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Effects of the preservation medium and storage duration of domestic cat ovaries on the maturational and developmental competence of oocytes *in vitro*

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Abstract. We examined the effectiveness of saline, Euro-Collins solution (EC), and ET-Kyoto solution (ET-K) as preservation media for the cold storage of feline ovaries. Ovaries were maintained in these media at 4°C for 24, 48, or 72 h until oocyte retrieval. The ET-K group exhibited a higher oocyte maturation rate than the saline group after 72 h of storage. Moreover, ET-K could sustain the competence of the feline oocytes to cleave after 48 h, and the morula formation rate of the ET-K group was higher than that of the other groups after 24 and 48 h. Furthermore, the ET-K group exhibited a higher blastocyst formation rate than the other groups after storage for 24 h, and only ET-K retained the developmental competence in blastocysts after 48 h of storage. In addition, regarding the cell numbers of the blastocysts, there was no significant difference among the tested groups. In conclusion, our results indicate that ET-K is a suitable preservation medium for feline ovaries.

Key words: Blastocyst, Feline oocyte, *In vitro* culture, *In vitro* maturation, Preservation

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According to the International Union for the Conservation of Nature Red List, most feline species are currently endangered. Therefore, felines are conserved using assisted reproductive technology (ART) [1, 2]. In most ART cases for wild cats, ovaries are collected postmortem by performing ovariohysterectomy in wild habitats or zoos. These ovaries are subsequently transported to ART centers and used for research and embryo production. However, stress caused by long shipping periods to ART centers degenerates oocytes.

To date, researchers are still determining the optimum method for preserving cat ovaries. Feline oocytes have been reported to possess the ability to meiotically mature after preservation for at least 72 h at 4°C and develop into blastocysts after 24 h of preservation at 4°C [3]. However, when ovaries were preserved in phosphate-buffered saline (PBS) at 4°C for more than 24 h, their ability to develop into blastocysts significantly decreased [3]. This may be attributed to the lack of necessary components for ovary preservation in PBS. However, sufficient research on determining the components of an optimal solution for ovary preservation has not yet been performed. Using an optimum solution to preserve ovaries will enable efficient ART operations in various animal facilities.

Several organ preservation solutions, including the Euro-Collins solution (EC) and ET-Kyoto solution (ET-K), have been developed for organ transplantation in humans. EC consists of glucose and an electrolyte content similar to that of intracellular fluid (high K/low Na) (Supplementary Table 1) [4, 5]. In contrast, ET-K is a preservation medium with an electrolyte content similar to that of extracellular fluid (low K/high Na) along with trehalose, gluconate, and hydroxyethyl starch (HES) (Supplementary Table 1) [4–6]. Therefore, they have been used practically for the preservation of various human organs.

The present study aimed to evaluate more suitable preservation solutions for the prolonged storage of cat ovaries. We examined the meiotic maturation rate in Experiment 1 and the cleavage rate, morula, and blastocyst development capability in Experiment 2 after preservation of cat ovaries in saline, EC, or ET-K at 4°C for 24, 48, and 72 h.

The results of Experiment 1 are shown in Fig. 1. None of the tested groups exhibited any significant differences in maturation rates compared to the control group after 24 and 48 h of storage. However, after 72 h of storage, the maturation rates of all tested groups were lower than those of the control group ($P < 0.05$). The proportion of oocytes that reached metaphase II (MII) in the ET-K group was significantly higher than that in the saline group after storage at 4°C for 72 h ($P < 0.05$).

Figs. 2a–c shows the results of Experiment 2. Although the cleavage rate of each of the three test groups was significantly lower than that of the control group after preservation for 72 h ($P < 0.05$, Fig. 2a), only the ET-K group exhibited no significant reduction in the cleavage rate after storage for 48 h. As shown in Fig. 2b, the number of oocytes that developed into morulae in the ET-K group was significantly higher than that in any other tested groups after 24 or 48 h of storage ($P < 0.05$). Moreover, after 72 h of storage, the rate of morula formation in the ET-K group was higher than that in the saline group ($P < 0.05$). Compared with the control group, the proportion of blastocysts was significantly decreased in all three test

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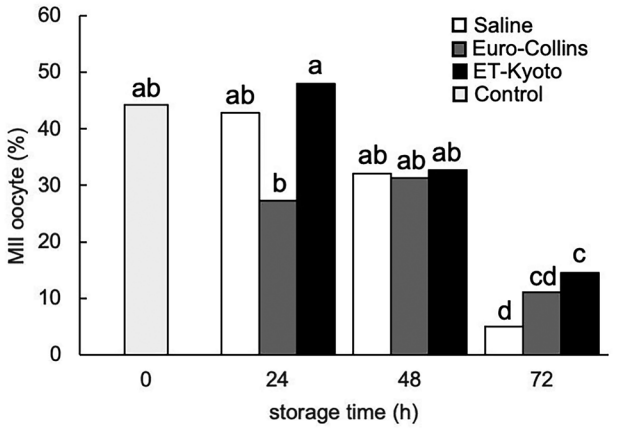

Fig. 1. The rate of oocyte maturation to metaphase II (means) after 28 h *in vitro* maturation. From a total of 1094 oocytes collected from 92 female cats, the number of oocytes examined in each group ranged from 83−140 [control: 113 oocytes; saline: 126 (24 h), 140 (48 h), and 101 (72 h) oocytes; EC: 110 (24 h), 102 (48 h), and 91 (72 h) oocytes; ET-K: 127 (24 h), 101 (48 h), and 83 (72 h) oocytes]. Within the graph, means marked with different letters are significantly different ($P < 0.05$).

groups after preservation for 72 h ($P < 0.05$). However, there was no significant difference in the rate of blastocyst formation between the control and ET-K groups after storage for 48 h. Furthermore, after storage for 24 h, the proportion of blastocysts in the ET-K group was higher than that in the saline and EC groups ($P < 0.05$). Regardless of the duration of preservation, no statistically significant differences were observed between the cell numbers of blastocysts in any of the test and control groups (data not shown).

The oocyte maturation rate of the ET-K group was higher than that of the saline group after 72 h of refrigeration. The inhibition of cell swelling is important for organ preservation [7]. After organ removal, ischemic cells switch from aerobic to anaerobic metabolism and increase lactate production, causing acidosis. Acidosis allows the influx of Cl^- into cells [8]. Moreover, low enzyme activity due to cold storage decreases ATP production. Since decreased ATP production leads to Na^+/K^+-ATPase dysfunction, Na^+ flows into the cells [9]. Along with the influx of Na^+ and Cl^-, water enters the cytoplasm, resulting in edema. Hence, the maturation rates of feline and bovine oocytes decrease by exposing them to sub-optimal medium because of cell edema [10, 11]. Oxidative stress also reduces oocyte maturation capacity [7]. Oxidative stress oxidizes membrane lipids, and the resulting lipid peroxides injure proteins and DNA. The preservation of feline ovaries in PBS for over 48 h has been observed to increase ROS and degenerate oocyte DNA [12]. Moreover, preservation in PBS for 72 h decreased the nuclear maturation rates [12]. The PBS

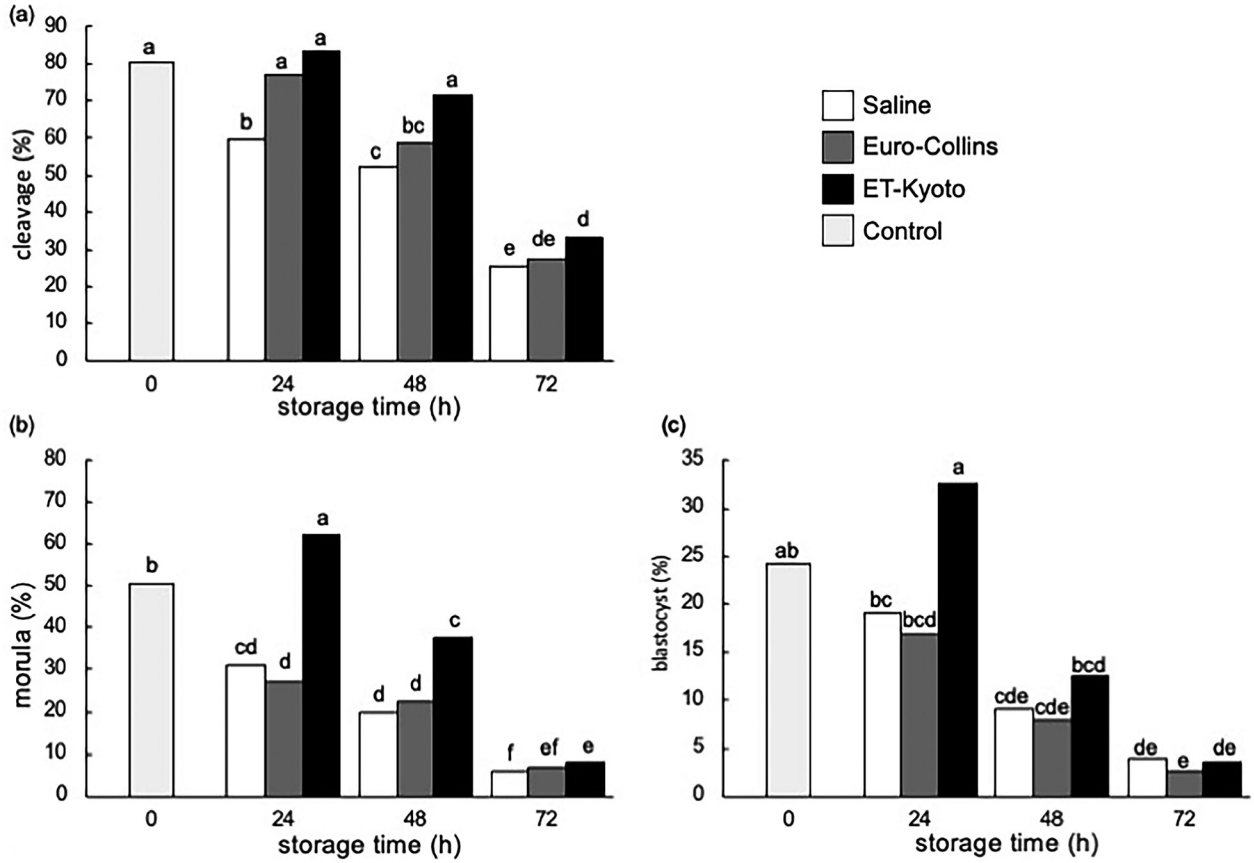

Fig. 2. a) The cleavage rates, b) rates of development to morulae, and c) rates of development to blastocysts (means) after *in vitro* culture. From a total of 1168 oocytes collected from 103 female cats, the number of oocytes examined in each group ranged from 102−141 [control: 111 oocytes; saline: 141 (24 h), 111 (48 h), and 102 (72 h) oocytes; EC: 118 (24 h), 112 (48 h), and 117 (72 h) oocytes; ET-K: 132 (24 h), 112 (48 h), and 112 (72 h) oocytes]. Within the graphs, means marked with different letters are significantly different ($P < 0.05$).

and saline used in this study do not contain substances that can inhibit cell edema and oxidative stress. Although the glucose in EC can produce enough osmotic pressure to suppress cell swelling, the ability of these components lasts only for 48 h. Eventually, it decreases after 72 h due to the breakage of the cell membrane, as observed in a previous study regarding kidney preservation [13]. In this study, no differences were observed between the oocyte maturation rates of the EC and control groups after 48 h of storage, suggesting that glucose suppresses cell swelling. In contrast, ET-K does not contain Cl^- but includes gluconate and HES, which can suppress cell and interstitial edema [14, 15]. Moreover, ET-K contains trehalose, which stabilizes the cell membrane, has an antioxidant effect, and creates enough osmotic pressure to suppress cell edema. Hence, it is suggested that the sufficient action of these components prolonged the preservation time of cat oocytes in the ET-K group.

In this study, the rate of morula formation in the ET-K group was significantly higher than that in the other tested groups after storage for 24 and 48 h. Similar to the maturation rate, cell edema and oxidative stress negatively affected oocyte competence [10, 16, 17]. Although the effect of cell edema on embryonic development competence has not been examined in feline species, cell edema caused by hypotonic treatment has been observed to reduce the blastocyst formation rate of porcine oocytes and the 8-cell to morula development and blastocyst formation rates of bovine oocytes by causing aberrant spindle formation [10, 16]. A previous study showed that adding the antioxidant superoxide dismutase (SOD) to PBS increased morula development rates after 24, 48, and 72 h of preservation [17]. In that study, the addition of SOD resulted in an increase in the expression of the anti-apoptotic gene *Bcl-2*, and a decrease in the expression of the pro-apoptotic gene *Bax* in the cat ovary tissue [17]. Based on the above findings, we believe that gluconate, HES, and trehalose create ambient osmotic pressure, and trehalose protects oocytes from oxidative stress. This prevented the formation of abnormal spindles in feline oocytes and protected them from apoptosis. Therefore, ET-K could prolong the preservation time of feline oocytes and sustain their developmental competence to form morula.

In this study, only the ET-K group did not exhibit a significant reduction in the rate of blastocyst formation after storage for 24 and 48 h compared to the control. Moreover, the blastocyst formation rate of the ET-K group was higher than that of the saline and EC groups after 24 h of storage. In another study, when feline ovaries were stored in PBS containing the antioxidant relaxin at 4°C for 24 h, a blastocyst formation rate of 16% was observed [18]. Additionally, it was reported that the blastocyst formation rate was 9.1% to13% after 24 h of preservation in PBS, and that oocytes failed to develop into blastocysts after 48 h of storage in PBS [3, 17]. Although a simple comparison is impossible because of differences in culture conditions, in this study, the blastocyst formation rates of the ET-K group after 24 h (32.6%) and 48 h (12.5%) of preservation were relatively higher than those previously reported [3, 17, 18]. Our results collectively suggest that gluconate, HES, and trehalose positively affect the cold storage of feline oocytes in terms of their capacity to develop into blastocysts and morulae.

Although the ET-K group exhibited a higher rate of morula formation than any other tested group, there was no significant difference in the rate of blastocyst formation between ET-K and any other tested group after 48 h of storage. Therefore, we speculate that this may have been caused by a developmental block at the morula-to-blastocyst stage, which is often reported in feline embryos [19]. Hence, oocytes in the ET-K group may have developed into the morula stage but not into the blastocyst stage because of the morula block. From these observations, it may be inferred that prolonged storage weakens the developmental competence of feline oocytes. Therefore, oocytes may not overcome the morula block after prolonged storage due to this lack of competence.

The number of blastomeres is used as an indicator of embryo quality [20]. In this study, the preservation medium and storage time did not affect the number of blastomeres; hence, it may be inferred that the quality of blastocysts does not deteriorate even after prolonged storage. However, the number of blastocysts used in this study was too small to derive a statistically significant conclusion. Hence, further investigations are warranted to establish this possibility conclusively.

This study demonstrates that ET-K can maintain the nuclear maturation ability and competence of oocytes to develop into morula and blastocyst after fertilization *in vitro* longer than saline and EC. Furthermore, the blastocyst formation rate of the ET-K group was higher than that of the saline and EC groups after preservation for 24 h. Based on these results, we conclude that ET-K is suitable for the prolonged preservation of feline ovaries.

Materials and Methods

In this study, cat ovaries and testes with epididymides were collected from a local veterinary clinic (Osaka, Japan) following routine ovariohysterectomies and castrations. The animals were neither operated on nor killed for the purposes of this study. The use of tissues was approved by a local veterinary clinic. All cats were privately owned, and each owner's consent was obtained before sample collection.

Ovarian storage, collection of cumulus–oocyte complexes (COCs), and in vitro maturation (IVM)

The ovaries of domestic cats (≥ 5 months) were randomly preserved in physiological saline (Otsuka Pharmaceutical, Tokushima, Japan), EC (Irom Pharmaceutical, Tokyo, Japan), or ET-K (Otsuka Pharmaceutical) for 24, 48, or 72 h and stored at 4°C until use. The COCs were then collected from fresh and individually stored ovaries and matured *in vitro* as described by Alam *et al*. [21]. The collected COCs were rinsed three times with IVM medium, which contains Medium 199 (Thermo Fisher Scientific, MA, USA) supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Corp., Mo, USA), 10 IU/ml 17β-estradiol (Sigma-Aldrich), 100 µg/mL gentamycin (Sigma-Aldrich), 137 µg/ml sodium pyruvate (Sigma-Aldrich), 0.02 IU/ml Follistim® (human recombinant follicle-stimulating hormone; MSD, Tokyo, Japan), and 25 ng/ml epidermal growth factor (Sigma-Aldrich). The COCs were then incubated in IVM medium droplets covered with mineral oil (Sigma-Aldrich) at 38.5°C in humidified air (5% CO_2) for 28 h.

Assessment of nuclear maturation

Oocyte maturation was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I, telophase I, or MII. Oocytes in the MII stage, with the first polar body, were characterized as mature oocytes. The meiotic stage of oocytes was determined following the procedure described by Kanegi *et al*. [22] with minor modifications. Cumulus cells were removed from the oocytes via gentle pipetting. The oocytes were washed twice with PBS(–) (PBS without Ca^{2+} and Mg^{2+}, Nacalai Tesque, Kyoto, Japan) and were subsequently permeabilized and fixed with 3.7% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) and 1% (v/v) Triton X-100 (Merck) in PBS(–) at room temperature (22–25°C) for 15 min. The oocytes were then placed in PBS(–)

containing 0.3% (w/v) polyvinylpyrrolidone (Sigma-Aldrich) at room temperature (22–25°C) for 15 min. To determine the meiotic stages, the fixed oocytes were transferred into a small drop containing PBS(–) supplemented with 90% (v/v) glycerol (Sigma-Aldrich) and 10 µg/ml Hoechst 33342 (bisbenzimide, Sigma-Aldrich) for 5 min, mounted on a slide, overlaid with a coverslip, and incubated at 4°C until assessment under a fluorescence microscope.

Sperm collection and cryopreservation

Feline testes were obtained from a local veterinary clinic. After removing external tissues from the testes, we separated the epididymides. The epididymides were placed in PBS(–), cut into small pieces, and incubated at 38.5°C under 5% CO_2 in humidified air for 10 min. After filtering through a 20-µm filter (Nipro, Osaka, Japan), the semen was centrifuged for 5 min at 500 × *g*, and the seminal plasma was removed by aspirating the supernatant.

The pelleted sperm was resuspended in EYT-FC solution, which contains egg yolk supplemented with 13 µg/ml citric acid (Nacalai Tesque), 10 µg/ml D-fructose (Nacalai Tesque), 24 µg/ml Tris aminomethane (Nacalai Tesque), 1000 IU/ml penicillin (Sigma-Aldrich), and 1 mg/ml streptomycin (Sigma-Aldrich), and stored at 4°C. After 1 h, an EYT-FC solution containing 14% (v/v) glycerol (Nacalai Tesque) was added to the existing solution to obtain a final cell density of 12.5 × 10^6 cells/ml and a final glycerol concentration of 7% v/v. This solution was then loaded into 0.25-ml straws (Fujihira, Tokyo, Japan). After sealing, the straws were laid horizontally on a rack, placed 4 cm above the surface of liquid nitrogen for 5 min, plunged into liquid nitrogen, and stored in a liquid nitrogen storage tank until further analyses.

In vitro fertilization (IVF)

The straws containing cryopreserved sperm were thawed by soaking in warm water (37°C) for 30 sec. The sperm were then released into modified human tubal fluid (m-HTF, Nippon Medical & Chemical Instruments Co. Ltd., Osaka, Japan) and centrifuged for 5 min at 500 × *g*. After centrifugation, the sperm were resuspended in HTF (Nippon Medical & Chemical Instruments Co. Ltd.), centrifuged for 5 min at 500 × *g*, and the cell density was adjusted to 1.5 × 10^6 cells/ml in IVF medium (HTF with 0.3% BSA). After 28 h of IVM, 5–10 COCs were washed twice in IVF medium and cultured in 100 µl IVF medium droplets covered with mineral oil at 38.5°C in humidified air (5% CO_2) for 18 h.

In vitro culture (IVC)

After IVF, cumulus cells were removed from the COCs by gentle pipetting, and the resulting denuded oocytes were used for IVC. Approximately 10 oocytes were incubated in 100 µl of IVC I medium for 2 days and then in IVC II medium for 5 days in humidified air (5% O_2, 5% CO_2, and 90% N_2). The IVC I and II media were composed of Only-One Medium (Nippon Medical & Chemical Instruments Co. Ltd.) supplemented with 0.3% (w/v) BSA and 5% (w/v) fetal bovine serum (Biosolutions International, Melbourne, Australia), respectively. All morulae and blastocysts were stained as described in our previous studies [22]. We classified embryos with more than 16 cells as morulae and designated embryos with blastocoel as blastocysts.

Statistical analyses

All experiments were independently repeated at least seven times. As for the estimation of the oocyte maturation rate and zygote development rates (cleavage, morula, and blastocyst), the results are expressed as the mean. The results were analyzed using the chi-square test for independence and Ryan's procedure for statistical differences. As for the cell numbers in the blastocysts, results are expressed as the mean ± standard error of the mean (SEM). Homogeneity of variance was determined using the Bartlett test (Statcel 3; OMC Publishing Inc., Saitama, Japan). Statistical differences were evaluated via analysis of variance and the Tukey-Kramer method (Statcel 3). In all cases, P-values < 0.05 were considered statistically significant.

Conflict of Interests: The authors declare that there are no conflicts of interest.

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