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—Original Article—

Effects of vitrification and a Rho-associated coiled-coil containing protein kinase 1 inhibitor on the meiotic and developmental competence of feline oocytes

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Abstract. Oocyte cryopreservation is the technique of choice for the long-term storage of female gametes. However, it induces an irreversible loss of oocyte viability and function. We examined the effects of vitrification and a Rho-associated coiled-coil containing protein kinase 1 (ROCK1) inhibitor (ROCKi) on the meiotic and developmental competence of feline oocytes. We examined the expression of LIM kinase (*LIMK*) 1 and 2, with and without ROCKi treatment. Cumulus oocyte complexes (COCs) were matured *in vitro* with 0, 10, 20, and 40 μM ROCKi. The oocytes were subsequently assessed for maturation rate and embryo development following *in vitro* fertilization. We repeated the COC experiment, but vitrified and warmed the COCs prior to culture. We detected *LIMK1* and *LIMK2* expression in feline oocytes, which could be downregulated by ROCKi treatment. The ROCKi at 10 μM affected neither meiotic nor developmental competence ($P > 0.05$, versus control). However, high concentrations of ROCKi during maturation induced meiotic arrest at metaphase I. Appropriate concentrations of ROCKi significantly improved the normal fertilization rate of vitrified warmed oocytes (49.4 ± 3.4%) compared with that of the control (42.8 ± 8.6%, $P < 0.05$). The ROCKi also significantly improved the embryo cleavage rate (36.1 ± 3.8%) as compared with the non-treated control (27.4 ± 2.5%, $P < 0.05$). Thus, this study revealed that the main mediators of the ROCK cascade (LIM kinases) are expressed in feline oocytes. The ROCKi (10 μM) did not compromise the meiotic or developmental competence of feline oocytes. In addition, 10 μM ROCKi improved the cytoplasmic maturation of vitrified–warmed oocytes as indicated by their fertilization competence.

Key words: Domestic cat, Embryo development, Oocyte, ROCK inhibitor, Vitrification

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Oocyte cryopreservation is the method of choice for genetic preservation. This technique has predominantly been used in human and veterinary medicine for addressing the premature loss of fertility due to gonadal-toxic cancer therapy and for the genetic banking of desired animals, such as valuable or endangered species [1]. However, successful oocyte cryopreservation has been hampered by oocyte structural complexity and the limited availability of samples for study. Oocytes are the largest cells in the body, with a sophisticated structure and low membrane permeability to water and cryoprotectants [2]. As a result, freezing and thawing frequently result in irreversible cryodamage at the cellular and subcellular levels. Although several types of cryoinjuries have been identified, such as disruption of cell organelles [3, 4], the cytoskeleton [5–8], and the plasma membrane [7, 9, 10], the exact mechanisms that cause cell injury remain largely unknown. Freezing techniques including controlled-rate cryopreservation and vitrification are frequently used for oocyte cryopreservation. However, extensive intracellular ice formation renders irreversible cell damage to mammalian oocytes during cooling at a suboptimal rate [2, 3, 6, 9]. Therefore, a novel approach for ice-free cryopreservation by means of vitrification has been increasingly used. Vitrification is beneficial as it is simple and less time-consuming compared to controlled-rate freezing. However, high cryoprotectant concentrations and extremely high rates of freezing are required for this method [2, 3, 11]. These factors contribute to the poor quality of oocytes after vitrification, as the high cryoprotectant concentration is frequently toxic and can cause excessive osmotic stress [6, 11]. This method of cryopreservation has also been reported to activate apoptotic pathways, including intrinsic and extrinsic mechanisms [7].

In the domestic cat, information regarding the cryopreservability of immature oocytes is limited and overall success is currently poor [12–15]. The types of cryoinjury that occur during freezing and thawing are also poorly characterized. Of the potential cryoinjuries, cryopreservation-induced apoptosis and cell death have been reported [16–21]. Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is a downstream target of the small GTP-binding protein Rho, which has been identified as a central regulator of apoptosis via activation of the lipase modulator (LIM) kinases and cofilin. These elements act synergistically to balance actin cytoskeleton dynamics and functions [5, 8, 22]. The cell cytoskeleton plays an essential role during

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oocyte maturation [5, 8, 22, 23], and preservation of its structure and function is essential for oocyte developmental competence following cryopreservation [10, 24]. ROCK1 and cryopreservation could also synergistically activate caspase-dependent apoptotic pathways and cell death following freezing and thawing.

Treatment with an inhibitor of ROCK (ROCKi; Y-27632) has been shown to improve the viability of frozen–thawed human embryonic and induced pluripotent stem cells [25–27]. Although the protective mechanism of this small molecule is not entirely clear, it has also been reported to be beneficial for bovine oocyte and blastocyst cryopreservation [28].

This study aimed to determine the effects of vitrification and a ROCKi (Y-27632) on the meiotic and developmental competence of feline oocytes. We examined the protective effects of this small molecule on oocytes against cryoinjury during vitrification and warming.

Materials and Methods

Experiment 1: Identification of a LIMK-associated ROCK pathway in feline COCs

COCs were collected from feline ovaries. The oocytes (n = 75, 3 replicates) were completely separated from the cumulus cells. The presence of *LIMK1* and *LIMK2* mRNA was investigated using RT-PCR. In addition, oocytes matured *in vitro* with (n = 90) and without ROCKi (10 μM ROCKi, n = 75) for 12 h were used to quantitatively demonstrate the effects of ROCKi on *LIMK1* and *LIMK2* mRNA levels.

Experiment 2: Determination of optimal concentrations of ROCKi to promote oocyte meiotic and embryo competence

Experiment 2.1: The COCs were randomly subjected to IVM with different concentrations of ROCKi (10, 20, and 40 μM; n = 90 per group). Oocytes subjected to IVM without ROCKi (0 μM) served as the control group. After 24 h of IVM, the oocytes were assessed for their stage of nuclear maturation. The stages of nuclear maturation of the feline oocytes were classified as germinal vesicle stage (GV), metaphase I (MI), metaphase II (MII), and degenerate oocytes.

Experiment 2.2: This experiment was performed as Experiment 2.1, except that the IVM oocytes (n = 90 per group) were further fertilized *in vitro*. After IVF, embryo development was classified as cleaved, morula, and blastocyst stages on days 2, 5, and 7 of development, respectively.

Experiment 3: Effects of different concentrations of ROCKi on oocyte meiotic and developmental competence following vitrification and warming

Vitrified–warmed oocytes were cultured in maturation medium containing different concentrations of ROCKi (0, 10, 20, and 40 μM; n = 90 per group). The meiotic competence was assessed after 24 h of IVM. The fertilization competence of the oocytes was assessed at 18 h post-IVF, in terms of the percentages of sperm penetration and pronucleus formation. The developmental competence was determined based on the cleavage, and morula and blastocyst formation rates. Blastocyst quality was examined by cell count.

Reagents

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Source of ovaries

Ovaries from domestic cats (*Felis catus)* were collected after routine ovariohysterectomy and generously provided by the Veterinary Public Health Division of the Bangkok Metropolitan Administration, according to a protocol approved by the Chulalongkorn University of Animal Care and Use. The ovaries were collected in 0.9% (w/v) normal saline solution with 100 IU/ml penicillin and 100 IU/ml streptomycin. Then, the ovaries were transported at approximately 26°C to the laboratory within 2 h.

Oocyte isolation

The ovaries were maintained in a holding medium (HM) (HEPES-buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 4 mg/ml bovine serum albumin (BSA; embryo-tested)). The tissues were minced in HM to release cumulus oocyte complexes (COCs). The COCs were morphologically classified under a 40 × magnified stereomicroscope (SMZ645; Nikon, Tokyo, Japan). Only grade A and B oocytes were used in this study. Grade A and B oocytes were fully surrounded by more than 5 layers of compacted cumulus cells and had homogenously darkened cytoplasm.

In vitro maturation and fertilization

In vitro oocyte maturation (IVM) and fertilization (IVF) were performed as previously described [29, 30] with some modifications. For IVM, a group of COCs (approximately 30–40 COCs) was cultured in 500 μl of IVM medium ($NaHCO_3$-buffered M199 with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml gentamicin, 4 mg/ml BSA, 0.05 IU/ml recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand), and 25 ng/ml epidermal growth factor). After 24 h of IVM, COCs were transferred to 50-μl droplets of IVF medium comprising synthetic oviductal fluid (SOF) with 6 mg/ml BSA. The frozen–thawed semen used in this study was obtained from a fertility-proven male cat. Semen was collected via electroejaculation, then equilibrated at 4°C with an egg yolk TRIS buffer containing 5% (v/v) glycerol for 1 h. The equilibrated sperm were then loaded into freezing straws and layered onto liquid nitrogen vapors for 10 min. The straws were plunged and kept in liquid nitrogen until use. The frozen semen was thawed at 37°C for 30 sec and evaluated subjectively. Aliquots of sperm with more than 50% progressive motility were added to each IVF droplet for a final concentration of 5×10^5 sperm/ml. Oocyte culture was performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro embryo culture

After 24 h of IVF, the presumptive zygotes were gently denuded and cultured for 24 h in 50-μl droplets of SOF containing 4 mg/ml BSA and antibiotic (100 IU/ml penicillin and 100 IU/ml streptomycin). Cleaved embryos (day 0 = day of IVF) were further cultured in SOF supplemented with 10% (v/v) fetal calf serum. The culture medium was changed every other day. This culture system was maintained at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

Table 1. Primers for qRT-PCR

Gene	Primer (5′-3′ orientation)	Amplicon size (kb)	Reference/ Accession No.
LIMK1	Forward: CTGGTCCGAGAGAACAAGAA Reverse: ATCTCACACAGGACGATTCC	227	XM_003998592.3
LIMK2	Forward: GTTCAAGTACCACCCAGAGT Reverse: CACTTCCCACAGTAAAGGGT	108	XM_003994813.3
YWHAZ	Forward: GAAGAGTCCTACAAAGACAGCACGC Reverse: AATTTTCCCCTCCTTCTCCTGC	115	[32]

Assessment of fertilization competence

After 18 h of IVF, the fertilization competence of vitrified–warmed oocytes was assessed by observation of pronucleus formation. The presumptive zygotes were denuded and fixed in 4% (w/v) paraformaldehyde and stained with 4, 6-diamidino-2-phenylindole (DAPI). Pronucleus formation was examined using an epifluorescence microscope. The IVF treated oocytes or presumptive zygotes were classified, and the percentages categorized as total fertilization (sperm-penetrated oocytes), normal fertilization, abnormal fertilization, and degenerate oocytes were calculated in relation to the total oocytes inseminated. The presence of sperm or a male pronucleus within the ooplasm following IVF indicated a fertilized oocyte. The presumptive zygotes with 2 pronuclei (male and female pronuclei) were classified as normal fertilization. Any abnormalities of pronucleus formation, such as single or multiple pronuclei (> 3), indicated abnormal fertilization. Contracted oocytes without chromatin and/or with dispersed chromatin were classified as degenerate oocytes.

Assessment of embryo development

All cleaved embryos were morphologically observed under an inverted microscope (40 × magnification) to evaluate morulae and blastocysts on day 5 and day 7, respectively (day of IVF = day 0). On day 7, all embryos were fixed in 4% (w/v) paraformaldehyde and stained with 0.1 µg/ml DAPI. Total cell counts were performed using an epifluorescence microscope (Olympus, Japan). The percentages of morulae (> 16 cells without blastocoels) and blastocysts (> 50 cells with blastocoel formation) were recorded.

Oocyte cryopreservation

Open pulled straw (OPS) vitrification of COCs was performed as previously described [15]. The 4-step cryoprotective agent (CPA) exposure technique was performed by incubating the COCs in vitrification media containing different concentrations of ethylene glycol. The COCs were finally loaded into the OPS by capillary effect and immediately submerged into liquid nitrogen. Warming of vitrified COCs was carried out at 37°C by immersing the OPS in HM supplemented with gradually decreasing concentrations of sucrose (0.25 M, 0.125 M, and 0.0625 M, 5 min/step). The vitrified–warmed COCs were kept in HM at 37°C until the next procedure.

RNA extraction

Cumulus cells were mechanically denuded by gentle pipetting. The denuded oocytes were washed in phosphate-buffered saline and kept for RNA extraction [30]. Total RNA was extracted using the Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions, at room temperature (25–30°C). The extracted RNA was assessed for quality using a spectrophotometer (NanoDrop™ 2000, Wilmington, DE, USA) and immediately stored at –80°C.

Quantitative reverse transcription PCR

Reverse transcription (RT) was performed using a First Strand cDNA Synthesis Kit (Superscript III Kit, Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The products were stored at –20°C for quantitative PCR (qPCR). The relative expression levels of LIM kinase 1 (*LIMK1)* and LIM kinase 2 (*LIMK2)* were normalized to the endogenous control gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) [31]. The primers used in this study are listed in Table 1. The *LIMK1* and *LIMK2* primers were designed based on the sequences of *Felis catus LIMK1* and *LIMK2,* using an NCBI primer design tool, Primer3 software v 4.0.0.

We used an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Luminaris Color HiGreen qPCR Master Mix, High ROX (Thermo Scientific, Carlsbad, CA, USA) for qPCR. Each qPCR sample (10 µl) consisted of 2.5 µl of DNA template mixture (2 µl of reverse transcription product or no template as a control, with 0.5 µl of yellow sample buffer) and 7.5 µl of a mixture containing 5 µl of Luminaris Color HiGreen qPCR Master Mix, High ROX, 0.3 µl of each forward and reverse primer, and 1.9 µl of nuclease-free water. The thermal cycling conditions were as follows: pretreatment with uracil-DNA glycolysase (UDG) with 50°C 2 min, activate Tag DNA polymerase with 95°C for 10 min, and 55 cycles of 95°C for 15 sec for denaturation, 60°C for 30 sec for annealing, and 72 for 60 sec for extension. The relative expression levels were analyzed by Sequence Detection System (SDS) Software (Applied Biosystems). *LIMK1* expression in fresh oocytes prior to oocyte maturation served as a control, in order to compare its relative expression to *LIMK2*. The relative expression of these kinases after 12 h of maturation were also compared with control (no ROCKi) and 10 µM ROCKi-treated oocytes (Fig. 1A and 1C).

The PCR products were confirmed by electrophoresis on a 1% (w/v) agarose gel (Bio-Rad, Hercules, CA, USA) containing 5% (v/v) RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea) in TBE (Tris/Borate/EDTA) buffer. The amplified products (Fig. 1B) were examined under UV light using a gel

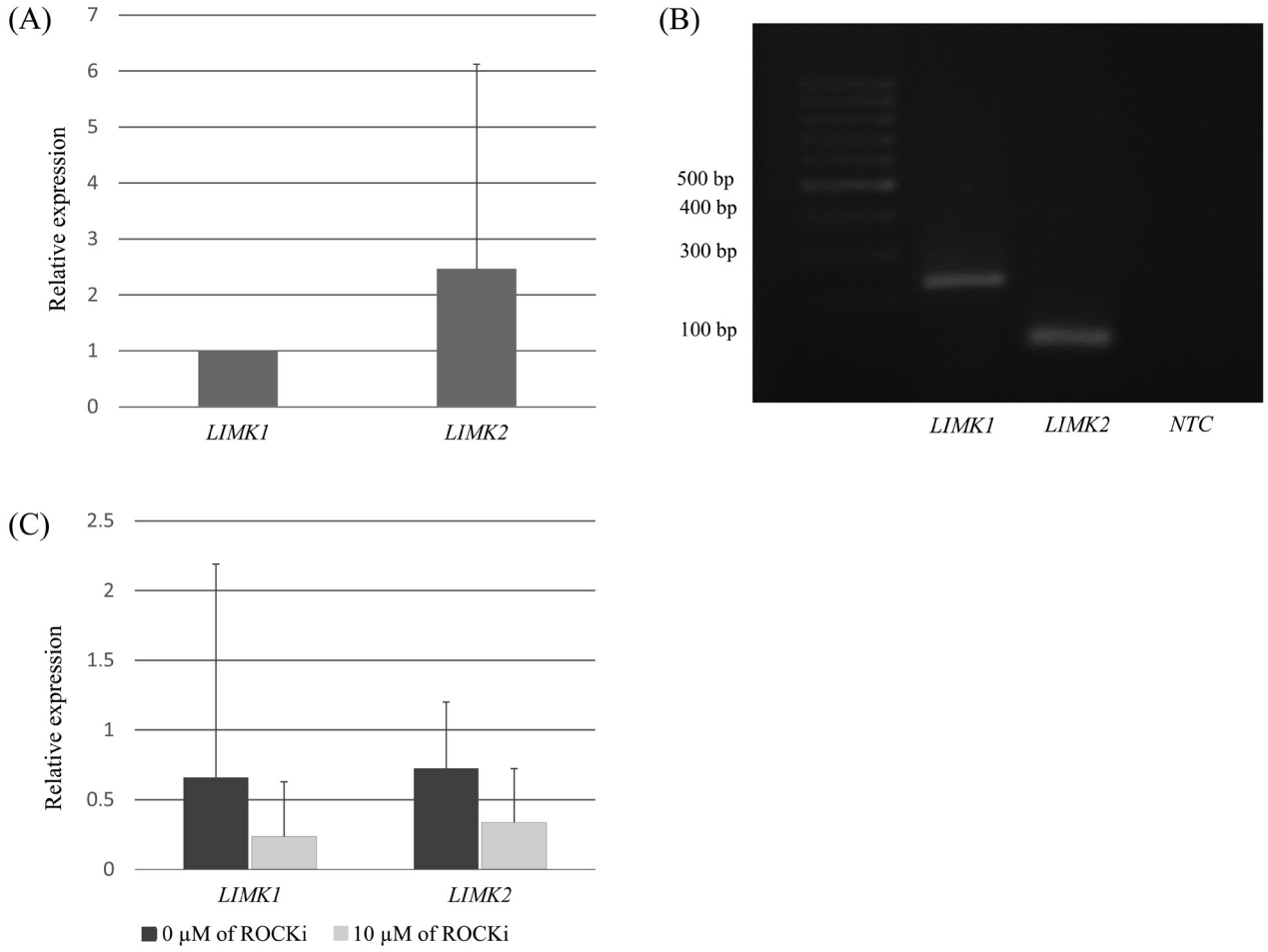

Fig. 1. The mRNA expression of *LIMK1* and *LIMK2* in feline oocytes (A). The PCR products were confirmed by electrophoresis (B). qRT-PCR expression of *LIMK1* and *LIMK2* in feline oocytes matured for 12 h with or without ROCKi (10 μM) (C).

documentation system (Syngene, Cambridge, UK).

Statistical analysis

All experiments were performed in triplicate or quadruplicate. Data were tested for normality and equal variance using the median test, and are expressed as mean ± standard deviation (SD) for each experiment. The fertilization competence of vitrified-warmed oocytes was defined based on the percentages of total fertilization, normal fertilization, abnormal fertilization, and degenerate oocytes. The percentage of cleaved embryos was defined as the number of cleaved embryos relative to the total number of oocytes in each group. The oocytes that were fertilized by sperm (sperm penetration) and the early stage embryos prior to cellular division at 48 h post-fertilization were excluded. The proportions of morulae and blastocysts were relative to the total number of cleaved embryos. Logistic regression was used to test the differences in the maturation stage of the oocytes, fertilization competence, and developmental rate of the embryos (cleavage, and morula and blastocyst formation rates). Multiple analysis of variance (ANOVA) and least significant difference post-hoc tests were used for statistical comparisons of the cell numbers within the blastocysts. Differences in quantitative mRNA expression were assessed by Student's t-test (relative expression between *LIMK1* and *LIMK2* in fresh oocytes) and ANOVA (Experiment 1). All statistical analyses were performed with SPSS version 22.0.0 (IBM, Armonk, NY, USA). P values less than 0.05 were considered statistically significant.

Results

Experiment 1: Identification of a LIMK-associated ROCK pathway in feline COCs

Feline oocytes expressed *LIMK1* and *LIMK2*, as shown in Fig. 1A and 1C. qRT-PCR revealed that *LIMK2* mRNA was expressed at a greater level than *LIMK1* ($P > 0.05$, Fig. 1A). The sizes of the PCR products were confirmed by electrophoresis (Fig. 1B). ROCKi supplementation during IVM downregulated *LIMK1* and *LIMK2* in feline oocytes, compared with the levels in the control (0 μM ROCKi). This finding indicated the bioactivity of the ROCKi in feline oocytes.

Table 2. The effect of the ROCKi on IVM and embryo development of non-cryopreserved feline COCs

ROCKi concentration (μM)	Stage of nuclear maturation				Stage of embryo development			Blastocyst cell number
	Degenerate (%)	GV (%)	MI (%)	MII (%)	Cleavage *	Morula **	Blastocyst **	
0	6.9 ± 0.5	12.2 ± 0	22.4 ± 0.5	58.0 ± 4.3 a)	55.7 ± 4.4	59.3 ± 22.1	52.5 ± 12.7	147.2 ± 11.0
10	7.7 ± 0.4	4.8 ± 3.7	21.1 ± 7.6	66.3 ± 6.2 a)	53.3 ± 9.5	60.3 ± 7.0	40.9 ± 22.8	176.3 ± 17.6
20	7.3 ± 0.4	4.8 ± 4.0	35.8 ± 8.6	47.8 ± 6.9 b)	58.6 ± 14.4	53.3 ± 9.5	35.1 ± 6.4	135.4 ± 12.8
40	7.8 ± 0.4	7.8 ± 1.4	49.4 ± 10.1	35.1 ± 12.7 c)	48.6 ± 11.6	58.7 ± 15.1	37.0 ± 1.5	143.8 ± 14.8

Data represent mean ± SD. a), b), c) within the same column, different superscripts denote values that differ significantly ($P < 0.05$). *, ** in relation to the number of oocytes and cleaved embryos, respectively.

Table 3. The effect of the ROCKi on the fertilization competence of vitrified-warmed feline oocytes

ROCKi concentration (μM)	Total oocyte	% total fertilization	% normal fertilization	% abnormal fertilization	% degeneration
0	55	50.5 ± 0.9	42.8 ± 8.6 a)	9.1 ± 1.5	48.4 ± 2.8
10	55	52.8 ± 0.5	49.4 ± 3.4 b)	3.6 ± 0.6	47.2 ± 0.5
20	57	50.9 ± 1.5	45.8 ± 6.1 c)	5.3 ± 1.0	49.1 ± 1.5
40	46	47.6 ± 4.1	39.3 ± 3.1 d)	13.0 ± 1.7	48.2 ± 7.7

Data represent mean ± SD. a), b) within the same column, different superscripts denote values that differ significantly ($P < 0.05$).

Table 4. The effect of ROCKi treatment on IVM and embryo development of vitrified–warmed feline COCs

ROCKi concentration (μM)	Stage of nuclear maturation				Stage of embryo development			Blastocyst cell number
	Degenerate (%)	GV (%)	MI (%)	MII (%)	Cleavage *	Morula **	Blastocyst **	
0	11.3 ± 2.5 a)	16.0 ± 7.9	20.0 ± 7.3 a)	53.6 ± 9.9 a)	27.4 ± 2.1 a)	16.1 ± 4.3	6.4 ± 4.5	66.0 ± 18.4
10	13.1 ± 4.0 a)	10.2 ± 1.2	29.3 ± 10.7 b)	46.6 ± 6.6 a)	36.1 ± 3.1 b)	24.2 ± 6.2	12.1 ± 6.6	68.4 ± 12.2
20	7.4 ± 2.6 a)	21.9 ± 13.3	26.4 ± 6.7 b)	43.8 ± 13.6 a)	33.2 ± 7.5 b)	22.3 ± 3.8	16.2 ± 5.2	96.3 ± 35.8
40	21.1 ± 3.9 b)	10.3 ± 5.5	31.5 ± 10.8 b)	36.2 ± 29.8 b)	25.6 ± 5.1 a)	12.4 ± 11.3	6.4 ± 4.5	81.0 ± 36.8

Data represent mean ± SD. a), b) within the same column, different superscripts denote values that differ significantly ($P < 0.05$). *, ** in relation to the number of oocytes and cleaved embryos, respectively.

Experiment 2: Determination of toxicity of ROCKi on meiotic and embryo competence

The effects of the ROCKi on oocyte meiotic and embryo competence are shown in Table 2. The ROCKi affected the meiotic resumption of the feline oocytes during IVM in a dose-dependent manner. ROCKi at 10 μM did not improve MII rates compared to the control (66.3 ± 6.2% *vs*. 58.0 ± 4.3%). High concentrations of the inhibitor (20 μM and 40 μM) significantly decreased meiotic competence, which appeared to be caused by a high incidence of MI arrest (Table 2). By contrast, treating the oocytes with the ROCKi during IVM did not affect embryo developmental competence, in terms of cleavage, or morula or blastocyst formation rates.

Experiment 3: Effects of different concentrations of ROCKi on the development of oocytes and embryos following vitrification and warming

Vitrification negatively affected the meiotic resumption and developmental competence of immature feline oocytes. The addition of the ROCKi in the maturation medium did not significantly improve the rate of oocyte entry into MII. ROCKi treatment of vitrified–warmed oocytes significantly induced meiotic arrest at MI, irrespective of the ROCKi concentration ($P < 0.05$). In addition, the highest concentration of the inhibitor (40 μM) significantly reduced MII rates when compared with the lower concentrations and the control (Table 4). Supplementation with the ROCKi (40 μM) also significantly increased the generation of degenerate oocytes compared with the control and lower ROCKi treatment conditions ($P < 0.05$).

Following fertilization for 18 h, the percentages of sperm-penetrated oocytes (total fertilization) did not significantly differ among the experimental groups ($P > 0.05$, Table 3). The ROCKi at concentrations of 10 μM and 20 μM significantly improved the percentage of normal fertilization (2-pronucleus zygotes) compared with the control treatment ($P < 0.05$). Similar to its effects on oocyte maturation, the highest concentration of ROCKi (40 μM) adversely affected the percentage of normally fertilized oocytes ($P < 0.05$). Our study revealed that vitrification and warming induced degeneration of the oocytes rather than abnormal fertilization (Table 3). However, the different concentrations of the ROCKi did not statistically affect the percentages of degenerate oocytes.

Similar to its effects on the fertilization competence of the vitri-

fied–warmed oocytes, the ROCKi at 10 μM and 20 μM was found to promote developmental competence, in terms of the cleavage rate (36.1 ± 3.1 and 33.2 ± 7.5%, respectively), compared to the 40 μM dose (25.6 ± 5.1%) and the control treatment (27.4 ± 2.1%) ($P < 0.05$, Table 4). Although the differences were not statistically significant, the cleaved embryos from the 10 μM and 20 μM ROCKi treatment groups developed into morulae and blastocysts at higher rates than the control. There was no effect of the ROCKi on blastocyst quality as assessed by embryonic cell number.

Discussion

In the present study, we examined the main mediators of the ROCK cascade (LIM kinases) and the effects of a specific ROCKi on feline oocyte meiotic and developmental competence. The ROCK cascade and its roles in somatic cells have been intensively studied [31–33]. However, knowledge on the presence and functions of the LIM kinases in oocytes is fairly limited. In experiment 1, we identified *LIMK1* and *LIMK2* mRNA expression in feline oocytes. This expression indicated that, as observed in other species such as swine and mouse, the ROCK cascade plays an important role in oocyte viability and competence [5, 8, 34], particularly the homeostasis of the oocyte cytoskeleton [5, 8, 21, 35]. In addition, ROCK signaling is involved in the caspase-3-dependent apoptotic cascade [31, 36–38]. ROCK1 is phosphorylated by caspase-3, then regulates the phosphorylation of the downstream LIMK proteins, which are associated with membrane stability [31, 33, 39]. LIMK1 and LIMK2 are serine/threonine kinases that work cooperatively to control polymerization of the actin cytoskeleton via phosphorylation and inactivation of cofilin, an actin-depolymerizing factor [31, 33]. This finding is in agreement with previous reports indicating that *LIMK1* plays an essential role in controlling microtubule function [25, 32]. In the mouse, *LIMK1* was gradually detected after germinal vesicle breakdown and peaked at MI and MII [40]; it localized to the microtubule organizing center of the spindle pole of MII oocytes. Although the activity of the LIM kinases has yet to be examined throughout the time course of feline oocyte maturation, we determined the effect of the ROCKi on the levels of LIM kinase mRNA after 12 h of maturation in this study, since MI stage oocytes were expected to present predominantly at this maturation time [41].

In this study, addition of the ROCKi (10 μM) during oocyte maturation downregulated LIM kinases, but it did not significantly arrest meiotic resumption compared with the control treatment. However, the higher ROCKi concentrations (20 μM and 40 μM) used in this study proportionally increased the meiotic arrest of oocytes. This finding is similar to other studies that indicated that a high concentration (50 μM) of a ROCKi negatively affected oocyte maturation in swine and mice [5, 8, 42]. It is possible that higher concentrations of the ROCKi could further downregulate the LIM kinases, which are essential for meiotic resumption [40] and cytoskeletal reorganization [39, 43]. Interestingly, it appears that a small proportion of the MI-arrested oocytes induced by the ROCKi treatment (20 μM and 40 μM) could resume meiosis, progress to MII, and become fertilized during IVF, as the percentages of MII oocytes were lower than the cleavage rates (Table 2). This result contradicts a previous report that indicated that a ROCKi irreversibly affected porcine oocyte maturation and blocked cumulus expansion [44]. In addition, ROCKi treatment did not significantly improve the cytoplasmic maturation of the non-vitrified oocytes in terms of cleavage and blastocyst formation rates (Table 2).

In experiment 3, the nuclear and cytoplasmic maturation of feline oocytes following vitrification and warming were significantly compromised when compared with the non-frozen control oocytes. Oocytes are the largest cells in the body and contain complex structures that render them susceptible to injuries during the freezing procedure. Cryoinjuries affect meiosis, fertilization, and pregnancy rates [11–13, 15, 28, 45, 46]. The poor fertilization competence and early embryo development following vitrification (Table 3 and 4) suggest that the cryoinjuries predominantly induced oocyte degeneration rather than defective fertilization.

Our results revealed that supplementation of ROCKi in the oocyte maturation medium did not significantly improve the nuclear maturation of vitrified–warmed oocytes, although it improved as assessed by the fertilization competence and cleavage rate. Although the mechanism of the ROCKi-mediated reduction in or protection against cryoinjury during vitrification and warming is not clear, it is possible that optimal concentrations of ROCKi (10 μM to 20 μM) may influence the quality of cumulus cells and oocytes by supporting the mitochondrial and cytoskeletal dynamics of vitrified–warmed oocytes [5, 8, 47, 48]. In addition, the positive effects of the ROCKi may also be related to previous findings that a ROCKi suppressed caspase-3, -8, and -10 activity in pluripotent stem cells [36, 49–51]. Further study should be performed to clarify these pathways in order to improve the freezing technique and developmental competence of feline oocytes.

This study demonstrated the expression of members of the ROCK cascade (LIM kinases) in feline oocytes. An appropriate dose of ROCKi (10 μM) did not negatively affect oocyte and embryo development. The optimal concentration of the ROCKi promoted cytoplasmic maturation of vitrified–warmed oocytes, which is essential to their fertilization competence.

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