expulsion. The cumulus-free oocytes were stained with 10 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, Tokyo, Japan) and the plasma membrane integrity of the oocytes was assessed. The oocytes with a disrupted plasma membrane were dyed red with PI (Fig. 1b). Additionally, the kinetics of the sample temperature in the cryotop and cryotube were monitored with a thermometer (EB22005; Chino, Tokyo, Japan). The thermometer probe was plunged into liquid nitrogen directly (no-holder) or into a cryotube with 100 μL DAP213 solution (cryotube), on a cryotop sheet with 2 μL DAP213 solution (cryotop). For warming, the thermometer probe was taken from the liquid nitrogen (no-holder) and allowed to stand at room temperature (23 ± 2°C) for 60 s, and then diluted with 900 μL of PB1 medium (37°C) containing 0.25 M sucrose (cryotube). In the cryotop method, the thermometer probe on a cryotop was transferred from $LN_2$ into PB1 containing 0.25 M sucrose (cryotop).

*Statistical analysis*

Data on oocyte survival were compared using the $\chi^2$ test as presented by StatView software (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at a level of $P < 0.05$.

## Results

In all the vitrification groups, some of the vitrified-warmed COCs exhibited partly dispersed cumulus cells and disrupted cytoplasm, but the majority of vitrified-warmed COCs in each group were morphologically intact. The recovery rates of COCs in these groups were in the range of 93.8%–98.2%. In all the vitrification groups, more than 65% of the vitrified oocytes displayed a normal morphology (E30S-top, 65.6%; DAP-tube, 67.3%; DAP-top, 80.0%), although the percentage of normal oocytes after vitrification was significantly lower than that before vitrification (Table 1). However, as shown in Table 1 and Fig. 1, the percentages of oocytes maintaining plasma membrane integrity with a plasma membrane that was not stained by PI differed significantly among the three groups. The viability of oocytes in the DAP-top group (43.6%) was higher than that in the E30S-top group (21.3%, $P < 0.05$). Furthermore, the viability of the oocytes in the DAP-top group (43.6%) was higher than that in the DAP-tube group (4.1%, $P < 0.05$). As shown in Fig. 2, the cooling rate in the cryotop between 0 and −196°C was higher than the rate in the cryotube (6,158 and 697°C/min, respectively), and

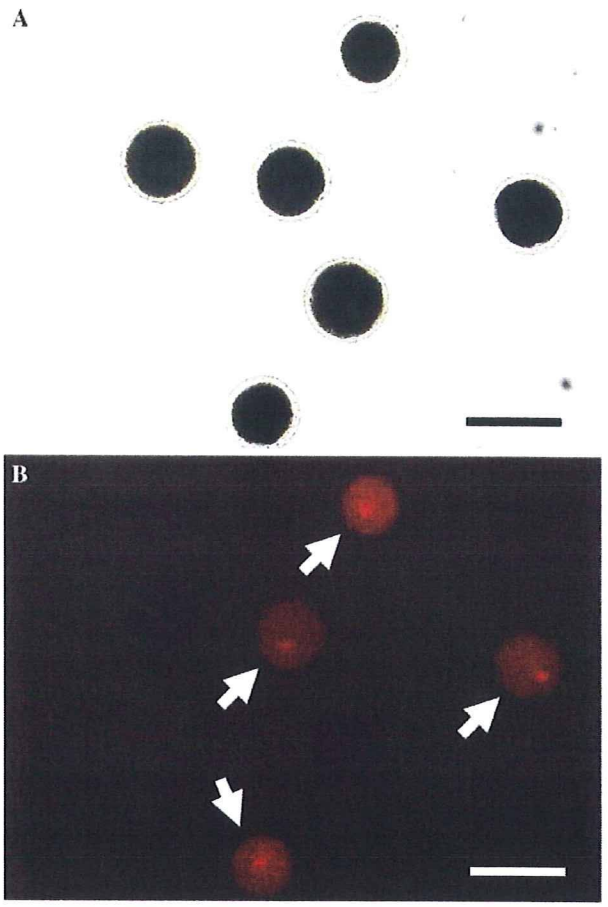


**Fig. 1** Morphological appearance of canine oocytes after vitrification by the DAP-tube method. **a** All oocytes show a morphologically normal appearance under light microscopy; **b** however, propidium iodide (PI) staining revealed that the oocytes stained *red* were damaged (*arrows*). *Bar* 200 μm

was not significantly different from that of the no-holder (6,000°C/min). On the other hand, the warming rates in the no-holder and cryotop between −196 and 0°C were 2,240 and 1,809°C/min, respectively. In the cryotube, the temperature rose to −95°C during standing at room temperature, and the warming rates before (0–60 s) and after dilution with PB1 medium containing 0.25 M sucrose (60 s) were 101 and 3,800°C/min, respectively (between −196 and 0°C, 190°C/min). These results suggest that a combination with DAP213 as the cryoprotectant and a cryotop sheet as the holder is suitable for the cryopreservation of canine oocytes by vitrification.

## Discussion

We have recently reported that canine oocytes were able to be preserved by vitrification in both DAP213 and E30S

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

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

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

* Percentages of vitrified oocytes examined

** Percentages of normal oocytes that were examined for morphology or integrity of plasma membrane

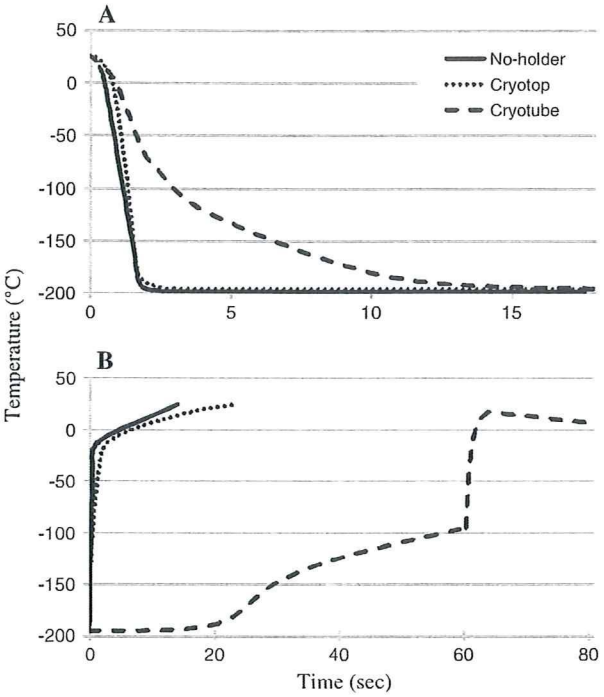


**Fig. 2** **a** Cooling and **b** warming rates in the cryotop and cryotube holders. The temperature in the holders was measured using a thermo recorder

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 ofa 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 $LN_2$ 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 $LN_2$ 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].

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