—Mini Review—

Development of a culture medium for rat 1-cell embryos

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Abstract: Rat 1-cell embryos fertilized *in vivo* or *in vitro* show a complete developmental block at the 2- to 4-cell stage in conventional culture media. To develop a culture medium supporting early embryonic development in rats, we examined the effects of some chemical and physical factors on the development of rat 1-cell embryos. The results indicated that (1) the developmental block is caused by phosphate, (2) glucose and amino acids are necessary, (3) the optimal osmolarity is 244–246 mOsm, and (4) the presence of bovine serum albumin and relatively high (110.0–130.0 mM) NaCl concentrations in the early stages preceding pronucleus formation is necessary. On the basis of these results, novel culture media designated as modified rat 1-cell embryo culture medium (mR1ECM) and mR1ECM for *in vitro* fertilization (mR1ECM-IVF) were developed. In cultures using these media, 90% or more of rat 1-cell embryos fertilized *in vivo* or *in vitro* successfully developed to the blastocyst stage *in vitro*.

Key words: Rat, Embryo, Medium, *In vitro* culture, *In vitro* fertilization

Introduction

In mammals, early embryonic development after fertilization takes place in the oviducts and uteri of females. Therefore, development of culture media to support early embryonic development in mammals is extremely important not only for basic research clarifying the mechanisms controlling the development of embryos, but also for the application of newly developed technologies for obtaining transgenic and knockout animals. Although rats and mice are the principal laboratory animals, culture media capable of supporting complete preimplantation development of rat embryos did not become available until the 1990s. One of the greatest obstacles to the development of complete culture media was that embryo development was blocked *in vitro* at the 2- to 4-cell stage [1–3].

In 1988, Schini and Bavister [4] found that phosphate and glucose, which are common components of culture media, are associated with the *in vitro* block of the development of hamster 2-cell embryos [5, 6]; therefore, they developed a phosphate/glucose-free medium. This medium, designated as hamster embryo culture medium-1 (HECM-1), supported the early development of hamster embryos *in vitro* beyond the 2-cell and 4-cell stages to the blastocyst stage [4, 7–9]. Although HECM-1 was applied to the culture of rat 1-cell embryos, the proportion of embryos that developed to the blastocyst stage was very low with this medium [10, 11]. Therefore, we conducted a series of experiments to modify HECM-1 and examined the effects of some chemical and physical factors on the early development of rat 1-cell embryos. This review discusses how a culture medium for rat 1-cell embryos was developed and improved.

Effects of Phosphate and Glucose

Initially, we examined the effects of phosphate and glucose on the development of rat 1-cell embryos, because the *in vitro* developmental block of hamster embryos at the 2-cell stage can be overcome by omitting these components [4]. We found that rat 1-cell embryos develop consistently to the blastocyst stage in HECM-1 without amino acids, and this medium was designated as modified (m) HECM-1 (Table 1). The addition of glucose did not inhibit or promote the development of rat 1-cell embryos to the morula stage in mHECM-1, but adequate concentrations (7.5–10.0 mM) of glucose improved the development of morulae to the blastocyst stage [12]. Very low concentrations (0.001–0.01 μM) of phosphate did not inhibit the development of 1-cell embryos to the blastocyst stage in mHECM-1 supplemented with 7.5 mM glucose. However, as the concentrations of phosphate increased, the developmental blocks started occurring at

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Table 1. Composition of mHECM-1, mR1ECM, mKRB, and mR1ECM-IVF

Component	mHECM-1	mR1ECM	mKRB	mR1ECM-IVF
NaCl	98.0 mM	76.7 mM	94.6 mM	110.0 mM
KCl	3.2 mM	3.2 mM	4.78 mM	3.2 mM
$CaCl_2$	2.0 mM	2.0 mM	1.71 mM	2.0 mM
KH_2PO_4	–	–	1.19 mM	–
$MgCl_2$	0.5 mM	0.5 mM	–	0.5 mM
$MgSO_4$	–	–	1.19 mM	–
$NaHCO_3$	25.0 mM	25.0 mM	25.07 mM	25.0 mM
Glucose	–	7.5 mM	5.56 mM	7.5 mM
Sodium pyruvate	0.5 mM	0.5 mM	0.5 mM	0.5 mM
Sodium lactate	10.0 mM	10.0 mM	21.58 mM	10.0 mM
PVA	1.0 mg/ml	1.0 mg/ml	–	–
BSA	–	–	4.0 mg/ml	4.0 mg/ml
Glutamine	–	0.1 mM	–	0.1 mM
MEM-AAS[a] (50×)	–	2% (v/v)	–	2% (v/v)
MEM-NAAS[b] (100×)	–	1% (v/v)	–	1% (v/v)
Streptomycin sulfate	–	–	50.0 µg/ml	–
Potassium penicillin	–	–	75.0 µg/ml	–
Osmolarity	276 mOsm	246 mOsm	310 mOsm	>310 mOsm

[a]Minimal essential medium amino acid solution. [b]Minimal essential medium nonessential amino acid solution.

earlier stages; the development of 4-cell embryos to the morula stage and 2-cell embryos to the 4-cell stage was inhibited at 0.1 and 1.0 *µ*M, respectively, and the addition of 10 *µ*M phosphate completely blocked the development of 1-cell embryos beyond the 2-cell stage [12]. The effect of phosphate on the *in vitro* development of rat embryos also varies with the developmental stage. We found that 0.4 mM phosphate inhibited the development of embryos before they reached the 8-cell stage; however, adequate concentrations (0.4–1.2 mM) of phosphate did not inhibit, but rather accelerated, the development of embryos at the 8-cell to morula stage, resulting in an increase in the number of cells in blastocysts [13].

These results clearly indicated that the *in vitro* developmental block of rat embryos is caused by phosphate, and that glucose is necessary for the development of rat 1-cell embryos *in vitro*.

Effects of Osmolarity and Amino Acids

The proportions of rat 1-cell embryos that develop to the morula (0–17.4%) and blastocyst (0–9.9%) stages are very low when embryos are cultured in HECM-1 [10, 11]. In contrast, we found that comparatively high proportions of rat 1-cell embryos developed to the morula (43–60%) and blastocyst (23–34%) stages in mHECM-1 [12]. Since HECM-1 includes 20 amino acids that increase the osmolarity (by ~30 mOsm) of the medium, we hypothesized that some amino acids or increased osmolarity might have a negative effect on the development of rat embryos. When rat 1-cell embryos were cultured in mHECM-1 containing 5.0 mM glucose at a variety of osmolarities, produced by adjusting the NaCl concentration, the development of embryos beyond the 2-cell stage was found to be largely dependent on the osmolarity of the medium. A higher proportion of embryos developed to the blastocyst stage at 244 mOsm (with 80.05 mM NaCl) than at 212–235 mOsm (with 65.05–72.25 mM NaCl) or 276–304 mOsm (with 95.50–110.05 mM NaCl) [12]. Similar results were obtained when 1-cell embryos were cultured in mHECM-1 containing 7.5 mM glucose at a variety of osmolarities produced by reducing the NaCl concentration to 63.8 mM and adding different concentrations of sorbitol; the highest proportion of blastocysts was obtained at 246 mOsm [14]. These results indicated that the osmolarity, and not the NaCl concentration, is responsible for maintaining the development of rat 1-cell embryos, and that the optimal osmolarity of the medium is 244–246 mOsm. Although 43–45% and 32–34% of 1-cell embryos developed to the morula and blastocyst stages, respectively, at 276–278 mOsm, no embryos developed to the morula and blastocyst stages at 304–306 mOsm in our studies [12, 14]. Since the osmolarity of mHECM-1 is 276 mOsm, the poor development of rat 1-cell embryos in HECM-1 in previous studies [10, 11] may be caused by the high osmolarity of the medium. The addition of 20

amino acids (0.1 mM glutamine, minimal essential medium (MEM) amino acids, and MEM nonessential amino acids) was neither beneficial nor detrimental to the development of rat 1-cell embryos to the morula stage in mHECM-1 containing 7.5 mM glucose [14]. However, these amino acids were found to significantly promote the morula to blastocyst transition and hatching of blastocysts, indicating that amino acids are necessary for the development of rat 1-cell embryos *in vitro*.

On the basis of the results described above, a medium was developed for the culture of rat 1-cell embryos after modification of mHECM-1 by adding 7.5 mM glucose, 0.1 mM glutamine, MEM amino acids, and MEM nonessential amino acids, and by reducing the NaCl concentration to 76.7 mM to obtain an osmolarity of 246 mOsm. Almost all (90%) 1-cell embryos developed to the blastocyst stage in this medium [14], and it was therefore designated as modified rat 1-cell embryo culture medium (mR1ECM, Table 1) [15, 16].

Development of Embryos Soon after Penetration *in vivo* or *in vitro*

In hamsters, the *in vitro* development of 1-cell embryos collected from oviducts soon after penetration, or obtained after *in vitro* fertilization, was found to be difficult [17]. These results indicate that the oviduct has an important role in the first few hours after penetration. It was unclear whether this observation was also applicable to rats, because we had recovered 1-cell embryos only at the pronuclear stage in the studies leading to the development of mR1ECM [12–14]. Therefore, we next examined the development of rat 1-cell embryos in mR1ECM soon after penetration *in vivo* and *in vitro*.

In our earlier studies [12–14], after female rats at proestrus had been naturally mated overnight with male rats, 1-cell embryos were recovered from mated females between 15:00 and 17:00 h on the following day. However, high proportions of embryos recovered between 10:00 and 13:00 h on the day after mating were also found to develop to the blastocyst stage [18]. In contrast, the blastocyst formation rates of 1-cell embryos recovered between 06:00 and 09:00 h on the day after mating were significantly low [18], indicating that the time of recovery of rat 1-cell embryos is a very important factor for their development in mR1ECM. These results suggested that (a) rat embryos may be exceptionally sensitive to the culture environment in the first few hours after penetration, when they contain a swollen sperm head, and that (b) the oviduct plays an important role during this critical period. When unfertilized cumulus-enclosed rat eggs were cultured for different periods with spermatozoa in modified Krebs-Ringer bicarbonate solution (mKRB, Table 1) [2], which is a commonly used medium for the *in vitro* fertilization of rat eggs, and the cumulus-free fertilized eggs were subsequently transferred into mR1ECM, similar results were obtained. The blastocyst formation rates of eggs transferred from mKRB into mR1ECM after 1–3 h of insemination were significantly lower than those of eggs transferred after 4–30 h of insemination [18]. Therefore, we examined the effects of preculture in mKRB on the development of 1-cell embryos recovered soon after penetration *in vivo*. The results showed that preculture in mKRB until 12:00–13:00 h improved the development in mR1ECM for embryos recovered between 06:00–08:00 h [18].

On the basis of these results, we hypothesized that one or more factors present in mKRB could compensate for the oviductal environment in terms of the ability to maintain the development of rat embryos at the early stages preceding pronucleus formation.

Factors Affecting *in vitro* Fertilization and the Development of Eggs

As described in the previous section, the results suggested that one or more factors in mKRB are required during pronuclear formation to achieve successful development of rat embryos. However, rat embryos fertilized *in vitro* showed a complete developmental block at the 2- to 4-cell stage in mKRB [2]. Only 45–64% of embryos fertilized *in vitro* developed to the blastocyst stage even when transferred from mKRB to mR1ECM at 4–30 h after insemination [18]. Therefore, although mKRB appeared to contain critical factor(s) required for sperm penetration and pronucleus formation, it may also contain factor(s) detrimental to early embryonic development. If sperm penetration and pronuclear formation could be achieved during coculture of cumulus-enclosed eggs with spermatozoa in mR1ECM, the development of rat embryos fertilized *in vitro* would be improved. Therefore, we next examined the effects of differences between mKRB and mR1ECM, the presence or absence of bovine serum albumin (BSA) and phosphate, and the concentration of sodium lactate and/or osmolarity on the *in vitro* fertilization of rat eggs in mR1ECM.

Although no sperm penetration occurred in mR1ECM and modified mR1ECM in which 1.0 mg/ml polyvinyl alcohol (PVA) was replaced with 4.0 mg/ml BSA (mR1ECM-BSA), the presence of BSA seemed to be necessary for the *in vitro* fertilization of rat eggs, because no penetrated eggs were obtained in mKRB that contained 1.0 mg/ml PVA instead of 4.0 mg/ml BSA [19]. Adding 1.19 mM

phosphate or increasing the concentration of sodium lactate from 10.0 mM to 21.58 mM did not result in successful sperm penetration in mR1ECM-BSA. However, when the osmolarity of mR1ECM-BSA was adjusted to the same level as in mKRB (310 mOsm) by increasing the NaCl concentration to 106.7 mM, 72% of eggs were penetrated and this proportion was not significantly different from that in mKRB. In contrast, the incidence of sperm penetration was extremely low when the osmolarity of mR1ECM-BSA was increased by adding 60.0 mM sorbitol. This means that a relatively high NaCl concentration, rather than high osmolarity itself, was beneficial for sperm penetration in rats. Therefore, we investigated the optimal concentration of NaCl in mR1ECM-BSA for the *in vitro* fertilization of rat eggs. The results indicated that, for mR1ECM-BSA containing 100.0–130.0 mM NaCl, the proportion of eggs penetrated was high and no differences were noted among these concentrations; also, the proportion of eggs penetrated did not differ from those in mKRB. In addition, the proportions of male and female pronuclei formed and of monospermic penetration were not different among these media. Moreover, when 1-cell embryos fertilized in mR1ECM-BSA containing 110.0–130.0 mM NaCl were transferred to mR1ECM at 10 h after insemination, the blastocyst formation rates were higher than those of embryos fertilized in mKRB and transferred to mR1ECM at the same time point [19].

These results indicated that the factors affecting the developmental ability of rat embryos during pronucleus formation and missing in mR1ECM are the presence of BSA and relatively high NaCl concentrations. Insemination performed in mR1ECM-BSA containing 110.0 mM NaCl (mR1ECM for *in vitro* fertilization [mR1ECM-IVF]; Table 1), followed by transfer to mR1ECM, allows 96% of the rat 1-cell embryos fertilized *in vitro* to develop to the blastocyst stage.

Viability of Embryos Developed *in vitro*

An unequivocal test to examine the normality of *in vitro*-cultured embryos is the production of fetuses or offspring. Therefore, we transferred rat morulae or blastocysts that had developed from 1-cell embryos in culture into the uteri of pseudopregnant female rats to examine their developmental ability *in vivo*.

When morulae or blastocysts that had developed in mR1ECM from 1-cell embryos recovered at the pronuclear stage after *in vivo* fertilization were transferred, only 10% of embryos developed into full-term fetuses [14]. Similar results were obtained when morulae or blastocysts developed in mR1ECM from 1-cell embryos fertilized in mKRB were transferred: 17% of embryos developed into full-term fetuses or offspring [18]. The offspring development rate (7%) did not improve even when morulae or blastocysts developed in mR1ECM from 1-cell embryos fertilized in mR1ECM-IVF were transferred [19]. In contrast, 58% of morulae recovered from rats 4 days after mating developed into offspring after transfer to pseudopregnant females [14]. These results indicated that rat morulae and blastocysts developed in mR1ECM from 1-cell embryos fertilized *in vivo* or *in vitro* are capable of normal development in the uteri of pseudopregnant females, but that their ability to develop into fetuses or offspring is lower than that of *in vivo*-developed embryos. About 70% of embryos developed in mR1ECM implant after transfer, but an extremely high proportion was lost after implantation [14, 18, 19]. Currently, the cause of this failure has not been explained. Further investigation will be required to improve the development of rat embryos that are cultured in mR1ECM and then transferred.

Table 2. Stock solutions used for preparing culture media for rat embryos

	Component	Quantity
Stock A1[a]	Milli-Q water	50 ml
	NaCl	3.2142 g
	KCl	0.1193 g
	$CaCl_2 \cdot 2H_2O$	0.147 g
	$MgCl_2 \cdot 6H_2O$	0.0508 g
Stock A2[a]	Milli-Q water	50 ml
	NaCl	2.2412 g
	KCl	0.1193 g
	$CaCl_2 \cdot 2H_2O$	0.147 g
	$MgCl_2 \cdot 6H_2O$	0.0508 g
Stock B[b]	Milli-Q water	9.81 ml
	Glucose	0.1352 g
	Sodium pyruvate	0.0055 g
	Sodium lactate (60% syrup)	0.19 ml
Stock C[c]	Milli-Q water	5 ml
	Glutamine	0.0073 g
Stock D[d]	Milli-Q water	2 ml
	$NaHCO_3$	0.0259 g

[a]Can be stored at 4 °C for up to 1 month. [b]Aliquot as 1.1 ml per 1.5 ml tube; can be stored at −20 °C for up to 3 months. [c]Aliquot as 0.2 ml per 0.6 ml tube; can be stored at −20 °C for up to 3 months. [d]Prepare immediately before use.

Preparation of mR1ECM and mR1ECM-IVF

Preparation of mR1ECM and mR1ECM-IVF from stock solutions is a practical and useful approach. Examples

Table 3. Preparation of culture media from stock solutions for rat embryos

Component	mR1ECM-IVF	mR1ECM
BSA	0.02 g	–
PVA	–	0.005 g
Milli-Q water	2.988 ml	2.988 ml
Stock A1	0.5 ml	–
Stock A2	–	0.5 ml
Stock B	0.5 ml	0.5 ml
Stock C	0.05 ml	0.05 ml
MEM amino acid solution (50×)	0.1 ml	0.1 ml
MEM nonessential amino acid solution (100×)	0.05 ml	0.05 ml
Stock D	0.812 ml	0.812 ml

of stock solutions used in our studies are shown in Table 2. For preparation of Stocks A1 and A2, the indicated amounts of NaCl, KCl, $CaCl_2{\cdot}2H_2O$, and $MgCl_2{\cdot}6H_2O$ are dissolved in 50 ml Milli-Q water. Stocks A1 and A2 can be stored at 4 °C for up to 1 month. For preparation of Stock B, the indicated amounts of glucose, sodium pyruvate, and sodium lactate (60% syrup) are dissolved in 9.81 ml Milli-Q water. After dissolution, Stock B is aliquoted as 1.1 ml per 1.5 ml tube and can be stored at –20 °C for up to 3 months. For preparation of Stock C, the indicated amount of glutamine is dissolved in 5 ml Milli-Q water. After dissolution, Stock C is aliquoted as 0.2 ml per 0.6 ml tube and can be stored at –20 °C for up to 3 months. For preparation of Stock D, the indicated amount of $NaHCO_3$ is dissolved in 2 ml Milli-Q water. Stock D is prepared immediately before use.

Table 3 shows the procedure for preparing 5 ml mR1ECM or mR1ECM-IVF; these media are prepared immediately before use. After mixing and filtration, 400-*μ*l drops of each medium are placed in 35-mm petri dishes (1 drop per dish), covered with paraffin oil, and equilibrated in an atmosphere of 5% CO_2 in air at 37 °C for at least 3 h. Precipitation often appears in drops after equilibration. To avoid this phenomenon, we inject 100% CO_2 gas into Stock D for 20–30 s immediately before mixture and reduce the pH of media in advance. mR1ECM can also be purchased from ARK Resource Co. Ltd. (Kumamoto, Japan).

Conclusion

On the basis of the results obtained in our studies, novel culture media designated as mR1ECM and mR1ECM-IVF were developed for rat 1-cell embryos. Using these media, 90% or more of rat 1-cell embryos fertilized *in vivo* or *in vitro* successfully develop to the blastocyst stage *in vitro*. Thus, these media enable more detailed investigations of the factors involved in the *in vitro* development of rat embryos [20], and also facilitate evaluation of the normal development of manipulated rat embryos [21]. However, the low developmental ability noted after implantation of morulae and blastocysts cultured in mR1ECM and mR1ECM-IVF, indicates that their compositions are not yet optimal. This remains an obstacle to the use of these media in animal science and biomedical applications.

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