

# Open-pulled straw vitrification differentiates cryotolerance of *in vitro* cultured rabbit embryos at the eight-cell stage

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## Abstract

The objective was to determine cryotolerance of *in vitro* cultured rabbit embryos to the open-pulled straw (OPS) method. Overall, 844 rabbit embryos at pronuclear, 2- to 4-cell, 8-cell, and morula/blastocyst stages were vitrified, and  $\geq 1$  mo later, were sequentially warmed, rehydrated, and subjected to continuous culture ( $n = 691$ ) or embryo transfer (ET,  $n = 153$ ). Embryos vitrified at the 8-cell stage or beyond had greater survival, expanded blastocyst and hatched blastocyst rates *in vitro*, and better term development than those vitrified at earlier stages. The 8-cell group had 70.1% expanded blastocysts, 63.7% hatched blastocysts, and 25.7% term development, as compared to 1.5–17.7%, 1.5–4.3% and 2.8–3.7% in the pronuclear, 2-cell and 4-cell embryos, respectively ( $P < 0.05$ ). The expanded and hatched blastocyst rates in vitrified morula/blastocyst post-warming were higher than that in the 8-cell group; however, their term development after ET was similar (8-cell vs morula/blastocyst: 25.7 vs 19.4%,  $P > 0.05$ ). Development after ET was comparable between vitrified-warmed embryos and fresh controls at 8-cell and morula/blastocyst stages (19.4–25.7 vs 13.7–26.6%,  $P > 0.05$ ). For embryos at pronuclear or 2- to 4-cell stages, however, term rates were lower in the vitrified-warmed (2.8–3.7%) than in fresh controls (28.6–35.6%,  $P < 0.05$ ). Therefore, cultured rabbit embryos at various developmental stages had differential cryotolerance. Under the present experimental conditions, the 8-cell stage appeared to be the critical point for acquiring cryotolerance. We inferred that for this OPS cryopreservation protocol, rabbit embryos should be vitrified no earlier than the 8-cell stage, and stage-specific protocols may be needed to maximize embryo survival after vitrification and re-warming.

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## 1. Introduction

Conventional slow freezing and vitrification are common methods to preserve mammalian embryos. Vitrification, defined as the solidification of a solution at low temperature by extreme elevation in viscosity during cooling without ice crystallization [1,2], has been increasingly used to replace slow freezing in the

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past decade [3]. The glass state formed during vitrification has the same ionic and molecular distribution as the liquid phase, thus avoiding both chemical and mechanical damage to embryos. The open-pulled straw (OPS) method has been one of the most widely used protocols for embryo preservation by thinning the straw wall to render high cooling and warming rates ( $> 20,000$  °C/min) [4].

Various vitrification protocols have been reportedly applied to rabbit embryos at late preimplantation stages, i.e. morula or blastocyst, and resulted better *in vitro* and *in vivo* development than in the programmed freezing procedures [5–15]. Using a modified OPS method, Lopez-Bejar et al (2002) obtained higher survival rate (88%) and term development (52%) after warming the embryos that were vitrified at morula to blastocyst stages, than those in the slow freezing group [11]. Naik et al (2005) reported a 91% survival rate *in vitro* and 29% term development (kits/total embryos transferred) after transfer of vitrified-morula stage embryos [9]. In contrast, there were limited reports of vitrification on rabbit embryos at earlier developmental stages (pronuclear to cleavage) and results were generally unsuccessful [14,16], except that one study with 94% *in vitro* survival and 36% birth rates was reported on pronuclear stage embryos vitrified by Cryoloop [5]. Yet to date, live birth from vitrified-warmed cleavage stage embryos in rabbits has apparently not been reported.

The rabbit is an important agricultural species and a useful model animal for biomedical research [17]. As a preferred laboratory species for the development of several reproductive technologies, such as IVF [18], embryonic stem cells [19], transgenesis [20] and animal cloning [21], rabbits have also been used for the study of embryo cryopreservation, including both slow freezing and vitrification [14,22].

Considering its ease of use and the superior efficiencies in several species [3,4,9,23–27], we adapted the OPS vitrification method to investigate *in vitro* development of rabbit embryos vitrified from pronuclear to early blastocyst stages, as well as their *in vivo* development.

## 2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (DPBS; 15240-013, Gibco, Grand Island, NY, USA) containing 0.1% polyvinyl alcohol (PVA; P-8136) was

used for flushing oocytes from the oviducts (DPBS-PVP). The basic manipulation medium was Medium 199 (M199) with Earle's salts, L-glutamine, 2.2 g/L sodium bicarbonate, and 25 mM HEPES (Gibco, 12340-014, Grand Island, NY, USA) supplemented with 10% FBS (SH0070.03, Hyclone, Logan, UT, USA). Embryos were cultured at 38.5 °C in an incubator containing 5% CO<sub>2</sub> and humidified air.

Sexually mature (6- to 12-mo-old) New Zealand White (NZW) rabbits were used as embryo donors and recipients in the present work. All animal maintenance, care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Connecticut, National Chung Hsing University, and National Taiwan University.

### 2.1. Embryo collection

Embryo donors were superovulated with our standard regime [28], consisting of two 0.3 mg, two 0.4 mg and two 0.5 mg injections of FSH (Folltropin-V, Bioniche Animal Health Canada, Belleville, ON, Canada) at 12 h intervals, with 200 IU of hCG (Chorulon, Intervet Inc, Millsboro, DE, USA) to induce ovulation, immediately followed by mating with NZW males. At 18 h post-hCG, reproductive tracts of the donors were flushed to harvest embryos.

### 2.2. Embryo culture

Presumptive zygotes were recovered and checked for polar body extrusion to ensure successful fertilization. Viable embryos were cultured in B2 medium (Laboratories CCD, Paris, France) supplemented with 2.5% FBS in a standard culture condition until being selected for vitrification at pronuclear, cleavage (2-, 4-, or 8-cell), and morula/blastocyst stages. Two-celled embryos were collected 20–22 h post-hCG, whereas 4-celled and 8-celled embryos were at 30–34, and 40–46 h post-hCG, respectively. Morulae and early blastocysts were collected on Day 3 after *in vitro* culture, and pooled as one group.

### 2.3. Vitrification and warming of embryos

Embryos of various stages were all subjected to the same vitrification and warming protocols; the OPS-vitrification procedure was described previously [9]. Solutions for vitrification were prepared as holding solution (HEPES-buffered DPBS containing 20% FBS and 50 µg/mL Genamicin), OPS-I (HEPES-buffered DPBS supplemented with 16% FBS, 10% ethylene glycol (EG), and 10% DMSO), and OPS-II (HEPES-

buffered DPBS supplemented with 0.6 M sucrose, 8% FBS, 20% EG, and 20% DMSO). Warming solutions were prepared as cryoprotective diluents I and II (CPD-I and CPD-II); CPD-I was prepared by mixing equal volumes of OPS-II and holding solution, and CPD-II was prepared by mixing OPS-I and holding solution in a 1:3 ratio. All solutions were warmed to 38.5 °C before use. The OPS straws were either purchased from Minitube (Cat #19050/0025, Verona, WI, USA) or were fabricated. For manual pulling, French mini straws (0.25 mL) were heated slightly and hand-pulled to a diameter half their original size. Later, 3 to 5 embryos were first equilibrated for 5 min in holding solution, and then transferred to OPS-I for 2 min. Finally, embryos were washed through three drops of OPS-II for an average of 20 s in each drop (total time in OPS-II was 1 min). Immediately after embryos were loaded, straws were directly immersed in liquid nitrogen and stored in liquid nitrogen for at least 1 mo before being rewarmed.

For warming, the straws containing vitrified embryos were taken out of liquid nitrogen and directly immersed in pre-warmed (38.5 °C) CPD-I. The embryos were flushed into CPD-I by gently blowing on one end of the straw. One min later, the embryos were transferred to CPD-II for 5 min, and then washed twice in the holding solution for 5 min each. The warmed embryos were either cultured in a standard *in vitro* culture condition as described above, or immediately transferred to recipient does.

#### 2.4. Embryo transfer

Embryo recipients were asynchronously induced ovulation (a 22 h delay after embryo donors) by im administration of 15 µg GnRH analogue (Cystorelin, Abbott Laboratories, North Chicago, IL, USA) per doe, according to the protocol described previously [29]. The day of GnRH injection was defined as Day 0. Embryo transfer was performed surgically 16 h after GnRH administration. Embryos (10 to 17) were transferred into the oviducts (5–9 embryos each side) of each pseudopregnant doe by midventral laparotomy. Pregnancy was monitored by transabdominal palpation at Days 14–16 post-transfer. All pregnancies were allowed to proceed to term (Days 31–33), with some deliveries performed by Caesarean section to ensure live births.

*In vitro* cultured fresh embryos at different developmental stages were used as controls for the vitrified-warmed embryos of the same stage.

#### 2.5. Measurements of surface area to volume (S/V) ratios of blastomeres

The S/V ratios were calculated as described previously [30]. For measuring the perimeter (P) and the surface area (A), embryos at pronuclear, 2-cell, 4-cell and 8-cell stages were photographed under an inverted microscope (Nikon Eclipse Ti-S). The values of P and A for blastomeres, inner and outer limits of the zona pellucida were determined by Image J (Version 1.40g, National Institutes of Health, Bethesda, MD, USA). The S/V ratio was calculated using the following equation:  $S/V = 4P/\pi A$  (P was mean perimeter and A was mean surface area).

#### 2.6. Statistical analysis

The effects of vitrification at various developmental stages on embryo survival, expanded blastocysts (including expanding and hatching embryos), hatched blastocysts, S/V ratios and term rates were analyzed using the SPSS software (SPSS 11.0, Chicago, IL, USA). Percentage data in each replicate were arc-sine transformed before subjected to one-way ANOVA. Means were compared by Fisher's least significant difference test (PLSD test). The Pearson's correlation (*r*) between the S/V ratio and the cell number was also determined using the SPSS software. For all analyses,  $P < 0.05$  was considered significant.

### 3. Results

#### 3.1. *In vitro* development of embryos vitrified at various stages

A total of 691 rabbit embryos collected from 30 does ( $23 \pm 2$  embryos/donor) were vitrified at pronuclear, 2-cell, 4-cell, 8-cell or morula/blastocyst stages and stored in liquid nitrogen. After being warmed and cultured, the survivability and developmental competence were assessed (Table 1). The intact rates of embryos vitrified at 4-cell (86.9%) stage or beyond (95.6–97.3%) were greater compared to those of pronuclear (61.8%) and 2-cell stages (44.3%). However, only a small proportion (17.7%) of intact 4-cell stage embryos developed to expanded blastocysts, which was much lower than that of the 8-cell (70.1%) and the morula/blastocyst stage embryos (91.7%), but even lower percentages of pronuclear (13.2%) and 2-cell stage (1.5%) embryos developed into expanded blastocysts. Hatched blastocyst rates from the survived embryos were also greater in both the 8-cell and the morula/blastocyst (63.7–86.2%) stages than in the pronuclear (4.3%), 2-cell (1.5%) and 4-cell

Table 1

Mean ( $\pm$  SEM) rates of intact embryos and *in vitro* development of rabbit embryos after vitrification and warming.

Embryonic stage	Total no.	Intact embryos (%)	Expanded BL (%)*	Hatched BL (%)**
Pronuclear	166	61.8 $\pm$ 7.8 <sup>a</sup>	13.2 $\pm$ 3.0 <sup>a</sup>	4.3 $\pm$ 2.0 <sup>a</sup>
2-cell	128	44.3 $\pm$ 6.6 <sup>a</sup>	1.5 $\pm$ 1.5 <sup>b</sup>	1.5 $\pm$ 1.5 <sup>a</sup>
4-cell	123	86.9 $\pm$ 0.7 <sup>b</sup>	17.7 $\pm$ 4.3 <sup>a</sup>	3.4 $\pm$ 2.2 <sup>a</sup>
8-cell	143	97.3 $\pm$ 2.2 <sup>b</sup>	70.1 $\pm$ 9.7 <sup>c</sup>	63.7 $\pm$ 8.9 <sup>b</sup>
Mor/BL	131	95.6 $\pm$ 3.2 <sup>b</sup>	91.7 $\pm$ 3.2 <sup>d</sup>	86.2 $\pm$ 4.1 <sup>c</sup>
Control (Pronuclear)	104	N/A	83.8 $\pm$ 3.9 <sup>c,d</sup>	70.1 $\pm$ 6.0 <sup>b</sup>

Mor/BL, morula/blastocyst.

<sup>a–d</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).

\* Expanded blastocysts (BL) were recorded on Day 4 of culture.

\*\* Hatched BL were recorded on Day 5.

(3.4%) stage groups. The intact embryo rates post-warming between 8-cell and morula/blastocyst groups were similar (95.6–97.3%); however, the expanding (91.7 vs 70.1%) and hatching (86.2 vs 63.7%) rates were significantly higher in the morula/blastocyst group compared to those in the 8-celled embryos. In the control group, 83.8% of 104 fresh pronuclear stage embryos developed to expanded blastocysts, and 70.1% hatched, both similar to that of vitrified-warmed 8-celled embryos.

### 3.2. Term development of embryos vitrified at various stages

A total of 153 rabbit embryos vitrified at various developmental stages were warmed and transferred to recipients. Fresh embryos of corresponding stages were transferred serving as the control ( $n = 184$ ; Table 2). Vitrified 2-celled and 4-celled embryos were pooled prior to transfer due to a similar hatching rate and low developmental competence. Term rates (number of kits born/total embryos transferred) of vitrified-warmed embryos were very poor for both pronuclear (3.7%) and 2–4 cell (2.8%) groups, much lower than their fresh

control groups (28.6–35.6%). In contrast, term rates of the 8-cell and the morula/blastocyst groups were much higher (25.7 and 19.4 %, respectively), similar to their fresh control groups (13.7–26.6%). Representative images for the vitrified-warmed 8-celled embryos developed into hatching blastocysts and live offspring are shown in Figure 1.

### 3.3. S/V ratios of the blastomeres at different embryonic stages

The mean S/V ratio of blastomeres and zona pellucidae (inner and outer limits) of pronuclear, 2-cell, 4-cell and 8-cell stage embryos were measured (Table 3). It was not possible to measure such ratios beyond these developmental stages, due to compaction of the embryos and the difficulty to clearly identify individual blastomeres with the equipment used. The mean S/V ratio of the blastomere increased steadily from pronuclear (0.0448/ $\mu\text{m}$ ), 2-cell (0.0808/ $\mu\text{m}$ ), 4-cell (0.098/ $\mu\text{m}$ ), and to 8-cell (0.1285/ $\mu\text{m}$ ) stages. The correlation coefficient ( $r$ ) between the S/V ratio and the cell number at the same stage was 0.94. The S/V ratios of *zona pellucida*

Table 2

Live births from rabbit embryos vitrified at various developmental stages.

Embryo stage	Treatment	Total no.	Embryos per recipient (Mean $\pm$ SEM)	Pregnant recipients No. (%)	Total live kits	Litter size (Mean $\pm$ SEM)	% kits/embryos (Mean $\pm$ SEM)
Pronuclear	Vitrified	37	12.3 $\pm$ 2.0	1 (33.3)	1	1	3.7 $\pm$ 3.7 <sup>a</sup>
	Fresh	40	13.3 $\pm$ 0.7	2 (66.7)	12	6.0 $\pm$ 3.0	28.6 $\pm$ 18.9 <sup>b,c</sup>
2–4-cell	Vitrified	32	10.7 $\pm$ 1.3	2 (66.7)	1	1	2.8 $\pm$ 2.8 <sup>a</sup>
	Fresh	51	17.0 $\pm$ 1.5	3 (100)	18	6.0 $\pm$ 1.5	35.6 $\pm$ 8.4 <sup>c</sup>
8-cell	Vitrified	40	13.3 $\pm$ 1.3	3 (100)	10	3.3 $\pm$ 0.3	25.7 $\pm$ 4.2 <sup>b,c</sup>
	Fresh	50	16.7 $\pm$ 0.7	3 (100)	13	4.3 $\pm$ 1.5	26.6 $\pm$ 9.5 <sup>b,c</sup>
Mor/BL	Vitrified	44	14.6 $\pm$ 3.8	3 (100)	9	3.0 $\pm$ 1.0	19.4 $\pm$ 4.1 <sup>b,c</sup>
	Fresh	43	14.3 $\pm$ 0.3	2 (66.7)	6	3.0 $\pm$ 1.0	13.7 $\pm$ 7.7 <sup>b</sup>

Mor/BL, morula/blastocyst.

<sup>a–c</sup> Within a column, values without a common superscript differed ( $P < 0.05$ ).



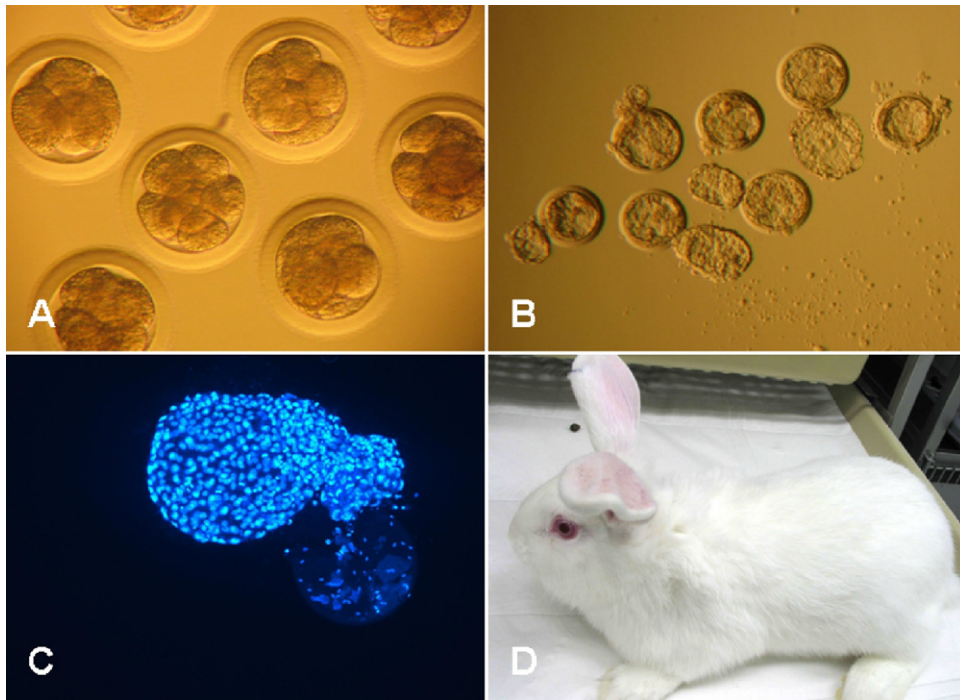


Fig. 1. *In vitro* and *in vivo* development of vitrified 8-cell stage rabbit embryos after warming. Vitrified 8-cell embryos were warmed (A) and cultured *in vitro* for 2 d to reach hatching blastocyst stage (B), or were transferred immediately after warming. A representative hatched blastocyst was stained with Hoechst 33342 (C). Representative healthy kit (15 wk old) produced from a vitrified-warmed 8-cell stage embryo after embryo transfer (D).

inner and outer limits changed little from the pronuclear ( $0.0410/\mu\text{m}$  and  $0.0434/\mu\text{m}$ ) to the morula ( $0.0322/\mu\text{m}$  and  $0.0332/\mu\text{m}$ ) stages.

#### 4. Discussion

Vitrification is an ultra-rapid cryopreservation procedure based on replacement of intracellular water molecules with vitrification solution containing cryoprotective agents (CPAs) and liquid nitrogen [2,31]. Vitrification requires a high concentration of CPAs; their toxicity is minimized by a rapid cooling rate achieved by plunging oocytes or embryos into liquid nitrogen with various carrier vehicles, such as a chilled solid surface [32], electron microscopy grids [33], Cryoloops [34,35], or an OPS [4]. The CPAs in the vitrification medium of OPS are DMSO and EG. Ethylene glycol was used for cryopreserving mouse and bovine embryos [27,36], due to its low toxicity and high permeability [1,36]. The mixture of EG and DMSO improved post-warming survival of oocytes and early embryos of various stages [4,37]. In addition to the optimized CPA compositions used in the vitrification and warming media, OPS renders very high cooling and warming rates

(>20,000 °C/min) [4]. Such high cooling and warming rates are believed to enhance survival of vitrified embryos, and to account for the better results compared to conventional slow freezing methods [4,38,39].

Rabbits are genetically and physiologically closer to humans than mice in the characteristics of their cardiovascular systems and lipoprotein metabolisms [17]. They are thus more appropriate than mice for the study of human diseases such as atherosclerosis and lipid metabolism. Efforts have been attempted to generate gene targeted transgenic rabbits for human disease models using embryonic stem cells or somatic cell nuclear transfer without success [29,40,41]. A reliable cryopreservation protocol for rabbit embryos would add a convenient tool into the ART (assisted reproductive technologies) box, which may in particular facilitate the advancement of rabbit cloning and transgenic technologies. To date, the efficiency of rabbit cloning remains low [29], due to, at least in part, the low number of viable embryos available for transfer. In the present study, there was a prominent increase in survival and blastocyst rates of embryos vitrified at and beyond the 8-cell stage after warming. Furthermore, embryo transfer confirmed the *in vitro* data that the

Table 3  
Mean ( $\pm$  SEM) surface area/volume (S/V) ratios ( $\mu\text{m}$ ) of various stages of rabbit embryos.\*

	Pronuclear		2-cell		4-cell		8-cell		Morula	
	No.	S/V	No.	S/V	No.	S/V	No.	S/V	No.	S/V
Mean blastomere	13	$0.044767 \pm 0.00024$	6	$0.080751 \pm 0.00305$	5	$0.098029 \pm 0.00327$	6	$0.128535 \pm 0.00165$	8	N/A
<i>Zona pellucida</i> inner limit	13	$0.040990 \pm 0.00019$	5	$0.042669 \pm 0.00040$	5	$0.042205 \pm 0.00022$	6	$0.044828 \pm 0.00032$	8	$0.043407 \pm 0.00024$
<i>Zona pellucida</i> outer limit	13	$0.032178 \pm 0.00018$	5	$0.032920 \pm 0.00046$	5	$0.033229 \pm 0.00010$	6	$0.034908 \pm 0.00048$	8	$0.033168 \pm 0.000231$

\* No significant differences were detected in all parameters measured.

highest term rates (19–26%) were obtained from transfer of the vitrified-warmed 8-celled and morula/blastocyst embryos. Development from 8-cell and morula/blastocyst stage embryos appeared undistinguishable from fresh control embryos. With this success, adequate number of cloned embryos at 8-cell stage or beyond may be pooled for transfer to increase pregnancies and live births. A similar strategy was successful in pig cloning [42–45].

Ideally, vitrification protocols should be optimized for embryos at various developmental stages, since cryological properties, e.g. membrane permeability and S/V ratios, change during embryo development. For example, mouse morula embryos have better CPA permeability than those at earlier stages [46,47], which require longer exposure time to the same concentration of CPAs. In mouse blastocysts, the blastocoel allows only even shorter exposure to CPAs, due to a higher chance of ice crystal formation in the blastocoel, although the membrane permeability was not greater than that of morulae [48]. In the present work, the same vitrification protocol was tested in various stages of rabbit embryos. Those at or beyond the 8-cell stage increasingly acquired cryotolerance under the present OPS method, suggesting that the OPS procedure could be used for preserving rabbit embryos at the 8-cell stage or beyond.

Embryos vitrified in the present OPS procedure were not pre-equilibrated with impermeable solutes, such as sucrose and trehalose, before transfer to CPA solutions. In an early study, Renard et al reported that better survival and term rates were achieved in frozen-thawed 2-celled rabbit embryos, by sucrose-assisted equilibration and a holding time at subzero environment ( $-30\text{ }^{\circ}\text{C}$ ) for 30 to 240 min [49]. These appeared to be critical steps to drive water molecules extracellularly and shorten the time of embryo exposure to CPAs prior to freezing, which in turn reduced intracellular ice formation and potential cytotoxicity to the embryos [50]. Therefore, better development in vitrified pronuclear, 2-celled and 4-celled rabbit embryos might have been achieved if a pre-equilibration procedure had been included in the present study.

Improved cryotolerance of embryos after the 8-cell stage in the present study may be associated with embryonic genome activation (EGA) [51], membrane permeability, and other biological properties of embryos [48]. In the rabbit, the EGA occurs around the 8- to 16-cell stages [51], which might cause intrinsic changes of the membrane property of embryos as the genes associated with membrane structures might have turned

on. For instance, a small channel membrane protein aquaporin (AQP) functioned to transport extracellular fluids [52] was expressed in mouse morula-stage embryos and played a role in transporting CPAs [53]. Furthermore, forced expression of AQP improved the survival of mouse oocytes after vitrification and re-warming [54]. Therefore, expression of genes like AQP might help embryos to hasten equilibration of vitrification solution. We speculate that similar membranous changes might have also occurred in rabbit embryos. The number of blastomeres in an embryo may also affect the cryotolerance at a certain developmental stage. Obviously, damage to one blastomere is much more important in pronuclear or 2-celled embryos than in 8-celled, morula or blastocyst embryos. Perhaps microfilament depolymerization agents, such as propanediol, may improve survival and development of 1-celled rabbit embryos by preventing fracture of the cytoskeleton [55]. In addition, the S/V ratio had a direct influence on transporting water and CPAs through the membranes [56]. Higher S/V ratios are often associated with faster cooling/warming rates. However, there was a lack of such information in rabbit embryos. Characterization of S/V ratios of blastomeres in various embryonic stages (pronuclear to 8-cell) in the present study may be useful in future studies on rabbit embryogenesis, although little change was observed during the period of *in vitro* development (Table 3).

Term development was similar between the 8-celled (25.7%) embryos and the morula/blastocyst stage (19.4%) embryos, whereas expanded and hatched blastocyst rates were higher in the morula/blastocyst group, perhaps due to a closer stage of morula/blastocyst to expanding and hatching. Similarly, in the control embryos, the 8-cell group had higher term rates than the morula/blastocyst group (Table 2). Such observations in the 8-celled and the morula/blastocyst embryos may have been due to differences between *in vitro* and *in vivo* environments in supporting embryonic development. The transferred 8-celled embryos were exposed earlier and stayed longer time in oviducts than were their morula and blastocyst counterparts. Longer *in vitro* culture would presumably compromise embryo quality and developmental competence to term [57]. Furthermore, presence of a mucin coat surrounding rabbit embryos also contributed to embryo survival after transplantation [58–60]. In this study, all embryos were recovered at the zygote stage, with minimal mucin coating. After vitrification and embryo transfer, 8-celled embryos would be re-coated with thicker mucin layers due to their longer retention in oviductal and

uterine environments, which would be beneficial for their subsequent implantation [58]. In rabbits, live kits were previously produced only from vitrified pronuclear and morula/blastocyst stage embryos using various protocols [5–9]. Excitingly, a total of 11 live kits were born from vitrified-warmed cleavage stage embryos in the present study. This was the first report of live births from vitrified 2- to 8-celled rabbit embryos.

In summary, we demonstrated the feasibility of using the OPS method to vitrify rabbit embryos at various developmental stages. Based on both *in vitro* and *in vivo* studies, we inferred that the 8-cell stage was a critical point for acquiring cryotolerance of the OPS-vitrified rabbit embryos, as cryotolerance of embryos at or beyond the 8-cell stage increased. To maximize re-warming survivability and term development, it is recommended that rabbit embryos are OPS-vitrified for cryopreservation no earlier than the 8-cell stage. Further refinement of the present vitrification procedure may be required to improve cryotolerance of rabbit embryos at various developmental stages.

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