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Development and subsequent cryotolerance of domestic cat embryos cultured in serum-free and serum-containing media

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ABSTRACT

The objectives of this study were to examine the effects of the presence or absence of serum during the in vitro culturing period of domestic cat embryos on their developmental potential into blastocysts as well as their tolerance to cryopreservation using a slow-freezing method. In vitro-fertilized cat oocytes were incubated in a modified synthetic oviduct fluid (mSOF) containing 4 mg/mL bovine serum albumin (BSA) throughout culturing (BSA group) or in mSOF containing 4 mg/mL BSA for the first 3 days followed by mSOF containing 5% fetal bovine serum (FBS group). The developmental potential of the embryos to the blastocyst and expanded blastocyst stages was evaluated 7 days after in vitro fertilization. The blastocysts were frozen-thawed by the slow-freezing method and cultured for 3 days to examine their viability in vitro. There were no differences in the formation rates of blastocysts or expanded blastocysts, or number of cells in the embryos between the two groups. After cryopreservation, the hatching rates of the expanded blastocysts in the BSA group were significantly higher ($P < 0.05$) than those of the FBS group. The postthaw viability of blastocysts was lower than that of expanded blastocysts irrespective of culture medium. These results indicate that the developmental potential of cat embryos cultured in serum-free medium is comparable to those cultured in serum-containing medium. Furthermore, expanded blastocysts produced without serum exhibit better postthaw viability than those produced with serum.

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Introduction

Assisted reproduction technologies including in vitro embryo production and somatic cell nuclear transfer for cats have progressed [42,35,38,15]. These technologies are expected to play potential roles in the conservation of endangered felids. In these procedures, successful cryopreservation of the generated embryos is essential for their widespread use in embryo transfer. However, it is well known that in vitro produced (IVP) embryos are much more sensitive to cryopreservation than their in vivo produced counterparts, particularly in ruminants [17,24].

In the domestic cat, the birth of live kittens from cryopreserved embryos produced by in vitro fertilization (IVF) from both in vivo matured [36,43] and in vitro matured (IVM) oocytes has been reported [14]. This is a useful model for applying the cryopreservation procedure to nondomestic cats [38]. On the other hand, serum has widely been used in culture media of cat embryos because of embryotrophic factors [38,18,10]. Serum has a biphasic effect on embryo development, inhibiting the early cleavage divisions while accelerating development when it is present from the initiation of compaction [34]. Although cat embryos develop to the blastocyst stage in a wide range of media, they still appear to be reliant on one or more unidentified components of serum for optimal blastocyst development [18].

Serum has been shown to contain beneficial substances for embryo development such as growth factors, antioxidants, and heavy-metal chelators [11,5,2]. In contrast, previous studies in ruminants indicate that embryos produced with serum exhibit altered developmental kinetics and morphology, including premature blastulation [20], excess lipid droplet accumulation [45,1], and altered gene expression [39,9]. Moreover, bovine blastocysts produced in the absence of serum are more successfully cryopreserved than those produced with serum [1,39,41,33,29,16,12]. These findings indicate that besides the intrinsic qualities of oocytes, the conditions of embryo culture play a crucial role in determining the resultant blastocyst quality. Furthermore, the efficiency of embryo cryopreservation can be improved by modifying the in vitro culturing system, including the removal of serum from media. Such modifications could also result in healthier offspring [45]. However,

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there is little information about the effects of the presence or absence of serum during the post-fertilization culture period on the quality of cat embryos as assessed by survival after cryopreservation.

In this study, domestic cat embryos derived from IVM–IVF were incubated in medium containing bovine serum albumin (BSA) throughout culturing or in medium containing BSA for the first 3 days followed by medium containing fetal bovine serum (FBS). The subsequent developmental potential of the embryos cultured in either serum-free or serum-containing media into blastocysts and their viability after cryopreservation using a slow-freezing method were evaluated.

Materials and methods

Oocyte recovery and in vitro maturation

The methods used for in vitro embryo production were modified from those described previously [23]. Ovaries were collected from sexually mature queens at various stages of the estrous cycles following routine ovariohysterectomy at local veterinary clinics. The ovaries were kept in physiological saline at room temperature (25 °C) and brought to the laboratory within 1–3 h. The ovaries were placed in a 90-mm culture dish containing prewarmed modified PBS (mPBS, Embryo-tech; Nihon-zenyaku, Fukushima, Japan) supplemented with 50 μg/mL gentamicin (Sigma, St. Louis, MO, USA) on a warm plate at 38 °C and sliced repeatedly with a scalpel blade to release cumulus–oocyte complexes (COCs). Only good-quality COCs with uniform dark-pigmented ooplasm and intact cumulus cell investment were transferred to preequilibrated in vitro maturation (IVM) medium, which consisted of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA) supplemented with 4 mg/mL BSA (Sigma), 0.1 IU/mL human menopausal gonadotropin (Teikoku-zoki, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Teikoku-zoki), 1 μg/mL estradiol-17β (Sigma), and 50 μg/mL gentamicin. The COCs were washed and cultured for 24 h in 100-μL droplets of IVM medium in mineral oil (Sigma) at 38 °C in a humidified atmosphere of 5% CO_2 in air ($\leqslant$10 oocytes per droplet).

Sperm collection and cryopreservation

Testes were collected from mature male cats following castration at local veterinary clinics. They were kept in physiological saline at room temperature and brought to the laboratory within 2 h. The epididymides were removed from the testes, washed, and placed in a 90-mm culture dish containing mPBS supplemented with 50 μg/mL gentamicin on a 38 °C warm plate. Each epididymis was then sliced repeatedly with a scalpel blade to release sperm. The sperm in mPBS were transferred to a 15-mL conical tube and centrifuged at 500*g* for 5 min at room temperature. After centrifugation, the supernatant was discarded and the resultant sperm suspension (~50 μL) was diluted with 450 μL first extender, which consisted of 8.8% (w/v) lactose (Wako, Osaka, Japan), 200 μg/mL ampicillin (Mitaka, Tokyo, Japan), and 20% (v/v) egg yolk in distilled water. The tube containing the diluted sperm suspension was transferred to a water bath at room temperature, which was set at 4 °C for 2 h. After equilibration, 250 μL second extender [the first extender supplemented with 6% (v/v) glycerol and 1.48% (v/v) orvus ES paste (Miyazaki-kagaku, Tokyo, Japan)] was added to the sperm suspension at 4 °C. After being kept at 4 °C for an additional 5 min, an additional 250 μL of the second extender was added to the sperm suspension at 4 °C. The sperm suspension was then immediately loaded into 0.25-mL plastic straws (Fujihira Co., Tokyo, Japan), which were then frozen after being placing on a styrofoam plate in liquid nitrogen (LN_2) vapor (4 cm above the surface of the LN_2). The straws were kept on the plate for 20 min and plunged into LN_2 for storage.

In vitro fertilization and culture

The straws of frozen sperm were thawed in a 38 °C water bath and washed twice in Brackett–Oliphant (BO) medium [4] supplemented with 137 μg/mL sodium pyruvate (Sigma), 3.5 mM taurine (Wako), 25 mM HEPES (Sigma), and 50 μg/mL gentamicin by centrifugation at 500*g* for 5 min at room temperature. The sperm pellet was resuspended in 500 μL BO medium, and the concentration was adjusted to 2×10^6 sperm/mL. The sperm suspension was diluted twice with the BO medium containing 6 mg/mL BSA and 20 μg/mL heparin to a final concentration of 1×10^6 sperm/mL. The COCs cultured for IVM were placed into 100-μL sperm droplets in mineral oil ($\leqslant$5 COCs per droplet, day 0) and coincubated for 12 h. After coincubation, the COCs were denuded of cumulus cells by being pipetted through a fine pipette and washed with modified synthetic oviduct fluid (mSOF) (Table 1) containing 4 mg/mL BSA. These presumptive zygotes were randomly assigned to one of the following two groups and cultured accordingly in 500 μL medium at 38 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. In the BSA group, the zygotes were cultured in mSOF containing 4 mg/mL BSA throughout culturing until day 7. In the FBS group, the zygotes were cultured in mSOF containing 4 mg/mL BSA for the first 3 days of culturing followed by mSOF containing 5% FBS until day 7. In both groups, cleaved embryos were transferred to 500 μL of their respective fresh medium on day 3. The embryos that became compact morula and blastocysts were evaluated morphologically on day 7. In this study, the blastocysts that had an intact zona pellucida were defined as the blastocyst stage, and the good-quality blastocysts that had a clear blastocoel cavity and a thin enlarged zona pellucida were defined as the expanded blastocyst stage.

Assessment of total cell numbers

Day 7 embryos at the blastocyst and expanded blastocyst stages were collected randomly from the BSA and FBS groups. They were fixed and permeabilized in Dulbecco's PBS (DPBS; Invitrogen Co.) containing 3.7% (w/v) paraformaldehyde (Wako) and 1% (v/v)

Table 1
Composition of mSOF used for cat embryo culture.

Component	Concentration
NaCl	107.63 mM
KCl	4.69 mM
KH_2PO_4	1.19 mM
$CaCl_2$	1.71 mM
$MgSO_4$	1.18 mM
$NaHCO_3$	25.07 mM
Sodium lactate	3.30 mM
Sodium pyruvate	0.33 mM
Glutamine	1.00 mM
Glycine	10.00 mM
Alanine	1.00 mM
Taurine	3.50 mM
Myoinositol	2.77 mM
Sorbitol	3.50 mM
Citric acid	0.50 mM
BME–EAA [a]	1×
MEM–NEAA [b]	1×
Apo-transferrin	10 μg/mL
Insulin	5 μg/mL
Gentamicin	50 μg/mL

[a] BME, essential amino acids solution.
[b] MEM, non-essential amino acids solution.

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