Effect of duration of in vitro maturation on nuclear maturation and fertilizability of feline oocytes

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Abstract

This study was conducted to improve in vitro production of embryos from domestic cats using TCM-199 as an IVM medium. The time sequence of nuclear maturation and the optimal timing of in vitro insemination were examined. Most oocytes were at the germinal vesicle stage immediately after collection; however, 8.3% had already resumed meiosis before IVM culture. After 30 h of IVM culture, the percentage of oocytes at metaphase II (MII) reached a peak (75.5%) and did not change ($P>0.05$) from 30 to 48 h after IVM culture. The percentage of oocytes with two pronuclei was higher ($P<0.05$) for oocytes matured for 30 and 36 h (38.2 and 33.0%, respectively) than for those after IVM culture for only 24 h (18.5%). Total sperm penetration rate was highest ($P<0.05$) for oocytes that had been matured for 30 h (46.1%). After 30 h of IVM and 18 h of IVF culture, 66.3 and 24.8% of inseminated oocytes had cleaved and developed to the blastocyst stage, respectively. We concluded that IVM of feline oocytes for 30 h in TCM-199 resulted in optimal nuclear maturation and sperm penetration.

Keywords: Domestic cat; Oocyte; IVC; IVF; Duration of IVM

1. Introduction

The domestic cat is an important experimental model for assisted reproductive technologies for non-domestic felidae; development of reliable methods for IVM and IVF of oocytes collected from domestic cat ovaries can be subsequently used to preserve endangered species. Recently, research on IVM, IVF, and IVC of oocytes derived from the domestic cat has increased rapidly [1]. However, information regarding IVM of feline oocytes is fragmentary, as researchers have used various culture media and intervals for IVM. Several studies investigated the time sequence of in vitro nuclear maturation of oocytes from domestic cats [2-7]. Although the relationship between the duration of IVM culture using Eagle's minimum essential medium (MEM) and subsequent development after IVF was investigated by both Johnston et al. [2] and Wolfe and Wildt [5], the timing of insemination was not consistent. The former concluded that 52 h of IVM was best, whereas the latter reported that 32 h of IVM was better than other culture periods to obtain high rates of cleavage and blastocyst development.

Recently, in vitro production of feline embryos was conducted after IVM using TCM-199 as a basal maturation medium for 24 h [8-11] or 24 to 28 h [12]. However, in these reports, there was no adequate rationale for the duration of IVM culture. Although the timing of nuclear maturation was determined in feline oocytes cultured in TCM-199 [4,7], the relationship between the duration of IVM culture and fertilization has not been studied.

Optimizing the duration of IVM culture improved subsequent development in bovine oocytes [13-17]. To improve the in vitro embryo production system in domestic cats using TCM-199 as an IVM medium, the time sequence of nuclear maturation and the optimal timing of in vitro insemination need to be examined. Therefore, the present study was

conducted to determine the optimal duration of IVM culture for oocytes from domestic cats, and to confirm the developmental ability of these oocytes after IVF.

2. Materials and methods

2.1. Oocyte recovery

Ovaries from domestic cats were obtained following ovariohysterectomy at the veterinary teaching hospitals of Tottori and Hokkaido universities, and from local animal hospitals near Hokkaido University. These ovaries were collected throughout the year (from March, 2001 to February, 2007), and the stage of the estrous cycle of the queen was not considered. All queens weighed more than 2.2 kg. Ovaries were kept in normal saline at room temperature, delivered to the laboratory within 4 h after collection, and subsequently transferred to TCM-199 (pH 7.4; Gibco Laboratories, Grand Island, NY, USA) containing 0.1% polyvinylalcohol, 25 mM HEPES, 0.85 mg/mL of $NaHCO_3$, and 50 μg/mL of gentamicin sulfate (isolation medium) [18]. Ovaries were cut (with scissors) along their long axis. The ovarian medulla was removed and the cortex cut (to form small pieces) in the isolation medium, and antral follicles were dissected (with a 25-gauge needle) under a stereomicroscope. Cumulus oocyte complexes (COCs) were collected, transferred to fresh isolation medium, and their morphological characteristics determined with the stereomicroscope (15 to 50 X). Only COCs that were tightly surrounded by several layers of cumulus cells, with evenly granulated black ooplasm, were used for experiments. Overall, 745 COCs were collected from 42 pairs of ovaries.

2.2. In vitro maturational culture

The COCs were washed once in maturation medium, composed of 25 mM HEPES-buffered TCM-199 (Earle's salt; Gibco Laboratories) supplemented with 0.3% fatty-acid free BSA (Sigma Chemical Co., St. Louis, MO, USA), 0.02 units/mL of FSH (Sigma Chemical Co.), 1 μg/mL of estradiol-17ß (Sigma Chemical Co.), 0.2 mM sodium pyruvate, and 50 μg/mL of gentamicin sulfate. They were then cultured in 50 μL droplets of maturation medium (10 to 15 COCs/droplet), covered with paraffin oil at 39 °C, in a humidified atmosphere of 5% CO_2 in air.

2.3. Sperm recovery and cryopreservation

Feline sperm were collected from cauda epididymides from two toms (castrated at Tottori University) and frozen as previously described [19]. Briefly, the epididymides were cut (at the corpus) into two equal parts; all portions of cauda epididymides (from both cats) were pooled and minced in 1 mL of egg yolk tris-fructose citrate solution (EYT-FC) [20] to liberate the sperm. The resulting solution was filtered through a sterile nylon mesh (pore size, 100 μm) and sperm were recovered (~55% of sperm were progressively motile). After recovery, the sperm suspension was subjected to the first dilution (in EYT-FC at 23 ˚C; concentration, 25 x 10^6 cells/mL), and subsequently cooled (in a refrigerator for 1.5 h) to 4 ˚C. Thereafter, the second dilution was performed by dripping EYT-FC supplemented with 14% glycerol and 1.5% Equex STM paste (Nova Chemical Sales Inc., Scituate, MA, USA) at 4 ˚C. The sperm suspension was diluted with an equal volume of extender (final concentration, 12.5 x 10^6 cells/mL). After equilibrating for 5 min at 4 ˚C, the sperm suspension was loaded in 0.25-mL straws (Fujihira Co. Ltd., Tokyo, Japan) and heat-sealed. Immediately after loading,

straws were placed 7 cm above the surface of liquid nitrogen for 5 min, and then they were immersed into liquid nitrogen (for freezing and storage).

2.4. In vitro insemination

Frozen sperm were thawed by immersing straws in a waterbath (37 °C for 30 s) and the contents were expelled into pre-warmed 5 mL modified Brackett & Oliphant (mBO) medium [21]. Sperm was washed twice by centrifugation (500 x g) with 5 mL of mBO medium for 5 min. After thawing, ~30% of sperm were progressively motile.

After IVM culture, approximately 10 to 15 COCs were co-incubated with frozen-thawed sperm (15 X 10^6 sperm/mL) in a 100-µL droplet of mBO medium for 18 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. The mBO medium contained 3 mg/mL of fatty-acid free BSA and 2.5 mM theophylline (Sigma Chemical Co.), and the droplets were covered with paraffin oil.

2.5. Evaluation of nuclear maturation and fertilization by whole- mount procedure

Before and after IVF culture, COCs were vortexed to remove cumulus investments. They were then fixed with a mixture of ethanol: acetic acid (3:1), stained with 1% aceto-orcein solution, and nuclear status and the presence of pronuclei were evaluated using a phase-contrast microscope, as described previously [22,23]. Briefly, the presence of two pronuclei was regarded as normal fertilization, whereas three or more pronuclei and/or enlarged sperm heads within an oocyte was judged as polyspermy. When oocytes had already cleaved or developed to >2-cell stage immediately after IVF, they were judged as

parthenogenetically activated (parthenotes).

2.6. In vitro culture and evaluation of subsequent embryonic development

After removal of the cumulus cells by vortexing, presumptive zygotes were washed three times with culture medium. The culture medium was a modified synthetic oviduct fluid (mSOF) [24], with 3 mg/mL of BSA in lieu of polyvinyl alcohol. All presumptive zygotes were cultured for 150 h (7 d after starting IVF culture) as groups of 15 to 30 embryos per 50-μL droplet of mSOF covered with paraffin oil at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

2.7. Evaluation of the numbers of cells in the blastocysts

Blastocysts were fixed and the number of live cells enumerated using an air-drying method, as reported previously [25]. Briefly, blastocysts were treated with 1% (w/v) sodium citrate and fixed with a mixture of methanol: acetic acid: distilled water (10:3:7) and a mixture of methanol and acetic acid (3:1). After staining with 3% (v/v) Giemsa's solution (Kanto Chemical Co., Inc., Tokyo, Japan), the total number of live cells in blastocysts was determined (light microscopy).

2.8. Experimental design

In Experiment 1, the effects of duration of IVM culture on the nuclear status of feline oocytes were evaluated by culturing COCs for 0, 24, 30, 36, or 48 h in maturation medium.

After IVM culture for varying durations, the nuclear status of oocytes was examined by the whole-mount procedure. In Experiment 2, effects of duration of IVM culture (24, 30, and 36 h; at the periphery of the nuclear maturation peak) on the fertilizability of feline oocytes were evaluated. After IVF culture, all oocytes were prepared as whole-mount specimens and fertilization was assessed. In Experiment 3, presumptive zygotes after 30 h IVM (presumptive optimal culture period) and 18 h IVF were further cultured to assess embryonic development. Cleavage, development to blastocysts, and the numbers of cells in blastocysts were examined at the end of culture. Oocytes cultured in mBO medium without sperm and subsequently cultured in mSOF were used as controls (to assess parthenogenetic activation).

All data on nuclear status after IVM and fertilization were analyzed using a Chi-square test (JMP Version 5; SAS Institute Inc., Cary, NC, USA); $P<0.05$ was considered significant.

3. Results

In Experiment 1, most oocytes were at the germinal vesicle stage immediately after collection; however, 8.3% of oocytes had resumed meiosis before IVM culture (Table 1). After 30 h of IVM culture, the percentage of oocytes at metaphase II (MII) reached a peak of 75.5%, with no further increase ($P>0.05$) after 36 or 48 h of IVM culture. Conversely, the percentage of degenerated oocytes increased ($P<0.05$) after 48 h of IVM culture, compared with culture for shorter intervals.

In Experiment 2, the percentage of feline oocytes with two pronuclei was higher ($P<0.05$) for oocytes after 30 or 36 h of IVM culture, than for those cultured for 24 h (Table 2). The total sperm penetration rate was the highest ($P<0.05$) in oocytes after 30 h of IVM culture

(46.1%). In all experimental groups, the rate of parthenogenesis was approximately 10%.

In Experiment 3, 66.3% (67/101, pooled data from four replicates) of feline presumptive zygotes had cleaved; 24.8% (25/101) developed to the blastocyst stage after 7 d of culture. The mean number of cells in the blastocysts was 138.2 ± 56.2 (mean ± SD; range, 59 to 227). Conversely, seven out of 30 oocytes (23.3%) that served as controls for parthenogenetic activation cleaved, but none developed to the blastocyst stage.

4. Discussion

In the present study, there was an association between the duration of IVM culture and the proportion of feline oocytes undergoing nuclear maturation and successful IVF; the optimal culture period for IVM (in TCM-199 medium) was 30 h. Furthermore, the proportion of oocytes that reached MII was higher after 30, 36, or 48 h of IVM culture than after 24 h. Similarly, in a previous study, the maturation rate (56.0%) after 28 to 30 h of IVM was not significantly different from that (67.3%) after 42 to 45 h of IVM in TCM-199 medium [7]. Total sperm penetration rate in the present study was highest for the oocytes after 30 h of IVM, and tended to be higher than those after 36 h of IVM (P=0.16). We inferred that feline oocytes were not fully competent after 24 h of IVM, whereas after 36 h, many oocytes were too aged to be fertilized in vitro. Our results were also comparable to a previous report [5] that the cleavage rate (69.4%) of feline oocytes after 32 h of IVM in MEM was higher than that after 16, 24, 40, or 48 h of IVM. There is one report [2] that 52 h of IVM in MEM was best for production of feline embryos; that the cleavage rate (36%) was very low and no embryos developed to the blastocyst stage, we inferred that the oocytes may have been aged due to prolonged culture.

Under our culture system, 66.3 and 24.8% of embryos derived from oocytes matured for 30 h in TCM-199 cleaved and developed to the blastocyst stage, respectively. There are several reports of feline IVM using TCM-199 for 24 h [8-11]. When Tyrode's solution was used as an IVC medium, there was 54% cleavage after IVF and 31% blastocyst formation after 7 d of culture [8]; however, this percentage of blastocyst formation was based on cleaved embryos, and was lower than our result. Other studies using modified Earle's balanced salt solution as an IVC medium reported that cleavage rates, rates of blastocyst formation and mean numbers of cells in blastocysts after 8 d culture were 49.0 to 72.5%, 6.7 to 32.0%, and 94.1 to 124.0 [9-11], respectively, similar to our data. However, their culture periods (180 to 192 h) were longer than ours (168 h). Therefore our culture system was probably better for in vitro production of feline embryos from IVM oocytes. Conversely, one study using mSOF for IVC, the same as the present study, reported 50.2% cleavage after IVF, as well as 25.9 and 30.9% blastocyst formation after 6 and 8 d of culture, respectively [12]. In that study, feline oocytes were matured for 24 to 28 h in TCM-199. The apparently lower cleavage rate compared to that of the present study was probably due to the poor fertilizability of immature oocytes, due to the shorter duration of IVM. Based on this finding and the present results, we inferred that application of the optimal duration of IVM culture and mSOF as the IVC medium may improve feline embryo production in vitro.

In the present study, the polyspermy rate tended to be higher in oocytes after 30 h of IVM ($P=0.09$) than after 36 h; perhaps oocytes after 30 h of IVM were more easily penetrated by sperm than after 36 h IVM. There was an association among sperm concentration, the interval that COCs were co-cultured with sperm, and the polyspermy rate in a bovine IVF system [26]. Therefore, in future studies of feline IVF, the optimal concentration of sperm and the interval that COCs were co-cultured with sperm should be critically assessed.

In the present study, the proportion of parthenotes after 18 h of IVF following 24, 30, or 36 h of IVM was 16.9, 9.2 and 8.0%, respectively; cultured oocytes of parthenogenetically activated controls had a high incidence of cleavage (23.3%). Murakami et al. [10] also reported a high incidence of parthenogenetically activated oocytes (0 to 31.6%) similar to our results. The present and previous studies [10] used BO medium for IVF. Conversely, only 1.9% of oocytes had parthenogenetic activation after 48 h of IVM in the present study, and several reports that did not use BO medium had relatively low percentages of parthenogenetic activation (≤4%) [2,5,6,8,12,27]. Therefore, we inferred that unfertilized feline oocytes were activated in BO medium. In future studies, the conditions for IVF of IVM feline oocyte need to be critically examined.

Although fertilization rates in bovine IVF were significantly different among bulls [28], we used only one batch of frozen sperm (pooled from two cats). Therefore the effect of sperm source on fertilization rate was not tested. In the present study, we used only oocytes that were tightly surrounded by several layers of cumulus cells, with evenly granulated black ooplasm, as previously reported [27]. However, intrinsic properties of the oocytes [27] and the stage of the estrous cycle of the donor queen [2,9,12] could have also affected fertilization rate. Therefore, in future studies, the effect of the stage of the estrous cycle on the fertilizability of feline IVM oocytes should be determined.

In conclusion, the proportion of feline oocytes undergoing nuclear maturation and successful fertilization was optimal following IVM for 30 h in TCM-199. However, in future studies, the effects of sperm concentration, duration of IVF, and IVF medium should be examined to further improve in vitro production of feline embryos.

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Table 1. Relationship between progression of nuclear status of feline oocytes and duration of IVM culture.

Duration of culture (h)	No. oocytes (replicates)	Oocytes in each nuclear status after IVM (%)				
		GV	MI	MII	Parthenote	Degeneration
0	60 (4)	91.7[a]	5.0	3.3[c]	0	0
24	91 (5)	30.8[b]	14.3	50.5[b]	2.2	2.2[b]
30	98 (6)	11.2[c]	6.1	75.5[a]	4.1	3.1[b]
36	62 (4)	16.1[c]	12.9	67.7[a]	0	3.2[b]
48	104 (6)	5.8[c]	6.7	69.2[a]	1.9	16.3[a]

[a-c] Within a column, percentages without a common superscript differed ($P<0.05$).

GV: germinal vesicle, MI: metaphase I, MII: metaphase II, Parthenote: more than two blastomeres after IVM culture.

Table 2. Rate of in vitro fertilization of feline oocytes following IVM for varying intervals.

Duration of culture (h)	No. oocytes (replicates)	Oocytes after IVF (%)				
		2PN	Polyspermy	Total	Parthenote	Degeneration
24	65 (4)	18.5[b]	7.7	26.2[b]	16.9	6.1
30	76 (4)	38.2[a]	7.9	46.1[a]	9.2	2.6
36	88 (3)	33.0[a]	2.3	35.2[a,b]	8.0	1.1

[a,b]Within a column, percentages without a common superscript differed ($P<0.05$).

2PN: two pronuclei, Total: 2PN + polyspermy, Parthenote: more than two blastomeres after IVF culture.