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 (20-25°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.

E30S mehod

The COCs or embryos 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 (20-25°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 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.

Acknowledgements

This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Funds from the Ministry of Health, Labour and Welfare of Japan.

5

References

1) Rodrigues, Bde A., dos Santos, L.C. and Rodrigues, J.L. (2007): Effect of maturation medium on in vitro cleavage of canine oocytes fertilized with fresh and cooled homologous semen. Zygote, 15, 43-53.

2) Otoi, T., Shin, T., Kraemer, D.C. and Westhusin, M.E. (2004): Influence of maturation culture period on the development of canine oocytes after in vitro maturation and fertilization. Reprod. Nutr. Dev., 44, 631-637.

3) Nakagata, N. (1993): Survival of mouse morulae and blastocysts derived from in vitro fertilization after ultra rapid freezing. Jikken Dobutsu, 42, 229-231.

4) Ishijima, T., Kobayashi, Y., Lee, D.S, Ueta, Y.Y, Matsui, M., Lee, J.Y., Suwa, Y., Miyahara, K. and Suzuki, H. (2006): Cryopreservation of canine ovaries by vitrification. J. Reprod. Dev., 52, 293-299.

5) Fujihira, T., Kishida, R. and Fukui, Y. (2004): Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. Cryobiology, 49, 286-290.

6) Whittingham, D.G. (1974): Embryo banks in the future of developmental genetics. Genetics, 78, 395-402.

7) Katayama, K.P., Stehlik, J., Kuwayama, M., Kato, O. and Stehlik, E. (2003): High survival rate of vitrified human oocytes results in clinical pregnancy. Fertil. Steril. 80, 223-224.

8) Rall, W.F. (1987): Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology, 24, 387-402.

9) Abe, Y., Hara, K., Matsumoto, H., Kobayashi, J., Sasada, H., Ekwall, H.,

Rodriguez-Martinez, H. and Sato, E. (2005): Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biol. Reprod., 72, 1416-1420.

10) Aono, N., Naganuma, T., Abe, Y., Hara, K., Sasada, H., Sato, E. and Yoshida, H. (2003): Successful production of blastocysts following ultrarapid vitrification with step-wise equilibriation of germinal vesicle-stage mouse oocytes. J. Reprod. Dev., 49, 501-506.

11) Aono, N., Abe, Y., Hara, K., Sasada, H., Sato, E. and Yoshida, H. (2005): Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified stepwise method, SWEID. Fertil. Steril., 84, Suppl 2, 1078-1082.

12) Kuwayama, M., Fujikawa, S. and Nagai, T. (1994): Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1,2-propanediol using 2-step and 16-step procedures. Cryobiology, 31, 415-422.

13) Mtango, N.R., Varisanga, M.D., Dong, Y.J., Otoi, T. and Suzuki, T. (2001): The effect of prefreezing the diluent portion of the straw in a step-wise vitrification process using ethylene glycol and polyvinylpyrrolidone to preserve bovine blastocysts. Cryobiology, 42, 135-138.

14) Fuku, E.J., Liu, J. and Downey, B.R. (1995): In vitro viability and ultrastructural changes in bovine oocytes treated with a vitrification solution. Mol. Reprod. Dev., 40, 177-185.

15) Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. and Machida, T. (1990): A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. Mol. Biotech., 89, 91-97.

16) Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T. and Callesen, H. (1998): Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51, 53-58.

17) Lane, M., Bavister, B.D., Lyons, E.A. and Forest, K.T. (1999): Containerless vitrification of mammalian oocytes and embryos. Nat. Biotechnol., 17, 1234-1236.

18) Hochi, S., Terao, T., Kamei, M., Kato, M., Hirabayashi, M. and Hirao, M. (2004): Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. Theriogenology, 61, 267-275.

19) Ushijima, H., Yoshioka, H., Esaki, R., Takahashi, K., Kuwayama, M., Nakane, T., and Nagashima, H. (2004): Improved survival of vitrified in vivo-derived porcine embryos. J. Reprod. Dev., 50, 481-486.

20) Hara, K., Abe, Y., Kumada, N., Aono, N., Kobayashi, J., Matsumoto, H., Sasada, H., and Sato, E. (2005): Extrusion and removal of lipid from the cytoplasm of porcine oocytes at the germinal vesicle stage: centrifugation under hypertonic conditions influences vitrification. Cryobiology, 50, 216-222.

Figure Legends

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.

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.

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 $P<0.05$.

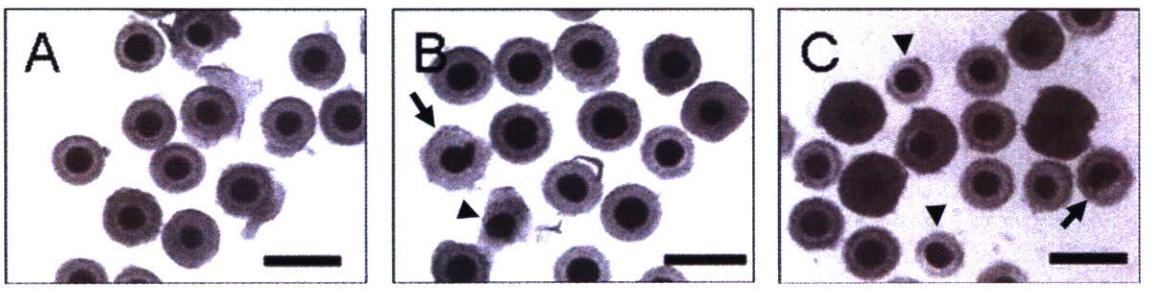

Fig. 1

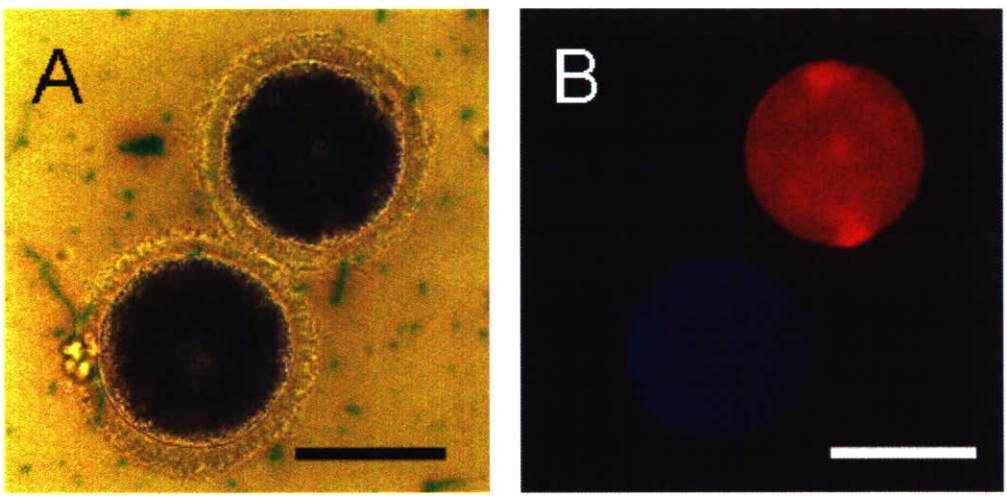

Fig. 2

イヌ卵子のガラス化保存

阿部靖之[1]・Dong-Soo Lee[1]・Sang-Keun Kim[2]・鈴木宏志[1,3]

[1]帯広畜産大学原虫病研究センター, 帯広市　〒080-8555,　[2]College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea, [3]東京大学大学院医学系研究科　〒113-0033

イヌ卵子を凍結保存するにあたって、２種類のガラス化法について比較検討した。卵巣から採取した卵子卵丘細胞複合体(COC)を30%エチレングリコール、0.5Mシュクロースを保存液としてクライオトップを用いてガラス化する方法(E30S)と2M DMSO、1Mアセトアミドおよび3Mプロピレングリコールを保存液としてクライオチューブでガラス化保存する方法(DAP213)を比較した結果、統計学的な有意差は認めなかったものの、E30Sのほうが DAP213 よりも正常な卵子の割合が高い傾向であった。イヌ卵子の保存にガラス化保存が適用可能であることが示唆された。

キーワード：イヌ、卵子、ガラス化保存

Advance Publication

Journal of Reproduction and Development

Accepted for publication: November 28, 2007
Published online: January 17, 2008

-Research Note-

Preimplantation Development of Embryos in Labrador Retrievers

Yasuyuki ABE[1)], Yoshinori SUWA[2)], Yoshiko YANAGIMOTO UETA[1)] and Hiroshi SUZUKI[1, 3)]

[1)]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, [2)]Hokkaido Guide Dog Association, Minami-ku, Sapporo, Hokkaido, 005-0030, and [3)]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, 113-0033, Japan

Running title: PREIMPLANTATION DEVELOPMENT IN DOGS /ABE *et al.*

Correspondence: H. Suzuki (e-mail: hisuzuki@obihiro.ac.jp)

Abstract. Preimplantation development of canine embryos is not well understood. To understand the timing of preattachment embryogenesis relative to the LH surge, early embryonic development was examined in Labrador Retrievers after artificial insemination. The embryos migrated from the oviduct to the uterus beginning on day 11 after the LH surge. This transport must be completed within 24 h. By day 13 after the LH surge, all of the embryos had moved and were localized in the uterus. The embryos developed to the morula stage within 11-13 days and to the blastocyst stage within 14 days after the LH surge, respectively. These findings add to the current understanding concerning the physiology of preimplantation development and should help further develop assisted reproductive techniques in canine species, such as cryopreservation and subsequent embryo transfer.

Key words: Dog, Embryo, Preimplantation development.

The dog is a monoestral polyovulatory nonseasonal species. Canine reproductive physiology is considerably different from other mammalian species. The plasma progesterone concentration of the dog begins to increase a few days before ovulation. Preovulatory luteinization is typical in dogs. The oocytes of dogs are ovulated at the germinal vesicle stage (an immature diploid stage), and they complete 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, have not been fully elucidated for canine species compared with many other domestic mammalian species [1].

On the other hand, large numbers of canines are produced and used as working dogs as well as companion animals in many parts of the world. It seems likely that application of assisted reproductive techniques, such as *in vitro* fertilization, embryo transfer, artificial insemination and cryopreservation of gametes and zygotes, would be useful for improved breeding of working dogs, such as guide dogs for the blind, as has proven to be the case in other domestic animals. However, development of assisted reproductive techniques for canines has been poor, with the exception of artificial insemination [2]. In order to develop methods of transfer and cryopreservation of embryos, it is essential to understand early embryonic development *in vivo* in all mammals. However, very little information has been reported regarding the preimplantation development of embryos after mating in dogs. It is believed that fertilized eggs develop to the 2-cell stage 6-10 days after the LH surge and that they migrate to the uterus 11-12 days after the LH surge in domestic dogs [3, 4]. However, the timing of preattachment embryogenesis relative to ovulation has yet to be

determined [5]. Thus, to obtain information regarding early embryonic development in the Labrador Retriever, one of the most utilized working dog breeds in such roles as guide dogs for the blind, embryos were recovered from excised reproductive tracts after artificial insemination, and then the stages and localizations of the embryos were determined.