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Introduction

In vitro fertilization and development of mouse ova have been studied extensively during the last four decades. Hammond[3] was the first to cultivate 8-cell mouse embryos to blastocysts, using a medium containing egg white. *In vitro* fertilization of mouse ova was successfully accomplished by Brinster and Biggers[1] in expanded oviducts. These processes were reproducible but the fertilization and early embryo development occurred within the oviductal lumen or in the undefined medium containing unknown substances. Later, many scientists fertilized mouse ova in a simple (chemically defined) medium using epididymal sperm and the fertilized ova were successfully cultured to blastocysts in a simple medium[4-8,11,12].

In spite of the abundance of reports concerning *in vitro* fertilization and development of mouse ova, the supportive functions of proteins (serum or bovine serum albumin, BSA) in the culture medium have not been clarified, yet, and proteins are routinely supplemented to culture medium. Recently, Goto *et al.*[2] have reported that mouse ova can be fertilized and be developed in protein-free medium up to blastocysts though there could be a contaminating amount of proteins from oviductal fluid and from epididymal sperm used in the fertilization but not in the growth medium used for culture from one-cell to blastocysts. Their study clearly indicated that mouse embryos did not require any protein in order to develop from one-cell to blastocyst *in vitro*. However, the ability of embryos cultured in protein-free medium *in vitro* to hatch has not been examined. Therefore, the present study was conducted to examine whether or not mouse ova fertilized and developed in protein-free medium *in vitro* can hatch in protein-free medium *in vitro*.

Materials and Methods

In vitro fertilization and culture of fertilized ova (*Experiment* 1)

JCL-ICR strains of mice were maintained on a 14 h light : 10 h dark schedule with food and water *ad libitum*. Eight to 16 weeks old female mice were superovulated by injection of 5 iu PMSG (Teikoku-zoki, Tokyo, Japan) followed by 5 iu hCG (Teikoku-zoki, Tokyo, Japan) 48 h later. Media and methods for fertilization and subsequent culture *in vitro* were similar to those described by Goto *et al.*[2] TYH medium (Toyoda *et al.*[11]) in which sodium pyruvate was modified to 0.5mM (Toyoda and Takasugi[10]) was used for preincubation of sperm and for *in vitro* fertilization. Original report of TYH medium contained bovine serum albumin (BSA, 4 g/l) but we used it without BSA. Therefore, the compositions of medium used for preincubation of sperm and for *in vitro* fertilization consist of NaCl (119.37mM), KCl (4.78mM), $CaCl_2 \cdot 2H_2O$ (1.71mM), KH_2PO_4 (1.19mM), $MgSO_4 \cdot 7H_2O$ (1.19mM), $NaHCO_3$ (25.07mM), sodium pyruvate (0.5mM), glucose (5.56mM),

streptomycin sulphate (0.05g/l) and penicillin potassium (100,000iu/l). We call this medium TYH(-BSA).

TYH-280 medium (Kasai *et al.*[6]) was used for the development of fertilized ova. Again, original report of TYH-280 medium contained BSA (4g/l) but no EDTA. We used it with or without BSA (4g/l) and with ethylene-diaminetetraacetic acid 2Na-salt (EDTA, 100 μM, Nakarai Chemicals Ltd, Kyoto, Japan). Therefore, the compositions of medium used for the development of fertilized ova consist of NaCl (72.17mM), KCl (4.78mM), calcium lactate (1.12mM), $MgSO_4$ (0.85mM), KH_2PO_4 (1.19mM), $NaHCO_3$ (25.07mM), glucose (5.56mM), sodium pyruvate (0.3mM), sodium lactate (30mM), BSA (0 or 4g/l, Lot. 36F-9360, Sigma, St. Louis, MO, USA), streptomycin sulphate (0.05g/l), penicillin potassium (100,000 iu/l) and EDTA (100 μM). We call these media TYH-280-EDTA and TYH-280 (-BSA)-EDTA for BSA-supplemented and non-supplemented medium, respectively.

The female mice were killed 15h after the hCG injection and their oviducts were removed and put into the polystyrene culture dish (35×10mm, Corning, NY, USA) containg 0.4 ml medium under paraffin oil (Lot. V5K5471, Nakarai chemicals Ltd, Kyoto, Japan), maintained at 39℃ under 5% CO_2 in air. The wall of the ampullar portion of the oviduct was dissected with a needle and the ova surrounded by cumulus cells were introduced into the medium.

Sperm was obtained from the cauda epididymis of 16-20 weeks old JCL-ICR male mice and suspended into 0.4 ml of the medium under paraffin oil. After incubation for 1-2 h at 39℃ under 5% CO_2 in air, a small volume (10-20 μl) of sperm suspension was added to the medium containing ova. The final sperm concentration was adjusted to be 100 cells/μl. After 6 h incubation the ova which extruded second polar body were picked up from the medium, washed 3times with the growth medium. After washing, the ova were put into the polystylene culture dish (60×15mm, Corning, NY, USA) containing 9ml of the medium under paraffin oil (4ml) and cultured for 168h at 39℃ under 5% CO_2 in air. All of the media used were sterilized through a Millipore Filter (0.22 μm average pore size, Millipore Crop., MA, USA) and the paraffin oil used was sterilized by autoclave (120℃, 30 min). The paraffin oil was washed twice with twofold of the ultra-pure water before putting into autoclave. The development rate was calculated by determining the portions of the cultured embryos which reached the 2-cell, 4-8-cell, morula, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages 24, 48, 72, 96, 120, 144 and 168h after insemination, respectively.

Collection and culture of in vivo fertilized ova (Experiments 2 and 3)

In vivo fertilized ova were collected from the oviducts of superovulated JCL-ICR mice 18 h after hCG injection and mating. The cumulus cells surrounding ova were removed by hyaluronidase (0.1%, 5min, Lot. 115F-8165, Sigma, St. Louis, MO, USA) treatment. The ova extruded second polar body were picked up and then washed three times with growth medium. The growth media used were TYH-280-EDTA and TYH-280(-BSA)-EDTA. The fertilized ova were cultured for 168 h at 39℃ under 5% CO_2 in air (Experiment 2). The development rate was calculated by determining the portions of the cultured ova which reached 2-cell, 4-8-cell, morula, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages 24, 48, 72, 96, 120, 144 and 168h in culture, respectively.

In some trials, embryos developed to morula or blastocyst stage were used for embryo transfer (data is not shown). Therefore, the numbers of denominators after morula or blalstocyst stage in Tables 1 and 2 became smaller than those of the start of culture.

In some trials, the *in vivo* fertilized ova which developed into morulae or blastocysts in the

TYH-280(-BSA)-EDTA were transferred to TYH-280-EDTA medium in order to examine the effects of BSA on the hatching of embryos (Experiment 3). The development rate was calculated by determining the portions of cultured morulae or blastocysts which reached blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst. Observation of embryos was done every 24h in culture.

Statistical analysis

X^2 test was used for the statistical significance of differences between groups.

Results

Table 1 shows the *in vitro* development of mouse ova fertilized *in vitro*. The percentages of embryos which developed into 2-cell-stage were 94.2 and 77.8% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed into 4~8-cell stage were 76.9 and 63.9% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed into morula stage were 71.2 and 50.0% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which develped into blastocyst stage were 58.7 and 47.2% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to expanded blastocyst stage were 32.0 and 44.4% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to hatching blastocyst stage were 8.0 and 36.1% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to hatched blastocyst stage were 0 and 25.0% for BSA-free and BSA-supplemented groups, respectively.

Table 2 shows the *in vitro* development of mouse one-cell fertilized *in vivo*. The percentages of embryos which develped into 2-cell-stage were 96.2 and 100% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed into 4~8-cell stage were 88.0 and 88.6% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed into morula stage were 82.2 and 86.4% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed into blastocyst stage were 68.0 and 79.5% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to expanded blastocyst stage were 59.1 and 72.7% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to hatching blastocyst

Table 1. *In vitro* fertilization of mouse ova in protein-free medium followed by *in vitro* development of fertilized one-cell in either protein-free or protein-supplemented medium (Experiment 1).

BSA in growth medium (g/l)	No. of fertilized ova used[1]	No. and (%) of embryos developed to :						
		2-cell (24h)[2]	4-8-cell (48h)	Morula (72h)	Blastocyst (96h)	Expanded Blastocyst (120h)	Hatching Blastocyst (144h)	Hatched Blastocyst (168h)
0	156	147/156 (94.2)	120/156 (76.9)	111/156 (71.2)	44/75 (58.7)	24/75 (32.0)	6/75 (8.0)	0/75 (0)
4	36	28/36 (77.8)**	23/36 (63.9)	18/36 (50.0)*	17/36 (47.2)	16/36 (44.4)	13/36 (36.1)**	9/36 (25.0)**

1) No. of ova which extruded second polar bodies.
2) Hours after insemination.
**P<0.01, *P<0.05.

stage were 4.5 and 47.7% for BSA-free and BSA-supplemented groups, respectively. The percentages of embryos which developed to hatched blastocyst stage were 0.9 and 29.5% for BSA-free and BSA-supplemented groups, respectively.

Table 3 shows the *in vitro* development of morula or blastocyst stage of mouse embryos which were fertilized *in vivo* and which developed *in vitro*. In this experiment ova which developed into morulae or blastocysts in BSA-free medium were transferred to BSA-supplemented medium. The percentages of morulae which develped to blastocyst, expanded blastocyst, hatching blastocyst, and hatched blastocyst were 90.3, 82.3, 48.4 and 40.3%, respectively. The percentages of blastocysts which develped to expanded blastocyst, hatching blastocyst, and hatched blastocyst were 95.7, 78.7, 66.0%, respectively.

Discussion

The results of this study have clearly indicated that BSA is required for the hatch of mouse embryos derived from both *in vivo* and *in vitro* fertilization. Furthermore, the present results have indicated that morulae and blastocysts developed from one-cell in BSA-free medium were capable of hatching if they were transferred to BSA-supplemented medium. These results appear to suggest that BSA-free medium used in this study has no detrimental effects on the viability of mouse embryos up to blastocyst stage. Schneider[9] reported the development of mouse 2-cell embryos up to hatched blastocyst stage in a medium which was lacking any proteins and consists of a basic salt solution with

Table 2. *In vitro* development of mouse one-cell fertilized *in vivo* in either protein-free or protein-supplemented medium (Experiment 2)

BSA (g/l)	No. of one-cell used[1]	No. and (%) of embryos developed to :						
		2-cell (24h)[2]	4-8-cell (48h)	Morula (72h)	Blastocyst (96h)	Expanded Blastocyst (120h)	Hatching Blastocyst (144h)	Hatched Blastocyst (168h)
0	208	200/208 (96.2)	183/208 (88.0)	171/208 (82.2)	104/153 (68.0)	65/110 (59.1)	5/110 (4.5)	1/110 (0.9)
4	44	44/44 (100)	39/44 (88.6)	38/44 (86.4)	35/44 (79.5)	32/44 (72.7)	21/44** (47.7)	13/44** (29.5)

1) No. of ova which extruded second polar bodies.
2) Hours in culture.
**P<0.01

Table 3. *In vitro* development[1] of morula or blastocyst stage of mouse embryos in protein-supplemented medium (Experiment 3)

No. and stage of embryos used[2]		No. and (%) of embryos developed to :			
		Blastocyst	Expanded Blastocyst	Hatching Blastocyst	Hatched Blastocyst
Morula	62	56/62 (90.3)	51/62 (82.3)	30/62 (48.4)	25/62 (40.3)
Blastocyst	47	-	45/47 (95.7)	37/47 (78.7)	31/47 (66.0)

1) Observation was done every 24 h in culture.
2) Ova fertilized *in vivo* that developed to morula or blastocyst stage after their culture in protein-free medium were used.

amino acids and vitamins added. However, he did not examine the development of mouse embryos from one-cell stage. The medium used in this study was not supplemented with any vitamins. Therefore, vitamins seem to be excluded from the culture medium of mouse embryos.

However it remains to be examined that BSA is substituted by amino acids. If it is possible for us to substitute BSA by amino acids we will be able to culture mouse ova from one-cell stage to the hatched blastocyst stage in a completely chemically-defined medium. This may open a valuable road for the study of the energy metabolism occurring in the mouse embryos as they develop.

Summary

In vivo or *in vitro* fertilized mouse one-cells were cultured in either BSA-free or BSA-supplemented medium. Mouse one-cells could develop to blastocyst stage both in BSA-free and in BSA-supplemented media, but the blastocysts could not hatch in BSA-free medium.

The result of this study shows that BSA is required for mouse embryos to hatch.

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