

Mouse Oocyte Maturation and Blastocyst Culture In Vitro in Medium Adjusted to Human Follicular Fluid Composition

Kazutomo Ohashi^{1*}, Teruki Nakazawa², Akiko Kawamoto³, Koichiro Shimoya³, Chihiro Azuma³ and Yuji Murata³

¹Department of Obstetrics and Gynecology, Kansai Rosai Hospital, 3-1-69 Inabaso, Amagasaki, 660-8511, Japan

²Research and Development Center, Fuso Pharmaceutical Industries LTD, 2-3-30 Morinomiya, Joto-ku, Osaka, 536-8523, Japan.

³Department of Obstetrics and Gynecology, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, 565-0871, Japan.

Abstract: A new medium which contains amino acids, electrolytes, glucose, lactate and pyruvate at concentrations adjusted to those in human follicular fluid was prepared and named human follicular fluid (HFF) medium. We compared the developmental ability of mouse embryos cultured in HFF and in three commercially available media, i.e., human tubal fluid (HTF), α -modified minimal essential medium (α MEM) and Ham's F-10. When mouse maturing oocytes obtained from ovarian follicles were cultured in HFF or α MEM/10% fetal calf serum (FCS), extrusion of the first polar body was observed in 71 and 72% of the oocytes, respectively. The oocytes matured in HFF/10% FCS showed good developmental competence to the blastocyst stage compared with those matured in HTF or Ham's F-10/10% FCS after *in vitro* fertilization. The supplementation of amino acids in follicular fluid to HTF medium (HTF(FF)) did not improve the development to the blastocyst stage after IVF of maturing oocytes. The HFF medium was applied to *in vitro* culture of blastocysts using mouse embryos obtained from oviducts. After 80 hours of incubation in each medium containing 0.5% bovine serum albumin (BSA), the percentages of expanded blastocysts in HFF and HTF(FF)/0.5% BSA were significantly higher than those in HTF and α MEM (89% and 78% vs. 39% and 34%, respectively). Blastocysts and morulae were transferred to the medium CMRL 1066/20% FCS, and their developmental competence was assessed. Embryos derived from blastocysts and morulae cultured in HFF and HTF/0.5% BSA appeared to have good developmen-

tal competence, with the formation of an inner cell mass and outgrowing trophoblast, but those cultured in HTF(FF) and α MEM/0.5% BSA did not. Amino acids at high concentrations supplemented in α MEM produced a large amount of ammonium which is harmful to embryo development in extended cultures. These results indicate that the HFF medium was beneficial for both *in vitro* oocyte maturation and embryo development in the extended culture.

Key words: Amino acids, Blastocyst culture, Follicular fluid, Oocyte maturation, Simple culture medium

Ovarian hyperstimulation syndrome (OHSS) has been noted as a major side effect in clinical *in vitro* fertilization and embryo transfer (IVF-ET). Patients with polycystic ovarian syndrome (PCOS) often suffer from OHSS when ovulation is strongly induced. To prevent OHSS, it is important to recover immature oocytes from ovarian follicles following ovulation induction when clomiphene citrate or human menopausal gonadotropin is administered at a reduced dose. Trounson *et al.* [1] reported a successful pregnancy and normal birth after IVF-ET with *in vitro* oocyte maturation using immature oocytes obtained from a PCOS patient. Since immature oocytes are often obtained from fully grown follicles at the time of their retrieval, the immature oocytes must mature *in vitro* for fertilization. Therefore, the rationale for the oocyte maturation *in vitro* is not only the prevention of OHSS, but also the avoidance of the time-consuming and expensive step of controlled ovarian hyperstimulation. However, the optimal culture conditions for *in vitro* maturation of human immature oocytes remain to be established. The fundamental

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*To whom correspondence should be addressed.

requirement of *in vitro* maturation is to mimic the environment of the *in vivo* maturation of oocytes in the follicles. Since oocytes are bathed in follicular fluid during *in vivo* maturation, we developed a new culture medium based on the composition of human follicular fluid (HFF medium), and we examined the effects of this medium on the *in vitro* oocyte maturation of mouse follicular oocytes.

Another challenge in human IVF is the avoidance of multiple pregnancies. Culture to the blastocyst stage *in vitro* may be useful for the assessment of the ability of embryos to achieve further development and for selecting a single embryo for embryo transfer [2]. Blastocyst culture and transfer techniques have also improved the pregnancy success rate [3]. To induce human embryo development to the blastocyst stage, co-culture systems using somatic cells (such as Vero cells and oviductal epithelial cells) were developed [4–6], and the transfer of blastocysts achieved pregnancies in women with prior IVF failure [7–11]. However, these culture systems are not optimal for conventional human IVF, because the systems may contain undesirable components such as viruses, and their use requires a laboratory that is well equipped for somatic cell cultures. It is also necessary to establish a culture medium for the blastocyst stage *in vitro*. We found that a supplement of amino acids at concentrations present in human serum and follicular fluid induced development of embryos of an F1 hybrid mouse strain (CBF1) to the blastocyst stage and alleviated the 2-cell block of embryos of a closed colony mouse strain (ICR) [12]. In the present study, therefore, we applied the new HFF medium to the mouse blastocyst culture *in vitro* as a pre-clinical trial for establishing an optimal medium for human IVF, because it is difficult to apply a new medium to human oocytes and embryos before confirming its efficacy and safety.

Materials and Methods

Measurement of free amino acids, electrolytes, glucose, pyruvate and lactate in human preovulatory follicular fluid

The collection of uncontaminated human follicular fluid was described previously [12]. Briefly, follicular fluid was collected from 21 female patients without ovulatory disorder and antisperm antibodies in sera, in the IVF program at Osaka University Hospital (Osaka, Japan) with informed consent. The patient's mean age was 31.6 years and fertilization occurred in their oocytes. After ovulation induction by the administration of human menopausal gonadotropin and buserelin acetate, folli-

cular growth was monitored by ultrasonography. When the size of the leading follicle reached 18 mm in diameter, 5,000 IU of human chorionic gonadotropin (hCG) was administered. Oocytes were recovered transvaginally 36 hours after the hCG injection. The follicular fluid was centrifuged at 4°C for 10 minutes at 400 × g, and the supernatants were stored below –20°C until use. The follicular fluid was deproteinized, and the individual concentrations of 21 amino acids were measured with an amino acid analyzer (L-2500, Hitachi, Tokyo, Japan). The concentrations of Na, K, and Cl ions were measured by a blood gas/electrolyte analyzing system (Ciba-Corning Diagnostics, Tokyo). The concentrations of Ca, Mg, P and glucose were measured by the colorimetric methods (Wako Pure Chemicals, Osaka), and those of pyruvate and lactate were assayed by enzymatic methods (Boehringer Mannheim, Mannheim, Germany).

Preparation of culture media

The human follicular fluid (HFF) medium was based on the composition of human follicular fluid with an osmolarity adjusted to that of human follicular fluid by elevating the concentrations of components other than amino acids. The human tubal fluid (HTF; solution) [13], α minimal essential medium (α MEM; powder) [14] and Ham's F-10 (powder) [15] were purchased from Gibco (Grand Island, NY). The compositions of these four media are shown in Tables 1 and 2. We also prepared HTF containing amino acids in follicular fluid, HTF(FF), as described previously [12]. Each medium was supplemented with 10% fetal calf serum (FCS) or 0.5% bovine serum albumin (BSA) for *in vitro* oocyte maturation, fertilization (IVF) and embryo culture. CMRL1066 medium (Gibco) [16] supplemented with 20% FCS was used to assess the viability of developed embryos.

Collection of mouse oocytes and embryos

CBF1 mice (8-week-old males and 6- to 12-week-old females) were purchased from Japan SLC (Shizuoka, Japan). The female mice were given an intraperitoneal (ip) injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Teikoku Zoki, Tokyo) and 5 IU of hCG (Teikoku Zoki) 48 hours after the PMSG injection.

(1) Experiment 1 (follicular oocytes): Female mice were sacrificed 6 hours after the hCG injection. Oocytes were collected from follicles in the ovaries, washed twice with fresh medium, and cultured in one of the five media containing 10% FCS. Ten to twenty cumulus-enclosed oocytes were cultured in 100 μ l droplets of each pre-

Table 1. Mineral concentrations, glucose, pyruvate, and lactate in human follicular fluid (HFF) medium and three conventional media

component	HFF	HTF	α MEM	Ham's F-10
(g/L)				
NaCl	6.190	5.938	6.800	7.400
KCl	0.230	0.350	0.400	0.285
CaCl ₂ H ₂ O	0.320	0.300	–	–
CaCl ₂	–	–	0.200	0.033
MgSO ₄ H ₂ O	0.215	0.049	0.200	–
MgSO ₄	–	–	–	0.075
KH ₂ PO ₄	0.137	0.050	–	0.083
NaH ₂ PO ₄ H ₂ O	–	–	0.140	–
Na ₂ HPO ₄	–	–	–	0.156
NaHCO ₃	2.150	2.100	2.000	1.200
glucose	0.753	0.500	1.000	1.100
Na-pyruvate	0.041	0.036	0.110	0.110
Na-lactate	1.849	2.398	–	–
(mM/L)				
Na ⁺	148.4	148.3	142.2	144.1
K ⁺	4.1	5.1	5.4	4.4
Ca ²⁺	2.2	2.1	1.8	0.3
Cl ⁻	113.4	110.4	125.4	131.0
HPO ₄ ²⁻	1.0	0.4	1.0	0.6
HCO ₃ ⁻	25.6	25.0	23.8	14.3
Na/K ratio	36.2	29.3	26.5	32.8

HTF: human tubal fluid. α MEM: α -modified minimal essential medium.

equilibrated medium with 10% FCS under a layer of paraffin oil in a petri dish (65mm in diameter) at 37°C in 5% CO₂ for 3 hours before in vitro fertilization (IVF).

(2) Experiment 2 (one-cell embryos): Female mice were mated with male mice at the time of the hCG injection and sacrificed 18 hours after the hCG injection to recover one-cell embryos from the oviducts.

In vitro fertilization

Spermatozoa aspirated from the cauda epididymis of a sacrificed male mouse were washed with 2 ml of phosphate-buffered saline (PBS) and suspended in each medium at a concentration of 1×10^6 /ml. Each sperm suspension was kept at 37°C in 5% CO₂ for 1.5 hours. After an adequate preincubation period, a droplet of the same medium with oocytes was inseminated with 10 μ l of sperm suspension. The subsequent culture for the IVF of follicular oocytes was 15 hours under 5% CO₂ in air at 37°C. The first polar body extrusion was observed in the IVF procedure of follicular oocytes when the oocytes were inseminated with spermatozoa and cumulus cells were dispersed.

Table 2. Amino acid concentrations in HFF medium and three conventional culture media

Amino acid	HFF	HTF	α MEM	Ham's F-10
(mg/L)				
Eagle essential				
Phe	6.9	–	33.0	5.0
Trp	6.7	–	10.2	0.6
Lys	23.8	–	73.1	29.3
Thr	14.8	–	47.6	3.6
Val	16.4	–	46.9	3.5
Met	2.1	–	14.9	4.5
Ile	4.5	–	52.5	2.6
Leu	7.9	–	52.5	13.1
Cys-Cys	1.4	–	24.0	–
Cys	–	–	100.0	35.1
Tyr	7.8	–	36.2	1.8
His HCl H ₂ O	16.4	–	41.9	21.0
Arg HCl	11.2	–	126.4	210.7
Glu	26.2	–	292.0	146.2
Eagle non-essential				
Pro	11.6	–	40.0	11.5
Gly	11.6	–	50.0	7.5
Ala	26.6	–	25.0	8.9
Tau	3.9	–	–	–
Asp	0.8	–	30.0	13.3
Ser	7.8	–	25.0	10.5
Asn H ₂ O	9.8	–	50.0	15.0
Gln	13.7	–	75.0	14.7

–: not included. Phe: phenylalanine, Trp: tryptophan, Lys: lysine, Thr: threonine, Val: valine, Met: methionine, Ile: isoleucine, Leu: leucine, Cys: cysteine, Cys-Cys: cystine, Tyr: tyrosine, His: histidine, Arg: arginine, Glu: glutamine, Pro: proline, Gly: glycine, Ala: alanine, Tau: taurine, Asp: aspartic acid Ser: serine, Asn: asparagine, Gln: glutamate.

Embryo culture

The embryos were treated with 0.1% hyaluronidase (Sigma, St. Louis, MO) in each medium/0.5% BSA to remove cumulus cells. They were rinsed with the same medium, placed in 100 μ l drops of pre-equilibrated medium in a petri dish (65 mm diameter), and cultured under 5% CO₂ in air at 37°C. Embryo development was observed with a phase-contrast microscope. In Experiment 2, morulae and blastocysts developed in each medium were cultured in a drop of 50 μ l of CMRL1066 containing 20% FCS in a petri dish, covered with paraffin oil, for 7 days. The attachment, the formation of an inner cell mass (ICM) and trophoectoderm (TE) cell outgrowth, and endoderm and ectoderm differentiation were assessed after 4 and 7 days of the culture. The assessment of the growth of ICM and TE was made according to the method of Spindle and Pederson [17] with some modifications.

Determination of ammonium production

Each medium was pre-equilibrated in 100 μ l droplets under a layer of paraffin oil overnight and incubated with CBF1 mouse embryos for 72 hours at 37°C in 5% CO₂ in air. The determination of ammonium production was performed by enzymatic analysis (Ammoniak, Boehringer Mannheim).

Statistical analysis

The percentages of embryos developed were calculated for every experiment, and the data were subjected to a one-way analysis of variance (ANOVA) using the StatView statistics package. When ANOVA showed significant differences, differences between individual media were assessed by Fisher's protected least significant difference (Fisher's PLSD). Significance was accepted at $p < 0.05$.

Results

A new culture medium based on the composition of human follicular fluid was developed and was referred to as HFF medium. As a model for the IVF of immature oocytes, we used oocytes from mouse ovarian follicles 6 hours after an hCG injection. After preincubation of the cumulus-enclosed oocytes for 3 hours in the 5 media containing 10% FCS, the oocytes undergoing maturation were inseminated with spermatozoa and cultured for an additional 15 hours in the same media. The first polar body extrusion was observed in the IVF program when cumulus cells dispersed 3 hours after insemination (Table 3). The percentages of oocytes with the first polar body cultured in HFF, HTF, HTF(FF) and α MEM/10% FCS after the 15-hour incubation were

significantly higher than that in Ham's F-10/10% FCS. Oocytes were cultured in the same medium/0.5% BSA, and the development of embryos was observed every 24 hours for 96 hours. The percentages of embryos developed to morulae or blastocysts in HFF/0.5% BSA were significantly higher than those in HTF and Ham's F-10/0.5% BSA. The supplementation of amino acids present in human follicular fluid to HTF medium did not improve the development rates to blastocysts in comparison with HTF/0.5% BSA.

In culture of one-cell embryos in HFF and three media containing 0.5% BSA and observation of the embryo development after an 80-hour incubation (Table 4), the percentages of total blastocysts developed in three media, HFF, HTF and HTF(FF)/0.5% BSA, were higher than those developed in α MEM/0.5% BSA. However, more embryos developed to expanded blastocysts in HFF and HTF(FF)/0.5% BSA than in HTF/0.5% BSA, suggesting that embryos in these two media developed more rapidly to expanded blastocysts. After the *in vitro* culture of one-cell embryos for 80 hours in each medium, the blastocysts and morulae that had developed were transferred to CMRL 1066/20% FCS for the assessment of their developmental competence. We observed the trophoblast spreading and the formation of ICM at 96 hours of incubation (Table 5). The trophoblast spreading and the formation of an ICM were observed in 96, 81 and 82% of the embryos which were derived from morulae and blastocysts in HFF, HTF and HTF(FF)/0.5% BSA, respectively. These percentages were significantly higher than those of the embryos derived from blastocysts and morulae in α MEM/0.5% BSA (62%). We then continued to culture these embryos in fresh CMRL 1066/20% FCS for 3 more days and ob-

Table 3. *In vitro* fertilization of oocytes recovered from follicles 6 hours after hCG injection and the development of embryos cultured in HFF, HTF, HTF(FF), α MEM or Ham's F-10 medium

	No. of oocytes	1st PB extrusion (%)	2-cell (%)	morulae (%)	blastocyst (%)
HFF	66	71 ^{ab}	67 ^a	48 ^a	44 ^a
HTF	72	46 ^a	26 ^{bc}	10 ^{bc}	8 ^{bc}
HTF(FF)	57	46 ^{ab}	42 ^{ac}	22 ^{ac}	14 ^{bc}
α MEM	77	72 ^b	52 ^{ac}	33 ^{ac}	29 ^{ac}
Ham's F-10	75	17 ^c	0 ^b	0 ^b	0 ^b

PB: polar body. Experiments were conducted five times. The data are the percentages of oocytes that excreted the first polar body and progressed to 2-cell, morulae or blastocyst within 96 hours of culture. Values with different superscripts within each column are significantly different (ANOVA, Fisher's PLSD, $p < 0.05$).

Table 4. Development of one-cell embryos after an 80-hour incubation in HFF, HTF, HTF(FF) and α MEM medium.

	No. of embryos	morulae (%)	early blastocysts (%)	expanded blastocysts (%)	total blastocysts (%)
HFF	92	0 ^a	9 ^a	89 ^a	98 ^a
HTF	91	10 ^a	48 ^b	39 ^b	87 ^a
HTF(FF)	62	5 ^a	15 ^{ac}	78 ^a	94 ^a
α MEM	92	31 ^b	31 ^c	34 ^b	65 ^b

Experiments except HTF(FF) were conducted five times. Experiment of HTF(FF) were conducted three times. The data are the percentages of embryos that progressed to morulae to blastocyst within 80 hours of culture. Values with different superscripts within each column are significantly different (ANOVA, Fisher's PLSD, $p < 0.05$).

Table 5. Development of blastocysts incubated in HFF, HTF, HTF(FF) and α MEM medium, after 96 and 168 hours of incubation in CMRL1066 medium

	No. of blastocysts	96 hours			168 hours
		A (%)	B (%)	C (%)	D (%)
HFF	60	0	4	96 ^a	35 ^a
HTF	56	9	8	81 ^a	33 ^a
HTF(FF)	31	7	6	82 ^a	16 ^b
α MEM	57	16	13	62 ^b	6 ^b

Experiments except HTF(FF) were conducted five times. Experiment of HTF(FF) were conducted three times. The data are the percentages of blastocysts that developed trophoblasts and inner cell mass in CMRL1066 medium. Values with different superscripts within each column are significantly different (ANOVA, Fisher's PLSD, $p < 0.05$). A; attachment (hatched). B; trophoblast outgrowth without inner cell mass. C; trophoblast outgrowth with inner cell mass. D; endoderm and ectoderm differentiation.

served the differentiation of the ICM, ectoderm and endoderm. Embryos derived from blastocysts and morulae in HTF(FF) and α MEM/0.5% BSA showed endoderm and ectoderm differentiation in significantly reduced populations (16% and 6%) compared with those in HFF and HTF/0.5% BSA (35% and 33%).

Since ammonium in the media is produced by the spontaneous breakdown of amino acids, mainly glutamine, we measured the ammonium production in four media, HFF, HTF, α MEM and Ham's F-10 (Fig. 1). Each medium was pre-equilibrated under a layer of paraffin oil overnight at 37°C in 5% CO₂ in air before the embryo cultures. At the beginning of these cultures, α MEM and Ham's F-10 revealed a significantly high level of ammonium production, and the ammonium produced in these media increased linearly with the

incubation time. A significantly higher ammonium production was observed in α MEM in comparison with the other three media; the level of ammonium produced over 72 hours in α MEM was 595 mg/dl, which was 4.8 times higher than that in HFF (123 mg/dl).

Discussion

Because mammalian oocytes are matured in ovarian follicles, we used the HFF medium, based on the composition of human follicular fluid, for the *in vitro* maturation of mouse immature oocytes. Cha *et al.* [18] reported the first pregnancy after the IVF of human follicular oocytes collected from a non-stimulated cycle. To mature the oocytes, they used Ham's F-10 with 20% FCS or 50% mature follicular fluid. Trounson *et al.* [1] deter-

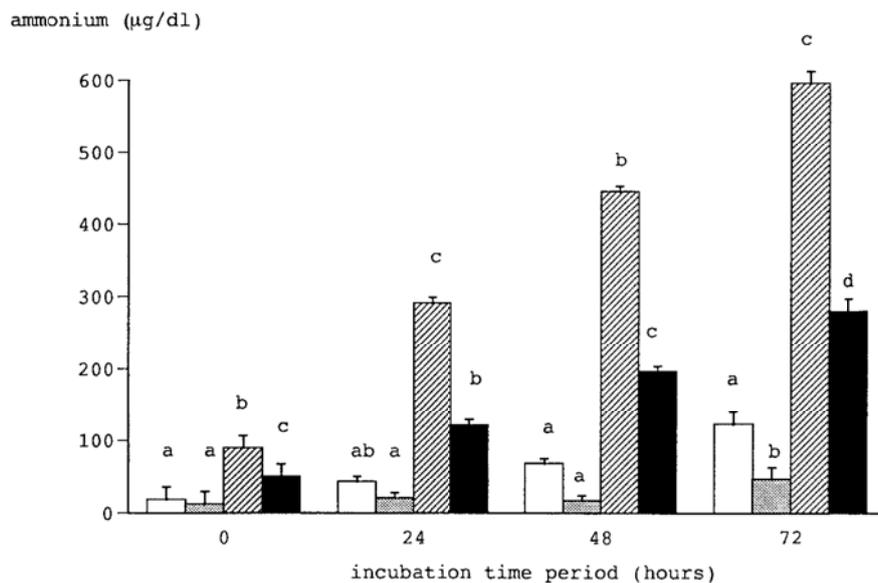


Fig. 1. Production of ammonium in each medium during incubation at 37°C, 5% CO₂ in air with mouse embryos. Values with different superscripts within each column show significant differences (ANOVA, Fisher's PLSD, $p < 0.05$). Each column represents the mean \pm SD of five determinations. Media: open bars, HFF; dotted bars, HTF; hatched bars, α MED; closed bars, Ham's F-10.

mined the *in vitro* maturational and developmental competence of immature oocytes recovered from untreated PCOS patients. Barnes *et al.* [19] also reported three cases of blastocyst development and birth after the *in vitro* maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. In the above two clinical reports, since immature oocytes were recovered with no ovarian stimulation, the maturation media were supplemented with 10% FCS, human follicular-stimulating hormone (FSH) and hCG. It was suggested that exogenous gonadotropins increased the oocyte maturation of immature mouse oocytes *in vitro* [20]. However, the optimal concentrations of FSH and leutenizing hormone (LH) supplemented in the culture medium for *in vitro* oocyte maturation are not yet known. Edirisinghe *et al.* [21] described the number of immature oocytes collected as being dependent on the individual, and the incidence of immature oocytes retrieved varied from 0 to 50%. Immature oocytes retrieved after hCG injection consisted of prophase I and metaphase I oocytes [22]. In the present study, oocytes undergoing maturation through division I of meiosis were recovered from ovarian follicles 6 hours after an hCG injection.

The maturation of oocytes consists of nuclear and cytoplasmic maturation. The former is estimated by the

polar body extrusion and the latter by the abilities of fertilization and the subsequent embryo development. The use of HFF/10% FCS resulted in high percentages of extrusion of the first polar body, fertilization, and embryo development to the blastocyst stage. The α MED/10% FCS also showed good results for *in vitro* oocyte maturation and fertilization, whereas HTF/10% FCS retarded the development to the morulae and blastocyst stages, suggesting that supplementation of amino acids in the maturation medium is necessary for the cytoplasmic maturation of maturing oocytes *in vitro*, because amino acids in the medium are brought to cumulus-enclosed oocytes through the gap junctional pathway between oocytes and cumulus cells [23]. The supplementation of amino acids improved the *in vitro* oocyte maturation in several species [24–26]. However, as shown in Table 3, the supplementation of amino acids at a concentration present in follicular fluid to HTF (HTF(FF)) did not improve the oocyte maturation or the subsequent development. The difference in the developmental competence of embryos between those matured in HFF and those in HTF(FF) was possibly due to differences in the level of glucose, lactate and potassium in each medium. Energy substrates, e.g., glucose, pyruvate, lactate and amino acids, are also required for the *in vitro* development of embryos to the blastocyst

stage, and their combinations and concentrations markedly change the developmental responses of embryos *in vitro* [27]. The HFF medium contains 1.85 g/L of lactate. When the concentration of lactate was elevated to 2.40 g/L, which is the level of lactate in HTF medium, the development of mouse embryos to the blastocyst stage was reduced (data not shown). Extracellular potassium affects the embryo development *in vitro* in cooperation with taurine and amino acids [28]. The level of potassium in tubal fluid was reported to be over 20 mM [29–31] and that in HTF was adjusted to 5.1 mM, which is higher than that in HFF (4.1 mM) and serum, as shown in Table 1. Despite its use in the first study on *in vitro* human oocyte maturation [18], Ham's F-10 was found not suitable for *in vitro* mouse oocyte maturation in this study. Ham's F-10 contains heavy metal ions (such as Zn²⁺, Fe²⁺ and Cu²⁺) [32] and hypoxanthine [33, 34], which are harmful to embryo development. Hypoxanthine was also identified as an inhibitory component of nuclear maturation in follicular fluid [35] and first polar body extrusions in Ham's F-10 medium were reduced in comparison with those in the other media, as shown in Table 3. These results indicate that such substances in Ham's F-10 are also harmful to *in vitro* oocyte maturation and embryo development.

Several media have been used for the *in vitro* culture of embryos and are considered sufficient for the current IVF procedures, in which 2- to 8-cell embryos are transferred into a human uterus 2 to 3 days after fertilization. The selection of developmentally competent embryos may be achieved by allowing embryo cleavage to progress to the blastocyst stage *in vitro*. The blastocyst transfer possibly prevents a multiple pregnancy and elevates the pregnancy success rate. Several single culture media for blastocyst development have been reported, with good results in culture medium alone in several species [36–40]. Walker *et al.* [41] reported that synthetic ovine oviductal fluid containing amino acids at oviductal concentrations facilitated the development of a high percentage of blastocysts and was beneficial for extended embryo culture *in vitro*. In our previous study [12], we used culture media based on HTF medium supplemented with amino acids at concentrations present in the follicular fluid, serum, and conventional media, and we examined the effect of amino acid supplementation on mouse embryo development *in vitro*. We found that supplementation with the 21 amino acids present in human follicular fluid and serum enhanced the development of CBF1 mouse embryos to the blastocyst stage and released a two-cell block in ICR mice. In the present study, we applied HFF me-

dium to the blastocyst culture *in vitro*. Using mouse one-cell embryos, we observed accelerated embryo development to the expanded blastocyst stage in HFF and HTF(FF)/0.5% BSA in comparison with that in HTF or α MEM/0.5% BSA. We also examined the quality of the blastocysts cultured in each medium. After a 96-hour incubation in CMRL 1066/20% FCS, the blastocysts cultured in HFF and HTF/10% BSA showed satisfactory potential for embryo development in comparison with that in HTF(FF) or α MEM/0.5% BSA. In clinical human IVF, because production of blastocysts should be established as quickly as possible, it is of clinical interest that a higher percentage of embryos developed to the expanded blastocyst stage in HFF/10% BSA than in HTF/10% BSA.

We prepared HFF medium supplemented with amino acids at concentrations adjusted to those in follicular fluid collected from patients with controlled ovarian hyperstimulation. The concentrations of amino acids in follicular fluid were also reported in patients during a natural cycle; the concentrations were influenced by a hormonal pretreatment [42–44]. However, these results and those of the present study indicate that follicular fluid contains lower amounts of amino acids, especially glutamine, in comparison with conventional culture media. The supplementation of an excess amount of amino acids was harmful to embryo development *in vitro*, as shown in our previous study [12] in which supplementation of amino acids in minimal essential medium (MEM) inhibited embryo development *in vitro*. MEM medium contains a high amount of glutamine, 11 times more than that in follicular fluid, and its inhibitory effect on embryo development *in vitro* may have been caused by elevated ammonium produced by the breakdown of glutamine [12, 45]. An experimental study of development after uterine transfer revealed that ammonium in the medium retards fetal development and induces the neural tube defect, exencephaly [46]. The amount of amino acids in HFF was smaller than that in conventional media. The concentration of glutamine in α MEM is 11 times higher than that in HFF, and the level of ammonium produced within 72 hours in α MEM was 4.8 times higher than that in HFF. As α MEM contains adenosine which blocks the development of mouse embryos at the two-cell stage [47], the high concentration of ammonium in α MEM may have caused the reduced development of embryos *in vitro*. The formation of an inner cell mass including endoderm and ectoderm differentiation was more severely reduced than the trophoblast spreading.

In the present study, we investigated a simple me-

dium for use in *in vitro* oocyte maturation, fertilization and extended embryo culture, and report that the HFF medium had good effects on maturation of mouse oocytes and embryo development after IVF *in vitro*. The composition of the HFF medium is the same as that of human follicular fluid, but the substrate requirements for human embryo development *in vitro* may be different from those of mouse embryos. Moreover, the supplementation of serum was required for *in vitro* oocyte maturation. To create optimal conditions of *in vitro* human oocyte maturation and blastocyst culture, we will apply the HFF medium as a basal medium after a quality check including toxicity and stability tests and we will determine the types and concentrations of other substrates, i.e., vitamins and gonadotropins.

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