Development of Rat Embryos at the 1- and 2-Cell Stage in Modified HECM-1 Medium after Exposure to a Medium that Contained Phosphate

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Abstract. The development of rat embryos after exposure to a medium that contained phosphate was examined. After exposure to a medium that contained phosphate, embryos were washed 10 times in watch glasses with 1 ml of modified HECM-1. Then they were rinsed 8 times with 100- μ l droplets of the same medium in an attempt to bring the concentration of phosphate after washing below 1.0 fM. After exposure for 0, 10 and 20 min to Ca^{2+} - Mg^{2+} and substrate-free Dulbecco's Phosphate Buffered Saline (D-PBS (-)), the development rates of 1-cell embryos to the blastocyst stage were 44%, 39% and 52%, respectively, and the mean numbers of blastomeres in the blastocysts were 20.0 17.7 and 19.4, respectively. After exposure for 0, 30 and 60 min to mHECM-1 supplemented with 1.19 mM KH_2PO_4 , the development rates to the blastocyst stage from the 1-cell stage were 47%, 60% and 55%, respectively, and mean numbers of blastomeres in the blastocysts were 16.3, 16.6 and 15.5, respectively. By contrast, 93% to 100% of 2-cell embryos developed to the blastocyst stage. In each six experiments, the development of embryos after brief exposure to phosphate was not significantly different from the group without exposure. When 2-cell embryos were exposed to phosphate for 15 h and 18 h, the rate of development to blastocysts decreased markedly to 60% and 22%, respectively. Embryonic development was completely inhibited by exposure to phosphate for more than 21 h.

Key words: Rat embryo, Developmental block, Phosphate, Exposure time.

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Mammalian embryos cultured *in vitro* cease development at a specific stage characteristic of each species. For example, rat embryos cease development at the 2-cell stage. This phenomenon is known as the "developmental block" and it has been overcome in embryos of livestock and experimental animals without in rat and hamster [1].

Recently, development of hamster 2-cell embryos to the blastocyst stage was achieved *in vitro* using hamster embryo culture medium-1 (HECM-1), which is free of phosphate and glucose [2].

Reportedly HECM-1 can also overcome developmental block in rat embryos [3] and concentrations of phosphate lower than 10 nM in this medium do not inhibit the rat embryonic development [4]. However, the reason phosphate causes mechanism of rat embryos to cease developing at the 2-cell stage is unclear, because phosphate is present in the culture medium from the start of cultivation. For this reason, timing applications of phosphate prior to the developmental block will help to identify the effects of phosphate on embryonic development.

In this study, the development of rat embryos was examined at the 1- or 2-cell stage after collecting them and washing them in Ca^{2+} -, Mg^{2+} -, and

substrate-free Dulbecco's Phosphate Buffered Saline (D-PBS (-)). The embryos were washed again after their exposure to a medium that contained phosphate. Furthermore, the development of 1- or 2-cell stage rat embryos was examined for various time intervals subsequent to exposure to D-PBS (-) or modified HECM-1 supplemented with 1.19 mM phosphate. The duration of exposure to phosphate was extended to examine the inhibitory effect at the 2-cell stage.

Materials and Methods

Animals

Wistar rats bred and maintained in The Laboratory of Animal Reproduction at Tohoku University were used for the experiments. The sexual cycle of females was monitored from vaginal smears. They were caged with males for mating at proestrus. The next morning was considered as day 1 of pregnancy when a vaginal plug and/or sperm in the vaginal smear was detected. Lights were kept on between 0600 h and 1800 h daily.

Cultivation and evaluation of embryos

All collected 1-cell or 2-cell stage embryos were subjected either to washing and culture (Experiment 1) or phosphate to exposure, washing, and culture (Experiment 2–5). The culture medium used was modified HECM-1 that contained 4 μ g/ml phenol red, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.5 mg/ml polyvinyl alcohol. Experiments 4 and 5, where the effects of KH_2PO_4 and the duration of exposure were examined, 5.56 mM glucose was added to the culture medium because glucose promotes the rat embryonic development [4]. The osmolality of the medium was checked in every experiment. Embryos were collected by flushing oviducts. One-cell embryos were freed of cumulus cells by treating them with 0.1% hyaluronidase in mHECM-1 or in D-PBS (-). Embryos were placed in 100 μ l of medium under a layer of mineral oil and cultured in a humidified atmosphere of 5% CO_2 in air at 37.5 C. In experiments 3, 4 and 5, the number of blastomeres in blastocysts was estimated by Giemsa staining after fixation as follows: a) they were treated with 1% trypsin in D-PBS (-) for 40 sec at 37 C; b) incubated in 60% fetal calf serum for 15 min at room temperature; c) fixed in a mixture of methanol, ace-

tic acid and distilled water (5:1:4, v/v) for 5 to 7 min at room temperature; d) mounted on a deproteinized and defatted, clean, glass slide; and e) fixed again by gentle immersion in a mixture of methanol and acetic acid (3:1, v/v).

Experiment 1

Effects of different flushing media on the development of the 1-cell embryo to the blastocyst stage were examined. One-cell embryos were collected at 1800 h on day 1 by flushing oviducts with mHECM-1 or D-PBS (-). After flushing, embryos were washed twice with flushing media in watch glasses and rinsed 2 to 3 times with 100- μ l droplets of mHECM-1. The total time required for flushing with mHECM-1 and D-PBS (-) was 25 min and 30 min, respectively.

Experiment 2

The effect of careful washing on embryonic development subsequent to flushing with D-PBS (-) was tested. One-cell embryos were collected at 1700 h on day 1. After flushing oviducts with D-PBS (-), embryos were washed 10 times in watch glasses with 1 ml each time of mHECM-1 and then rinsed 8 times in 100- μ l droplets of the same medium. On the basis of these washes, concentration of phosphate that was carried to the culture droplets should have been lower than 1.0 fM. In the control group, flushing and washing with mHECM-1 were performed as in Experiment 1. The total time required for flushing with mHECM-1 was 30 min, and the total time for flushing with D-PBS (-) and washing with mHECM-1 was 35 min.

Experiment 3

Effects of exposure to D-PBS (-) on development *in vitro* of 1-cell embryos were examined. One-cell embryos were collected at 1600 h on day 1 by flushing, washing with D-PBS (-) and exposed to the same medium for 10 or 20 min at 37.5 C. After exposure, the embryos were washed with mHECM-1 as in Experiment 2. In the control experiment, flushing and washing with mHECM-1 were performed in the same way as in Experiment 1.

Experiment 4

Conventional media (e.g. the media described by Whitten [5], Biggers [6] and Brinster [7]) con-

tain 1.19 mM KH_2PO_4 , and CZB medium contains 1.18 mM KH_2PO_4 [8]. But D-PBS (-) contains 9.55 mM phosphate. Therefore, the effects of exposure to mHECM-1 supplemented with 1.19 mM KH_2PO_4 on 1- and 2-cell embryonic development were examined. One-cell embryos were collected at 1600 h on day 1 and 2-cell embryos were collected at 1100 h on day 2 by flushing with mHECM-1. Embryos were exposed to the medium that contained phosphate for 30 or 60 min at 37.5 C.

Experiment 5

Development of rat embryos was blocked at the 2-cell stage by the exposure to the medium that contained phosphate. However, when the exposure was brief, development was not inhibited. Therefore, the duration of exposure was extended to examine the inhibitory effect of phosphate at the 2-cell stage. The embryos used in Experiment 5 were 2-cell embryos collected at 1100 h on day 2 by flushing with mHECM-1. Embryos were exposed to the mHECM-1 medium supplemented with 1.19 mM KH_2PO_4 for 3, 6, 9, 12, 15, 18, 21 or 24 h at 37.5 C.

Statistical analysis

Developmental rates were compared by the chi-square test. Numbers of blastomeres in blastocysts

were compared by Student's *t*-test.

Results

Experiment 1

When 1-cell embryos were flushed from oviducts with D-PBS (-), the rate of development beyond the 4-cell stage was significantly lower than in controls ($P < 0.05$), and no embryos developed to the blastocyst stage (Table 1).

Experiment 2

The rates of development of rat embryos beyond the 4-cell stage and to blastocysts did not differ significantly between flushing with mHECM-1 or flushing with D-PBS (-) (Table 2).

Experiment 3

The rates of development of rat 1-cell embryos beyond the 4-cell stage and to blastocysts, and the number of blastomeres did not differ significantly among the 3 exposure times of 0, 10 and 20 min (Table 3).

Experiment 4

Cultivation from the 1- and 2-cell stage following exposure for 30 or 60 min to 1.19 mM phosphate

Table 1. The effect of flushing media used for collection of embryos on development *in vitro* of rat 1-cell embryos

Flushing medium	No. of 1-cell embryos examined	No. of embryos developed to 2-cell stage	No. and (%) of embryos developed to:	
			≥ 4 -cell stage at 72 h	blastocysts at 120 h
mHECM-1	44	42	39 (93 ^a)	30 (71 ^a)
D-PBS (-)	40	32	3 (9 ^b)	0 (0 ^b)

The numbers in parentheses indicate percentages relative to the initial number of 2-cell embryos.
a, b Values with different superscripts within each column differ significantly ($P < 0.05$).
The osmolality of mHECM-1 was 283 mOsm.

Table 2. The effect of washing with mHECM-1 on development *in vitro* of rat 1-cell embryos collected with D-PBS (-)

Flushing medium	No. of 1-cell embryos examined	No. of embryos developed to 2-cell stage	No. and (%) of embryos developed to:	
			≥ 4 -cell stage at 72 h	blastocysts at 120 h
mHECM-1	31	31	29 (94)	9 (29)
D-PBS (-)	32	32	28 (88)	7 (22)

The numbers in parentheses indicate percentages relative to the initial number of 2-cell embryos.
The osmolality of mHECM-1 was 285–286 mOsm.

did not affect the development of embryos. Neither development nor numbers of blastomeres in blastocysts differed significantly among the 3 exposures of 0, 30 and 60 min (Tables 4 and 5).

Experiment 5

As the duration of exposure to phosphate increased, rates of development decreased significantly (Table 6; $P < 0.05$). Embryos at the 2-cell stage that had been exposed to mHECM-1 supplemented with phosphate for more than 18 h showed significantly decreased rates of development beyond the 4-cell stage. The rate of development of the 2-cell embryos to the blasto-

cyst stage was significantly reduced by exposure for more than 15 h. No embryos developed to blastocysts beyond 21 h of exposure. The number of blastomeres in the blastocysts also decreased as the duration of exposure to phosphate increased.

Discussion

In this study, the development of 1-cell embryos that were flushed and washed with D-PBS (-) without no other treatment was inhibited. This inhibition was probably caused by phosphate carried over to the culture droplets. By repeated

Table 3. The effect of the duration of exposure to D-PBS (-) on development *in vitro* of rat 1-cell embryos

Exposure time (min)	No. of 1-cell embryos examined	No. of embryos developed to 2-cell stage	No. and (%) of embryos developed to:		Mean no. of cells in blastocysts* (no. examined)
			≥4-cell stage at 72 h	blastocysts at 120 h	
0	27	27	26 (96)	12 (44)	20.0 ± 3.5 (9)
10	28	28	25 (89)	11 (39)	17.7 ± 3.9 (10)
20	31	31	31 (100)	16 (52)	19.4 ± 4.4 (14)

The numbers in parentheses indicate percentages relative to the initial number of 2-cell embryos.

The osmolarity of mHECM-1 was 285–286 mOsm.

*: Mean ± S.D.

Table 4. The effect of the duration of exposure to mHECM-1 supplemented with 1.19 mM phosphate plus 5.56 mM glucose on development *in vitro* of rat 1-cell embryos

Exposure time (min)	No. of 1-cell embryos examined	No. of embryos developed to 2-cell stage	No. and (%) of embryos developed to:		Mean no. of cells in blastocysts* (no. examined)
			≥4-cell stage at 72 h	blastocysts at 120 h	
0	51	51	43 (84)	24 (47)	16.3 ± 6.6 (15)
30	51	47	45 (96)	28 (60)	16.6 ± 4.6 (16)
60	51	51	48 (94)	28 (55)	15.5 ± 3.8 (22)

The numbers in parentheses indicate percentages relative to the initial number of 2-cell embryos.

The osmolarity of mHECM-1 was 289 mOsm.

*: Mean ± S.D.

Table 5. The effect of the duration of a brief exposure to mHECM-1 supplemented with 1.19 mM phosphate plus 5.56 mM glucose on development *in vitro* of rat 2-cell embryos

Exposure time (min)	No. of 2-cell embryos examined	No. and (%) of embryos developed to:		Mean no. of cells in blastocysts* (no. examined)
		≥4-cell stage at 48 h	blastocysts at 96 h	
0	29	29 (100)	29 (100)	22.3 ± 6.9 (27)
30	28	26 (93)	26 (93)	21.1 ± 7.3 (25)
60	29	29 (100)	29 (100)	21.8 ± 6.0 (21)

The osmolarity of mHECM-1 was 289 mOsm.

*: Mean ± S.D.

Table 6. The effect of the duration of prolonged exposure to mHECM-1 supplemented with 1.19 mM phosphate on development *in vitro* of rat 2-cell embryos

Exposure time (hr)	No. of 2-cell embryos examined	No. and (%) of embryos developed to:		Mean no. of cells in blastocysts* (no. examined)
		≥4-cell stage at 48 h	blastocysts at 96 h	
0	107	107 (100 ^a)	104 (97 ^a)	29.4 ± 8.5 (92 ^a)
3	35	35 (100 ^a)	33 (94 ^a)	28.3 ± 5.8 (23 ^{ab})
6	34	31 (91 ^a)	33 (97 ^a)	26.2 ± 5.7 (27 ^b)
9	47	46 (98 ^a)	45 (96 ^a)	26.3 ± 8.3 (32 ^b)
12	51	51 (100 ^a)	46 (90 ^a)	26.7 ± 8.7 (38 ^b)
15	48	46 (96 ^a)	29 (60 ^b)	25.4 ± 6.8 (19 ^b)
18	49	33 (67 ^b)	11 (22 ^c)	19.9 ± 5.3 (9 ^c)
21	38	8 (21 ^c)	0 (0 ^d)	
24	36	1 (3 ^d)	0 (0 ^d)	

^{a-d} Values with different superscripts within each column differ significantly ($P < 0.05$).

The osmolarity of the medium was 289–292 mOsm.

*: Mean ± S.D.

washings, the embryonic development was no longer inhibited. The assumed concentration of phosphate after these washings was below 1.0 fM. This results corresponded with earlier reports that said a concentration of phosphate below 10 nM in HECM-1 did not inhibit rat embryonic development [4].

Embryonic development was examined by exposing embryos for a limited time to the medium containing phosphate. After washing, exposure for 20 min to D-PBS (-) did not inhibit subsequent embryonic development nor reduce the number of blastomeres in blastocysts. Exposure for 30 or 60 min to mHECM-1 that contained 1.19 mM KH_2PO_4 also did not decrease embryonic development rates of embryos at the 1-or 2-cell stage either to blastocysts nor beyond the 4-cell stage. Thus, when the exposure was brief, development was not inhibited.

The duration of exposure was gradually extended. Embryos at the 2-cell stage were exposed to mHECM-1 that contained 1.19 mM KH_2PO_4 for 3, 6, 9, 12, 15, 18, 21 or 24 h. When embryos were exposed to phosphate for more than 18 h, the rates of development beyond the 4-cell stage and numbers of blastomeres per blastocyst decreased as the duration of exposure increased (Table 6; $P < 0.05$). The rate of development to the blastocyst stage was decreased by exposure to phosphate for more than 15 h.

Rat embryonic development at the 2-cell stage was inhibited by phosphate in certain conditions. This inhibition was related to the duration of ex-

posure. When embryos were exposed to phosphate for a marginal period (15 h), the development rate of the 2-cell stage to the 4-cell stage was not affected, but that to the blastocyst stage was significantly decreased. It is suggested that exposure to phosphate would delay some unknown metabolic steps necessary for the transition of embryos from the 2-cell to 4-cell stage as long as phosphate is present in the culture medium at effective concentration. The addition of an energy source to the culture medium allowed cleavage to the 4-cell stage [9]. This phosphate-caused delay of more than 15 h would have resulted in an irreversible consequence, such as desynchronization of metabolic cascade, to further promote embryonic development beyond the 4-cell stage.

Mammalian embryos cultured *in vitro* cease development at a specific stage that is characteristic for each species. However, it is unclear how the mechanism of development is caused at a species, specific cell stage. In mice, the influences of ampulla on embryonic development are restricted to a specific period at the 2-cell stage *in vitro* [10]. In rats, the initiation of embryonic gene activation occurs at the late 2-cell stage and the first two cleavage divisions can occur in the absence of transcription [11].

Cultivation of embryos *in vitro* has been achieved for many mammalian species by adding growth factors, amino acids and energy sources to the medium and/or co-culture systems thereby enhancing embryonic development. However, such additions and systems have failed to overcome the develop-

mental block in rat embryos. Using mHECM-1, which is free of phosphate and glucose, Kishi *et al.* were able to overcome the 2-cell block in rat embryos [3]. The presence of glucose and the absence of phosphate in mHECM-1 promoted rat embryonic development and concentrations of phosphate below 10 nM in HECM-1 did not inhibit development [4]. Nevertheless, phosphate is present in many culture media. Phosphate provides buffering action and inorganic phosphate acts as an intermediate in energy metabolism that involves ATP and ADP. Furthermore, phosphate is present in the oviductal fluids of ewes, rabbits and sows *in vivo* [12–14]. The presence of phosphorus has also been reported in mouse oviducts [15]. Therefore, the mechanism of the developmental block in rat 2-cell embryos in the presence of phosphate is not well understood.

As previously stated HECM-1 is free of phosphate and glucose, and glucose reportedly inhibits the hamster embryonic development in the presence of phosphate [2, 16–21]. Also, the conventional level of glucose in media reportedly inhibits embryonal development of mice [8, 22–24] and cattle [25, 26]. However, both phosphate and glucose are present in the oviductal fluids of ewes, rabbits and sows *in vivo* [12–14]. It is unclear, there-

fore, why the absence of glucose promotes embryonic development of some species *in vitro*. As reported previously, the development rate of rat embryos is increased by the presence of glucose [4] and the uptake of glucose by rat embryos increases during the blastocyst stage [27]. However, in the present experiment, glucose did not promote subsequent development when the embryos at 1-cell stage were exposure to phosphate following culture.

In conclusion, phosphate caused a developmental block in rat 2-cell embryos. This inhibition was caused by exposure to phosphate for 21 h at the 2-cell stage. Because developmental block occurs during cultivation of many mammalian embryos *in vitro* and methods for overcoming the block differ among species, overcoming the block in rat embryos will contribute to future studies regarding mammalian embryonic development.

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