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—Technical Note—

A Comparison of Cryotop and Solid Surface Vitrification Methods for the Cryopreservation of *In Vitro* Matured Bovine Oocytes

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Abstract. The aim of the present study was to compare the efficacies of the cooling systems of the solid surface (SSV) and Cryotop vitrification methods for cryopreservation of bovine oocytes at the metaphase II stage. The effects of vitrification on oocyte viability, *in vitro* fertilization (IVF), pronucleus formation and subsequent *in vitro* development were assessed. *In vitro* matured (IVM) bovine oocytes were subjected to equilibration and vitrification solutions according to the SSV method, and then the oocytes were vitrified either by dropping onto a cold dry metal surface (SSV group) or by plunging into liquid nitrogen on Cryotop sheets (Cryotop group). Warming was conducted according to the SSV method. Some oocytes were subjected to the cryoprotectants and warming regimen without cooling (Solution control group). The live/dead status of oocytes was evaluated by fluorescein diacetate staining. Live oocytes were subjected to IVF, and the resultant embryos were cultured *in vitro*. The rates of live oocytes were similar among the Fresh control, Solution control, SSV and Cryotop groups. There was no difference in the rates of fertilization, pronuclear formation and monospermy among these groups. The cleavage rates in the SSV and Cryotop groups (41.6 and 53.2%, respectively) were significantly lower than those in the Fresh control and Solution control groups (65.9 and 61.3%, respectively). The blastocyst rates in SSV and Cryotop groups did not differ (10.3 and 12.8%, respectively); however, they were significantly lower than those in the Fresh control and Solution control groups (36.4 and 24.8%, respectively). The inner cell mass, trophoblast and total cell numbers in blastocysts did not differ significantly among the Fresh control, Solution control, SSV and Cryotop groups. Our results indicate that IVM bovine oocytes could be cryopreserved successfully using the cooling systems of the Cryotop and Solid Surface Vitrification methods with similar efficacy.

Key words: Bovine, Cryotop, Oocyte, Solid surface vitrification, Vitrification

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Cryopreservation of bovine oocytes is of great importance in the preservation of female genetic resources. Cryopreserved oocytes can be used for *in vitro* fertilization (IVF), nuclear transfer and genetic manipulation. Rall and Fahy [1] were the first to report vitrification, a glass-like solidification, by ultra-rapid cooling of mouse embryos, showing an alternative to traditional freezing methods to avoid chilling injury and ice crystal formation. Vitrification has been widely applied to cryopreserve oocytes in other mammalian species including cattle [2], pigs [3], sheep [4], goats [5], horses [6], buffalo [7], cats [8] and humans [9].

In general, oocytes are more susceptible to cooling damage than zygotes because metaphase spindle microtubule integrity is disrupted during cooling and the high concentration of cryoprotectants affects oocytes during equilibration [10]. Also, differences in the membrane structures seem to make oocytes more sensitive to chilling compared with zygotes [11]. Although the toxicity of cryoprotectants can be minimized by increasing the cooling rate [2], other side effects of cooling and cryoprotectants such as zona

hardening and parthenogenetic activation may affect fertilization results and cause low development rates to the blastocyst stage [12, 13]. Successful vitrification of oocytes requires pretreatment with permeating cryoprotective agents (CPAs) at a relatively low concentration before the final treatment with a vitrification solution containing high concentrations of permeating and non-permeable CPAs. The low intracellular CPA level of the pretreated oocytes would be concentrated to a higher level by severe dehydration in the vitrification solution, which is a key factor in avoiding intracellular ice formation during the subsequent cooling using liquid nitrogen [14]. Practical use of vitrification to preserve bovine oocytes is still limited since vitrified oocytes seem to exhibit an impaired developmental competence after *in vitro* fertilization. Several methods had been developed to achieve rapid cooling by minimizing the volume of vitrification solution containing oocytes and embryos, such as vitrification with electron microscope grids [2], glass capillaries [15], open pulled straws [16], cryoloops [17], Cryotops [9] and solid surface vitrification (SSV) [18]. These methods differ in CPA composition and treatment regimen and also in the methods for cooling and storing the preserved specimens. Two methods, the SSV and Cryotop vitrification methods have been reported to be highly efficient for cryopreservation of bovine oocytes [18, 19]. These methods have different cooling strategies

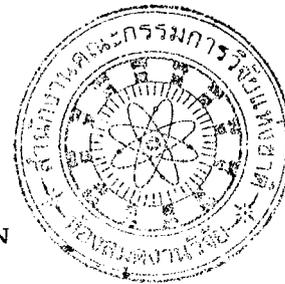
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and apply different cooling and carrier devices. The objective of this study was to compare the efficacies of the cooling systems of the SSV and Cryotop methods for cryopreservation of *in vitro* matured bovine oocytes in terms of oocyte survival fertilization and subsequent *in vitro* development of the resultant embryos.

Materials and Methods

Chemicals and media

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise indicated.

Oocyte collection and *in vitro* maturation (IVM)

Collection and IVM of bovine follicular oocytes were performed as previously described [20]. Ovaries from approximately 28–32-month-old Japanese Black heifers were collected at a local slaughterhouse and transported to the laboratory. Cumulus-oocyte complexes (COCs) were then aspirated from small follicles (2–6 mm in diameter) using a 5-ml syringe and a 19-gauge needle. The maturation medium consisted of 25 mM Hepes buffered TCM199 (GIBCO BRL, Grand Island, NY, USA) and 5% calf serum (CS, GIBCO BRL). COCs were washed twice with the maturation medium and cultured for 20 h in 600- μ l droplets (in groups of 80–100/droplet) of the maturation medium covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) in 35-mm plastic dishes (Nalge Nunc International, Roskilde, Denmark) at 38.5 C in a humidified atmosphere of 5% CO₂ in air.

Oocyte vitrification

In vitro matured oocytes were partially denuded by a brief treatment with 0.1% (w/v) hyaluronidase followed by gentle pipetting through a fine glass pipette. Equilibration and cryoprotectant (Solution control) treatment were performed by the methods previously described by Dinnyes *et al.* [18]. Briefly, the oocytes were washed three times in a basic medium (BM), which was composed of 25 mM Hepes buffered TCM199 supplemented with 20% (v/v) fetal bovine serum (FBS, GIBCO BRL), and then treated with an equilibration medium which was composed of BM supplemented with 4% (v/v) ethylene glycol (EG), for 12 to 15 min at 38.5 C. Groups of 5–10 equilibrated oocytes were rinsed three times in 20- μ l droplets of a vitrification solution, which was composed of BM supplemented with 35% (v/v) EG, 50 mg/ml polyvinyl pyrrolidone and 0.4 M trehalose, for 30 sec and then either directly dropped with about 2 μ l vitrification solution onto the chilled dry surface of an aluminum foil floating on LN₂ (SSV group) [21] or placed on a sheet of a Cryotop (Kitazato Supply, Tokyo, Japan; Cryotop group) and plunged into LN₂ [9]. Vitrified oocytes were warmed without storage by transferring microdrops/Cryotops into a warming solution (0.3 M trehalose in BM) at 38.5 C. One to 2 min later, the oocytes were consecutively transferred for 1 min into 500- μ l droplets of BM supplemented with each of 0.15, 0.075 and 0.0375 M trehalose, respectively. They were washed three times in BM at 38.5 C and then returned into their original droplets of maturation medium and incubated for an additional 2 h at 38.5 C in a humidified atmosphere of 5% CO₂ in air.

Evaluation of oocyte viability

At 2 h after warming, oocyte viability was evaluated by fluorescein diacetate (FDA) staining according to the method previously described by Mohr and Trounson [22]. Briefly, oocytes were treated with 2.5 μ g/ml FDA in PBS supplemented with 5 mg/ml bovine serum albumin (BSA) at 38.5 C for 2 min in a dark room and then washed three times in PBS supplemented with 5 mg/ml BSA and evaluated under an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with an excitation wavelength of 460–495 nm and emission at 510 nm. Oocytes expressing bright green fluorescence were regarded as living and used in subsequent experiments.

In vitro fertilization (IVF)

IVF of surviving oocytes was carried out as previously reported [20]. Briefly, frozen-thawed semen of a Japanese Black bull was thawed in a 37 C water bath for 30 sec and then centrifuged in 3 ml of a 90% Percoll solution at 740 \times g for 10 min. The pellet was re-suspended and centrifuged in 6 ml of sperm washing medium, which was composed of Brackett and Oliphant (BO) solution [23] supplemented with 10 mM hypotaurine and 4 U/ml heparin (Novo-Heparin Injection 1000, Aventis Pharma, Tokyo, Japan), at 540 \times g for 5 min. Then, the pellet was re-suspended with sperm washing solution and BO medium supplemented with 20 mg/ml BSA to achieve the final concentrations of 3 \times 10⁶ spermatozoa/ml, 5 mM hypotaurine, 2 U/ml heparin and 10 mg/ml BSA. One hundred μ l drops of this sperm suspension were prepared in 35-mm plastic dishes, covered with mineral oil and used as fertilization droplets. The oocytes were removed from the maturation medium, washed twice in BO medium supplemented with 10 mg/ml BSA, placed in the fertilization droplets (20 oocytes/droplet) and cultured for 6 h at 38.5 C in a humidified atmosphere of 5% CO₂ in air with saturated humidity.

In vitro culture (IVC)

IVC was performed in 100 μ l droplets of CR1aa medium [24] supplemented with 5% CS covered with paraffin oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting with a fine glass pipette in preincubated IVC medium. Fifteen to twenty five zygotes were placed in each culture drop and then cultured at 38.5 C in a humidified atmosphere of 5% CO₂ in air for 9 days. The day of IVF was considered to be Day 0.

Evaluation of fertilization events

At 16–18 h after insemination, some oocytes of each treatment group were mounted on glass slides and fixed with acetic alcohol (acetic acid 1:ethanol 3) for at least 3 days. The oocytes were stained with 1% (w/v) orcein in acetic acid, rinsed in glycerol:acetic acid:water (1:1:3) and then examined under a phase-contrast microscope. Oocytes with a female pronucleus were considered to be activated. Oocytes with a male pronucleus and/or sperm heads with the contributing sperm tails were classified as fertilized. The presence of one female and one male pronucleus was considered to indicate normal fertilization.

Evaluation of *in vitro* development

Embryo development was assessed by occasional viewing under a stereo microscope. Cleavage rates in each treatment group were recorded on Day 2. Since early developmental speed has been reported to predict developmental competence [25], the rates of embryos developing beyond the four cell stage were also recorded at this time. The rates of embryos developing to the blastocyst stage were recorded on Day 7, Day 8 and Day 9.

Blastocyst evaluation by differential staining of inner cell mass (ICM) and trophectoderm (TE) cells

Differential staining of ICM and TE nuclei in blastocysts was performed by the method previously described by Thouas *et al.* [26], with slight modifications. Briefly, blastocysts were simultaneously treated with 0.1 mg/ml propidium iodide (PI) and 0.2% Triton X-100 dissolved in Dulbecco's phosphate buffered saline for 60 sec to permeabilize the membrane and stain the nuclei of TE cells. The embryos were then treated with 25 µg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol for 5 min, mounted on glass slides in glycerol droplets, flattened by cover slips and examined under UV light with an excitation wavelength of 330–385 nm using an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan). The nuclei of TE cells labeled by both PI and Hoechst appeared pink or red, whereas the nuclei of ICM cells labeled only by Hoechst appeared blue. A digital image of each embryo was taken, and the cell numbers of both cell types were counted using the NIH Image J (v. 1.40) software [27]. Total cell numbers were counted in all embryos. The numbers of ICM and TE cells were counted separately in embryos that had clearly distinguishable populations of red and blue nuclei.

Experimental design

Four treatment groups were compared in this study; the Fresh control group consisted of *in vitro* matured oocytes without exposure to cryoprotectants or cooling. To assess cryoprotectant toxicity, oocytes were exposed to equilibration and vitrification treatments as described above, but without cooling, and subjected to the rehydration (warming) procedure (Solution control group). To compare cryodevice efficiency, *in vitro* matured oocytes were cryopreserved either by using Cryotop devices (Cryotop group) or SSV (SSV group). They were also warmed in the same manner as described above. The viability of oocytes in each group was compared 2 h after the treatments. Live oocytes were fertilized *in vitro*, and the resultant embryos were cultured *in vitro*. In each replication, a sample of 10 putative zygotes was selected randomly from each treatment group at 16–18 h after IVF to assess their fertilization status. The rest of the oocytes were subsequently cultured to compare *in vitro* development and blastocyst cell numbers.

Statistical analysis

Data for embryo development, fertilization and embryo cell numbers were arcsine transformed and analyzed by one-way ANOVA using the KyPlot package (Ver. 4.0, KyensLab, Tokyo, Japan). Five replications of the experiments were performed.

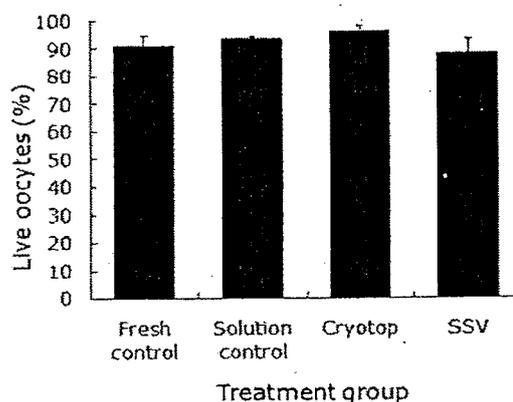


Fig. 1. Viability of oocytes after different treatments evaluated by FDA staining. The four treatment groups shown here are the Fresh control group (n=252), oocytes without treatment; Solution control group (n=249), oocytes pretreated with vitrification solution and recovered without cooling; Cryotop group (n=238), oocytes vitrified by the Cryotop method; and SSV group (n=239), oocytes vitrified by solid surface vitrification. Five replications were performed. No significant difference was detected among the treatment groups at $P < 0.05$ using one-way ANOVA.

Results

Effect of cooling device on viability of oocytes

The viability of oocytes selected by morphology was examined by FDA staining. The rates of live oocytes in the non-vitrified, Solution control and both vitrified groups were over 90% (Fig 1). There was no significant difference in the rates of live oocytes after treatment among the Fresh control, Solution control, Cryotop and SSV groups.

Effect of cooling device on fertilization results after IVF of surviving oocytes

As shown in Table 1, there was no significant difference among the Fresh control, Solution control, SSV and Cryotop groups in the rates of fertilization and monospermy after IVF. The total fertilization rates of oocytes vitrified in the Cryotop and SSV groups were similar (78.0 and 64.0%, respectively) and did not differ significantly from those in the Fresh control and Solution control groups (54.0 and 62.0%, respectively). There was no significant difference in the percentage of activated oocytes among the Fresh control, Solution control, SSV and Cryotop groups (50.0, 58.0, 48.0 and 62.0%, respectively). The rates of normal fertilization of oocytes in the Fresh control and Solution control exposed groups (36.0 and 40.0%, respectively) were not significantly different from those of oocytes in the Cryotop and SSV groups (34.0 and 36.0%, respectively).

Effect of cooling device on *in vitro* development of surviving oocytes

As shown in Table 2, the cleavage rate in the SSV group was significantly lower compared with that of the Solution control group but did not differ from those of the Fresh control and Cryo-

Table 1. Fertilization status of oocytes at 18 h after IVF

Treatment	No. of oocytes examined	No. (%) of oocytes		
		Fertilized	Activated	Normal fertilization
Fresh control	50	27 (54.0 ± 9.7)	25 (50.0 ± 8.9)	18 (36.0 ± 11.2)
Solution control	50	31 (62.0 ± 11.1)	29 (58.0 ± 9.1)	20 (40.0 ± 8.3)
Cryotop	50	39 (78.0 ± 8.0)	31 (62.0 ± 6.6)	17 (34.0 ± 8.1)
SSV	50	32 (64.0 ± 7.4)	24 (48.0 ± 8.6)	18 (36.0 ± 12.0)

Five replications were performed. Data in parentheses are presented as means ± SEM. No significant difference was detected among the treatment groups at $P < 0.05$ using one-way ANOVA. Solution control: Oocytes were pretreated with vitrification solution and recovered without cooling. Cryotop: Oocytes were vitrified by the Cryotop method. SSV: Oocytes were vitrified by the solid surface vitrification method. Oocytes with a female pronucleus were considered to be activated. Oocytes with a male pronucleus(i) and/or sperm heads with the contributing sperm tails were classified as fertilized. The presence of one female and one male pronucleus was considered to indicate normal fertilization.

Table 2. *In vitro* development of control, CPA and vitrified oocytes after IVF

Treatment	No. of oocytes cultured	Day 2 *		Oocytes (%) developed to the blastocyst stage on		
		Cleaved	> 4 Cell	Day 7	Day 8 [#]	Day 9 [#]
Fresh control	175	117 (65.9 ± 10.9) ^{ab}	79 (45.1 ± 3.2) ^a	44 (25.3 ± 7.4) ^a	59 (33.1 ± 7.3) ^a	65 (36.4 ± 7.6) ^a
Solution control	171	106 (61.3 ± 5.9) ^a	80 (46.7 ± 8.2) ^a	31 (18.1 ± 2.6) ^a	40 (23.2 ± 3.1) ^a	43 (24.8 ± 3.9) ^{ab}
Cryotop	162	88 (53.2 ± 9.4) ^{ab}	68 (41.9 ± 7.3) ^{ab}	10 (6.1 ± 3.7) ^b	14 (8.7 ± 5.1) ^b	21 (12.8 ± 5.0) ^b
SSV	140	55 (41.6 ± 4.7) ^b	37 (26.4 ± 6.6) ^b	13 (8.0 ± 3.6) ^b	16 (9.8 ± 5.0) ^b	17 (10.3 ± 5.1) ^b

Five replications were performed. Data in parentheses are presented as means ± SEM. ^{ab} Different superscripts in the same column indicate a significant difference at $P < 0.05$ (one-way ANOVA). *The day of IVF was considered to be Day 0. [#] Cumulative values. Solution control: Oocytes were pretreated with vitrification solution and recovered without cooling. Cryotop: Oocytes were vitrified by the Cryotop method. SSV: Oocytes were vitrified by the solid surface vitrification method.

top groups. The percentages of embryos that developed beyond the 4-cell stage on Day 2 did not differ significantly between the Cryotop and SSV groups (41.9 and 26.4%, respectively); however, the percentage in the SSV group was significantly lower than that of the Fresh control group (45.1%). The rates of cleavage and embryo development to the blastocyst stage on Day 7, Day 8 and Day 9 were not significantly different between the Fresh control and Solution control groups. The blastocyst rates did not differ between Cryotop and SSV groups on Day 7 (6.1 vs. 8.0%, respectively), Day 8 (8.7 vs. 9.8%, respectively) and Day 9 (12.8 vs. 10.3%, respectively) but they were significantly lower than those of the Fresh control group (25.3, 33.1 and 36.4%, respectively).

Effect of cooling device on blastocyst quality

As shown in Table 3, there was no significant difference among the Fresh control, Solution control, SSV and Cryotop groups in the total numbers of nuclei in blastocysts, which ranged between 73.9 ± 12.5 and 90.9 ± 3.1 on Day 9. Similarly, no significant difference was found in the numbers of ICM and TE cells among the Fresh control, Solution control, SSV and Cryotop groups.

Discussion

Vitrification is a simple, rapid and cost-effective method for cryopreservation of mammalian cells. Using this method cryopreserved cells are less likely to experience solution effects and intracellular ice formation compared with the traditional method of slow cooling [28]. On the other hand, due to the high concentrations of cryoprotective agents the cells may be exposed to detrimental osmotic effects. Therefore, the period of exposure to CPAs before plunging into LN₂ is critical and should not be extended. High concentrations of CPA used for vitrification have been proven to be toxic to cells [29, 30]. However, in our study, CPA treatment did not affect survival and development of oocytes, suggesting that the present CPA treatment regimen is suitable for vitrification of M-II bovine oocytes. Metaphase II oocytes are more difficult to cryopreserve than embryos due to the temperature sensitive metaphase spindle and its susceptibility to chilling injury [31]. Thus, successful vitrification requires high concentrations of permeable cryoprotectants and a very rapid cooling rate, which can be provided by both the Cryotop and SSV methods [9, 18]. The Cryotop method applies a thin plastic sheet on which oocytes/embryos are placed with a minimum amount of vitrification solution before plunging into LN₂ [9]. On the other hand, the SSV

Table 3. Cell numbers in blastocysts developed from control, CPA and vitrified oocytes

Groups	No. of blastocysts evaluated	No. of nuclei \pm SEM		
		Total cells	TE	ICM
Fresh control	52	90.9 \pm 3.1	65.5 \pm 2.5	25.3 \pm 3.9
Solution control	39	83.4 \pm 5.9	52.7 \pm 4.9	30.5 \pm 3.2
Cryotop	17	77.9 \pm 14.3	46.1 \pm 12.5	31.8 \pm 2.8
SSV	13	73.9 \pm 12.5	46.5 \pm 17.0	27.4 \pm 4.4

Five replications were performed. No significant difference was detected among the treatment groups at $P < 0.05$ using one-way ANOVA. Solution control; Oocytes were pretreated with vitrification solution and recovered without cooling. Cryotop, Oocytes were vitrified by the Cryotop method. SSV; Oocytes were vitrified by the solid surface vitrification method.

method achieves a high cooling rate by using a combination of microdrops and improved heat exchange by direct contact with a dry metal surface cooled by LN₂ [18]. In this study, high survival and fertilization rates were achieved for vitrified oocytes by both the Cryotop and SSV methods, which enabled their subsequent *in vitro* development after IVF. The high survival rates and normal morphology of the vitrified-thawed bovine oocytes could have been achieved due to high rates of cooling and warming. Previous reports have shown that treatment with CPAs or cooling of M-II oocytes may trigger biochemical reactions similar to those phenomena occurring during fertilization, which can result in hardening of the zona pellucida and/or parthenogenetic activation of the oocyte in several mammalian species [12, 13, 32–35]. However, we observed similar fertilization, monospermy and pronuclear formation rates among the Fresh control, Solution control, SSV and Cryotop groups, suggesting that the Solution control treatment, both cooling methods and the warming regimen used in the present study did not cause such phenomena in M-II bovine oocytes and allowed their normal fertilization by IVF. Also, we observed similar rates of subsequent blastocyst formation on Day 7, Day 8 and Day 9 for oocytes vitrified by the Cryotop and SSV methods. Taken together, these results indicate that the cooling strategies of the SSV and Cryotop vitrification methods are equally effective for cryopreservation of bovine IVM oocytes. In this respect, both of these cooling methods can be recommended for cattle oocyte vitrification. The choice between the two methods may depend on the advantage and convenience of each method and the actual circumstances (e.g., the number of oocytes for cryopreservation) of the work. SSV is a very cheap method that enables preservation of several microdrops of vitrification solution within a short period of time, each containing a large number of oocytes. On the other hand, controlling the droplet size by this method requires extensive practice, and there is a constant chance of oocyte loss since some oocytes tend to attach to the inner surface of the glass capillary during droplet formation. This method is therefore more suitable for rapid cryopreservation of large quantities (hundreds) of oocytes/embryos. More precise control of the volume of the vitrified solution and oocyte numbers can be achieved by Cryotop vitrification, and this method offers a convenient way of storage and handling after cooling. However, the numbers of oocytes that can be preserved on each Cryotop sheet is limited, and preservation of large numbers of oocytes by this method takes an

extended period of time.

The cleavage and blastocyst formation rates of bovine M-II oocytes cryopreserved by either the Cryotop and SSV methods in our study were higher than those reported by Roser *et al.* [36] using the Cryotop method. On the other hand, the cleavage and blastocyst formation rates of surviving vitrified oocytes in both the Cryotop and SSV groups were still reduced compared with those of the Fresh control and Solution control groups. Similar to previous studies of cattle and pig oocytes [13, 18], treatment of M-II stage oocytes with cryoprotectants without vitrification according to the SSV protocol did not influence fertilization and *in vitro* development. Taken together with the high survival rates, this suggests that the impaired developmental ability of vitrified oocytes was caused by sub-lethal damage that could be mainly related to the cooling and/or warming procedure. Such sub-lethal damage may include disruption of the meiotic spindle and other cytoskeletal elements [10, 37], damage and dysfunction of organelles such as mitochondria or endoplasmic reticuli [38–40] or degradation of cytoplasmic mRNA levels [41], which have been reported to occur in cryopreserved mammalian oocytes. Further improvements of the present vitrification protocols will be necessary to avoid such damage.

In conclusion, our data indicate that both the Cryotop and SSV vitrification methods could yield high rates of surviving oocytes that could develop to the blastocyst stage after IVF. The cooling systems of the Cryotop and SSV methods were proven to be equally effective for preservation of IVM bovine oocytes in terms of their survival and subsequent developmental competence after IVF and IVC. However, further studies are necessary to improve the developmental competence of cryopreserved oocytes.

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References

1. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature* 1985; 313: 573–575.

2. Martino AN, Songsasen S, Leibo SP. Development in to blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod* 1996; 54: 1059-1069.
3. Isachenko V, Soler C, Isachenko E, Perez-Sanchez F, Grishchenko V. Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. *Cryobiology* 1998; 3: 250-253.
4. Isachenko V, Alabart JL, Nawroth F, Isachenko E, Vajta G, Folch J. The open pulled straw vitrification of ovine GV-oocytes: positive effect of rapid cooling or rapid thawing or both? *Cryo Lett* 2001; 22: 157-162.
5. Begin I, Bhatia B, Baldassarre H, Dinnyes A, Keefer CL. Cryopreservation of goat oocytes and *in vivo* derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. *Theriogenology* 2003; 59: 1839-1850.
6. Hurtt AE, Landim-Alvarenga F, Seidel Jr GE, Aquires EL. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. *Theriogenology* 2000; 54: 119-128.
7. Dhali A, Manik RS, Das SK, Singla SK, Palta P. Vitrification of buffalo (*Bubalus bubalis*) oocytes. *Theriogenology* 2000; 53: 1295-1303.
8. Murakami M, Otoi T, Karja NW, Wongsrikao P, Agung B, Suzuki T. Blastocyst derived from *in vitro* fertilized cat oocytes after vitrification and dilution with sucrose. *Cryobiology* 2004; 48: 341-348.
9. Kuwayama M, Kato O. All-round vitrification method for human oocytes and embryos. *J Assist Reprod Genet* 2000; 17: 477 (abstract).
10. Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a review article. *Mol Cell Endocrinol* 2003; 202: 101-107.
11. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod* 2005; 12: 3385-3389.
12. Carroll J, Depypere H, Matthews CD. Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. *J Reprod Fert* 1990; 90: 547-553.
13. Somfai T, Ozawa M, Noguchi J, Kaneko H, Karja NWK, Farhudin M, Dinnyes A, Nagai T, Kikuchi K. Developmental competence of *in vitro*-fertilized porcine oocytes after *in vitro* maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology* 2007; 55: 115-126.
14. Hochi S, Terao T, Kamei M, Koto M, Hirabayashi M, Hirao M. Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. *Theriogenology* 2004; 61: 267-275.
15. Hochi S, Fujimoto T, Braun J, Oguri N. Pregnancies following transfer of equine embryos cryopreserved by vitrification. *Theriogenology* 1994; 42: 483-488.
16. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobson H, Greve T, Callesen H. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998; 51: 53-58.
17. Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol* 1999; 17: 1234-1236.
18. Dinnyes A, Dai Y, Jiang S, Yang X. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. *Biol Reprod* 2000; 63: 513-518.
19. Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured *in vitro* following vitrification. *J Reprod Dev* 2004; 50: 685-696.
20. Imai K, Tagawa M, Yoshioka H, Matoba S, Narita M, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S. The efficiency of embryo production by ovum pick-up and *in vitro* fertilization in cattle. *J Reprod Dev* 2006; 52: 19-29.
21. Li X, Su L, Li Y, Ji W, Dinnyes A. Vitrification of Yunnan cattle oocytes: work in progress. *Theriogenology* 2002; 58: 1253-1260.
22. Mohr LR, Trounson AO. The use of fluorescein diacetate to assess embryo viability in the mouse. *J Reprod Fert* 1980; 58: 189-196.
23. Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa *in vitro*. *Biol Reprod* 1975; 12: 260-274.
24. Rosenkrans Jr CF, Zeng GQ, Mcnamara GT, Schoff PK, First NL. Development of bovine embryos *in vitro* as affected by energy substrates. *Biol Reprod* 1993; 49: 459-462.
25. Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their *in vitro* viability and sex. *Theriogenology* 1998; 50: 1285-1299.
26. Thomas GA, Korfiatis NA, French AJ, Jones GM, Trounson AO. Simplified technique for differential staining of inner cell mass and trophoblast cells of mouse and bovine blastocysts. *Reprod Biomed Online* 2001; 3: 25-29.
27. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with image. *J Biophotonics International* 2004; 11: 36-42.
28. Fahy GM, McFralane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. *Cryobiology* 1984; 21: 407-426.
29. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. *Reprod Biomed Online* 2004; 9: 680-691.
30. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed* 2005; 300: 308 (abstract).
31. Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* 2000; 53: 59-72.
32. Van der Elst J, Van den Abbeel E, Nerinckx S, Van Steirteghem A. Parthenogenetic activation pattern and microtubular organization of mouse oocyte after exposure to 1,2-propanediol. *Cryobiology* 1992; 29: 549-562.
33. Larman MG, Sheehan CB, Gardner DK. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction* 2006; 131: 53-61.
34. Matson PL, Graefling J, Junk SM, Yovich JL, Edirisinghe WR. Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an *in vitro* fertilization programme. *Hum Reprod* 1997; 12: 1550-1553.
35. Tian SJ, Yan CL, Yang HX, Zhou GB, Yang ZQ, Zhu SE. Vitrification solution containing DMSO and EG can induce parthenogenetic activation of *in vitro* matured ovine oocytes and decrease sperm penetration. *Anim Reprod Sci* 2007; 101: 365-371.
36. Morató R, Izquierdo D, Paramio MT, Mogas T. Cryotop versus open-pull straw (OPS) as carriers for the cryopreservation of bovine oocytes: Effect on spindle and chromosome configuration and embryo development. *Cryobiology* 2008; 57: 137-141.
37. Albarracín JL, Morató R, Rojas C, Mogas T. Effects of vitrification in open pulled straws on the cytology of *in vitro* matured prepubertal and adult bovine oocytes. *Theriogenology* 2005; 63: 890-901.
38. Fuku E, Xia L, Downey BR. Ultrastructural changes in bovine oocytes cryopreserved by vitrification. *Cryobiology* 1995; 2: 139-156.
39. Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Mol Reprod Dev* 2002; 4: 464-470.
40. Lowther KM, Weitzman VN, Maier D, Mehlmann LM. Maturation, fertilization, and the structure and function of the endoplasmic reticulum in cryopreserved mouse oocytes. *Biol Reprod* 2009 Mar 18. [Epub ahead of print]
41. Succu S, Bebbere D, Bogliolo L, Arju F, Fois S, Leoni GG, Berlinguer F, Naitana S, Ledda S. Vitrification of *in vitro* matured ovine oocytes affects *in vitro* pre-implantation development and mRNA abundance. *Mol Reprod Dev* 2008; 3: 538-546.