Penetration In Vitro of Naturally Ovulated Rat Eggs and the Development of Eggs in a Chemically Defined Medium

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Abstract: *Naturally ovulated eggs from mature rats were inseminated with spermatozoa recovered from the cauda epididymidis of mature males in modified Krebs-Ringer bicarbonate solution. High proportions (77–100%) of eggs were penetrated with no statistical difference among different sperm concentrations (0.05–1.0 × 10^6 spermatozoa/ml) and 25–61% of penetrated eggs showed polyspermy. When penetrated eggs were transferred into modified rat 1-cell embryo culture medium (mR1ECM) 10 h after insemination, 58% of them developed to the blastocyst stage 130 h after insemination. These results indicate that rat eggs collected from naturally ovulated females can be penetrated in vitro by epididymal spermatozoa and the eggs penetrated in vitro can develop to the blastocyst stage in a chemically defined medium.*

Key words: *Rat, One-cell embryo, In vitro fertilization, In vitro development, Chemically defined medium.*

Recently, a chemically defined medium that does not contain glucose and phosphate, designated as hamster embryo culture medium 1 (HECM-1), was developed by Schini and Bavister [1]. This medium supported consistent development of hamster embryos *in vitro* beyond the 2-cell and 4-cell stages to the blastocyst stage [1–4]. Although this medium was also applied to the culture of rat 1-cell embryos, development to the blastocyst stage was supported only to a limited extent [5]. Using modified HECM-1, we have recently found that the presence of phosphate at a very low concentration (10 μ M) completely inhibited the development of *in vivo* penetrated rat 1-cell eggs beyond the 2-cell stage, but the osmolarity of the medium and adequate concentrations of glucose are important factors for the development to the blastocyst stage [6]. Thus the modified HECM-1 has been designated rat 1-cell embryo culture medium (R1ECM) [7]. When R1ECM was supplemented with 20 amino acids, as high as 90% of *in vivo* penetrated rat 1-cell eggs could develop to the blastocyst stage [7].

Although rat eggs fertilized *in vitro* are able to develop to fetuses [8–10], it is suggested that fertilization *in vitro* of rat eggs leads to defects in the embryos causing a delay in early development and a large number of preimplantation losses [11]. However, these experiments have been done by transferring 1-cell or 2-cell embryos to the oviducts of recipient females. If *in vitro* penetrated 1-cell eggs could develop to the preimplantation stage in culture, it is possible to delete the effects of transfer technique, and the difference in the developmental ability between eggs penetrated *in vivo* and *in vitro* would be determined more accurately. The present study was conducted to examine whether R1ECM is applicable to culture of *in vitro* penetrated rat 1-cell eggs.

Materials and Methods

Media: The medium used for fertilization of eggs was modified Krebs-Ringer bicarbonate solution (mKRB) composed of 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $HaHCO_3$, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 4.0 mg bovine serum albumin (BSA; No. A-7638, Sigma Chemical Co., St. Louis, MO, USA)/ml, 50 μ g streptomycin sulphate/ml and 75 μ g potassium penicillin G/ml. This medium was essentially the same as that used by Toyoda and Chang [8], except that phenol red was omitted. The medium used for culture of 1-cell embryos obtained after *in vitro* fertiliza-

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tion was R1ECM supplemented with 20 amino acids which was exactly the same as that used in our previous report [7]. This medium, designated mR1ECM, was composed of 76.7 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg polyvinylalcohol/ml, 2% (v/v) Minimal Essential Medium (MEM) essential amino acid solution (No. 320–1130, GIBCO Laboratories, Grand Island, NY, USA), 0.1 mM glutamine (Sigma Chemical Co.) and 1% (v/v) MEM nonessential amino acid solution (No. 320–1140, GIBCO Laboratories). The osmolarity of mR1ECM was about 246 mOsm. Fertilization and culture media (each 400 μl) were previously covered with warm paraffin oil (No. 261–17, Nacalai Tesque Inc., Kyoto) in a polystyrene culture dish (35 mm × 10 mm; No. 1008, Becton and Dickinson, NJ, USA) and equilibrated with the gas phase and temperature (5% CO_2 in air at 37 °C) in a CO_2 incubator overnight. Both media had a pH of 7.4 after equilibration.

Preparation of sperm suspension: Spermatozoa were obtained from Wistar rats as described by Toyoda and Chang [8]. Briefly the epididymis attached to the testis was isolated from mature male rats (10–12 months old) killed by cervical dislocation. After cutting the epididymal ducts with an iridectomy scissor, one drop of the dense mass of spermatozoa was picked up quickly with the tip of the fine glass rod and introduced into 400 μl mKRB which had been kept in a CO_2 incubator. About 5 min after the preparation, 4–100 μl of the sperm suspension was introduced to 400 μl mKRB in a different dish to give various sperm concentrations. The diluted sperm suspension was preincubated for 5 to 6 h under 5% CO_2 in air at 37 °C.

Collection of eggs and in vitro fertilization: Sexually mature female Wistar rats (2–3 months old) maintained under controlled lighting conditions (14 h light: 10 h darkness; lights on at 6:00 h) and at estrus, which was assessed by examination of vaginal smears, were killed between 5:00 and 6:00 h. The oviducts were isolated and put into a dish containing paraffin oil and the diluted sperm suspension. The cummlus-egg complexes were dissected out of the oviducts and introduced into the sperm suspension. The dishes were kept for 10 h under 5% CO_2 in air at 37 °C.

Examination of eggs: At the end of incubation, eggs were transferred into 400 μl mKRB supplemented with 0.1% hyaluronidase (No. H-3506, Sigma Chemical Co.) and freed from cumulus cells by repeated passage through a fine pipette. The denuded eggs were placed in the centre of 4 vaseline spots on a glass slide, compressed gently with a cover-slip, and fixed briefly with 2.5% glutaraldehyde in phosphate buffer solution (pH 7.4) and for 4–6 h at room temperature in 10% neutral formalin. After fixation, eggs were dehydrated with 95% ethanol, stained with 0.25% lacmoid in 45% acetic acid and examined under a phase-contrast microscope. Eggs were considered penetrated when they had male pronucleus(ei) and corresponding sperm tail(s) in the vitellus. Eggs with spermatozoa only in the perivitelline space were not considered penetrated.

Culture of eggs: Eggs inseminated with 0.5–1.0 × 10^6 spermatozoa/ml were freed from cumulus cells as described above, washed 3 times with mR1ECM, and observed under a phase-contrast microscope for evidence of sperm penetration 10 h after insemination. Eggs with female and male pronuclei and penetrating sperm tail(s) were transferred (10–15 eggs) into 400 μl mR1ECM and cultured under 5% CO_2 in air at 37 °C. Development of eggs was observed at 34, 82, 106 and 130 h after insemination under a phase-contrast microscope. Embryos showing compaction and blastocoel cavity formation were classified as morulae and blastocysts, respectively. Blastocysts in the process of emerging and emerged from the zonae pellucidae were classified as hatching and hatched blastocysts, respectively.

Statistical analysis: In the experiment which examined fertilization *in vitro*, the proportions of penetrated eggs and polyspermy were subjected to an arc-sin transformation before being assigned for one-way analysis of variance (ANOVA). When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's multiple range test.

Results

Penetration of eggs in vitro with spermatozoa preincubated at various concentrations

To examine whether naturally ovulated eggs can be penetrated *in vitro* under the present experimental conditions, spermatozoa preincubated at different concentrations were used for insemination. When examined 10 h after insemination, no eggs were penetrated with a swollen sperm head: all penetrated eggs were at the prenuclear stage. As shown in Table 1, high proportions (77–100%) of eggs were penetrated with no statistical difference among different sperm concentrations tested. However, a large variation in penetration rates (20–100%) was observed among different trials at the lowered sperm concentrations (0.05–0.1 × 10^6 cells/ml), while comparatively steady penetration rates (70–

100%) were obtained at 0.5–1.0 × 10^6 cells/ml. There was no significant difference in the proportions (25–61%) of polyspermy among different sperm concentrations. Although variations observed in polyspermic penetration was remarkable among different trials at any sperm concentrations tested, comparatively small variation (13–64%) was observed at 0.5–1.0 × 10^6 cells/ml.

Development in mR1ECM of in vitro penetrated eggs

As shown in Table 2, all penetrated eggs could cleave to the 2-cell stage 34 h after insemination. At 82 and 106 h after insemination, 73% and 69% of eggs developed to the ≧4-cell and ≧morula stages, respectively. Fifty-eight per cent of eggs developed to the blastocyst stage (Fig. 1) and 5 (19%) of 26 blastocysts were hatching or hatched 130 h after insemination.

Table 1. Effect of sperm concentration on *in vitro* penetration of naturally ovulated rat eggs[a]

Concentration of spermatozoa (10^6/ml)	No. of eggs examined	No. of eggs penetrated		No. of polyspermic eggs	
		Total (%)	Range (%)	Total (%)[b]	Range (%)[b]
0.05	57	44 (77)	20–100	17 (39)	17–100
0.1	64	54 (84)	31–100	33 (61)	0–79
0.5	53	48 (91)	70–100	19 (40)	13–64
1.0	55	55 (100)	100–100	14 (25)	17–36

[a]Experiments were repeated 5 times.
[b]Percentage of the total number of eggs penetrated.

Table 2. Development in a chemically defined medium (mR1ECM) of rat eggs penetrated *in vitro* 10 h after insemination[a]

No. of eggs cultured	No. (%)[b] of embryos developed to			
	2-cell (34)[c]	≧4-cell (82)[c]	≧Morula (106)[c]	Blastocyst (130)[c]
45	45 (100)	33 (73)	31 (69)	26 (58)

[a] Experiments were repeated 4 times. The sperm concentration was 0.5–1.0 × 10^6 cells/ml. [b] Percentage of the number of eggs cultured.
[c] Numbers in parenthesis indicate the time of examination (h after insemination).

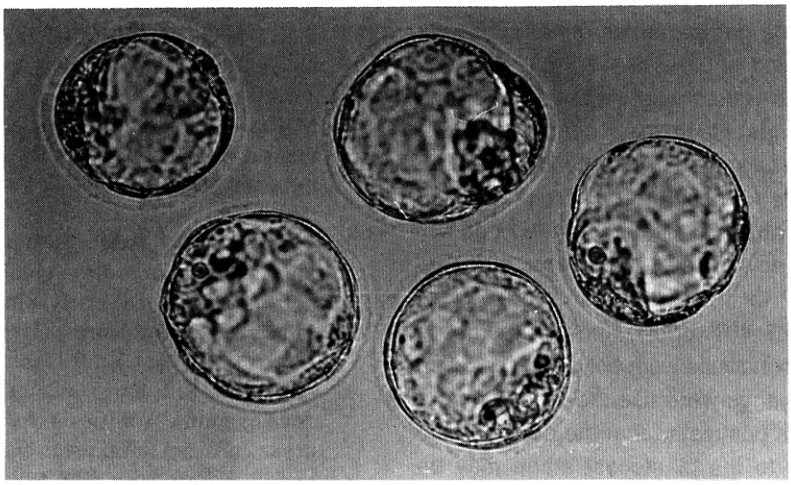

Fig. 1. Blastocysts obtained 120 h after culture in a chemically defined medium (mR1ECM) of rat 1-cell eggs penetrated *in vitro* 10 h after insemination. × 258.

Discussion

The results of the present study indicate that naturally ovulated eggs from mature rats can be penetrated by epididymal spermatozoa *in vitro* and the penetrated eggs can develop to the blastocyst stage in mR1ECM.

Niwa and Chang [12, 13] have reported that eggs from superovulated immature rats are easier to be penetrated *in vitro* than those from naturally ovulated mature rats. However, high proportions (77–100%) of naturally ovulated eggs were penetrated in the present study. Although CD strain rats were used in the previous studies [12, 13], the improved penetration rate for naturally ovulated eggs in the present study is unlikely to be due to the strain difference and is most probably related to technical improvement. It is reported that sperm concentration plays an important role for the capacitation of rat spermatozoa *in vitro* and maintenance of their fertilizing capacity [14]. The optimal sperm concentration for *in vitro* fertilization of superovulated eggs from immature rats was 0.5–1.5 $\times$ 10^6 cells/ml [13]. In the present study, there was no significant difference in penetration rates among different sperm concentrations (0.05–1.0 $\times$ 10^6 cells/ml). However, penetration rates varied greatly according to the different trials at 0.05–0.1 $\times$ 10^6 cells/ml, while comparatively steady penetration rates (70–100%) were obtained at 0.5–1.0 $\times$ 10^6 cells/ml. These results indicate that the optimal sperm concentration for *in vitro* fertilization of naturally ovulated eggs is similar to superovulated eggs from immature rats.

The incidence of polyspermy was also varied greatly according to the different trials at any sperm concentrations. However, since comparatively small variation (13–64%) was observed at 0.5–1.0 $\times$ 10^6 cells/ml, this range of sperm concentration was employed to obtain *in vitro* penetrated eggs which were used for culture experiment. The present results clearly indicate that mR1ECM is applicable for culture of *in vitro* penetrated rat 1-cell eggs. The proportion (58%) of the eggs developed to the blastocyst stage was lower than the reported value (90%) in the *in vivo* penetrated 1-cell eggs [7]. However, 25–40% of eggs penetrated in the present experimental conditions were polyspermic. Since all *in vitro* penetrated eggs were used for culture in the present study and it is postulated that the development of polyspermic eggs to the blastocyst stage may be difficult, the developmental ability of eggs penetrated *in vitro* with one spermatozoon would be expected to be similar to *in vivo* penetrated eggs.

In hamsters, it is reported that development of 1-cell eggs soon after penetration *in vivo* or *in vitro* is difficult [15]. We have observed a similar phenomenon in rats in which blastocyst formation in mR1ECM of 1-cell eggs collected from mated animals between 6:00–7:00 h (22%) on the following day of mating was greatly inhibited compared with those collected between 12:00–13:00 h (93%) on the same day (unpublished data). These results indicate that hamster and rat eggs may be exceptionally sensitive to the culture environment within the first few hours after penetration when they had swollen sperm head. In the present study, however, unpenetrated cumulus-enclosed rat eggs were cultured for 10 h with spermatozoa in mKRB until male and female pronuclei were formed and then cumulus-free penetrated eggs were transferred into mR1ECM; nevertheless a large proportion of penetrated eggs could develop to the blastocyst stage. It appears therefore that cumulus cells and/or factor(s) in mKRB could compensate for the oviductal environments which may be necessary to maintain the development of penetrated eggs at the early stage before pronuclear formation.

In conclusion, we have shown that a large proportion of naturally ovulated eggs from mature rats are penetrable with epididymal spermatozoa *in vitro* and a large proportion of the penetrated 1-cell eggs can develop to the blastocyst stage in a chemically defined, protein-free medium. These results will enable more detailed investigations of the factors involved in the development of *in vitro* penetrated rat eggs.

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ラット体外受精卵子の限定培地における体外発生

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Wistar系成熟雄ラットの精巣上体精子を修正KRB中に種々の濃度（0.05～1.0×10^6精子/ml）に希釈して前培養した後，同系成熟雌ラットから採取した自然排卵卵子を導入して授精した．授精10時間後に卵子を固定・染色して調べた結果，精子濃度は侵入率（77～100%）および多精子受精率（25～61%）に影響をおよぼさなかった．0.5～1.0×10^6精子/mlの精子濃度を用いて得られた侵入卵を授精10時間後に修正R1ECMに移して培養を継続した結果，58%の卵子が胚盤胞にまで発生した．以上の結果から，成熟雌ラットから採取された自然排卵卵子と精巣上体精子の体外受精が可能であり，このようにして得られた受精卵は限定培地で培養することにより胚盤胞にまで発生し得ることが明らかになった．

キーワード：ラット，1-細胞期胚，体外受精，体外発生，限定培地．