containing 110-130 mM NaCl (92.5 ± 4.5% to 95.7 ± 2.6% and 85.7 ± 7.8% to 95.6 ± 2.7%, respectively) as compared to mKRB (69.2 ± 7.3% and 62.3 ± 6.0%, respectively). A total of 66 morulae and 3 early blastocysts that were fertilized in mR1ECM-BSA containing 110 mM NaCl and developed in mR1ECM were transferred into 7 recipients. Four recipients failed to maintain their pregnancies and resumed cyclicity on Days 13, 14, 16, and 20, respectively. Upon examination for implantation sites, an average of 7.0 ± 0.6 scars were found. The remaining 3 recipients maintained the pregnancy to term and delivered a total of 5 offspring (1.7 ± 0.3 offspring per litter; 4 males and 1 female) with average weights of 6.2 ± 0.4 g.

DISCUSSION

In the present studies, we demonstrated that successful sperm penetration was achieved in mR1ECM with modifications in which PVA was replaced with BSA and the NaCl concentration was increased to 100-130 mM. In hamsters [8], mice [9], and cattle [10], sperm penetration of oocytes in vitro was achieved when cumulus-oocyte complexes were cocultured in media not containing BSA. In the rat, in contrast, previous findings showing that BSA is essential in order to result in sperm penetration [11, 12] have not yet been overturned. Miyamoto and Chang [13] also reported that in the presence of glucose, BSA was the most important component for in vitro fertilization of rat oocytes and that addition of lactate and pyruvate facilitated the process. In the present studies, however, neither simply replacing PVA with BSA, or replacing PVA with BSA and increasing the concentration of sodium lactate, resulted in successful sperm penetration in mR1ECM. Since we confirmed the necessity of supplementing with BSA in mKRB, we basically used mR1ECM-BSA in the other experiments. The fertilizability of spermatozoa also appears to be higher in hypertonic rather than hypotonic solution in the rabbit [14], mouse, and hamster [15]. In rats, although penetration in hypotonic solution has not been examined, a high proportion of oocytes were penetrated in standard isotonic (309 mOsm) and slightly hypertonic (317-397 mOsm) solution regulated by adding NaCl [16]. Therefore, epididymal rat spermatozoa appear to be more capacitated in media with elevated isotonic strength [16]. In the present studies, we found that sperm penetration was observed only when the osmolarity of mR1ECM-BSA was increased to the level in mKRB (310 mOsm) or more (see Table 5) by adding NaCl but not by adding sorbitol. This means that not high osmolarity itself, but rather relatively high NaCl concentrations (100-130 mM), are beneficial for sperm penetration in the rat. In the present studies, we did not determine whether a high NaCl condition affected each step of sperm penetration such as sperm capacitation, penetration through zona pellucida, and fusion with oocytes. It has been reported that the fertilizing ability of rabbit spermatozoa was improved by treatment with hypertonic (380 mOsm) medium before insemination [17]. It is possible that the effect of high NaCl condition could be mainly on sperm penetration.

Currently, more than 80% of 1-cell rat embryos can de-

TABLE 5. Sperm penetration of rat oocytes in mKRB or mR1ECM-BSA[1] containing various concentrations of NaCl.

Fertilization media	Oocytes			
	No. examined[a]	% penetrated[b]	% with male and female pronuclei[c]	% polyspermic[c]
mKRB	64	81.6 ± 3.8[e]	100 ± 0	24.0 ± 7.7
mR1ECM-BSA with				
90 mM NaCl	64	21.0 ± 5.1[d]	83.4 ± 9.6	10.4 ± 6.3
100 mM NaCl	53	88.8 ± 4.1[e]	90.8 ± 1.2	9.2 ± 4.3
110 mM NaCl	53	91.5 ± 6.5[e]	100 ± 0	16.4 ± 4.2
120 mM NaCl	56	92.0 ± 3.4[e]	97.9 ± 2.1	7.2 ± 4.2
130 mM NaCl	56	93.1 ± 5.1[e]	98.3 ± 1.7	8.5 ± 6.4
140 mM NaCl	62	58.8 ± 13.2[f]	77.5 ± 11.9	2.8 ± 2.8

[a] Oocytes were examined 10 h after insemination. Experiments were replicated 4 times.
[b] Percentage (mean ± SEM) of oocytes examined.
[c] Percentage (mean ± SEM) of oocytes penetrated.
[def] Means within columns with different superscripts are different (at least $p < 0.05$).

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TABLE 6. Early development in mR1ECM of rat embryos fertilized in mKRB or mR1ECM-BSA containing various concentrations of NaCl.

Fertilization media	No. of zygotes cultured[a]	%[b] of embryos developed to			
		2-cell (24)[c]	> 4-cell (72)[c]	> Morula (96)[c]	Blastocyst (120)[c]
mKRB	53	97.9 ± 2.1	86.0 ± 6.2[d]	69.2 ± 7.3[d]	62.3 ± 6.0[d]
mR1ECM-BSA with					
100 mM NaCl	41	100 ± 0	94.6 ± 5.4[de]	86.1 ± 2.7[de]	84.3 ± 4.4[de]
110 mM NaCl	48	100 ± 0	97.0 ± 1.8[de]	95.7 ± 2.6[e]	95.6 ± 2.7[e]
120 mM NaCl	48	100 ± 0	92.8 ± 3.2[de]	92.8 ± 3.2[e]	92.8 ± 3.2[e]
130 mM NaCl	44	100 ± 0	100 ± 0[e]	92.5 ± 4.5[e]	85.7 ± 7.8[e]

[a] Oocytes were examined 10 h after insemination and only zygotes were cultured; experiments were replicated 4 times.
[b] Data are expressed as means ± SEM.
[c] Numbers in parentheses indicate the time of examination (hours after insemination).
[de] Means within columns with different superscripts are different (at least $p < 0.05$).

velop to the blastocyst stage in a chemically defined mR1ECM [3]. However, the developmental ability of 1-cell rat embryos in mR1ECM is known to be very low when obtained soon after penetration in vitro [6]. This phenomenon has also been described in hamster embryos in a chemically defined medium, HECM-3 [7]. The low early embryonic development was also observed when rat zygotes were collected before pronuclear formation and then cultured in mR1ECM [3]. Low embryonic development appears to be due to the absence of essential factor(s) in mR1ECM during pronuclear formation. The low development of the embryos has been overcome by supplementation of HECM-3 with hypotaurine in the hamster [7] and by preincubation in mKRB [6]. In the present studies, the developmental ability of 1-cell rat embryos fertilized in mR1ECM-BSA containing 110-130 mM NaCl was not reduced but improved as compared to that of embryos fertilized in mKRB. Therefore, these results indicate that the factor(s) affecting the developmental ability of embryos during pronuclear formation and missing in mR1ECM was the presence of BSA and/or relatively high NaCl concentrations. Further studies are being directed toward the potential roles of BSA and/or relatively high NaCl concentrations during early development of the zygotes. According to Miyoshi et al. [6], only 45-64% of embryos fertilized in mKRB could develop to the blastocyst stage even when transferred from mKRB to mR1ECM at 5-30 h after insemination. In the present studies, we also demonstrated that more than 80% of embryos fertilized in mR1ECM-BSA containing 110-130 mM NaCl were able to develop to the blastocyst stage. Therefore, this evidence also suggested that factor(s) in mKRB had still reduced the developmental ability during sperm penetration and/or pronuclear formation.

In the present studies, we demonstrated successful development to term after transfer of morulae and early blastocysts that were fertilized in mR1ECM-BSA containing 110 mM NaCl and developed in a chemically defined medium, mR1ECM. However, the litter size was very small, and more than half the recipients had failed to maintain the pregnancy around Days 13-20. Similar results were obtained after transfer of morulae or early blastocysts that had been fertilized in vivo [3] and in vitro [6] and then developed in mR1ECM. Therefore, the small litter size and low efficiency of offspring production after transfer of the embryos appear not to be due to unsuitable conditions in mR1ECM-BSA containing 110 mM NaCl, but possibly in mR1ECM. In porcine embryos, both the number of cells in a blastocyst and the incidence of blastocysts hatched were dramatically improved by supplementation with fetal bovine serum by the morula stage [18]. Those characteristics of the embryos were not examined in the present studies. Further investigation will be required to improve the development of rat embryos cultured in mR1ECM and then transferred.

In summary, successful sperm penetration has been achieved in a suitable culture medium for rat embryos, mR1ECM, with modifications in which PVA was replaced with BSA and the NaCl concentration was increased to 100-130 mM. Although rat zygotes appear to be very sensitive to the culture condition(s) during pronuclear formation, the developmental ability of the zygotes is maintained in mR1ECM with BSA instead of PVA and containing 110-130 mM NaCl. In vitro fertilization in the medium and then early embryonic development in mR1ECM should be a suitable system for production of blastocysts in vitro from rat oocytes.

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