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Theriogenology

Feline embryo development in commercially available human media supplemented with fetal bovine serum

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ABSTRACT. Feline embryo development was examined for 7 days after fertilization using commercially available human media supplemented with 0.3% bovine serum albumin (BSA) or 5% fetal bovine serum (FBS). Cumulus-oocyte complexes were categorized as Grades 1, 2, and 3 according to morphology. Only-One Medium (OM) was used for *in vitro* culture (IVC) in OM + BSA, OM + FBS, and OM + BSA/FBS, with BSA supplementation for the first 2 days and FBS for the subsequent 5 days. Embryos cultured in Early Culture Medium (1–2 days) and Blastocyst Medium (3–7 days) were defined as EB + BSA and EB + BSA/FBS. The developmental rate until the blastocyst stage of Grade 1 and 2 oocytes cultured in OM + BSA/FBS was higher than for the other groups and was significantly higher than for the OM + BSA and EB + BSA groups ($P<0.01$). Grade 3 oocytes cultured in OM + BSA/FBS also showed the greatest proportion of blastocyst formation. However, FBS supplementation throughout the IVC period reduced blastocyst number. The percentage of 2 pronuclei after fertilization as well as blastocyst cell number were significantly higher in Grade 1 and 2 than Grade 3 oocytes when cultured in OM + BSA/FBS ($P<0.05$). These results indicate that commercially available OM supplemented with BSA for the first 2 days of culture and FBS for the subsequent 5 days is suitable for feline embryo development until the blastocyst stage.

KEY WORDS: feline, fetal bovine serum, human medium, *in vitro* culture

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In vitro reproductive technologies in the domestic cat (*Felis catus*) would be useful not only for this species but also for the conservation of endangered feline species [20]. As generation of feline blastocysts using *in vitro* culture (IVC) is very difficult because of the "morula block" [22], several studies have been undertaken to improve the IVC process [16, 21]. Significantly better results were obtained when a two-step culture was used and the culture medium was changed on day 2 or 3 than when a one-step culture was used [5, 21]. Specifically, the addition of fetal bovine serum (FBS) instead of bovine serum albumin (BSA) in the late period of culture was a key factor for the improved results.

In many species, ion concentrations and amino acid composition in the culture medium are important for embryo development [1, 9]. Many types of synthetic media have been developed especially for human embryos and are commercially available [11, 24]. Predefined, commercially available media are preferable as they are made under adequate quality control conditions and standardize embryo cultures among different laboratories. If these media could be used for feline embryo culture, they could contribute to the conservation of endangered feline species by *in vitro* embryo production. However, Herrick *et al*. [5] suggested that feline embryos maintain ionic homeostasis differently from rodent embryos. Therefore, the availability of commercially available media for IVC of feline embryos should be investigated. Recently, commercially available serum-free media have been developed for human embryo IVC, with two opposing views emerging on the ideal composition. These are the "single-step media", which contain all components embryos need for development; and the "sequential media", having a different composition that aims to fulfill the needs of the embryo during early or late development [24].

In the present study, we examined the period of FBS supplementation using alternative human IVC media of different compositions to identify the appropriate conditions for IVC of fertilized feline oocytes until blastocyst formation.

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Table 1. Media used for various phases of *in vitro* culture of cat embryos

Experiment	Group	Early culture (2 days)	Late culture (5 days)
I	OM + BSA	Only-One Med. + 0.3% BSA	Only-One Med. + 0.3% BSA
	OM + BSA/FBS	Only-One Med. + 0.3% BSA	Only-One Med. + 5% FBS
	EB + BSA	Early Culture Med. + 0.3% BSA	Blastocyst Med. + 0.3% BSA
	EB + BSA/FBS	Early Culture Med. + 0.3% BSA	Blastocyst Med. + 5% FBS
II	OM + BSA/FBS	Only-One Med. + 0.3% BSA	Only-One Med. + 5% FBS
	OM + FBS	Only-One Med. + 5% FBS	Only-One Med. + 5% FBS
III	OM + BSA/FBS	Only-One Med. + 0.3% BSA	Only-One Med. + 5% FBS

During the late culture period, half of the medium was changed every 2 days.

Table 2. Cleavage and developmental rates to morula + blastocyst and blastocyst stages of feline IVF oocytes after culture in human media with or without FBS supplementation (experiment I)

Oocyte grade	Group	No. of oocytes examined (replicates)	Cleavage (%)	Morula + Blastocyst (%) Based on oocytes	Blastocyst (%) Based on oocytes
1 and 2	OM + BSA	191 (14)	72.5 ± 15.9	44.6 ± 20.3	7.5 ± 7.6[a)]
	OM + BSA/FBS	317 (21)	74.2 ± 12.5	45.7 ± 20.1	20.9 ± 18.3[b)]
	EB + BSA	185 (15)	70.1 ± 12.3	41.1 ± 18.2	7.7 ± 7.9[a)]
	EB + BSA/FBS	240 (14)	69.8 ± 17.0	42.9 ± 22.9	13.1 ± 9.4[a,b)]
3	OM + BSA	148 (14)	43.7 ± 15.1	23.5 ± 17.4	4.7 ± 9.2[a)]
	OM + BSA/FBS	338 (21)	47.5 ± 15.9	31.6 ± 14.6	14.9 ± 12.2[b)]
	EB + BSA	165 (14)	44.9 ± 20.1	26.2 ± 26.9	7.6 ± 8.9[a,b)]
	EB + BSA/FBS	238 (15)	43.1 ± 23.2	21.9 ± 18.5	7.9 ± 10.7[a,b)]

Values represent the means ± SD. a, b) Different superscripts indicate statistical differences within a column (P<0.01).

Table 3. Cleavage and developmental rates to morula + blastocyst and blastocyst stages of feline IVF oocytes after culture in human media with or without FBS supplementation (experiment II)

Oocyte grade	Group	No. of oocytes examined (replicates)	Cleavage (%)	Morula + Blastocyst (%) Based on oocytes	Blastocyst (%) Based on oocytes
1 and 2	OM + BSA/FBS	289 (18)	71.9 ± 13.9	41.8 ± 19.7	20.4 ± 12.3[a)]
	OM + FBS	171 (11)	67.1 ± 18.5	31.9 ± 16.7	6.1 ± 5.3[b)]
3	OM + BSA/FBS	327 (18)	42.5 ± 11.6	17.4 ± 11.1	9.0 ± 9.3[a)]
	OM + FBS	190 (11)	39.6 ± 12.0	12.7 ± 10.9	1.7 ± 4.1[b)]

Values represent the means ± SD. a, b) Different superscripts indicate statistical differences within a column (P<0.01).

MATERIALS AND METHODS

Cat ovaries and testes with epididymis were collected from a local veterinary clinic following routine ovariohysterectomy and castration. The cats were anesthetized using a combination of xylazine and ketamine before the surgical procedures. The animals were neither operated on nor killed for this study. The use of tissues was approved by the committee of the local veterinary clinic. All the cats were privately owned and the owners' consent was obtained before sample collection.

Collection of oocytes and in vitro maturation

Cat ovaries were collected throughout the year from 117 pubertal queens at a veterinary clinic; the estrous stage of the queen was not considered. The samples were randomly selected several times in each experiment, and the replicate numbers are shown in Tables 2–5. The ovaries were immediately transported to the laboratory in physiological saline (0.85% [w/v] NaCl) at 37°C. The ovaries were cut into small pieces with scissors and stored in TCM 199 (Earle's salts) buffered with 25 mM Hepes buffer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Cumulus-oocyte complexes (COCs) were separated from the ovarian follicle with a 30-gauge needle. The collected COCs were washed twice in *in vitro* maturation (IVM) medium, consisting of TCM 199 supplemented with 0.4% (w/v) BSA (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.), 0.2 mg/m*l* 17β-estradiol (Sigma-Aldrich Corp.), 100 μg/m*l* gentamycin (Sigma-Aldrich Corp.), 137 μg/m*l* sodium pyruvate, 0.02 IU/m*l* Follistim® (human recombinant FSH; MSD, Tokyo, Japan), and 25 *n*g/m*l* human recombinant EGF (Sigma-Aldrich Corp.).

The oocytes were classified into Grades 1, 2, and 3 according to the method of Wood and Wildt [28]. Grade 1 oocytes had homogenous dark cytoplasm surrounded by several layers of compacted cumulus. Grade 2 oocytes also had homogenous dark cytoplasm, but fewer than five layers of supporting cumulus oophorus cells. Grade 3 oocytes lacked uniformity and had an almost

Table 4. Status of feline oocyte nuclei after 18 hr of co-culture with sperm (experiment III)

Oocyte grade	No. of oocytes examined (replicates)	Total penetration (%)	2PN (%)	Polyspermy (%)	Others (%)	Parthenogenesis (%)	Degenerated (%)
1 and 2	111 (5)	69.3 ± 9.9	38.4 ± 6.9[a)]	15.6 ± 5.7	15.3 ± 15.9	1.6 ± 3.6	10.1 ± 6.2
3	63 (5)	65.1 ± 15.8	22.3 ± 13.7[b)]	18.4 ± 10.6	24.3 ± 9.9	1.3 ± 3.0	5.3 ± 8.7

Values represent the means ± SD. 2PN, two pronuclei. a, b) Different superscripts indicate statistical differences within a column ($P<0.05$).

Table 5. Cleavage and developmental rates to morula + blastocyst and blastocyst stages of feline IVF oocytes after culture in OM + BSA/FBS (experiment III)

Oocyte grade	No. of oocytes examined (replicates)	Cleavage (%)	Morula + Blastocyst (%) Based on oocytes	Blastocyst (%) Based on oocytes	Blastocyst cell number (No. of blastcysts)
1 and 2	147 (11)	80.5 ± 14.2[a)]	54.4 ± 23.9[a)]	22.0 ± 17.7	235.4 ± 87.7[c)] (29)
3	139 (9)	47.1 ± 15.4[b)]	23.4 ± 14.0[b)]	9.3 ± 9.1	108.7 ± 60.1[d)] (15)

Values represent the means ± SD. a–d) Different superscripts indicate statistical differences within a column (a–b) $P<0.01$, c–d) $P<0.05$).

full complement of the corona radiata and 1–2 layers of cumulus oophorus cells that were less tightly compacted than in the higher grades. In addition, the oocyte diameter of Grade 1, 2, and 3 oocytes was also recorded according to the procedures described by Otoi *et al*. [18]. Oocytes with a diameter greater than 100 μm (excluding the zona pellucida) were used for IVM. Approximately 10 COCs were placed in 100 μl IVM medium, covered with mineral oil, and incubated for 28 hr at 38.5°C in a humidified atmosphere with 5% CO_2.

In vitro fertilization

In vitro fertilization (IVF) was performed using cryopreserved epididymal cat sperm. Testes with epididymis were collected from adult male cats following castration at a local veterinary clinic. The tissues were immediately transported to the laboratory in physiological saline (0.85% [w/v] NaCl) at 37°C. Approximately 2 hr after excision, the epididymis was separated from the testes and sliced repeatedly with a scalpel. The spermatozoa were released into a 35-mm Petri dish containing 1 m*l* of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Nacalai Tesque, Kyoto, Japan) and incubated at 37°C for 10 min. Spermatozoa cryopreservation was performed according to the method described by Karja *et al*. [7]. Frozen sperm were thawed at 37°C and washed twice in mHTF medium (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) by centrifuging at 500 × *g* for 5 min. The supernatant was removed and the sperm pellet was immediately subjected to motility analysis. Only samples with over 50% sperm motility were used for IVF.

After IVM culture, 8–10 COCs were co-incubated with frozen-thawed sperm (1×10^6 sperm/m*l*) in 100-μl drops of HTF medium (Nippon Medical & Chemical Instruments Co., Ltd.) supplemented with 0.3% BSA (Sigma-Aldrich Corp.), covered with mineral oil, and incubated for 12 hr at 38.5°C in a humidified atmosphere with 5% CO_2.

In vitro culture

After IVF, a narrow-bore glass pipette was used to remove the cumulus cells from the oocytes. The presumptive zygotes were cultured in 100-μl drops of IVC medium in lots of 8–10 per drop, covered with mineral oil, and incubated at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Post-IVF *in vitro* embryonic development was assessed by microscopic observation at 24-hr intervals for 7 days. Embryo development was assessed by morphologic characteristics, and morula stage embryos were ascertained by the presence of at least 16 cells [17, 27]. The percentages of cleavage, morula + blastocyst, or blastocyst only stage embryos were calculated on day 7 after the IVF culture.

Five types of IVCs were set as described in Table 1. The composition of the media used in this study is provided in Supplementary Table S1. In the OM + BSA group, for both the early (for 2 days) and late (for 5 days) cultures, the oocytes were incubated using Only-One Medium (Nippon Medical & Chemical Instruments Co., Ltd.) supplemented with BSA (0.3%). During the OM + BSA culture, half of the medium was replaced with identical fresh medium on days 2, 4, and 6 of IVC. In the OM + BSA/FBS group, the oocytes were incubated in the Only-One Medium with BSA during the early culture, and subsequently in the Only-One Medium with FBS (5%) during the late culture. For the EB + BSA culture, the oocytes were incubated using Early Culture Medium (Nippon Medical & Chemical Instruments) with BSA in the early culture, followed by Blastocyst Medium (Nippon Medical & Chemical Instruments) with BSA in the late culture. Similarly, in the EB + BSA/FBS group, Early Culture Medium with BSA and Blastocyst Medium with FBS were used in the early and late cultures, respectively. In the late culture, half of the medium was renewed on days 4 and 6.

We then performed the following three experiments using presumptive zygotes. In experiment I, presumptive zygotes were cultured in various single and sequential IVC media (Table 1). In experiment II, zygotes were selected for culture in OM + BSA/FBS and OM + FBS (Table 1) to investigate the effect of FBS in the early or whole period of IVC. Finally, we performed experiment III for assessment of parthenogenesis, fertilization rate, and blastocyst cell number in OM + BSA/FBS culture using Grade 1, 2, and 3 oocytes.

Assessment of oocyte parthenogenesis

In experiment III, we performed the IVM, IVF, and IVC in OM + BSA/FBS as described above in experiments I and II, except that sperm was used in the IVF step. After 7 days of IVC, we examined oocyte parthenogenetic development.

Assessment of fertilization rate

After 18 hr of IVF, oocytes were fixed and stained for assessment of the fertilization status. Briefly, the oocytes without cumulous cells were washed twice in Dulbecco's phosphate-buffered saline (DPBS) and then permeabilized and fixed in DPBS supplemented with 3.7% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) and 1% (v/v) Triton X-100 (Merck) at room temperature for 15 min. The cells were then placed in DPBS containing 0.3% (w/v) polyvinylpyrrolidone (Sigma-Aldrich Corp.) for 15 min at room temperature. The fixed oocytes were transferred in small drops consisting of DPBS supplemented with 90% (v/v) glycerol (Sigma-Aldrich Corp.) and 10 μg/m*l* bisbenzimide (Hoechst 33342; Sigma-Aldrich Corp.) for 5 min, mounted on a slide, overlaid with a coverslip, and incubated at 4°C. The oocytes were evaluated by fluorescence microscopy and classified into different groups according to Nagano *et al*. [13], with some modifications, such as: non-penetrate, when sperm could not penetrate the oocyte; 2PN, the presence of 2 pronuclei (male and female) was considered as normal fertilization; polyspermy, when three or four pronuclei were present; others, an enlarged sperm head with an anaphase II/telophase II chromosome or a male pronucleus and telophase chromosome; parthenogenesis, development of over 2 cell stage; and degenerated, oocytes where the pronuclei were unidentifiable or invisible.

Assessment of blastocyst cell number

In experiment III, all blastocysts were rinsed twice with DPBS, and then fixed and stained with 99.5% ethanol and 25 μg/m*l* bisbenzimide, respectively, for 2–3 hr at 4°C. The embryos were then washed twice in glycerol and placed on a slide with glycerol, overlaid with a coverslip, and incubated for 24 hr at 4°C. The cell number of each embryo was examined using a fluorescence microscope.

Statistics

All experiments were repeated independently more than five times. Data for experiments I and II were subjected to arcsine transformation, ANOVA, and Fisher's protected least significant difference *post-hoc* test (Stat View; Hulinks Inc., Tokyo, Japan). $P<0.05$ was considered statistically significant. Data for experiment III were subjected to the Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Experiment I

We first examined the development of feline embryos cultured in the alternative human IVC medium supplemented with FBS instead of BSA, in the late period of the culture, as reported by Pope *et al*. [21]. As shown in Table 2, the percentage of morula + blastocyst stage embryos among Grade 1 and 2 oocytes was not significantly different among the four IVC groups designated in this study. However, the OM + BSA/FBS group showed the highest rate of development to the blastocyst stage of and the rate was significantly higher than those in the OM + BSA and EB + BSA groups ($P<0.01$).

Compared to Grade 1 and 2, Grade 3 oocytes formed a smaller proportion of morula + blastocyst after IVC but the difference among the groups was not statistically significant (Table 2). However, Grade 3 oocytes in all the groups could develop to the blastocyst stage. Notably, as shown in Table 2, the OM + BSA/FBS group showed the highest proportion of blastocyst formation and the proportion was significantly higher than for the OM + BSA group ($P<0.01$).

Experiment II

From the findings of experiment I, we considered that the addition of FBS instead of BSA in the late IVC period elicited better results in terms of blastocyst formation. Therefore, it was essential to examine morula and blastocyst formation in the IVC supplemented with FBS for the entire period. As shown in Table 3, there was no difference in morula + blastocyst formation between the OM + BSA/FBS and the OM + FBS groups among any of the oocyte Grades. However, a significant reduction in blastocyst formation was observed in the OM + FBS group compared to the OM + BSA/FBS group in the IVC with oocytes of all Grades ($P<0.01$).

Experiment III

We evaluated the parthenogenesis rates of Grade 1 and 2 and Grade 3 oocytes in OM + BSA/FBS. The proportions of parthenotes in Grade 1 and 2 oocytes and in Grade 3 oocytes were 3.3 and 7.9%, respectively. Only one oocyte in both Grade 1 and 2 oocytes and Grade 3 oocytes developed to the 8-cell stage; no oocyte developed to the morula or blastocyst stage.

Next, we evaluated the fertilization rate of Grade 1 and 2 and Grade 3 oocytes. As shown in Table 4, the percentage of 2PN formation was significantly higher in Grade 1 and 2 than in Grade 3 oocytes ($P<0.05$), whereas there was no difference in polyspermy or rate of parthenogenesis among the three Grades of oocytes.

We also investigated morula + blastocyst and blastocyst formation rates as well as blastocyst cell number using Grade 1 and 2 oocytes and Grade 3 oocytes cultured in the OM + BSA/FBS. As shown in Table 5, we found that the morula + blastocyst

formation rate was significantly higher in Grade 1 and 2 oocytes than in Grade 3 oocytes ($P<0.01$). There was a trend ($P=0.069$) toward blastocyst formation in Grade 1 and 2 oocytes compared to Grade 3 oocytes. The blastocyst cell number was also significantly higher in Grade 1 and 2 oocytes than in Grade 3 oocytes ($P<0.05$).

DISCUSSION

To find a suitable condition to improve the development of feline embryos using a commercially available human IVC medium, we examined the effects of the period of FBS supplementation using alternative human IVC media. As reported by Pope *et al*. [21], the addition of FBS in the late period of IVC led to the formation of more blastocysts. Culture in OM + BSA/FBS, in particular, resulted in a significantly higher number of blastocysts than culture in OM + BSA, whereas no difference in the number of morulae + blastocysts was observed between these cultures. These results indicate that FBS contains components that can overcome the morula block in the late IVC period. Mun *et al*. [12] reported that the addition of FBS in the late IVC period of porcine embryos significantly increased blastocyst formation. They also found that FBS significantly reduced the levels of intracellular reactive oxygen species (ROS). The reduction of ROS prevents parthenogenetic activation and increases developmental competence parameters, such as blastocyst formation. In addition, Singh and Armstrong [23] reported that FBS contains insulin-like growth factor (IGF) which is essential for embryo growth. Indeed, supplementing IVC with IGF inhibited apoptosis and increased the blastocyst formation rate in rabbits [6], bovines [10], and humans [25]. Moreover, Herrick *et al*. [5] reported the extent to which unidentified components of FBS can promote optimal blastocyst development.

However, supplementation with FBS during the entire IVC period (the OM + FBS group) partially inhibited blastocyst formation. These results suggest that late period-specific enhancing factors act reversely in early embryo development. Mun *et al*. [12] also reported that reduction of intracellular ROS in the early period of IVC negatively affected developmental parameters.

The OM + BSA/FBS group showed the highest rate of blastocyst formation in the present study, and the rate of blastocyst formation in Grade 1 and 2 oocytes was 20.9%. Herrick *et al*. [5] defined the physiological requirements of domestic cat embryos and attempted to develop and optimize feline-specific culture media. However, they used *in vivo*-matured oocytes whereas ours were matured *in vitro*. Therefore, their optimized media cannot be compared to OM + BSA/FBS. In contrast, Naoi *et al*. [14] used *in vitro*-matured Grade 1 and 2 oocytes from ovaries of ovariohysterectomized cats and showed that the proportion of blastocysts was 6.7%. In the study, the oocytes were incubated in modified Earle's balanced salt solution (MK-1) with BSA during early culture and subsequently in the same medium but with added FBS (5%) during late culture. These results suggest that the commercial human single-step media used in the present study are highly beneficial for IVC of cat embryos. Moreover, the results obtained using Grade 3 oocytes indicate that the present culture condition can promote the development of poor-grade feline oocytes *in vitro*.

In the present study, using OM + BSA/FBS, the morulae + blastocyst and blastocyst rates in Grade 3 oocytes were reduced in experiments II and III compared to experiment I, whereas those of Grade 1 and 2 oocytes were not. Naoi *et al*. [15] indicated that the reproduction cycle stage of donor cat ovaries has no apparent effects on the *in vitro* development of Grade 1 oocytes after IVF, whereas for poor grade oocytes the rate of blastocyst formation was different in the estrous cycle. Since the estrous stage of the queens was not considered in this study, the differences in the results for Grade 3 oocytes among the experiments may have been due to the use of oocytes at different stages of the estrous cycle. Moreover, another group [26] has suggested that the size of the follicles/oocytes, as well as their qualities, may affect the *in vitro* maturation and subsequent development of cat oocytes. Therefore, future studies should evaluate the effects of the stage of the estrous cycle, as well as oocyte diameter, on the development of Grade 3 oocytes.

In a study of human oocytes using serum-free media, the single-step culture showed improved [19, 24] or comparable [11] results in blastocyst formation compared to the sequential culture. In the present study, the blastocyst formation rate of the OM + BSA group was similar to that of the EB + BSA group. This result may have been due to the medium exchange. Fujita *et al*. [4] reported that, during culture, embryo-secreted growth factors are essential for embryo development. Ammonia and ROS are generated as byproducts of cellular metabolism and, although a certain level of ROS is beneficial for embryo development, higher levels of ROS and ammonia are not suitable for embryo development *in vitro* [5, 12]. In addition, during culture, it is important to remove some of the old medium and replace it with fresh medium, as fresh medium contains almost all the important ingredients for embryo culture and old medium contains embryo-secreted growth factors. Therefore, we replaced half the old medium with fresh medium for better embryo development. However, the increased stability of alanyl-L-glutamine results in significantly reduced ammonium production with incubation at 37°C [8]. Only-One Medium contains alanyl-L-glutamine instead of L-glutamine and therefore the medium does not have to be changed when it is used for human embryo culture. In contrast, Blastocyst Medium contains L-glutamine. As embryos are stressed by medium exchange, embryo development using the Only-One medium may have been better if the medium had not been changed.

The OM + BSA/FBS group exhibited relatively, but not significantly, increased blastocyst formation compared to the EB + BSA/FBS group. As Biggers and Racowsky [2] reported that fertilized oocytes were sensitive to changes in environmental conditions in the early period of IVC, sensitivity may have affected embryo development. Moreover, Only-One Medium and Blastocyst Medium contain both essential and non-essential amino acids. In contrast, Early Culture Medium contains only alanyl-L-glutamine and taurine. Bavister and Arlotto [1] reported that addition of the 20 amino acids to hamster culture media overcame the one-cell block. The morula block normally observed in *in vitro* cat embryo development [22] may be due to the culture conditions of the oocytes and embryos [27]. Whereas Early Culture and Blastocyst media provide amino acids only after day 2, Only-One Medium provides

amino acids throughout the 7 days of culture. Therefore, Only-One Medium may be superior to Early Culture and Blastocyst media in terms of supporting cat embryo development during IVC.

These media also differ in terms of osmolarity. The osmolarity of Only-One Medium is 265 mOsm, and that of Early culture and Blastocyst media is 280 mOsm. The osmolarity of culture media is a major factor affecting *in vitro* embryo development [9]. Moreover, NaCl is the major component of culture media affecting osmolarity. As for other species [9], the development of feline embryos was improved under reduced NaCl concentrations [5]. Therefore, in our experiment, the low osmotic pressure of Only-One Medium may have had a positive effect on embryogenesis.

It is well known that feline oocytes frequently show parthenogenesis [13]. In this study, the proportion of parthenotes was 3.3 in Grade 1 and 2 oocytes and 7.9% in Grade 3 oocytes. Moreover, only one oocyte in each of the Grades developed to the 8-cell stage using OM + BSA/FBS, and no oocyte developed to the morula or blastocyst stage. We also evaluated the fertilization rate of Grade 1 and 2 oocytes and Grade 3 oocytes, and observed 2PN formation in both groups. The results indicate that Grade 3 oocytes develop to the blastocyst stage due to normal fertilization and not parthenogenesis.

Because blastocyst quality is evaluated in terms of blastocyst cell number [3], it is important to investigate cell numbers when evaluating an IVC medium. Although we cultured embryos in OM + BSA/FBS for 7 days, the average blastocyst cell number in the present study (235.4 ± 87.7) is at least similar to that reported by Herrick *et al*. [5] (121.0 ± 8.8) who cultured embryos for 6 days and used cat-optimized culture medium with serum in the late culture period.

In conclusion, the commercially available human Only-One Medium supplemented with BSA for the first 2 days of culture and with FBS for the subsequent 5 days of culture is suitable for feline embryo development until the blastocyst stage. Moreover, as the IVC condition was effective for IVF of poor-quality oocytes, it can be a promising tool for use in the conservation of endangered feline species. However, in the present study, we used media provided by only one company, and did not compare them to media provided by other companies and developed for other species. In the future, the effect of other commercially available media on feline IVC should be investigated.

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