Development of *in vitro* Fertilized Feline Embryos in a Modified Earle's Balanced Salt Solution: Influence of Protein Supplements and Culture Dishes on Fertilization Success and Blastocyst Formation

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ABSTRACT. The effects of protein supplements and culture dish type on *in vitro* fertilization (IVF) and embryo development in culture were examined in the domestic cat. In Experiment I, follicular oocytes were fertilized and cultured in either 1) modified Earle's balanced salt solution, designated MK-1, supplemented with one of the following: 10% human serum (HS), 10% FCS or 0.4% BSA, or 2) Medium 199 (M-199) supplemented with 10% FCS. Fertilization rates were lower ($P<0.01$) in MK-1 + BSA (74.4%), MK-1 + FCS (56.1%), and M-199 + FCS (51.4%) than in MK-1 + HS (94.7%). A greater ($P<0.01$) percentage of blastocysts was obtained in MK-1 + HS (50.0%) than in other treatment groups (range, 4.3–17.2%). In Experiment II, the effect of dish type (tissue culture dish, TCD, versus suspension culture dish, SCD) on embryo development was evaluated in MK-1 supplemented with either HS or BSA. Significantly higher proportions of IVF-derived embryos developed to blastocysts at 120 and 144 hr post-insemination, respectively, when cultured in HS/SCD (47.2 and 71.7%) than in BSA/SCD (11.4 and 27.3%) or BSA/TCD (10.4 and 25.0%). At 120 hr post-insemination, there was a lower ($P<0.01$) percentage of blastocysts in HS/TCD (22.2%) than in HS/SCD. In Experiment III, six embryos per cat were transferred to the uterine horns of 17 recipients at 144 hr after hCG treatment. Five of 7 recipients which received late morulae cultured in MK-1 + BSA (SCD) for 120 hr became pregnant (71.4%). Eight of 10 recipients which received early blastocysts cultured in MK-1 + HS (SCD) for 120 hr became pregnant (80.0%). We conclude that MK-1 containing HS is highly beneficial for overcoming the *in vitro* developmental block of IVF-derived feline embryos and increasing the success rate of IVF/ET. — KEY WORDS: developmental block, feline (domestic), human serum, *in vitro* culture, IVF-ET.

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Assisted reproductive techniques such as *in vitro* fertilization (IVF), *in vitro* culture (IVC), embryo cryopreservation and embryo transfer (ET) in the domestic cat are not only useful for maintaining genetic diversity of our closed cat colony for biomedical research but could be applied to artificially propagate endangered nondomestic field species. In addition, routine production of feline embryos from follicular oocytes would allow the domestic cat to be used more readily as a model for human genetic diseases and developmental biology research. Recently, successful transfer of IVF-derived feline embryos has been reported [10, 26, 31] but pregnancy and embryo survival rates following IVF/IVC were disappointingly low. Inadequate culture conditions contribute to this low success rate of IVF-ET. Despite a high rate of fertilization and successful growth to morulae, IVF-derived feline embryos show a partial but highly significant *in vitro* developmental block at the morula-to-blastocyst stage [10, 14, 15, 26–28, 32]. Moreover, conventional changes in culture conditions such as incubation temperature [15], gas atomosphere [15] and oviductal cell monolayer co-culture [27, 32] have all failed to circumbent the block.

In previous studies, complex media such as Ham's F-10 [10, 14, 15, 26–28, 32] and M-199 [17, 26] have been widely used for feline IVF and embryo culture. These media consist of many components, some of which have been shown to have detrimental effects on embryo development in culture [3, 22]. To investigate the regulatory mechanisms and the nutritional requirements of feline early embryonic development *in vitro*, therefore, simple media such as Krebs-Ringer's bicarbonate salt solution (KRB) and Tyrode's solution are more useful than these complex media. Johnston *et al.* [14] demonstrated that fertilization success *in vitro* of matured feline follicular oocytes was high and most embryos developed equally well to the morula stage in two simple media supplemented with BSA, modified KRB (mKRB) [34] and modified Tyrode's solution (TALP) without phosphate or glucose [2], but blastocyst formation was very low in both media. Likewise, Swanson *et al.* [32] evaluated the impact of the presence or absence of glucose and glutamine in mKRB, but the IVF-derived embryos also failed to develop into blastocysts (2–5%) under any culture conditions applied. Therefore, mKRB and TALP are inadequate as basal media for IVF and IVC in the cat. In this study, we designed a novel medium that can be easily prepared and would be expected to overcome the morula-to-blastocyst developmental block of IVF-derived feline embryos. This media was prepared by modification of Earle's balanced salt solution based on recent research on oviductal fluid [8, 12, 13, 20, 23] and many findings that had highlighted the inadequacies and limitations of conventional embryo culture media [3, 22, 25, 33].

The impact of protein supplementation on *in vitro* maturation (IVM) of ovarian oocytes, IVF and IVC was

analyzed in the cat. Johnston *et al*. [14] reported that a complex media (Ham's F-10) containing FCS produced a higher in rate of fertilization, and about 2-fold more of these embryos developed in culture to blastocysts compared to embryos supplemented with polyvinylalcohol (PVA) or BSA. In two recent studies [16, 38], in contrast, IVM and IVF rates of ovarian oocytes cultured in Eagle's minimum essential medium (MEM) were decreased by using FCS as protein supplement compared to BSA and PVA. Therefore, in Experiment I, the effects of protein supplements on feline IVF and IVC were evaluated using a simple medium, MK-1.

In standard IVF and IVC systems, cumulus cells are mechanically removed after IVF, then oocytes are cultured in tissue culture dishes, TCD (35 mm dish or 4-well dish). In previous studies [10, 14, 15, 27, 28, 32], most feline oocytes fertilized *in vitro* received the same treatment. Pope *et al*. [26] demonstrated that intact cumulus cells, some of which proliferated to form monolayers in TCD during the culture period, did not markedly affect the average number of cells per embryo. It appears that dish type affects attachment to the dish surface and growth of intact cumulus cells in culture. Therefore, in Experiment II, the effect of dish type on *in vitro* development of IVF-derived feline embryos with intact cumulus cells was evaluated using two kinds of culture dishes, TCD and suspension culture dishes, SCD. Additionally, in Experiment III, intrauterine ET was performed to investigate the ability to implant and develop to the fetuses of feline blastocysts produced IVF/IVC.

MATERIALS AND METHODS

Culture media and protein supplements: Media used in this study were M-199 containing Earle's salts (Gibco, Inc., U.S.A.) and modified Earle's balanced salt solution. The latter medium, designated MK, is characterized by exchange of $CaCl_2$ for L(+)Ca(lactate)$_2$ (Sigma, Inc., U.S.A.), reduction of $NaHCO_3$ (25.0 mM) and glucose (1.50 mM) and addition of sodium pyruvate (0.36 mM). Subsequent derivatives of this medium were MK supplemented with MEM essential and non-essential amino acids (MK-1), HEPES-buffered MK (Hep-MK) and Hep-MK supplemented with 0.2% hyaluronidase (Type I-S, Sigma) (Hyal-MK). The compositions of these media are shown in Table 1. Besides 19 common amino acids, MK-1 contains L-asparagine and M-199 contains L-cysteine and L-hydroxyproline. M-199 contains a further 31 components including vitamins and nucleic acids. Three kinds of protein supplements, which were 10% heat-treated fetal calf serum (FCS, cat. no. 16000; Gibco), 0.4% bovine serum albumin (BSA, cat. no. A-0281; Sigma) or 10% heat-treated human serum (HS) from a young male, were used. The concentration of glucose in MK-1 was reduced to 1.00 mM with the subsequent addition of 10% FCS or HS.

Animals: Twenty-two sexually mature female cats were used as oocyte donors and ten males were used as sperm donors. They were of mixed breed and housed singly in aluminum cages under nearly constant environmental conditions as previously described [17]. All cats were given a commercial cat diet (Science Diet, Hill's Pet Products, Inc., U.S.A.), and water *ad libitum.*

Table 1. Compositions (mM) of culture media used

Component	Medium 199[a)]	MK-1	Hep-MK	Hyal-MK
NaCl	116.36	116.36	116.36	116.36
KCl	5.37	5.37	5.37	5.37
$NaH_2PO_4 \cdot 2H_2O$	1.01	1.01	1.01	1.01
$MgSO_4 \cdot 7H_2O$	0.81	0.81	0.81	–
$Fe(NO_3)_3 \cdot 9H_2O$	0.002	–	–	–
$NaHCO_3$	26.19	25.00	5.00	5.00
$CaCl_2$	1.80	–	–	–
L(+)Ca(lactate)$_2$	–	1.80	1.80	–
Sodium pyruvate	–	0.36	0.36	0.36
Glucose	5.55	1.50[b)]	1.50	1.50
HEPES	–	–	20.00	20.00
Phenol red (mg/m*l*)	0.02	0.01	0.002	0.002
BSA (mg/m*l*)	–	–	3.00	–
Heparin (units/m*l*)	–	–	10.00	–
Hyaluronidase (mg/m*l*)	–	–	–	2.00
Amino acids	+21	+20	–	–
Others	+31	–	–	–

Penicillin G (100 U/m*l*), streptomycin sulfate (100 μg/m*l*) and amphotericin B (0.25 μg/m*l*) were added in all media. a) With Earle's salts. b) The concentration of glucose in MK-1 supplemented with human serum or fetal calf serum was reduced to 1.00 mM.

Gonadotropin treatment and oocyte recovery: Follicular development in oocyte donors was hormonally stimulated by intramuscular injection of pregnant mare serum gonadotropin (PMSG, Nippon Zenyaku Industrial Co., Ltd.), with a total dose of 150 IU divided into 100 IU on the first day and 50 IU on the second day. On the sixth day, they were given 100 IU of human chorionic gonadotropin (hCG, Sankyo Co., Ltd.) intravenously to induce oocyte maturation. Oocytes were recovered by follicular aspiration from the donors after ovarian exposure via midventral laparotomy at 23 ± 1 hr after hCG treatment. Laparotomy was performed under sterile conditions and general anesthesia induced by intramuscular injection of propiopromazine (0.5 mg/kg) followed by a similar injection of ketamine hydrochloride (20 mg/kg) 5–10 min later. Mature ovarian follicles (≥2 mm in diameter) were aspirated using 20-gauge × 1/2" needles (Nipro, Inc.) attached to 1 m*l* syringes containing Hep-MK adjusted to pH 7.4. Follicular contents were transferred to 60 × 15 mm polystyrene Petri dishes at each follicular aspiration and rinsed with 5 m*l* of Hep-MK. To separate each oocyte from cumulus oophorus and viscous follicular fluid, the follicular contents were incubated in Hyal-MK for 2 to 3 min. Oocytes were immediately washed with Hep-MK and examined morphologically by phase-contrast microscope. Only oocytes with darkly pigmented, uniform cytoplasm and surrounded by an expanded cumulus and distinct corona radiata were used for IVF.

Collection and processing of spermatozoa: Adult male cats were anesthetized by the same regimen as in the oocyte

donors, and one epididymis with the ductus deferens was removed with the testis through a scrotal incision. After cutting a part of the cauda epididymis with a surgical knife, the dense mass of spermatozoa was taken up with the tip of a fine glass rod, introduced into a 1.5 m*l* centrifuge vial containing 500 μl of Hep-MK and centrifuged for 5 min at 350 × g. After removal of the supernatant, the sperm pellet was overlaid with 400 μl of Hep-MK and the spermatozoa were allowed to swim up for 2 hr at 38°C in air. The swim-up layer was carefully recovered, and small aliquots were used to evaluate sperm concentration and percentage motility. The samples were diluted with Hep-MK or M-199 supplemented with 10% FCS to a concentration of 5×10^6 motile sperm per milliliter and used immediately for IVF.

In vitro fertilization and embryo culture: Five to 10 oocytes per well were transferred into 4-well dishes for tissue culture (cat. no. 176740, Nunclon®, Nunc, Inc., Denmark), containing 480 μl of equilibrated medium per well according to protocols for Experiments I and II. Twenty-microliter sperm samples were added to each well, resulting in a final concentration of 2×10^5 motile sperm per milliliter. After 3 hr co-incubation with processed spermatozoa under 5% CO_2 in air at 38°C, oocytes were transferred into 4-well dishes containing 500 μl of sperm-free fresh medium or 35 mm Petri dishes for suspension culture (cat. no. MS-1135R, Sumilon, Sumitomo Bakelite Co., Ltd.) containing 2.5 m*l* of medium and cultured for an additional 21 hr. At 24 hr post-insemination (3 hr co-incubation + 21 hr culture), oocytes were freed from only loosely attached cumulus cells and spermatozoa by gently passing through a fine pipette and were then transferred into drops of about 20 μl (1 embryo per drop) of fresh medium and cultured under 5% CO_2 in air at 38°C. Each culture drop had previously been covered with light mineral oil (Sigma) in a 35 mm Petri dish for tissue culture (cat. no. 153066, Nunclon®) or for suspension culture (Sumilon) and equilibrated with the gas phase and temperature in a CO_2 incubator overnight. Each medium had a pH of 7.3 to 7.4 after equilibration. All oocytes were assessed for fertilization on the basis of evidence of cleavage to at least the 2-cell stage at 24 hr and again 48 hr post-insemination. Embryos were examined morphologically, freed from cumulus cells by gently pipetting and transferred into fresh and equilibrated medium at 24 hr intervals for 144 hr post-insemination. Embryos having over 16 blastomeres were considered as morulae, and those showing a blastocoel cavity were classified as blastocysts.

Experiment I: This preliminary study was performed to determine whether *in vitro* fertilized feline ova could develop in relatively simple medium, MK-1, compared to more complex medium, M-199. Using these two media, the effects of protein source on IVF and embryonic growth were evaluated. The oocytes collected from individual females were randomly allocated to (1) MK-1 + 10% HS; (2) MK-1 + 0.4% BSA; (3) MK-1 + 10% FCS; or (4) M-199 + 10% FCS groups, then inseminated and cultured in TCD.

Experiment II: Based on the findings of Experiment I, MK-1 supplemented with 10% HS and/or 0.4% BSA were chosen as the optimal fertilization and culture media for Experiment II. In Experiment II, the effect of dish type (TCD versus SCD) on IVF and embryonic growth was compared between the four treatment groups (2 different protein supplements × 2 different types of culture dishes). The oocytes after insemination in each medium were randomly divided into either the tissue culture or suspension culture system.

Evaluation of embryo development: At 144 hr post-insemination, all embryos (in Experiments I and II) were evaluated the developmental stage and then fixed using an air-dry technique modified from Dyban [4] and Pope *et al.* [26]. Briefly, after hypotonic treatment in 0.5% KCl for 30 min, embryos were treated for 1 to 2 min in a mixture of ethanol:glacial acetic acid:distilled water (8:3:1) and fixed in ethanol:glacial acetic acid (3:1) overnight. These procedures were conducted at 0 to 4°C. Embryos were then mounted on clean and cooled microslides, air-dried and stained for 30 min in 0.5% Giemsa in phosphate buffer solution (pH 6.8). After rinsing in distilled water and air drying, the embryos were mounted in Eukitt (O. Kindler, Inc., Germany) and covered with a coverslip. The number of cell nuclei per embryo was counted using a bright-field microscope at a magnification of × 200 or × 400.

Experiment III: Embryo transfer was performed to investigate the ability to implant and develop to the fetuses of embryos cultured for 120 hr in HS/SCD and/or BSA/SCD. Six embryos per cat used as oocyte donors were transferred to the uterine horns of 17 recipients at 144 ± 2 hr after hCG treatment. Recipients were selected from 22 cats used as oocyte donors. Each recipient received homologous and/or heterologous embryos derived from one donor. Embryo transfer was performed by laparotomy based on the previously described protocol [17]. A 24-gauge intravenous catheter (Surfuro®, Terumo, Inc.) and a transfer pipette with a diameter of about 400 μm made by drawing a hard glass capillary were used. Embryos were transplanted into the lumen of the cranial end of the uterine horn on the side of the ovary containing the larger number of corpora lutea (CL). Pregnancy was determined by abdominal palpation 2 and 3 weeks after embryo transfer, and pregnant recipients were allowed to complete gestation so that the birth rate of live kittens could be determined.

Statistical analysis: The proportions of fertilization and embryos developing to morulae and blastocysts were analyzed between treatment groups using the Chi-square or Fisher's test. The mean numbers (± SEM) of cell nuclei in morulae, blastocysts and total embryos were determined for each treatment group and evaluated using analysis of variance. Differences between treatment groups were assessed with the Schéffe's multiple comparison test. Experiments I and II were conducted in five replicates, respectively. In Experiment II, 6 embryos (2 in HS/SCD and 4 in HS/TCD) were lost during fixing or staining.

Table 2. Cleavage and development of *in vitro* fertilized feline oocytes cultured in MK-1 and Medium 199 supplemented with different protein sources

Culture medium	Protein[a] supplement	No. of oocytes		No. of embryos developed to		No. of nuclei per embryo (mean ± SEM)
		Inseminated	Cleaved (%)[b]	Morula (%)[c]	Blastocyst (%)[d]	
MK-1	HS	38	36 (94.7)[e]	33 (91.7)	18 (50.0)[e]	111.5 ± 8.8[e]
MK-1	BSA	39	29 (74.4)[f]	28 (96.6)	5 (17.2)[f]	125.9 ± 7.9[e]
MK-1	FCS	41	23 (56.1)[f,g]	20 (87.0)	1 (4.3)[f]	61.5 ± 6.2[f]
M-199	FCS	37	19 (51.4)[g]	16 (84.2)	1 (5.3)[f]	50.2 ± 7.2[f]

a) HS, heat-inactivated (56°C for 30 min) human serum (10% v/v); FCS, heat-inactivated fetal calf serum (10% v/v); BSA, bovine serum albumin (4 mg/m*l*). b) By 48 hr post-insemination. c) By 96 hr post-insemination. d) By 144 hr post-insemination. e-g) Values with different superscripts within each column are significantly different (at least P<0.05).

Table 3. Effects of protein supplement and culture dish type on cleavage and development of *in vitro* fertilized feline oocytes cultured in MK-1 medium

Culture treatment[a]		Cleavage rate (%)	No. and (%) of embryos developed to			Mean no. of nuclei (± SEM)	
Protein	Dish		Morula (96)[b]	Blast. (120)[b]	Blast. (144)[b]	per morula	per blastocyst
HS	SCD	53/58 (91.4)[c]	51 (96.2)	25 (47.2)[c]	38 (71.7)[c]	79.8 ± 6.1[c,e]	171.9 ± 4.8
HS	TCD	54/58 (93.1)[c]	51 (94.4)	12 (22.2)[d]	30 (55.6)[c]	69.5 ± 5.2[c]	168.2 ± 8.7
BSA	SCD	44/59 (74.6)[d]	43 (97.7)	5 (11.4)[d]	12 (27.3)[d]	115.2 ± 6.1[d]	187.4 ± 8.9
BSA	TCD	48/59 (81.4)[c,d]	46 (95.8)	5 (10.4)[d]	12 (25.0)[d]	110.2 ± 7.9[d,e]	181.7 ± 8.3

a) The inseminated oocytes were cultured either in suspension culture dishes (SCD) or in tissue culture dishes (TCD) with MK-1 medium supplemented with either human serum (HS) or bovine serum albumin (BSA). b) Numbers in parentheses indicate the time of examination (hours post-insemination). c-e) Values with different superscripts within each column are significantly different (at least P<0.05).

RESULTS

Experiment I: The fertilization rate by 48 hr post-insemination was significantly higher in MK-1 + HS (94.7%) than in the other culture conditions (74.4% in MK-1 + BSA, 56.1% in MK-1 + FCS and 51.4% in M-199 + FCS, Table 2). Development of cleaved embryos to morulae was similar (P>0.05) for all treatment groups (range, 84.2–91.7%) but a greater (P<0.01) percentage of blastocysts were formed in MK-1 + HS (50.0%) than in other treatment groups (range, 4.3–17.2%). No differences in the mean number (± SEM) of nuclei per embryo were detected in MK-1 + HS (111.5 ± 8.8) or MK-1 + BSA (125.9 ± 7.9). However, these values were significantly different (P<0.01) from those obtained in MK-1 + FCS (61.5 ± 6.2) and M-199 + FCS (50.2 ± 7.2). The mean number of nuclei per morula cultured in MK-1 + BSA (122.3 ± 7.5) was greater (P<0.01) than those in MK-1 + HS (70.3 ± 5.5), MK-1 + FCS (61.5 ± 5.9) and M-199 + FCS (51.6 ± 6.5).

Experiment II: Dish type had no effect (P>0.05) on *in vitro* fertilization success, because the cleavage rates were 82.9% (97/117) for SCD and 87.2% (102/117) for TCD (Table 3). However, cleavage rate was higher (P<0.01) using HS (92.2%; 107/116) as a protein supplement compared to the BSA (78.0%; 92/118). In the HS/TCD treatment group, intact cumulus cells attached to the culture dishes and proliferated to form monolayers (Fig. 1-a). However, in other treatment groups, cumulus cells did not attach to the dishes (Fig. 1-b) and came off gradually during culture (Fig. 2). Cleaved embryos from each treatment group were equally capable of developing to the morula stage (Figs. 2-b, c) (range, 94.4–97.7%). A significantly (P<0.01) greater proportion of embryos developed to the blastocyst stage at 120 hr post-insemination (Fig. 2-d) when cultured in HS/SCD (47.2%) than in other treatment groups (range, 10.4–22.2%). SCD enhanced (P<0.01) blastocyst formation at 120 hr post-insemination of embryos cultured in MK-1 + HS compared to TCD. The highest proportion (71.7%) of embryos developed to blastocysts by 144 hr post-insemination (Fig. 2-e) in HS/SCD (P<0.01), although this value was not significantly different from that obtained in HS/TCD (55.6%). A greater (P<0.01) percentage of blastocysts were formed in MK-1 + HS (63.6%; 68/107) than in MK-1 + BSA (26.1%; 24/92). The mean numbers of nuclei per embryo were 148.5 ± 6.9 in HS/SCD, 124.7 ± 8.8 in HS/TCD, 134.9 ± 7.0 in BSA/SCD and 128.1 ± 7.7 in BSA/TCD, respectively, and no differences were detected in each treatment group. The mean numbers of nuclei per morula were greater (P<0.01) in BSA/SCD (115.2 ± 6.1) than those in HS/SCD (79.8 ± 6.1) and in HS/TCD (69.5 ± 5.2). No differences were detected in the mean number of nuclei per blastocyst in each treatment group. Dish type had no effect (P>0.05) on *in vitro* development of embryos cultured in MK-1 + BSA.

Experiment III: The results of IVF/ET are presented in Table 4. In the group using MK-1 + 0.4% BSA as the

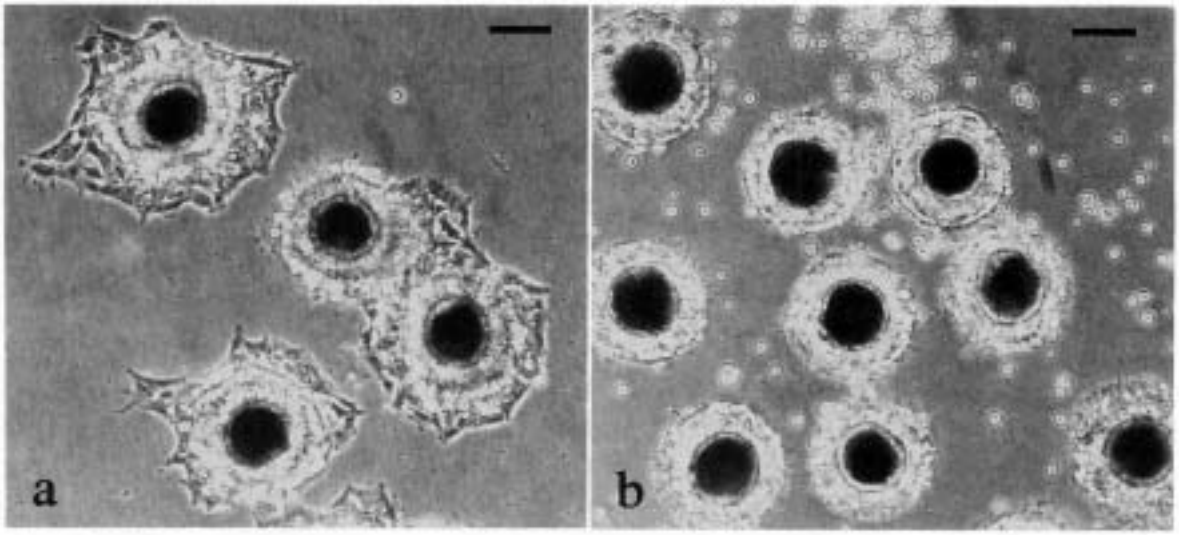

Fig. 1. Feline ova cultured in MK-1 + 10% HS at 20 hr post-insemination. Bar=100 μm. a) Intact cumulus cells attached to the 4-well dish for tissue culture and proliferated to form a monolayer. Thus, oocytes were fixed to the dish. b) Cumulus cells could not attach to the 35 mm Petri dish for suspension culture, and thus oocytes were free-floating.

medium for IVF/IVC, 5 of 7 recipients which received late morulae cultured for 120 hr became pregnant (71.4%). All of the pregnant cats delivered live offspring (mean 2.0; range 1–3) at full term. In the group using 10% HS as the protein supplement, 8 of 10 recipients which received early blastocysts cultured for 120 hr became pregnant (80.0%). Seven of 8 pregnant cats delivered a total of 21 live offspring, while the remaining one animal expelled only placentae on day 73 of pregnancy. Four of 6 (66.7%) recipients in homologous ET, and 9 of 11 (81.8%) recipients in heterologous ET became pregnant. There were no significant differences ($P>0.05$) in pregnancy rate or mean litter size between MK-1 + BSA and MK-1 + HS, or between homologous and heterologous ET. The mean gestation period was 68.9 (range, 68–71) days, with the time of hCG administration designated as day 0 of pregnancy.

DISCUSSION

The establishment of basal media for feline embryo culture is indispensable to investigate regulatory mechanisms and nutritional requirements of feline early embryonic development. The results of this study demonstrated that our culture medium, MK-1, was useful as a basal medium for supporting feline IVF and IVC. The mean number of nuclei per embryo cultured in MK-1 containing BSA at 144 hr post-insemination was 4-fold greater than those in another simple medium, mKRB, containing BSA (31.9 ± 1.3) in a recent study [32]. Furthermore, adding HS to MK-1 consistently increased the fertilization success and blastocyst formation compared with MK-1 containing other proteins. Although serum factors were needed for blastocoel cavity formation, MK-1 was also beneficial for overcoming the *in vitro* developmental block of feline embryos.

MK-1 was prepared by modification of Earle's balanced salt solution which is the basal salt composition of M-199, based on recent research on oviductal fluid. In MK-1, Ca^{2+} was supplied by L(+)Ca(lactate)$_2$ instead of $CaCl_2$. The concentration (3.6 mM) of L(+)lactate was based on measurements in mouse [8], rabbit [20], pig [23] and human [12] oviductal fluid. The concentration (0.36 mM) of pyruvate was arranged to adjust the lactate/pyruvate ratio to 10.0, which approximates the physiological values in mouse [8], rabbit [20] and human [12] oviductal fluid. The glucose concentration (1.5 mM) of MK-1 was decreased based on measurements in rabbit [20], sheep [13] and human [12] oviduct fluid, and was set as that in SOF medium for cow and sheep embryo culture [33].

Many simple media for embryo culture [34, 37] are characterized by high levels of lactate (>20 mM) compared to physiological levels in oviductal fluid. However, the finding in the mouse that embryo development up to the blastocyst *in vitro* was enhanced in the presence of lower levels of lactate supports the observations of Pomp *et al.* [25], who found that a reduction of lactate from 21.6 to 11.65 mM in Whitten's medium [37] was beneficial to the development of zygotes to the blastocyst stage in culture. Interestingly, Swanson *et al.* [32] reported that IVF-derived cat embryos developed readily to the morula stage but most morulae failed to develop into blastocysts (2–5%) in mKRB supplemented with 0.4% BSA which containing high levels of lactate (21.58 mM). These studies question the suitability of employing high lactate levels in culture medium formulated to support blastocyst development.

In the domestic cat IVF system, it is known that the type of protein supplement has a marked impact on fertilization

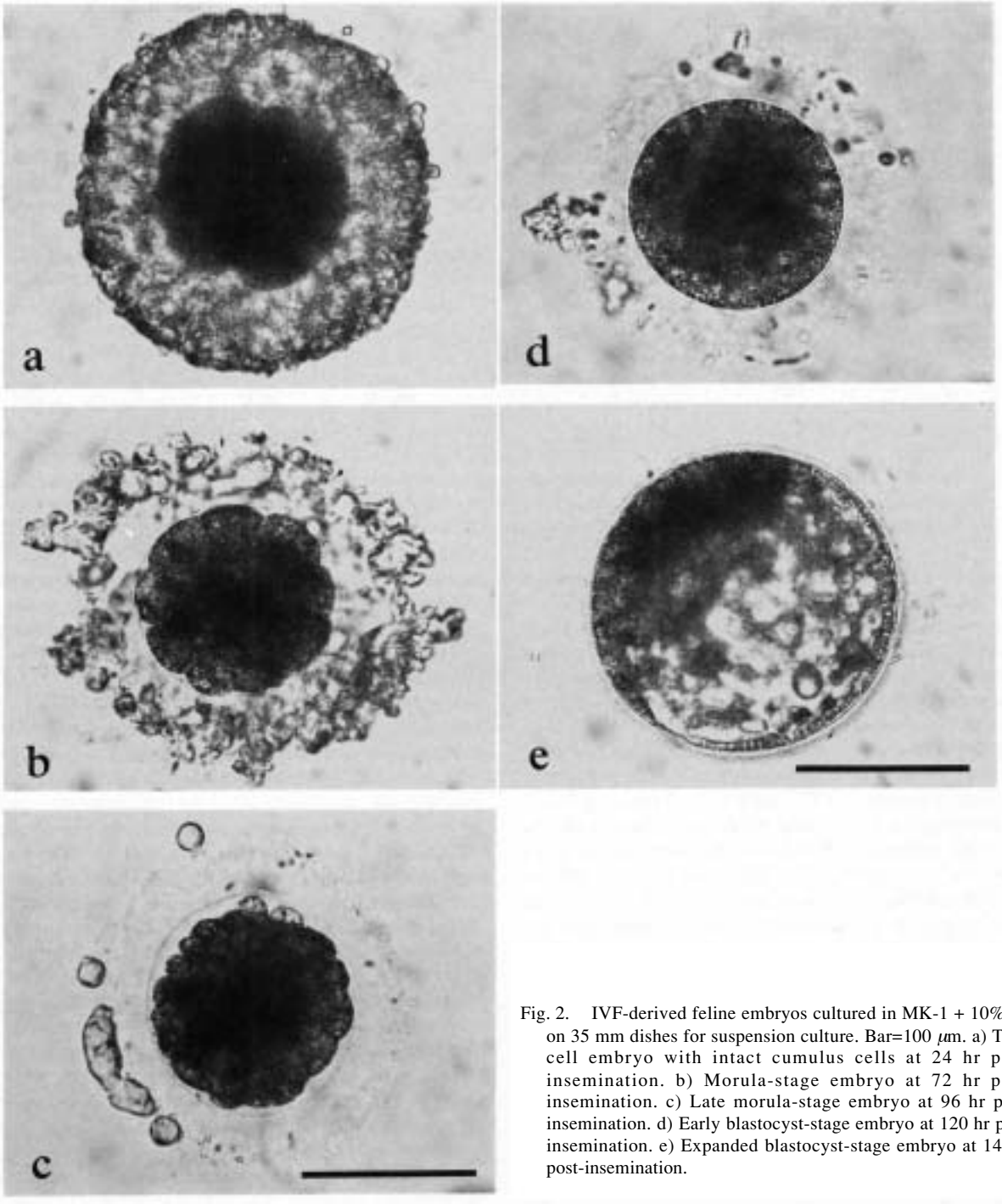

Fig. 2. IVF-derived feline embryos cultured in MK-1 + 10% HS on 35 mm dishes for suspension culture. Bar=100 μm. a) Two-cell embryo with intact cumulus cells at 24 hr post-insemination. b) Morula-stage embryo at 72 hr post-insemination. c) Late morula-stage embryo at 96 hr post-insemination. d) Early blastocyst-stage embryo at 120 hr post-insemination. e) Expanded blastocyst-stage embryo at 144 hr post-insemination.

success. Johnston *et al*. [14] demonstrated that fertilization was greater in a complex medium (Ham's F-10) supplemented with 5% FCS (84.0%) or 5% estrus cat serum (85.2%) than with 0.2% PVA (67.3%), while that with 0.4% BSA (76.1%) was intermediate. On the other hand, Swanson *et al*. [32] reported that fertilization rate was lower for oocytes inseminated in Ham's F-10 containing 10% FCS (42.6%) than in mKRB containing 0.4% BSA (73.4%). In our study (Experiment I), interestingly, fertilization rate was much lower ($P<0.01$) for oocytes inseminated in MK-1 supplemented with FCS than with HS, while that with BSA was intermediate between these two rates. In a recent study [16], *in vitro* maturation and fertilization rates of ovarian oocytes cultured in both MEM and Waymouth MB 753/1

Table 4. Uterine transfer of IVF-derived feline embryos cultured in MK-1 medium supplemented with different protein sources

	Protein supplement		Total
	HS	BSA	
Stage of embryos[a)]	Blastocyst	Morula	
No. of recipients	10	7	17
No. and (%) of pregnancies	8 (80.0)	5 (71.4)	13 (76.5)
No. and (%) of deliveries	7 (87.5)	5 (100)	12 (92.3)
Litter size[b)] Mean (range)	3.0 (1–5)	2.0 (1–3)	2.6 (1–5)
Gestation period[c)] Mean (range)	68.9 (68–70)	69.0 (68–71)	68.9 (68–71)

a) Six embryos per recipient were transferred to one of the uterine horns. b) Delivery only. c) The time of hCG administration was designated as day 0 of pregnancy.

medium were decreased by using FCS as protein supplement compared to BSA. Based on these findings, although serum factors can promote IVM, IVF and IVC, FCS probably contains inhibitory elements for feline oocytes during the early growth phase until first cleavage. With bovine IVF-derived embryos, FCS has been shown to inhibit the first cleavage division without affecting blastocyst formation of the cleaved embryos [24]. In contrast, feline embryos fertilized and cultured in MK-1 supplemented with FCS failed to develop to the blastocyst stage (Experiment I). These results suggested that FCS used as a protein supplement during early culture until the first cleavage contributes to the later morula-to-blastocyst block in feline embryo culture.

The protein source used to supplement MK-1 significantly affected development of early feline embryos *in vitro*. In Experiment I, a greater ($P<0.01$) percentage of blastocysts were formed in MK-1 supplemented with HS than with BSA, interestingly, no differences were detected in the mean number of nuclei per embryo between these treatment groups. Commercial preparations of serum albumin are contaminated with proteins other than albumin, as well as fatty acids and other small molecules. These small molecules include an embryotrophic growth factor, which stimulates cell division and growth in rabbit morulae and blastocysts [18]. Since the BSA used in this study was highly purified (≥99%) and free of essential fatty acids, such unidentified embryotrophic growth factors might have been lost. In contrast, human serum contains a heterogeneous mixture of many proteins and other factors. It has been demonstrated that human serum enhances blastocyst formation in cow [19], goat [1], sheep [9] and pig [30] embryos in culture, while on the other hand it inhibits embryo development in mice [21]. In feline embryos, it appears that unidentified factors in human serum stimulate blastocoel cavity formation without effect on cell division. Further studies are needed to examine the effects of PVA as a non-protein supplement and/or growth factors for the culture of feline embryos using MK-1.

In Experiment I, fertilization rate was lower for oocytes inseminated in M-199 containing FCS than in MK-1 containing BSA and/or HS, and was equivalent to that in MK-1 containing FCS. Further, when additionally cultured in M-199 + 10% FCS, these IVF-derived embryos showed a higher failure rate in reaching the blastocyst stage (5.3%) than *in vivo* fertilized 1-cell embryos cultured in M-199 + 20% FCS (39.1%) in our previous study [17]. This lower blastocyst formation rate of IVF-derived embryos was similar to that of embryos (0–6.9%) cultured in another complex medium, Ham's F-10, containing FCS [28, 32]. Further studies are necessary to examine whether the low fertilization and blastocyst formation were caused by the medium itself or detrimental effects of FCS, and whether adding HS to M-199 would improve fertilization success and blastocyst formation.

In standard IVF and IVC systems, cumulus cells are mechanically removed after IVF, then oocytes are cultured in TCD (35 mm dish or 4-well dish). In previous studies [10, 14, 15, 27, 28, 32], most feline oocytes fertilized *in vitro* received the same treatment. Pope *et al*. [26] reported that intact cumulus cells, some of which proliferated to form monolayers during culture, did not improve the rate of cell division over that of embryos from which cells were removed after IVF. They used 4-well TCD for IVF and IVC. To ensure excellent cell attachment, cell spreading and cell growth, the surface of TCD is treated to make the plastic hydrophilic and negatively charged. In contrast, SCD are not treated to increase cell attachment to the dish surface. In Experiment II, feline embryos cultured in serum containing MK-1 were prevented from fixing to the dishes by suspension culture which provided a co-culture environment with retention of cumulus cells during the early culture period. A higher proportion of feline embryos developed to blastocysts at 120 hr post-insemination were formed in HS/SCD than in HS/TCD. Thus, it was suggested that embryos with own cumulus cells needed to be free from the surface of culture dishes for *in vitro* growth.

Growth blocks *in vitro* can be mitigated by co-culture of embryos with different kinds of somatic cells such as oviductal epithelial cells in other species including cows [6], sheep [7] and pigs [36]. In the cat, however, previous studies demonstrated that IVF-derived feline embryos consistently failed to form blastocysts in co-culture with either feline oviductal cell monolayers [27, 32] or intact cumulus cells [26]. Goto *et al*. [11] found that co-culture with bovine cumulus cell monolayers was a useful system for long-term culture of bovine IVF-derived embryos. Additionally, Saito *et al*. [29] reported that co-culture of IVF-derived human embryos with their own cumulus mass (cumulus cells and cumulus matrix) beneficially affected embryo growth and improved pregnancy rate of IVF-ET. Also in this study, the highest proportion of feline embryos developed to blastocysts in the HS/SCD treatment group, in which such an environment was provided. This enhancement may be related to the production of stimulatory

components and/or the removal of inhibitory compounds in the culture medium. Further studies are necessary to evaluate the usefulness of this co-culture method on blastocyst formation by using both cumulus-intact and cumulus-free feline embryo.

Despite the number of published papers on *in vitro* embryonic development in the cat, a few experiments have been performed to examine IVF-ET. Goodrowe *et al.* [10] found a high pregnancy rate (5/6, 83.3%) following transfer of IVF-derived embryos cultured in mKRB containing BSA to the oviducts of oocyte donors. However, these embryos were at the 2- to 4-cell stage at 42–52 hr post-insemination. Unfortunately, pregnancy rates following intrauterine transfer of embryos beyond the morula stage were exceptionally low in two previous studies [26, 32]. Swanson and Godke [31] attemped nonsurgical transfer of IVF-derived morulae via uterine cervices. That was a unique method but resulted a low conception (1/7, 14.3%). Pope *et al.* [26] reported that more pregnancies occurred following intrauterine transfer of ≥12 embryos (42.3%) per cat than if <12 embryos (26.1%) were transferred. According to Tsutsui *et al.* [35], the average number of CL was 5.6 ± 1.9 (mean ± SD; range, 2–11) and the average number of fetuses was 4.5 ± 1.4 (range, 1–8) in a total of 169 pregnant cats presented for ovariohysterectomy. In litter-bearing animals including the cat, sufficient spacing is necessary for physiological development and implantation of embryos in the uterine cavity [5, 35]. It is not recommended to transfer too many embryos as this may cause early embryonic death or fetal losses [17]. Following transfer of only six blastocysts per cat into the uterus, approximately the physiological number of ovulations in spontaneous estrous cats, a higher rate (80.0%) of conception was achieved in our study than in previous studies [26, 32]. This is the first report of successful feline IVF-ET with only blastocyst-stage embryos overcoming the developmental block. Additionally, embryos cultured in MK-1 containing BSA, staying at the late morula stage, had an equal number of nuclei to embryos in MK-1 containing HS and equivalent developmental competence. These finding suggested that our culture system maintained embryo quality over prolonged culture periods and increased the success rate of IVF-ET.

In conclusion, an improved culture media, designated MK-1, was useful as a basal medium for supporting feline IVF and IVC. Furthermore, human serum was highly beneficial for overcoming the *in vitro* developmental block and enhancing blastocyst formation. The culture system described in this paper is a simple and practical way to obtain kittens following IVF-ET and may be applicable to sustain the development of embryos obtained from feline animal models of human genetic diseases and endangered nondomestic felid species. The establishment of such a simple medium as MK-1 will facilitate investigations into the actions of particular compounds, *e.g.* energy substrates, growth factors, different molecular weight fractions of human serum, *etc.* This approach will facilitate investigations into the regulatory mechanisms and the nutritional requirements of feline early embryonic development.

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