Vitrification of Canine Oocytes

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Abstract: *The objective of the present study was to compare the vitrification method for cryopreservation of canine oocytes. Canine cumulus-oocyte complexes (COCs) were collected from ovaries, and were vitrified by ethylene glycol based (E30S) or DMSO based (DAP213) methods. In the E30S method, COCs were exposed to the vitrification solution, composed of 30% ethylene glycol and 0.5 M sucrose, step-wise transferred onto a cryotop holder, then plunged directly into liquid nitrogen. In the DAP213 method, COCs were exposed to 1 M DMSO and DAP213 solution in a cryotube, and thereafter plunged directly into liquid nitrogen. Although vitrified-warmed COCs in the E30S method showed fewer morphological abnormalities, and higher viability than those in the DAP213 method, there was no significant difference in between. These results indicate that either method of vitrification is available and statistically comparable for cryopreservation of canine oocytes.*

Key words: *Dog, Oocyte, Vitrification, Cryopreservation*

Introduction

Assisted reproductive techniques (ART) of canine species such as *in vitro* maturation (IVM), culture and cryopreservation of the genetic resource materials have limited application *per se*, when compared to those for other experimental and domestic animals. However, they can be useful for improved breeding of companion and working dogs, including guide dogs for the blind. Although guide dogs remarkably contribute to the improvement of the quality of life of blind people in the world, many countries suffer from an acute shortage of guide dogs. Even among Labrador Retrievers, which are particularly suited to the role, only 30–40% of the dogs that are trained become guide dogs in Japan. Current figures indicate that approximately 950 dogs are actively engaged in guiding blind people, however, this number is low in light of the estimated demand, which ranges between 4,800–7,800, including latent needs in Japan. ART would help make it possible to overcome one of the problems. Although there are some reports on IVM of oocytes and culture of embryos in [1, 2], no attempt has been made to cryopreserve canine oocytes and embryos, and then perform embryo transfer (ET). Vitrification has been widely developed to apply to cryopreservation of mammalian embryos. In the mouse, embryo cryopreservation by a vitrification method utilizing a sampling tube with DAP213 solution (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) as a vitrification solution (DAP213 method) has been proven successful [3]. Moreover, it is possible to vitrify canine ovarian tissues by the DAP213 method [4]. Porcine oocytes were vitrified successfully using a cryotop sheet following exposure to vitrification solution by the step-wise method (E30S method) [5]. However, the suitability of both vitrification methods for canine embryos has not been investigated.

The objective of the present study was to compare the DAP213 with E30S methods for vitrification of canine germinal vesicle (GV) stage oocytes, to improve the breeding management programs for guide dogs for the blind.

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Materials and Methods

Collection of cumulus oocyte complexes (COCs)

Ovaries within the ovarian bursa from bitches of mixed breed at random stages of the estrous cycle were collected at slaughterhouses and transported to the laboratory in a thermos flask containing sterile saline at approximately 37°C. Each ovary was cleaned of fat and blood vessels and placed in a Petri dish containing TCM199 medium (Gibco-Invitrogen Life Technologies, NY, USA) supplemented with 10% fetal calf serum, 100 units/ml penicillin G potassium (Meiji, Tokyo, Japan) and 100 μg/ml streptomycin sulfate (Meiji, Tokyo, Japan), for further dissection. Ovarian tissue was sliced by a surgical blade (Feather, Osaka, Japan) repeatedly to collect COCs. Only COCs with more than two layers of cumulus cells and a homogeneous dark cytoplasm ≥100 μm in diameter were used in this study. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA) except for those specifically described.

The tissues and cells derived from animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Vitrification and Thawing

1) DAP213 method

The COCs were pretreated with PB1 medium [6] containing 1 M dimethyl sulfoxide (DMSO) at room temperature (23 ± 2°C). The COCs were transferred into a 1 ml cryotube (Nalge Nunc International, Tokyo, Japan) containing 5 μl of 1 M DMSO, which was then placed in ice water for 5 min to allow DMSO to thoroughly bathe the COCs. Subsequently, 95 μl of DAP 213 solution (2 M DMSO, 1 M acetamide, and 3 M propylene glycol) [3], maintained at 0°C, were added to each cryotube. After the cryotubes had been placed in ice water for 5 min, they were plunged directly into liquid nitrogen and stored until use. For thawing, the samples were taken from the liquid nitrogen and allowed to stand at room temperature (23 ± 2°C) for 60 sec, and then diluted with 900 μl of PB1 medium (37°C) containing 0.25 M sucrose. The recovered COCs were transferred to PB1 medium and washed 5 times.

2) E30S method

The COCs were exposed to PB1 containing 5, 10 and 20% ethylene glycol (EG), and 30% EG containing 0.5 M sucrose for 5, 2, 2 and 1 min, respectively, at room temperature (23 ± 2°C) [5]. They were then placed on a cryotop sheet (Kitazato Supplies, Japan) [7], and the cryotop was immediately plunged into liquid nitrogen. The COCs were warmed at 37°C, and the cryoprotectants were removed in a step-wise manner at 37°C: the cryotop holder was transferred from LN_2 into PB1 with a sequential series of 0.5, 0.25, and 0.125 M sucrose, 1 min in each solution at 37°C, and finally transferred into PB1 for 5 min at 37°C.

Examination of vitrified-warmed GV oocytes

After thawing, the oocytes were denuded of cumulus cells in PB1 using a fine-bore pipette by repeated aspiration and expulsion. The cumulus-free oocytes were stained with 20 μg/ml propidium iodide (PI) in phosphate buffered saline containing 0.1% polyvinyl alcohol and incubated for 15 min in darkness. The oocytes were examined under ultraviolet light using an epifluorescence microscope (Nikon, Japan) and plasma membrane integrity of oocytes was assessed. The oocytes with disrupted plasma membrane were dyed red with PI.

Statistical analysis

Data on oocyte survival were compared using the chi-square test as presented by StatView software (Abacus Concepts, Inc., Berkeley, CA, USA). Differences were considered significant at a level of $P<0.05$.

Results

Morphology of fresh COCs and vitrified-warmed COCs by the DAP213 and E30S methods are shown in Fig. 1. In both the vitrification methods, some vitrified-warmed COCs had partly dispersed cumulus cells and disrupted cytoplasm, however, the majority of vitrified-warmed COCs were morphologically intact. The recovery rates of COCs in the DAP213 and E30S groups were 97.5 and 92.7%, respectively (Table 1). In both the vitrification methods, about 60% of the vitrified-warmed oocytes showed normal morphology. However, there was a tendency for the viability of oocytes, as assessed by PI stain, in the step-wise group vitrified with E30S to be higher than those in the DAP213 group (17.6% and 5.1%, respectively, $P>0.05$) (Table 1 and Fig. 2).

Discussion

Although there were no significant differences in the recovery rates and morphological normality of the denuded canine oocytes between the E30S and DAP213 methods (Fig. 1 and Table 1), the percentage

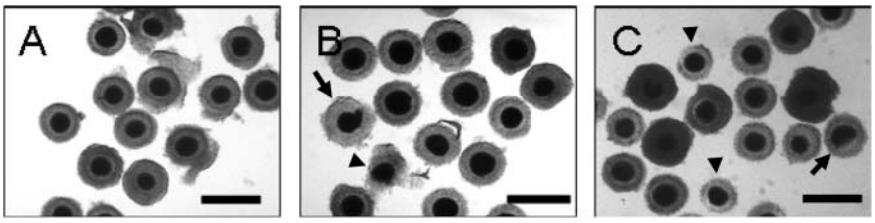

Fig. 1. Morphological figures of the cumulus oocyte complexes (COCs). (A) Fresh; (B) vitrified-warmed by the DAP213 method; (C) vitrified-warmed by the E30S method. Some vitrified-warmed COCs had partly dispersed cumulus cells (arrow head) and disrupted cytoplasm (arrow). Bar = 200 μm.

Table 1. Viability of canine GV oocytes after vitrification

Method	No. of vitrified oocytes	No. of oocytes examined (%)*	No. (%) of normal oocytes**	
			Morphology	PI stain
Fresh	–	20	20 (100)[a]	19 (95.0)[a]
E30S	55	51 (92.7)	30 (58.8)[b]	9 (17.6)[b]
DAP213	40	39 (97.5)	24 (61.5)[b]	2 (5.1)[b]

*The percentages of examined oocytes of vitrified oocytes. **The percentages of normal oocytes of oocytes that were examined for morphology or by PI stain. Values with different superscripts in the same column are significantly different at $P<0.05$.

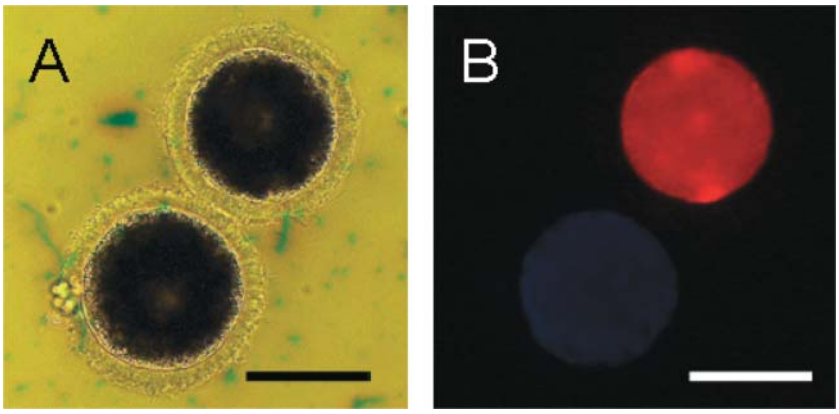

Fig. 2. Morphological appearance of canine oocytes after vitrification by the E30S method. Both oocytes showed morphologically normal appearance under light microscopy (A); however, PI stain revealed that one of the oocytes (upper) was damaged (B). Bar = 100 μm.

of oocytes with integral plasma membrane as measured by PI stain in the E30S group was higher than that in the DAP213 group ($P>0.05$). These results suggest that the integrity of oocytes after cryopreservation can not be evaluated by morphology alone, and it may be necessary to make histochemical examinations such as with PI stain. Further study is required to examine the potential of maturation and subsequent fertilization in vitrified canine oocytes. Canine reproductive physiology is considerably different from other mammalian species. For example, oocytes are ovulated at the germinal vesicle stage (an immature diploid stage) and complete their meiotic maturation in the oviduct. Thus, canine oocytes and embryos spend a long time prior to implantation in the reproductive tract. Due to these singular reproductive features, the actual situation and mechanisms of early development such as oocyte maturation, fertilization and subsequent

embryogenesis are not fully understood in canine species, as compared to many other domestic mammalian species. Thus, the efficiency of in vitro maturation of canine oocytes remains very low compared with that of other domestic animals.

Because vitrification is a non-equilibrium cryopreservation method that needs relatively high concentrations of cryoprotectants, a step-wise addition of cryoprotectants may reduce the toxic effect of cryoprotectants and is considered to minimize damage due to extreme cell-volume expansion [8]. In fact, for vitrification of bovine GV oocytes, three-step exposure to cryoprotectants showed less damage than the single-step procedure [9]. Aono *et al.* [10, 11] reported high survival rates and subsequent production of blastocysts of mouse GV oocytes after vitrification by a step-wise manner with permeable cryoprotectants for treatment of oocytes. They suggested that osmotic injury to cells occurring in the GV oocytes is due to the osmotic stress accompanying treatment with permeable cryoprotectants. On the other hand, the injury to the cells in the process of cryopreservation can be due to osmotic effects accompanying treatment with permeable cryoprotectants [12, 13]. Fuku *et al.* [14] and Kasai *et al.* [15] proposed that the supplementation of saccharides such as sucrose into the vitrification medium would reduce toxicity to the embryos by reducing the extracellular concentration of the cryoprotectant. In our present study, the DAP213 and E30S methods were used with two and four-step exposures to vitrification solutions, respectively. Moreover, sucrose was used as the cryoprotectant only in the E30S method. Thus, it may have been the cause of the difference in membrane integrity between the vitrification methods.

High cooling and warming rates are required to prevent ice crystal formation and reduce severe chilling injury when oocytes are cryopreserved by vitrification. In efforts to increase cooling and warming rates during vitrification, modification of the methods has taken place, especially the development of various containers, such as open-pulled straws [16], cryoloops [17] and cryotops [18]. In the present study, the E30S method using the cryotop required very small volumes of oocyte suspension liquid (less than 1 μl), while the oocytes were suspended in 100 μl of vitrification solution in the DAP213 method. Minimizing the volume of the solution in which oocytes are vitrified might result in higher viability of oocytes after vitrification. Moreover, the lower temperature of the vitrification solution may have decreased the viability of the oocytes in the DAP213 group, since oocytes were treated with vitrification solution at room temperature in the E30S method, and oocytes were kept at 0°C for 10 min in the DAP213 method. However, both methods of vitrification were statistically comparable for morphology for the cryopreservation of canine oocytes.

This study also showed that vitrified-warmed oocytes in both methods were damaged and had decreased integrity of the plasma membrane. The sensitivity of canine oocytes to cryopreservation may be related to their high lipid content and they may become tolerant to cryopreservation if their lipid content is reduced. In porcine, the high sensitivity of oocytes and embryos to cryopreservation has been ascribed to their high cytoplasmic lipid content, and the removal of cytoplasmic lipid droplets improved the survival of porcine oocytes and embryos [19, 20]. Thus, further study seems to be required to develop an optimal cryopreservation method for canine oocytes.

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