

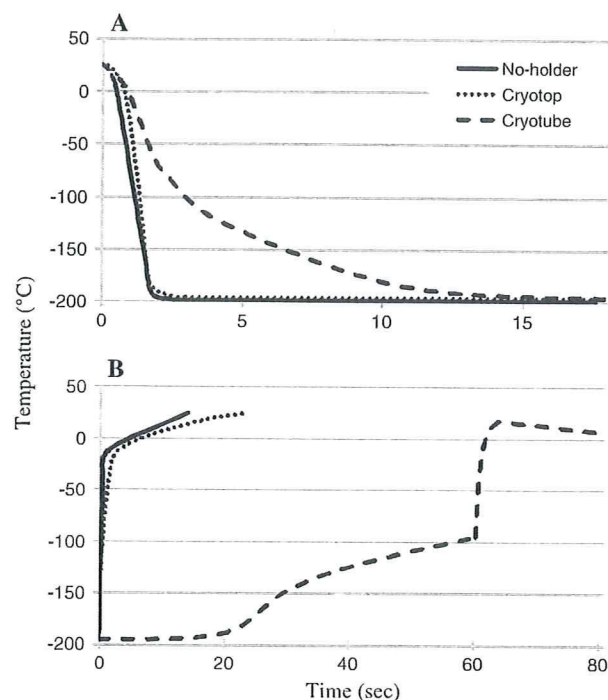
**Table 1** Viability of canine germinal vesicle (GV) oocytes after vitrification

Method	Solution	Container (holder)	No. of vitrified oocytes	No. of oocytes examined (%) <sup>*</sup>	No. of normal oocytes (%) <sup>**</sup>	
					Morphology	Plasma membrane
Fresh	—	—	—	37	37 (100) <sup>a</sup>	36 (97.3) <sup>a</sup>
E30S-Top	E30S	Cryotop	65	61 (93.8)	40 (65.6) <sup>b</sup>	13 (21.3) <sup>b</sup>
DAP-Tube	DAP213	Cryotube	50	49 (98.0)	33 (67.3) <sup>b</sup>	2 (4.1) <sup>c</sup>
DAP-Top	DAP213	Cryotop	56	55 (98.2)	24 (80.0) <sup>b</sup>	24 (43.6) <sup>d</sup>

Values with different superscripts in the same column are significantly different, at  $P < 0.05$

<sup>\*</sup> Percentages of vitrified oocytes examined

<sup>\*\*</sup> Percentages of normal oocytes that were examined for morphology or integrity of plasma membrane



**Fig. 2** a Cooling and b warming rates in the cryotop and cryotube holders. The temperature in the holders was measured using a thermo recorder

solution [3]. Either method of vitrification was suitable and the methods were statistically comparable in terms of cryopreservation, but the plasma membrane integrity of the vitrified oocytes, as assessed by PI staining, was lower with the DAP213 method than with the E30S method. Because the DAP213 method uses a cryotube as the container for the DAP213 solution containing the oocytes, while the E30S method employs a cryotop holder, the difference in viability between the DAP213 and E30S methods seems to be associated with the shape and/or the constituents of the material. Thus, in the present study, the effect of the container (holder) on the vitrification of canine germinal vesicle (GV)-stage oocytes in DAP213 and E30S solution was examined, to improve the cryopreservation method for

canine oocytes. As shown in Table 1, vitrification in a combination with DAP213 solution and the cryotop sheet was much more effective than the conventional DAP213 method for the preservation of canine GV stage oocytes. Although there were no significant differences in the morphological normality of the denuded canine oocytes among the three experimental groups (Table 1), the percentage of oocytes maintaining plasma membrane integrity in the E30S-top and DAP-tube groups, as measured by PI staining, was significantly lower than that in the DAP-top group ( $P < 0.05$ ). Although we did not examine a method using E30S solution with a cryotube, these results suggest that DAP213 as the cryoprotectant and a cryotop sheet as the holder are more suitable, compared with E30S and a cryotube, for the vitrification of canine GV oocytes. Additionally, the integrity of oocytes after cryopreservation could not be evaluated by morphology alone, and it is necessary to perform a histochemical examination, such as with PI staining, as was done in the present study.

Freezing injury is caused in the event that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or damage may result from secondary effects via changes in the composition of the liquid phase. Cryoprotectants reduce the amount of ice formation. To be biologically acceptable, they must be able to enter the cells with low toxicity. Many compounds have such properties, including glycerol, dimethyl sulfoxide (DMSO), ethanediol, and propanediol. Kartberg et al. [11] showed that DMSO-containing vitrification solutions did not lead to cell membrane damage and death as quickly as DMSO-free vitrification solutions in mouse embryos. Our results in the present study have similarly shown that DAP213 solution had higher protective abilities against cryoinjuries during vitrification compared with E30S solution, even when both methods used the cryotop holder. These results may have been caused by the characteristics or the method of treatment with cryoprotectants. Glass formed during vitrification is known to undergo relaxation when stored at a temperature below its glass temperature, and this process will affect the preservation quality of biological materials

embedded in the glassy matrix [12]. Takeda et al. [13] reported that differences in glass transition and (or) enthalpy relaxation depended on cryoprotectants, such as glycerol, propylene glycol, and EG. In the present study, the exposure times to the final vitrification solution were different between the DAP-top and E30S-top groups (5 min vs. 1 min); thus, dehydration may have been insufficient in the E30S-top group. Additionally, we can commercially utilize a vitrification kit for human oocytes (Kitazato Supplies), which consists of a cryotop sheet as the holder and vitrification medium containing EG and DMSO. This medium may also be suitable for the vitrification of canine oocytes, and further study will be required. Furthermore, damage such as ice crystal formation or toxicity caused by cryoprotectants is reportedly dependent on the cell type, cooling rate, and warming rate [14]. High cooling and warming rates are required when oocytes are cryopreserved by vitrification. A number of innovations have been introduced to achieve an increase in the cooling rate. A minimal volume of the cryoprotectant solution containing the oocyte is exposed directly to LN<sub>2</sub> in a thin open straw [7], which has since been modified to the cryotop [9]. In the present study, vitrification using the cryotop required only very small volumes of oocyte suspension liquid (less than 1 µl) when the oocytes were suspended in 100 µl of vitrification solution in the cryotube, and direct contact of oocytes with LN<sub>2</sub> may increase the viability of the vitrified oocytes. Minimizing the volume of the solution in which oocytes were vitrified gave a faster cooling rate (Fig. 2) and resulted in higher viability of oocytes after vitrification (Table 1). Additionally, the cryotop sheet is an open holder in which direct contact between the liquid nitrogen and the solution containing the oocytes is required. The cryoTip (Irvine Scientific, Santa Ana, CA), which is similar, but has a closed holder so there is no direct contact with liquid nitrogen, results in slightly reduced subsequent developmental rates of human vitrified oocytes, compared with the cryotop sheet [15, 16]. These results suggest the holder is an essential factor for the efficient vitrification of oocytes.

In conclusion, we demonstrated the feasibility of using DAP213 as the cryoprotectant and a cryotop sheet as the holder in the vitrification of canine GV oocytes, resulting in a higher percentage of oocytes maintaining plasma membrane integrity (as examined by PI staining) among the vitrified oocytes, compared with results using E30S and a cryotube. However, the ratio of cells maintaining plasma membrane integrity was still comparatively low in the vitrified oocytes, including the DAP-top group. The sensitivity of canine oocytes to cryopreservation may be related to their high lipid content. They may become much more tolerant to cryopreservation if their lipid content were to be reduced such as porcine oocytes and embryos [17, 18]. Thus, further study will be required to develop an optimal

cryopreservation method for canine oocytes, and to examine the potential for maturation and subsequent fertilization in vitrified canine oocytes, although a method for IVM in canine oocytes has not been fully established as yet [1, 2].

**Acknowledgments** 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; Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists; and Funds from the Ministry of Health, Labour and Welfare of Japan.

## References

- Rodrigues BA, Rodrigues JL. Responses of canine oocytes to in vitro maturation and in vitro fertilization outcome. *Theriogenology*. 2006;66:1667–72.
- Songsasen N, Wildt DE. Oocyte biology and challenges in developing in vitro maturation systems in the domestic dog. *Anim Reprod Sci*. 2007;98:2–22.
- Abe Y, Lee DS, Kim SK, Suzuki H. Vitrification of canine oocytes. *J Mammal Ova Res*. 2008;25:32–6.
- Nakagata N. Survival of mouse morulae and blastocysts derived from in vitro fertilization after ultra rapid freezing. *Jikken Dobutsu*. 1993;42:229–31.
- Ishijima T, Kobayashi Y, Lee DS, Ueta YY, Matsui M, Lee JY, et al. Cryopreservation of canine ovaries by vitrification. *J Reprod Dev*. 2006;52:293–9.
- Fujihira T, Kishida R, Fukui Y. Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. *Cryobiology*. 2004;49:286–90.
- Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev*. 1998;51:53–8.
- Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol*. 1999;17:1234–6.
- Hochi S, Terao T, Kamei M, Kato M, Hirabayashi M, Hirao M. Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. *Theriogenology*. 2004;61:267–75.
- Whittingham DG. Embryo banks in the future of developmental genetics. *Genetics*. 1974;78:395–402.
- Kartberg AJ, Hambiliki F, Arvidsson T, Stavreus-Evers A, Svalander P. Vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO. *Reprod Biomed Online*. 2008;17:378–84.
- Pikal JM. Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of the thermodynamics stabilization and glassy state relaxation dynamics. In: Rey L, May JC, editors. *Freeze-drying/lyophilization of pharmaceutical and biological products*. New York: Marcel Dekker; 1999. p. 161–98.
- Takeda K, Murata K, Yamashita S. Thermodynamic investigation of glass transition in binary polyalcohols. *J Non-Cryst Sol*. 1998;231:273–9.
- Pegg DE. Principles of cryopreservation. *Methods Mol Biol*. 2007;368:39–57.
- Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online*. 2005;11:608–14.

16. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the cryotop method. *Theriogenology*. 2007;67:73–80.
17. Ushijima H, Yoshioka H, Esaki R, Takahashi K, Kuwayama M, Nakane T, et al. Improved survival of vitrified in vivo-derived porcine embryos. *J Reprod Dev*. 2004;50:481–6.
18. Hara K, Abe Y, Kumada N, Aono N, Kobayashi J, Matsumoto H, et al. Extrusion and removal of lipid from the cytoplasm of porcine oocytes at the germinal vesicle stage: centrifugation under hypertonic conditions influences vitrification. *Cryobiology*. 2005;50:216–22.

