Early Embryonic Development *in vitro* and Embryo Transfer in the Cat

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ABSTRACT. Ten female cats were given a total dose of 200 IU PMSG over 3 days to induce superovulation. One to four-cell stage embryos were collected by flushing the oviducts 48 to 54 hr after the initial 250 IU dose of hCG. Some of the normal embryos collected were examined for culture in Medium-199 supplemented with 20% FCS. After 72 hr of culture, 222/248 (89.5%) had developed to the morula stage, and by 96–168 hr, 110 (64.7%) out of 170 morulae had developed into blastocysts. Four to 12 embryos cultured *in vitro* per cat were transferred to one of the uterine horns of 12 recipients in which synchronous ovulation had been induced with hCG. All 4 recipients of embryos which had developed to the morula stage on culture day 3, 3 of the 5 recipients of blastocysts on culture days 4–6, and none of the 3 recipients of blastocysts on culture day 7 became pregnant. It is concluded that early feline embryos are capable of efficiently developing into transferable morulae *in vitro* by ordinary culture methods, but that there is partial developmental arrest from the morula to the blastocyst stage.—KEY WORDS: embryo development, embryo transfer, feline, *in vitro* culture, pregnancy.

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The "cleavage block" that occurs in many species *in vitro* when ordinary culture methods are used has been a major obstacle to the *in vitro* study of early embryonic development. Such developmental blocks have been observed in the mouse [11], pig [5], cow [9], and sheep [10] with arrest at the 2-cell, 4-cell, and 8- to 16-cell stages, respectively.

Some papers have been published on the embryonic development of the cat either *in vivo* [6, 7, 23] or *in vitro* [2, 12, 13, 15, 16, 21]. Recently successful embryo transfer in the cat has been reported with cryopreserved embryos [8], *in vitro* fertilized embryos [12, 21], and during the non-breeding season [24]. However, the transfer of cat embryos cultured and developed *in vitro* had resulted in a low conception rate [21].

We think that developmental biotechnology including *in vitro* fertilization, embryo culture, embryo cryopreservation and embryo transfer are not only useful for managing our domestic cat colony for biomedical research but would also allow the alteration of the phenotype in the domestic cat through genetic engineering and could be used as a model for the study of genetic diseases. As the initial phase of this project, the present study assessed (1) the induction of superovulation by PMSG-hCG and the collection of ova in the cat, (2) early embryonic development *in vitro*, and (3) the possibility of embryo transfer with morulae and blastocysts which had developed *in vitro*.

MATERIALS AND METHODS

Animals: Twenty-two sexually mature female cats were used in the present experiment. Ten were used for embryo recovery and 12 were selected as embryo recipients. Five male cats were used for mating. The cats were of mixed breed and caged individually under nearly constant environmental conditions (room temperature 22±2°C, relative humidity 50±20%, fluorescent light cycle 12 hr). The cats were fed 70 g dry cat food (Hill's Pet Products, Inc., U.S.A.) per day, and water was provided *ad libitum*.

Induction of superovulation: Follicular development in the 10 queens used for embryo recovery was hormonally stimulated by intramuscular injection of pregnant mare serum gonadotropin (PMSG, Nippon Zenyaku Industrial Co., Ltd.), with a total dose of 200 IU divided into 100 IU on the first day (day 0) and 50 IU each on days 1 and 2. Ovulation was induced by 2 intravenous injections of 250 IU human chorionic gonadotropin (hCG, Sankyo Co., Ltd.) 16 hr apart on day 7 (16:00) and day 8 (8:00) in accordance with the procedure described in another report [25]. After administration of the initial dose of hCG, each queen was placed in a pen with a stud male overnight for mating. Successful mating was judged on the basis of the presence of sperm in vaginal smears.

Collection of ova: All ova were collected from the superovulated queens surgically 48 to 54 hr after the initial dose of hCG. Ovariohysterectomy was performed under general anesthesia induced by an intramuscular injection of propiopromazine (0.5 mg/kg) followed 5–10 min later by a similar injection of ketamine hydrochloride (20 mg/kg). Taking the location of embryos in the oviduct into consideration [6, 14], they were collected by flushing the oviducts twice with 2 m*l* of Dulbecco's phosphate-buffered saline (D-PBS) supplemented with glucose (1.0 mg/m*l*), sodium pyruvate (0.036 mg/m*l*), penicillin-G sodium (100 IU/m*l*), streptomycin sulfate (0.1 mg/m*l*) and bovine serum albumin (BSA; Fraction-V, 3.0 mg/m*l*). After collection, the ova were thoroughly washed with two changes of D-PBS, examined morphologically by stereo- and phase-contrast microscopy and classified according to the developmental stage.

Embryo culture and assessment: To examine the *in vitro* developmental capacity of these embryos, they were cultured in Medium-199 containing Earle's salts (Flow

Laboratories, Inc., Australia) supplemented with 20% heat-treated fetal calf serum (FCS), penicillin-G sodium (100 IU/m*l*) and streptomycin sulfate (0.1 mg/m*l*). Each embryo was cultured in approximately 50 μl droplets of the medium, overlaid with paraffin oil (Squibb & Sons, Inc., U.S.A.) and gassed with a mixture of 5% O_2, 5% CO_2, and 90% N_2 in an anaerobic jar (BBL®, Becton Dickinson, U.S.A.). The jar was placed in an incubator (37°C). The embryos were monitored for quality and stage of development by phase-contrast microscopy, and transferred to fresh medium at intervals of 24 hr. Embryonic development was evaluated with respect to the stage of development at initiation of the culture. The proportion of embryos that developed to the morula stage after 72 hr of culture was expressed as a percentage. Embryos with over 16 cells were considered as morulae. In addition, to examine the ability to develop to the blastocyst stage, some of these morulae were further cultured for 96 hr. Data were analyzed by chi-square analysis to determine differences between groups.

Recipient synchronization: Seven estrous induced (EI) queens treated with the same hormone regimens as embryo donors and five spontaneous estrous (SE) queens were used as embryo recipients. To induce ovulation synchronized with the donor, the recipients were treated with 250 IU of hCG twice, at the same time as the donors. The time of the initial dose of hCG was counted as day 0 of pregnancy.

Embryo transfer: Embryo transfer was attempted to investigate the developmental competence of morulae and blastocysts obtained through *in vitro* culture. Four to 12 embryos per cat were transferred to the uterine horns of 12 recipients. Each recipient received the embryos derived from one donor. Embryo transfer was performed by laparotomy through a mid-line incision under general anesthesia with the same regimen as in the donors. The ovaries were examined for corpora hemorrhagica (CH) and unovulated follicles. A 24- gauge Surfuro® intravenous catheter (Terumo, Inc.) and a transfer pipet made by drawing a hard glass capillary out to about 400 μm in diameter were used as the embryo transfer instruments (Fig. 3). The i.v. catheter was inserted into the lumen of the cranial end of the uterine horn on the side of the ovary containing the larger number of CH. Embryos were aspirated into the tip of the transfer pipet with a minimal volume of culture medium (approximately 5 μl). The transfer pipet was then inserted into the catheter in place of the inner needle, and the embryo-bearing medium was slowly injected into the lumen. Pregnancy was determined by laparotomy 12 to 17 days after embryo transfer.

RESULTS

Ovum recovery: A total of 364 ova were collected from the 10 embryo donors (range, 26-65/cat). Classification of these ova yielded 75 (20.6%) 1-cell ova including both unfertilized ova and zygotes (Fig. 1-a), 141 (38.7%) 2-cell embryos, 107 (29.4%) 4-cell embryos (Fig. 1-b), and 41 (11.3%) were categorized as degenerated ova. The average number of CH was 39.1±4.2 (mean ± SE; range 26-69), so that the overall ovum recovery rate was 93.1% (range 77.1–100%). The number of unovulated follicles (follicular size ≥2 mm) averaged 1.8±0.5 (range 0–5).

In vitro embryo development: By 72 hr of culture, 222/248 (89.5%) developed to the morula stage (Table 1). The rates of development of the morula classified according to recovery stages were 81/83 (97.6%) of 4-cell embryos, 103/115 (89.6%) of 2-cell embryos, and 38/50 (76.0%) of 1-cell ova. There were significant differences in the proportion of morulae among the three groups ($P<0.05$). However, the rate of development to morulae based on the number of cleaved embryos developing from 1-cell ova was 90.5% (38/42) and was not significantly

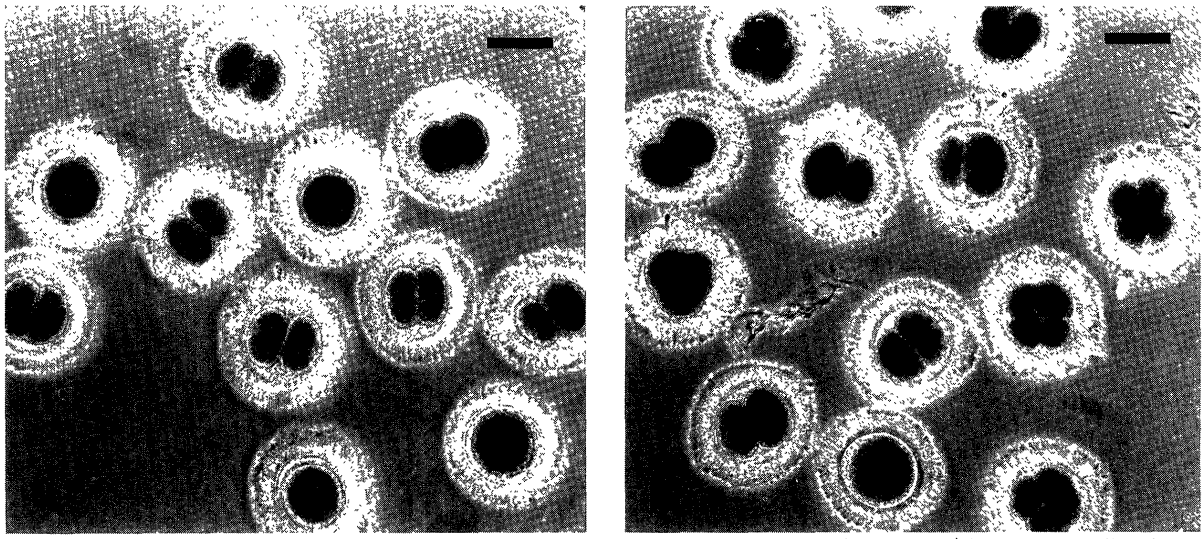

Fig. 1. Feline embryos, recovered from donor's oviducts observed by phase-contrast microscopy. a) Mono- to 2-cell embryos recovered at 48 hr post-hCG. b) 2- to 4-cell embryos recovered at 52 hr post-hCG. Bar=100 μm.

Table 1. Development of feline embryos derived from *in vivo* fertilization and cultured in medium-199 with 20% FCS for 72 hr

Stage[a] of embryos	No. of embryos cultured	No. and (%) of embryos that developed to					
		1-cell	2-cell	4-cell	8-cell	16-cell	Morula
1-cell	50	8[b]	2	1	0	1	38 (76.0)[c]
2-cell	115		2	2	6	2	103 (89.6)[d]
4-cell	83			0	0	2	81 (97.6)[e]
Total	248	8	4	3	6	5	222 (89.5)

a) Developmental stage of embryos at beginning of culture.
b) Underlined numbers mean embryos have not developed beyond the recovery stage.
Significant difference between c) and e) at $P<0.01$.
Significant differences between c) and d), d) and e) at $P<0.05$.

Table 2. Development of feline morulae continuously cultured in medium-199 with 20% FCS

Stage[a] of embryos	No. of morulae cultured	No. and (%)[b] of morulae that developed to blastocysts			
		96 hr[c]	120 hr	144 hr	168 hr
1-cell	35	1 (2.9)	8 (22.9)	15 (42.9)	18 (51.0)
2-cell	74	11 (14.9)	24 (32.4)	42 (56.8)	51 (68.9)
4-cell	61	7 (11.5)	20 (32.8)	31 (50.8)	41 (66.1)
Total	170	19 (11.2)	52 (30.6)	88 (51.8)	110 (64.7)

a) Developmental stage of embryos at beginning of culture.
b) Percentage of the number of morulae cultured.
c) Hours in culture.

Table 3. Results of embryo transfer

Cat No.	Transferred embryos			No. of implantations		Rate of implantation (%)	No. of kittens born (gestation period)
	Period of culture (days)	Develop. stage	No. of embryos	Left	Right		
R-1[a]	3	M[b]	7	1	[0][c]	14.3	abortion
R-2[d]	3	M	9	2	[5]	77.8	4 (65)
R-3[a]	3	M	6	2	[2]	66.7	abortion
R-4[d]	3	M	12	[3]	4	58.3	6 (68)
R-5[a]	4	B[e]	10	2	[3]	50.0	5 (69)
R-6[d]	5	B	4	—[f]		0	—
R-7[d]	5	B	5	—		0	—
R-8[a]	5	B	5	[1]	1	40.0	1 (69)
R-9[d]	6	B	5	1	[2]	60.0	3 (68)
R-10[a]	7	B	9	—		0	—
R-11[a]	7	B	10	—		0	—
R-12[a]	7	B	6	—		0	—

a) Estrous induced recipient. b) Morula. c) □: Transfer side of uterine horn.
d) Spontaneous estrous recipient. e) Blastocyst. f) Sterility.

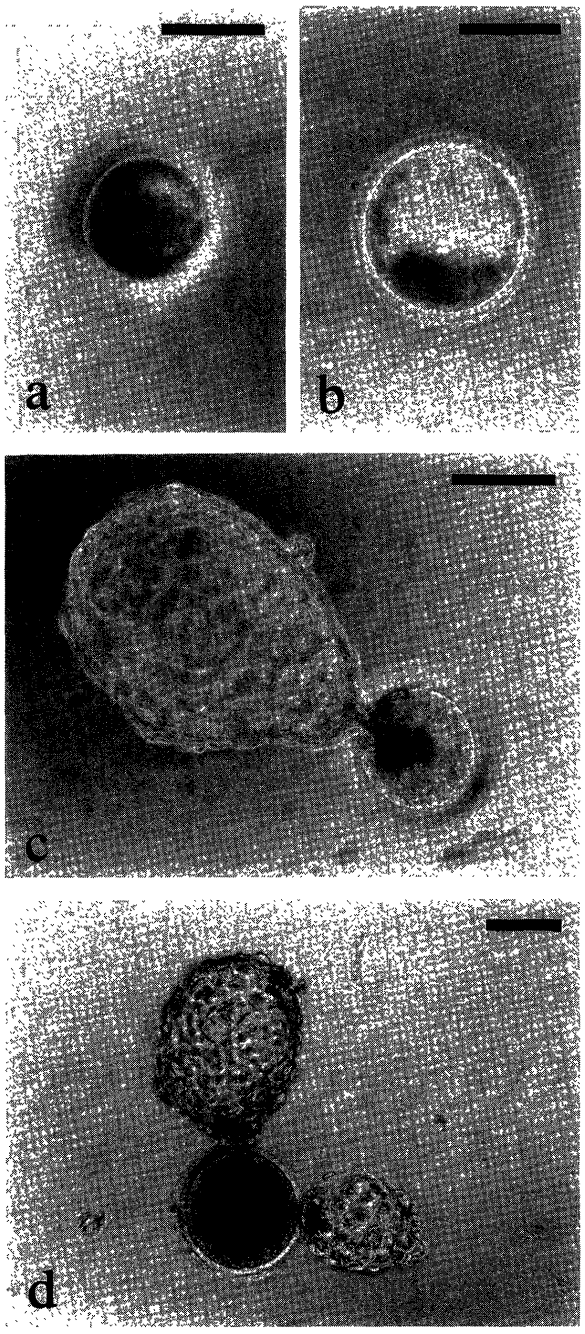

Fig. 2. Feline embryos, cultured *in vitro* observed by phase-contrast microscopy. Bars=100 μm. a) Early blastocyst-stage embryo at 96 hr of culture. b) Blastocyst-stage embryo at 120 hr of culture. c) Hatching blastocyst-stage embryo at 144 hr of culture. d) Hatching of blastocyst through two sites of the zona pellucida.

different ($P>0.05$) from those of the other groups. Initial blastocyst formation was observed after 96 hr of culture (Table 2, Fig. 2-a), and by 168 hr of culture, 110 (64.7%) out of 170 morulae had developed to the blastocyst stage (Fig. 2-b). There were no significant differences ($P>0.05$) in the rates of blastocyst development among the three groups. Blastocyst hatching was observed starting at 144 hr of culture (Fig. 2-c), and there were one or multiple hatching sites of trophectoderm cells (Fig. 2-d). Most of the hatching blastocysts stopped their development and attached to the culture dish during the hatching process from the zona pellucida, so that few embryos hatched through the zona pellucida completely.

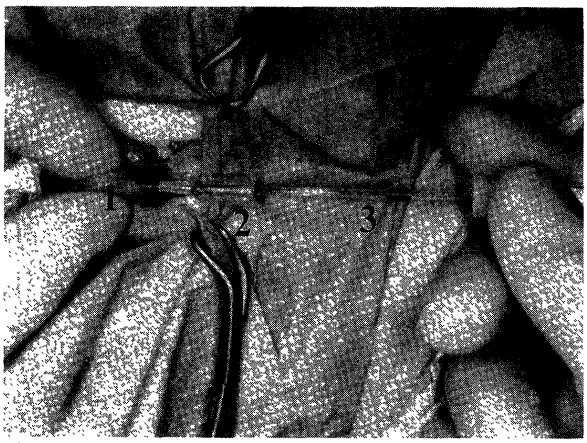

Fig. 3. Operation for embryo transfer. 1; cranial end of the uterine horn, 2; intravenous catheter, 3; transfer pipet.

Fig. 4. Pregnant uterus (21 days post-hCG) of the embryo recipient; R-2. The recipient, which had received 9 morulae into the right uterine horn, was found to have 7 implantations. Nos. 1 and 2 were on the left, 3 to 7 were on the right uterine horn.

Embryo transfer and determination of pregnancy: Table 3 shows the results of embryo transfer. All 4 recipients (SE:2, EI:2) which received morulae at day 3 of culture became pregnant. The ratios of implantations to transplanted embryos were 1/7, 7/9, 4/6, and 7/12, respectively. Three of five recipients (SE:3, EI:2) which received blastocysts at days 4 to 6 of culture were found to have become pregnant. No conceptions were detected in any of the 3 recipients (EI:3) transplanted with the blastocysts (culture day 7). Transuterine migration of the transplanted embryos was observed in all 7 of the pregnancies.

Pregnancy outcome: A total of 19 live offspring were delivered at full term from 5 of the 7 pregnancies (Table 3). The remaining 2 pregnancies (R-1, R-3) were aborted

on days 42 and 54 of pregnancy, respectively. The ratio of kittens to the number of implantations was 19/29 (65.5%), and fetal losses occurred in 5 of the 7 pregnancies.

DISCUSSION

In this study, the developmental stages of the ova recovered 48 to 54 hr after hCG administration, were 1- to 4-cell. Of the 50 one-cell ova, 42 cleaved to the ≥2-cell stage within 24 hr of culture. These ova should therefore be considered as normal zygotes, but the remainder that failed to cleave could not be judged as zygotes, unfertilized ova or others without staining. It was impossible to visualize intracellular structures in feline ova by conventional light microscopy. Yamada *et al.* [26] recovered 4-cell embryos 48 hr after hCG administration. According to several reports [19, 22, 26], ovulation is induced between 26 and 30 hr post-hCG. It has been reported that the first cleavage of *in vitro* fertilized feline ova is observed between 20 and 28 hr post-insemination [2, 12]. It is therefore assumed that ovulation might have occurred much earlier than 26 hr post-hCG in some of the embryo donors.

The proportion of feline embryos cultured *in vitro* which reached the morula stage was 89.5%, a satisfactory success rate, but there was an occurrence of some developmental arrest between the morula and the blastocyst stages. With the exception of human, rhesus monkey and some strains of mice, preimplantation embryos of most mammalian species are blocked *in vitro* at earlier stages than in the cat [5, 9, 10, 11]. The present study shows that the "semi-morula block" during the *in vitro* culture of feline embryos is not as severe as the "2-cell block" in the mouse [11] and "8- to 16-cell block" in the cow [9]. There is a similar or more severe morula-to-blastocyst developmental block during the culture of feline embryos fertilized *in vitro* [15]. In general, *in vitro* developmental blocks appear related to the activation of the embryonic genome [11], a transitional period when embryo metabolic requirements are demanding [4]. In some species, developmental blocks have been overcome by modifying the media [3] or establishing co-culture systems with uterine or oviductal epithelial cells [9, 10]. In the cat, Johnston *et al.* [15, 16] examined the influence of culture media (modified Krebs Ringer bicarbonate, modified Tyrode's solution, and Ham's F-10), protein supplements (polyvinylalcohol, BSA, FCS, and estrous cat serum), culture temperature (37, 38, and 39°C), and gas atmosphere (5% CO_2 in air; 10% CO_2 in air; and 5% CO_2, 5% O_2, 90% N_2) on *in vitro* fertilization and embryo development. Despite changing these culture conditions, over 90% of the 2-cell embryos developed to morulae *in vitro* (in Ham's F-10 with 5% FCS), and the rate was equal with ours (in M-199 with 20% FCS). However, the morula-to-blastocyst developmental block occurred. Similarly, Pope *et al.* [21] demonstrated that the average number of cells per embryo was not affected markedly either by the culture media (Ham's F-10 and M-199) or by the presence of intact cumulus cells, and that the rate of blastocyst development during culture periods of 168 hr after *in vitro* fertilization was only 18% (in Ham' F-10 with 10% FCS). The proportion was much lower than ours with *in vivo* derived fertilized embryos. In our study, a higher proportion of 2- & 4-cell embryos developed to blastocysts than 1-cell ova. Thus, 2- & 4-cell embryos, 1-cell ova fertilized *in vivo* and zygotes fertilized *in vitro* are arranged in the order of proportional development to the blastocyst stage in culture. This suggests that the embryonic growth phases starting from culture *in vitro* have a great impact on enhancing the development of cat embryos beyond the morula stage. Recently, Chatot *et al.* [3] demonstrated that the presence of glucose (in the presence of phosphate) during the first 48 hr of culture actually inhibits the development of mouse embryos advancing to the blastocyst stage. The deleterious effects of glucose or phosphate on rat [18], porcine [20], and bovine [17] embryo development has also been reported. On the other hand, AbuBaker *et al.* [1] reported that the addition of sodium pyruvate at a decreasing concentration and glucose at an increasing concentration enhances the early embryonic development of rabbit embryos. Also for cat embryos, the presence of glucose may affect embryonic development. Further detailed studies are planned involving the addition and removal of glucose during various early embryonic growth phases.

In the cat, the entry of embryos into the uterus is not clearly established but it is thought to occur on the fourth or fifth day after ovulation [14] or the sixth day after coitus [6]. In our unpublished data, feline embryos enter the uterus on the fifth to sixth day post-hCG, and the developmental stages observed at this time are compacted morulae or early blastocysts. In this study, the morulae exhibited cell compaction less clearly *in vitro* than *in vivo*. Thus it appears that indistinct compaction of morulae cultured *in vitro* plays an important role in this developmental arrest. Blactocysts hatching from the zona pellucida *in vivo* were reported to occur later than the 12th day post coitus [7, 23], but under the present culture conditions, the hatching of *in vitro* cultured embryos was observed after 144 hr of culture without full expansion. Moreover there were one or multiple hatching sites, and few embryos hatched through the zona pellucida completely. It is therefore also necessary to investigate *in vivo* development and hatching of transplanted embryos.

Our embryo transfer instruments designed for the present study were of practical use, as there was no significant bleeding from the site of uterine puncture. All 4 recipients transplanted with morulae and 3 of the 5 transplanted with blastocysts after 4 to 6 days of culture became pregnant, but none of the 3 recipients which had received blastocysts at day 7 of culture became pregnant. The causes of this failure appear to be (1) the time difference (≥3 days) between the recipients and the age of the transferred embryos, (2) that the blastocysts whose development had been delayed up to 3 days had already begun to degenerate, and (3) that the transplanted

blastocysts had not normally hatched from the zona pellucida. Besides our study, successful embryo transfer in the cat has also been reported by two groups using *in vitro* fertilized embryos [12, 21]. Of these studies, one was designed for embryo transfers into the oviducts using 2- to 4-cell embryos, and the other into the uterus using ≥96 hr cultured embryos. According to the report by Pope *et al.* [21], the percentage of recipients producing kittens after transfer of embryos cultured for 96 or 120 hr in Ham's F-10 was only 31 and 25, respectively. Developmental stages of these transplanted embryos were considered to be morulae or early blastocysts. A higher rate of conception (100%) with morulae was achieved in our study than theirs, though half of the pregnancies resulted in abortion.

In conclusion, early feline embryos recovered from the oviducts are capable of efficiently developing into transferable morulae *in vitro* by ordinary culture methods, but some developmental arrest occurred between the morula and the blastocyst stages. Further studies concerning nutritional and energy requirements are needed to ensure that these embryos develop into blastocysts *in vitro*, and it is also necessary to investigate the early embryonic development *in vivo* and the time course in detail as background data.

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