

Distinct effects of extra- and intracellular trehalose supplementation in freezing medium on the post-thaw developmental capacity of feline oocytes

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Abstract

Oocyte cryopreservation is a crucial technique for preserving the genetic material of female cats, yet maintaining post-thaw oocyte developmental competence remains a significant challenge. This study investigated the impact of intra- and extracellular trehalose on the developmental competence of feline oocytes following cryopreservation. In Experiment 1, immature feline oocytes were matured *in vitro* (IVM) with varying concentrations (0, 1.5, and 3 mM) of intracellular trehalose hexaacetate (Tre-(OAc)₆). The matured oocytes were then cryopreserved and thawed prior to *in vitro* fertilization and embryo culture. Experiment 2 examined the effects of extracellular trehalose supplementation at concentrations of 0, 0.125, 0.25, and 0.5 M in the freezing medium on the post-thaw developmental competence of matured oocytes. Experiment 3 evaluated the effectiveness of combining intra- and extracellular trehalose treatments in the freezing medium of matured oocytes. In all experiments, non-frozen oocytes served as a control group. The study revealed that cryopreservation generally reduced the developmental competence of embryos. However, adding 3 mM Tre-(OAc)₆ during IVM resulted in superior developmental competence of frozen-thawed feline oocytes compared with adding 0 and 1.5 mM of Tre-(OAc)₆. Additionally, 0.25 M extracellular trehalose was required to notably enhance the post-thaw developmental capacity of matured oocytes. Although the combination of 3 mM Tre-(OAc)₆ and 0.25 M extracellular trehalose in the freezing medium yielded the highest embryo developmental rates, the blastocyst formation rate was lower than when using 0.25 M extracellular trehalose alone ($24.4 \pm 2.0\%$ vs. $13.8 \pm 1.9\%$). In conclusion, intra- and extracellular trehalose supplementation during cryopreservation improved the developmental competence and quality of feline oocytes. However, further optimization of the combination of intra- and extracellular trehalose is recommended.

Keywords: cat, extracellular trehalose, freezing, intracellular trehalose, oocyte, embryo

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Introduction

Assisted reproductive technologies (ARTs) are technological processes that conserve the genetic biodiversity of endangered species. Various ARTs can be applied to animals, and those are especially beneficial for valuable animals and endangered species. Domestic cats have been used as a model for other felid species as they have physiological and biological reproductive functions similar to those of wild felids (Wildt *et al.*, 1993; Comizzoli *et al.*, 2000; Luvoni and Colombo, 2020). Cryopreservation is a method of choice for long-term preservation. Among the cryopreserved cells, oocytes are the most challenging cell types because they contain very complex structures with cryosensitive organelles and dynamic molecular changes during oocyte maturation and embryo development (Comizzoli *et al.*, 2004; Jewgenow and Zahmel, 2020). In cryopreserved feline oocytes, both immature and mature oocytes have been successfully cryopreserved by conventional controlled-rate freezing and vitrification. However, the post-thaw viability rates and developmental rates remain to be improved. Thus, optimization of freezing techniques aimed at minimizing cryodamage is required (Comizzoli *et al.*, 2004; Comizzoli *et al.*, 2009; Colombo *et al.*, 2021; Tharasanit and Thuwanut, 2021). During controlled-rate freezing, ice crystal formation occurs in intra- and extracellular compartments along with a gradual decrease in temperature. The excessive increase in ice crystals destroys normal cellular function through various processes, such as cytoskeletal disruption, cell membrane imperforation, and other cellular malfunctions, leading to cellular death (Comizzoli *et al.*, 2004; Arayatham *et al.*, 2017). To reduce ice crystal formation, cryoprotective agents (CPAs) are normally added to the freezing medium to avoid excessive cellular water during the freezing process. CPAs are divided into penetrating molecules and non-penetrating molecules. Penetrating CPAs are lipophilic molecules, such as dimethyl sulfoxide, 1,2-propanediol, and ethylene glycol, that can penetrate through the plasma membrane of cells, acting as intracellular free-water replacements. These CPAs are widely used to avoid ice crystal formation, which is lethal to cells. The unfavorable effects of penetrating CPAs are high toxicity to cellular functions (Fahy, 2010). Non-penetrating CPAs, such as yolk, protein, and sugar, are lipophobic molecules that act as osmotic controllers to prevent excessive extracellular ice crystal formation. These molecules are large and do not penetrate through the plasma membrane (Best, 2015). A combination of penetrating and non-penetrating CPAs consequently mitigates ice crystal formation during the freezing process.

Trehalose is a biological disaccharide sugar composed of two molecules of glucose at the α,α -(1,1) glycosidic bond. The trehalose molecule has lipophobicity and is chemically inert (Elbein *et al.*, 2003). Thus, trehalose has low cellular toxicity and rarely permeates through the cell membrane. During the freezing stage, a high concentration of trehalose acts as a protein- and cell membrane-stabilizing agent, preventing cellular damage and reducing ice crystal formation (Jain and Roy, 2009; Abazari *et al.*, 2015).

Recently, membrane-permeable trehalose hexaacetate (Tre-(OAc)₆) has been engineered as a penetrating trehalose and used as a penetrating CPA. Tre-(OAc)₆ self-permeates through the cytoplasm and cleaves by intracellular esterase enzymes to the natural trehalose form. Intracellular trehalose keeps its natural trehalose properties: lipophobicity, subcellular stabilization, and minimizing ice formation (Eroglu *et al.*, 2000; Arayatham *et al.*, 2022). However, the use of Tre-(OAc)₆ combined with natural trehalose as a non-penetrating CPA, has yet to be studied. This study, therefore, aimed to evaluate the effects of trehalose, in both intra- and extracellular forms and in a combination of both forms, on the freezing ability and developmental competence of frozen-thawed feline oocytes.

Materials and Methods

Ethics: The animal use protocol was followed and approved by the Chulalongkorn University of Animal Care and Use (Accession No. 2031002).

Experimental design:

Experiment 1: Effects of intracellular trehalose on the freezing ability of feline matured oocytes

The cumulus-oocyte complexes (COCs) were matured *in vitro* for 24 h in the presence of varying concentrations of trehalose hexaacetate (Tre-(OAc)₆) (1.5 and 3 mM). The oocytes were then conventionally cryopreserved and stored in liquid nitrogen until further steps. To examine the efficiency of intracellular trehalose, the frozen oocytes were thawed and evaluated for quality and developmental competence. Developmental competence was assessed following *in vitro* fertilization and culture. The percentages of embryos in the cleavage, morula, and blastocyst stages were examined. The quality of the blastocysts was further determined by cell number.

Experiment 2: Effects of extracellular trehalose on the freezing ability of frozen-thawed feline oocytes

The feline COCs were collected and matured *in vitro*. The matured oocytes were cryopreserved with a freezing medium containing different concentrations of trehalose: 0.125, 0.25, and 0.5 M. After thawing, the oocytes were subsequently *in vitro* fertilized. The developmental competence (cleavage rate, morula rate, blastocyst rate, and blastocyst cell number) of the treated oocytes was compared with the developmental competence of a control group consisting of non-treated oocytes (0 M trehalose).

Experiment 3: Effects of a combination of intra- and extracellular trehalose on the cryopreservability of feline oocytes

This study was performed similarly to Experiment 1 and Experiment 2. However, the COCs were matured in a maturation medium containing Tre-(OAc)₆ at 1.5 and 3 mM. After 24 h of maturation, the oocytes were subjected to cryopreservation in the presence of trehalose at 0, 0.125, and 0.25 M. The oocyte quality following cryopreservation was examined by means of post-thaw developmental competence.

***In vitro* maturation:** All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis,

Missouri, USA) unless otherwise specified. The *in vitro* maturation (IVM) medium consists of NaHCO_3 -buffered M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 50 $\mu\text{g}/\text{ml}$ 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. To assess the developmental competence of the oocytes, the COCs were cultured in the IVM medium and were then fertilized with frozen semen. The embryo developmental rates were determined on Days 2, 5, and 7 for cleavage, morula, and blastocyst rates, respectively. In all cases, the processes for the IVM and fertilization (IVF) of oocytes were performed as previously described by Sananmuang *et al.* (2010) and Thongkittidilok *et al.* (2014).

Oocyte cryopreservation and thawing: The matured oocytes subjected to cryopreservation were maintained at 24 °C in a freezing medium containing 10% (v/v) ethylene glycol. The freezing temperature was maintained and controlled by a programable freeze control system (CryoLogic CL-8800i, Australia). The freezing protocol was set as follows: holding at 24 °C for 5 minutes; cooling down to -6 °C at a rate of 1 °C/minute; holding for 5 minutes and seeding; holding for 5 minutes; cooling down to -33 °C at a freezing rate of 0.3 °C/minute. The straws containing oocytes were immediately plunged and stored in liquid nitrogen. For thawing, the oocytes were thawed in air for 10 seconds and rapidly submerged into a 37 °C water bath for 30 seconds. The thawed oocytes were pooled and serially diluted in a thawing medium (TM) containing 0.25 M sucrose in a holding medium (HM), followed by $\frac{1}{2}\text{TM}$, $\frac{1}{4}\text{TM}$, and HM. Finally, frozen-thawed COCs were pooled in HM at 37 °C until the next step.

In vitro fertilization and in vitro culture: For *in vitro* fertilization (IVF), 10 *in vitro* matured COCs were cultured in 50 μl droplets of *in vitro* culture medium 1 (IVC-1), consisting of synthetic oviductal fluid (SOF) with 4 mg/ml BSA and an antibiotic. Frozen semen used in this study was collected from a fertility-proven tom cat. The semen was thawed at 37 °C for 30 s and evaluated before it was co-cultured with COCs. Sperm with more than 50% progressive motility was used for IVF. The total concentration of sperm that was co-cultured in each COC droplet was 5×10^5 spermatozoa/ml. All culture steps were performed at 38.5 °C in a humidified atmosphere with 5% CO_2 in the air. After 24 h in the sperm-oocyte co-culture, all COCs were denuded by a mouth-controlled glass pasture pipette. Then, the oocytes were washed twice and further cultured for 24 h in 50 μl droplets of IVC-1. During the next 24 h, the cleaved embryos were collected, washed twice, and then cultured in IVC-2 medium (SOF supplemented with 10% v/v fetal calf serum). The culture medium droplets were completely changed every other day.

Assessment of embryo development: All cleaved embryos were observed under an inverted microscope (40x magnification) to evaluate the morula and blastocyst 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 kept overnight at 4 °C before they were stained with 4',6-diamidino-2-phenylindole (DAPI, 0.1 $\mu\text{g}/\text{ml}$) in a phosphate-buffered saline solution (PBS) for 10 min for a nucleus count. The fluorescently labeled embryos were then observed using an epifluorescence microscope. The percentage of morulas (>16 cells without blastocoels) and blastocysts (>50 cells with blastocoel formation) were examined on Day 5 and Day 7, respectively.

Statistical analysis: The statistical analysis was performed using SPSS version 22 (IBM Corp., USA). Data were tested for normality and equal variance using the proportion and reported in percentages. The percentage of cleaved embryos was defined as the number of cleaved embryos relative to the total number of oocytes in each group. The proportion of each embryo stage was expressed relative to the total number of cleaved embryos. The differences in the stage of oocytes and the developmental competence (cleavage, morula, and blastocyst formation rates) were tested with a chi-square test, and the statistical comparison of cell numbers within blastocysts was performed with a one-way ANOVA. A *P* value of less than 0.05 was considered significant.

Result

Experiment 1: Effects of intracellular trehalose on the freezing ability of feline matured oocytes: The freezing and thawing process negatively affected feline developmental competence compared with that of the non-frozen-thawed feline oocytes (*P* < 0.05). The effects of intracellular trehalose by Tre-(OAc)₆ supplementation on developmental competence are shown in Table 1. Compared with the frozen-thawed groups, the Tre-(OAc)₆ supplementation groups showed improvements in the developmental rate and the average cell numbers. The cleavage rate was significantly higher in the groups supplemented with 1.5 and 3 mM of Tre-(OAc)₆ than in the control group. However, no significant differences were observed between the two concentrations of Tre-(OAc)₆. The morula rate and blastocyst cell numbers were significantly improved only in the 3 mM Tre-(OAc)₆ group.

Experiment 2: Effects of extracellular trehalose on the freezing ability of frozen-thawed feline oocytes: Table 2 shows that the developmental rate and the average blastocyst cell numbers of the frozen-thawed oocytes were significantly lower than those of the non-frozen-thawed control group. The supplementation of extracellular trehalose during the freezing process of feline oocytes significantly improved the developmental competence of post-thaw oocytes compared with that of oocytes without supplementation, as indicated by higher cleavage, morula, and blastocyst rates, as well as increased average cell numbers. Furthermore, the findings indicated that 0.25 M trehalose notably enhanced embryo development, showing superior cleavage, morula, and blastocyst rates among the frozen-thawed groups. However, there was no difference in average

cell numbers among the trehalose supplementation groups, although the group without trehalose supplementation had the lowest average cell numbers.

Experiment 3: Effects of a combination of intra- and extracellular trehalose on the cryopreservability of feline oocytes: To evaluate the effects of combinations of intra- and extracellular trehalose, different concentrations of Tre-(OAc)₆ as intracellular trehalose

(1.5 and 3 mM) and extracellular trehalose (0, 0.125, and 0.25 M) were tested. The results indicated that 3 mM of Tre-(OAc)₆ combined with 0.25 M of trehalose yielded significantly the highest rates of cleavage, morula, and blastocyst formation, as well as the highest number of cells in the blastocysts. ($45.2 \pm 6.0\%$, $17.0 \pm 1.4\%$, $13.8 \pm 1.9\%$, and 96.08 ± 6.4 cells, respectively) among all the frozen-thawed groups.

Table 1 Effects of intracellular trehalose (Tre-(OAc)₆) on the freezing ability of feline matured oocytes.

Oocytes	Tre-(OAc) ₆ conc. (mM)	N	%Cleavage*	%Morula*	%Blastocyst*	Average cell no.
Non-frozen	0	122	61.4 ± 2.5^a	36.1 ± 1.3^a	36.1 ± 1.3^a	129.0 ± 7.5^a
	0	108	26.8 ± 1.0^b	9.3 ± 1.6^b	4.6 ± 1.5^b	63.0 ± 3.5^b
Frozen-thawed	1.5	128	28.9 ± 0.4^c	9.5 ± 2.4^b	4.8 ± 1.9^b	73.1 ± 3.9^c
	3	153	29.1 ± 0.9^c	11.9 ± 2.2^c	5.4 ± 2.0^b	85.7 ± 9.5^d

Data represent mean $\pm$ SEM. ^{a,b,c,d} Different superscripts in the same column denote significant differences ($P < 0.05$). * Development in relation to the total number of COCs.

Table 2 Effects of extracellular trehalose on the developmental competence of frozen-thawed feline matured oocytes.

Oocytes	Trehalose conc. (M)	N	%Cleavage*	%Morula*	%Blastocyst*	Average cell no.
Non-frozen	0	110	64.0 ± 3.9^a	42.3 ± 3.9^a	36.7 ± 2.3^a	131.3 ± 8.4^a
	0	110	26.4 ± 0.7^b	9.9 ± 2.8^b	5.4 ± 2.5^b	61.4 ± 3.2^b
Frozen-thawed	0.125	93	50.7 ± 1.9^c	19.6 ± 4.7^c	18.7 ± 2.7^c	110.8 ± 6.8^c
	0.25	74	58.3 ± 1.4^d	26.0 ± 2.3^d	24.4 ± 2.5^d	109.2 ± 4.7^c
	0.5	79	49.2 ± 2.3^c	19.3 ± 1.6^c	14.2 ± 2.0^e	103.8 ± 7.8^c

Data represent mean $\pm$ SEM. ^{a,b,c,d,e} Different superscripts in the same column denote significant differences ($P < 0.05$). * Development in relation to the total number of COCs.

Table 3 Effects of the combination of intra- and extracellular trehalose on the developmental competence of frozen-thawed feline oocytes

Oocytes	Tre-(OAc) ₆ conc. (mM)	Trehalose conc. (M)	N	%Cleavage*	%Morula*	%Blastocyst*	Average cell no.
Non-frozen	0	0	131	63.2 ± 2.8^a	38.8 ± 3.3^a	37.3 ± 1.5^a	125.8 ± 3.8^a
	0	0	119	26.8 ± 1.1^b	10.0 ± 4.9^b	5.3 ± 0.9^b	68.5 ± 1.5^b
	0	0	73	29.4 ± 2.1^c	5.8 ± 1.7^c	4.9 ± 2.5^b	73.7 ± 4.6^b
Frozen-thawed	1.5	0.125	79	33.6 ± 3.8^{cd}	11.5 ± 1.3^b	5.2 ± 1.6^b	94.7 ± 5.1^c
		0.25	54	36.9 ± 1.2^d	16.4 ± 2.4^d	9.1 ± 1.7^c	96.5 ± 7.4^c
		0	57	33.4 ± 1.9^{cd}	8.6 ± 2.4^{bc}	5.3 ± 0.6^b	99.7 ± 11.5^c
	3	0.125	91	33.0 ± 3.2^{cd}	9.9 ± 0.5^{bc}	5.5 ± 1.8^b	89.0 ± 11.5^c
		0.25	58	45.2 ± 6.0^e	17.0 ± 1.4^d	13.8 ± 1.9^d	96.1 ± 6.4^c

^{a,b,c,d,e} Different superscripts in the same column denote significant differences ($P < 0.05$). * Development in relation to the total number of COCs.

Discussion

This study examined the effects of intra- and extracellular trehalose as cryoprotective agents on the developmental competence of frozen-thawed feline oocytes. Cryopreservation is a useful method for long-term preservation, but successful results are still hard to obtain due to cellular damage induced by ice formation during the freezing process (Best, 2015). Ice formation induces several types of cellular damage, such as cytoskeletal disruption (Comizzoli *et al.*, 2004), lipid inhomogeneity (Okotrub *et al.*, 2018; Amstislavsky *et al.*, 2019), protein denaturation (Eroglu *et al.*, 2020), intracellular organelle and membrane perforation, and terminated cellular apoptosis (Comizzoli *et al.*, 2004). In our study, the developmental rate and the total cell number were significantly lower in the frozen-thawed groups than in the non-frozen-thawed groups. This is consistent with several reports showing that cryopreservation induced irreversible cellular damage, resulting in poor

embryo development (Arayatham *et al.*, 2017; Luvoni and Colombo, 2020).

Cryoprotective agents are generally used to reduce cryodamage. The penetrating CPAs are often hydrophobic chemicals that permeate and accumulate in the intracellular fluid. However, suboptimal concentrations may lead to subcellular toxicity (Fahy, 2010). On the other hand, non-membrane-permeable trehalose was reported as the most effective osmotic controller among non-penetrating CPAs (Best, 2015). However, trehalose can be synthesized only in some prokaryotes, plants, and insects, while most mammalian cells cannot synthesize it (Elbein *et al.*, 2003). Tre-(OAc)₆ is a novel synthetic self-permeable trehalose that has been demonstrated to be an effective penetrating CPA during cryopreservation (Abazari *et al.*, 2015; Arayatham *et al.*, 2022). To our knowledge, Tre-(OAc)₆ is hydrolyzed through *deacetylation* by cellular esterase enzymes into natural trehalose molecules. This natural trehalose played an effective role in reducing ice formation. Our findings showed

that the cleavage rate and the blastocyst quality were improved by 3 mM Tre-(OAc)₆ supplementation. This indicates that intracellular trehalose provides better protection of frozen-thawed oocytes, possibly by more effectively reducing ice formation within the cell during freezing. Likewise, extracellular trehalose played a role as an osmotic controller and reduced the damage caused by extracellular ice formation. According to a previous report, Tre-(OAc)₆ self-permeated and accumulated in feline ooplasm in natural trehalose form at approximately 0.15 g/l (0.0004 M), while other mammalian somatic cells preserved normal cellular function at least up to 0.2 M (Eroglu *et al.*, 2000). In addition, Tre-(OAc)₆ slowly permeated into the cytoplasm of feline oocytes, requiring at least 6 h to reach a sufficient amount (Arayatham *et al.*, 2022). This shows the limitation of the use of Tre-(OAc)₆ in feline oocytes. Therefore, different types and species of cryopreserved cells require different types and concentrations of cryoprotective agents (Best, 2015; Tharasanit and Thuwanut, 2021). Trehalose exchanges molecular hydrogen bonds with water and biological molecules, such as the lipid bilayers of cell membranes, cellular proteins, and many other cellular substances. These strong hydrogen bonding networks induce biological stabilization, especially unfolding proteins (Olgenblum *et al.*, 2020). This study supported the idea that extracellular trehalose enhanced the quality of frozen-thawed feline oocytes by means of increasing the embryo developmental rate compared with that of embryos without trehalose supplementation. However, different trehalose concentrations (0.125, 0.25, and 0.5 M) potentially induce different osmotic pressures (329 ± 2.6, 368.8 ± 7.6, and 875 ± 7.1 mOsm, respectively). In agreement with a previous report, feline oocytes tolerated osmotic pressures in the range of 250-750 mOsm (Comizzoli *et al.*, 2008), whereas 0.5 M trehalose resulted in extremely high osmolality. High osmotic pressure could cause over-dehydration, resulting in poor freezing ability and developmental competence of feline oocytes.

The combination of intra- and extracellular trehalose (Experiment 3) was selected based on the good embryo developmental rate of intra- and extracellular trehalose in Experiments 1 and 2. The rationale for combining these treatments was to enhance cryoprotection by using both intra- and extracellular mechanisms. The results emphasized that trehalose was necessary for feline oocyte cryopreservation. However, no further improvement was observed, as the combination did not outperform extracellular trehalose supplementation alone. This suggests that the combination of intra- and extracellular trehalose did not exhibit a synergistic effect. Although trehalose is well-known for its cryoprotective properties, its optimal application may vary depending on the cellular context. Furthermore, exploring the molecular mechanisms influenced by trehalose, both inside and outside the cell, could help refine cryopreservation techniques, ensuring better survival and developmental potential of oocytes.

In conclusion, the study highlighted that trehalose was an effective cryoprotectant for feline oocytes. Cryopreservation of matured feline oocyte

supplemented with Tre-(OAc)₆ improved post-thaw developmental competence. Incubation of oocytes with 3 mM intracellular trehalose during *in vitro* maturation for 24 h was the most effective treatment. As an extracellular cryoprotective agent, trehalose functioned optimally in a concentration of 0.25 M. Furthermore, a combination of 3 mM of Tre-(OAc)₆ and 0.25 M of trehalose was effective for oocyte cryopreservation. However, no synergistic effect was observed when they were combined.

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Conflict of interest: The authors declare that there is no conflict of interest.

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