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Original Article

Mouse *in vivo*-derived late 2-cell embryos have higher developmental competence after high osmolality vitrification and −80°C preservation than IVF or ICSI embryos

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Abstract. Mammalian embryos are most commonly cryopreserved in liquid nitrogen; however, liquid nitrogen is not available in special environments, such as the International Space Station (ISS), and vitrified embryos must be stored at −80°C. Recently, the high osmolarity vitrification (HOV) method was developed to cryopreserve mouse 2-cell stage embryos at −80°C; however, the appropriate embryo is currently unknown. In this study, we compared the vitrification resistance of *in vivo*-derived, *in vitro* fertilization (IVF)-derived, and intracytoplasmic sperm injection (ICSI)-derived mouse 2-cell embryos against cryopreservation at −80°C. The ICSI embryos had lower survival rates after warming and significantly lower developmental rates than the *in vivo* and IVF embryos. Further, IVF embryos had a lower survival rate after warming, but a similar rate to the *in vivo* embryos to full-term development. This result was confirmed by simultaneous vitrification of *in vivo* and IVF embryos in the same cryotube using identifiable green fluorescent protein-expressing embryos. We also evaluated the collection timing of the *in vivo* embryos from the oviduct and found that late 2-cell embryos had higher survival and developmental rates to full-term than early 2-cell embryos. Some early 2-cell embryos remained in the S-phase, whereas most late 2-cell embryos were in the G2-phase, which may have affected the tolerance to embryo vitrification. In conclusion, when embryos must be cryopreserved under restricted conditions, such as the ISS, *in vivo* fertilized embryos collected at the late 2-cell stage without long culture should be employed.
Key words: Cryopreservation, Embryo, High osmolarity vitrification (HOV) method

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Determining whether mammalian species can reproduce normally in space or on other planets, where the environment markedly differs from that on Earth, including high levels of space radiation [1, 2] and microgravity [3], is of particular interest. The International Space Station (ISS) is currently the only option for studying the actual effects of the space environment on mammalian reproduction; however, long-term breeding of mice is impossible [4]. Embryo culture experiments could be performed on the ISS; however, vitrified embryos must be launched on rockets and warmed by the ISS astronauts.

Currently, vitrification is the first choice for mouse embryo cryopreservation owing to its technical simplicity [5]. Conventional vitrification methods require liquid nitrogen (LN_2: −196°C) to avoid intracellular ice crystal formation, which causes irreversible damage to embryos [6]. However, as LN_2 is not available on rockets and the ISS, conventional vitrification methods cannot be used for this purpose. Furthermore, vitrified embryos should typically be kept supercooled below −130°C to avoid cryodamage to embryos [7, 8]. Mochida *et al*. recently modified the conventional cryopreservation method and found that mouse 2-cell embryos can be kept at −80°C for more than one month and can be successfully shipped overseas on dry ice [9, 10]. This modified method is called high osmolality vitrification (HOV) [11]. If mouse embryos were cryopreserved using the HOV method, the embryos could be transported to the ISS without compromising their quality of development.

Although the performance of embryo culture experiments on the ISS may be possible, a high cost will be associated with launching the rocket and conducting the experiment on the ISS. Thus, failure of the experiments is not an option. Further, the experiments will be performed by astronauts who are not specialists on assessments with mammalian embryos. Considering these factors, high-quality embryos must be launched to increase their survival and developmental rates after warming. In addition, if the launch is postponed after the vitrified embryos are placed in the −80°C freezer on the rocket, these embryos would need to be replaced with backup embryos stored in LN_2 to maintain the high quality of the embryos. Accordingly, a large number of high-quality vitrified embryos must be prepared before starting the experiments on the ISS. In general, embryos can be obtained using three different methods: *in vivo* fertilized embryos, *in vitro* fertilization (IVF)-derived embryos, and embryos generated by intracytoplasmic sperm injection (ICSI). Each method has advantages and disadvantages.

In this study, we first compared the viability and developmental rates of vitrified/warmed mouse 2-cell embryos that were derived via *in vivo*, IVF, and ICSI methods and preserved at −80°C. To eliminate the influence of subtle differences due to vitrification techniques, we also conducted an experiment using identifiable 2-cell embryos derived from enhanced green fluorescent protein-expressing transgenic (eGFP-Tg) mice. To prepare the highest quality 2-cell embryos, we

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compared early and late 2-cell embryos, as well as short and long culture periods, to determine the conditions for better survival and developmental rates after preservation at −80°C.

Materials and Methods

Animals

Female and male ICR mice were obtained from SLC Inc. (Hamamatsu, Japan) and bred in our mouse facility. The eGFP-Tg ICR mouse strain was generated and maintained in our laboratory. Mice at 8–10 weeks of age were employed for the study. Surrogate pseudo-pregnant ICR females, which were used as embryo recipients, were mated with vasectomized ICR males whose sterility was demonstrated previously. On the day of the experiment or upon completion of all experiments, mice were killed by CO_2 inhalation or cervical dislocation. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi (reference number: A29-24), which followed the ARRIVE guidelines.

Media

HTF medium [12] was used for spermatozoa capacitation and IVF. HEPES-CZB (H-CZB) medium [13] was used for oocyte/embryo manipulation *in vitro*, and CZB medium [14] was used for oocyte/embryo incubation in 5% CO_2 at 37°C. Vitrification solutions of EFS20 and EFS42.5, and warming solutions of 0.25 M and 0.75 M sucrose-PB1 were used for embryo vitrification [9].

Preparation of in vivo fertilized embryos

Superovulation in female mice was induced via an injection of five international units (IU) of pregnant mare serum gonadotropin (PMSG) (ASKA Animal Health Co., Ltd., Tokyo, Japan) followed by an injection of 5 IU of human chorionic gonadotropin (hCG) (ASKA Animal Health Co., Ltd., Tokyo, Japan) at 48 h later. Immediately after hCG injection, each female mouse was mated with a male mouse. The mated female mice were examined the following day for the presence of vaginal plugs and then separated from the males. On the next day, 2-cell embryos were recovered by flushing the oviducts with H-CZB medium. In the first series of experiments (Tables 1–3), 2-cell embryos were collected from the oviduct at 44–46 h after hCG injection. In the next series of experiments (Tables 4 and 5), 2-cell embryos were collected from the oviduct at 40 h (early stage) or 49 h (late stage) after hCG injection. The collected embryos were placed in CZB medium and incubated at 37°C with 5% CO_2 until use.

Preparation of IVF and ICSI embryos

Superovulation in female mice was induced via an injection of 5 IU of PMSG followed by an injection of 5 IU of hCG at 48 h later. Cumulus oocyte complexes (COCs) in the oviducts of females were collected 14–16 h later and transferred to dishes containing H-CZB media. To prepare the sperm, the cauda epididymides were removed from a mature male mouse. Blood and adipose tissue were removed from the surface, and the caudal (enlarged) portion was excised using a pair of fine scissors. The excised specimens were compressed to release a dense mass of spermatozoa from which drops (approximately 2 µl) were collected and placed in a dish containing 0.4 ml HTF medium and cultured at 37°C in a CO_2 incubator for 20–30 min. IVF was performed as previously described [15, 16]. Five to ten microliters of sperm suspension were collected and introduced into each drop containing COCs. After insemination for 5–6 h, pronuclear formation was verified, and the zygotes were washed several times with 20-µL drops of CZB medium. The washed zygotes were then cultured in CZB at 37°C with 5% CO_2 until use. ICSI was performed as described previously [13, 17, 18]. To disperse the cumulus cells, the COCs were treated with 0.1% bovine testicular hyaluronidase for 3 min. Prior to start of the ICSI, 1–2 µl of the sperm suspension in HTF medium was directly transferred to polyvinylpyrrolidone (PVP) (Sigma-Aldrich Co. LLC, Tokyo, Japan) in the injection chamber for microinjection. Several piezo pulses were used to separate the spermatozoa heads from the tail. The head was then injected into oocytes. Pronuclear formation was verified 5–6 h after ICSI. Thereafter, the zygotes were washed several times with 20-µl drops of CZB and cultured in CZB medium at 37°C with 5% CO_2 until use.

Vitrification and warming

For the first series of experiments (Tables 1–3), IVF and ICSI 2-cell stage embryos were vitrified 46–48 h (1500 h to 1700 h) after hCG injection (Fig. 1A, E). For the second series of experiments (Tables 4, 5), *in vivo* 2-cell stage embryos were vitrified for 41 h (1000 h) or 50 h (1900 h) after hCG injection (Fig. 1G). The cryopreservation method was described previously [9]. Briefly, 15 to 20 embryos at the 2-cell stage were suspended in EFS20 equilibrium solution for 2 min and then transferred into cryotubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) containing 50 µl of EFS42.5. After 1 min, the cryotubes were plunged directly into LN_2 and then kept in LN_2 (−196°C) until use. Prior to the experiment, the cryotubes were stored at −80°C for 3–7 days. On the day of the experiment, the cryotubes were retrieved from the −80°C freezer and kept at room temperature for 1 min 30 sec. Thereafter, 850 µl of 0.75 M sucrose-PB1 solution was added to the cryotubes and left to stand for 4 min 30 sec. The solution was then mixed 4–5 times using a pipette. The entire volume of the solution was transferred to a dish and embryos were transferred to 0.25 M sucrose-PB1. After 3 min of equilibration, the recovered embryos were washed with two drops of 0.25 M sucrose-PB1 medium, checked for the states of slight shrinkage, and transferred to CZB medium. Viable embryos were incubated at 37°C in 5% CO_2.

Aphidicolin treatment

Early 2-cell embryos or late 2-cell embryos collected from oviducts were cultured in CZB medium with 3 mg/ml aphidicolin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), a specific inhibitor of replicative DNA polymerases [19]. The next morning (1000 h), the developmental stage of the treated embryos was evaluated. Some embryos were cultured in CZB containing 0.1% DMSO (Sigma-Aldrich Co. LLC, Tokyo, Japan), the solvent used for aphidicolin, to serve as a control. Theoretically, if embryos are in the S-phase of the cell cycle, aphidicolin will arrest the cell cycle and the embryo will not be able to develop to the 4-cell stage.

Embryo transfer

The vitrified-warmed 2-cell embryos derived from *in vivo* or IVF were cultured for 1–2 h to recover from vitrification damage. Thereafter, some randomly selected embryos were transferred to day 0.5 pseudopregnant mice that had been mated with vasectomized males the night prior to the transfer [16]. Five to eight embryos were transferred to each oviduct. On day 18.5, the offspring was delivered by cesarean section and allowed to mature. The remaining unused (untransferred) embryos were cultured for up to four days to evaluate their potential for development into blastocysts.

Statistical analysis

The survival rates, developmental rates, birth rates, and implanted rates were evaluated using the chi-squared test or the Tukey-Kramer test. Statistical significance was set at $P < 0.05$.

Results

Comparison of the survival and developmental rates among in vivo, IVF, and ICSI embryos preserved at −80°C

To determine the highest tolerance of embryos to cryopreservation at −80°C for more than 3 days, survival and developmental rates were compared among *in vivo*, IVF, and ICSI 2-cell embryos (Fig. 1A). *In vivo* embryos had a significantly higher survival rate (62%) after warming than IVF embryos (40%) and ICSI embryos (35%) (Fig. 1B, Table 1). When vitrified-warmed embryos were cultured for 3 days, the *in vivo* and IVF embryos had significantly higher developmental rates to the blastocyst stage (50%–68%) than the ICSI embryos (10%) (Fig. 1C, D, Table 1). When vitrified-warmed *in vivo* and IVF 2-cell embryos were transferred into recipient females, live healthy offspring was obtained at similar success rates of 66% and 55%, respectively (Table 2). Body weight did not differ significantly between the *in vivo* and IVF embryos. Similar results were observed for vitrified embryos cryopreserved in LN_2 (Tables 1 and 2). Although in theory, cryopreservation in LN_2 will lead to a higher survival rate of embryos than cryopreservation at −80°C, the survival rate of ICSI embryos was lower in LN_2 than at −80 °C (21% *vs*. 35%, respectively). However, surviving embryos cryopreserved in LN_2 had significantly higher developmental rates to the morulae than embryos cryopreserved at −80°C (65% *vs*. 25%, respectively). In addition, we previously cultured 2-cell fresh embryos derived from *in vivo*, IVF, and ICSI, and most embryos were found to develop to the morula and blastocyst stages (100% [n = 20], 91% [n = 22], and 82% [n = 38], respectively, unpublished data).

Table 1. Survival and *in vitro* developmental rates of vitrified-warmed *in vivo*, IVF, and ICSI 2-cell embryos

Preservation temperature	Embryo type	No. of embryos vitrified	No. (%) of embryos retrieved	No. (%) of embryos survived	No. (%) of embryos developed to		
					4–8 cell	Morula	Blastocyst
−80 ºC	*in vivo*	404	398 (99)	247 (62) [a]	202 (82) [a]	188 (76) [a]	169 (68) [a]
	IVF	131	125 (95)	50 (40) [b]	37 (74) [a]	34 (68) [a]	25 (50) [b]
	ICSI	182	179 (98)	63 (35) [b]	24 (38) [b]	16 (25) [b]	6 (10) [c]
−196 ºC	*in vivo*	108	107 (99)	69 (64) [a]	48 (70) [a]	44 (64) [a]	40 (58) [a]
	IVF	206	193 (94)	77 (40) [b]	63 (82) [a]	56 (73) [a]	44 (57) [a]
	ICSI	84	81 (96)	17 (21) [c]	12 (71) [a]	11 (65) [a]	2 (12) [b]

Retrieval rate: number of retrieved embryos/vitrified embryos; Survival rate: number of surviving embryos/retrieved embryos; developmental rate: number of embryos at each stage/surviving embryo. Survival and developmental rates were evaluated using the Tukey-Kramer test. Statistically significant differences between variables were determined at $P < 0.05$.

Table 2. Full-term development of vitrified-warmed *in vivo* and IVF 2-cell embryos

Preservation temperature	Embryo type	No. of embryos vitrified	No. (%) of embryos retrieved	No. (%) of embryos survived	No. of embryos transferred [no. of recipients]	No. (%) of offspring	No. (%) of implanted	Mean SD of offspring body weight (g)
−80 ºC	*in vivo*	103	102 (99)	92 (90)	61 [5]	40 (66) [a]	46 (75) [a]	1.60 ± 0.26
	IVF	117	115 (98)	54 (47)	49 [4]	27 (55) [a]	36 (73) [a]	1.67 ± 0.17
−196 ºC	*in vivo*	103	100 (97)	85 (85)	72 [5]	38 (53) [a]	46 (64) [a]	1.65 ± 0.19
	IVF	52	52 (100)	31 (60)	30 [2]	13 (43) [a]	16 (53) [a]	1.71 ± 0.14

Retrieval rate: number of retrieved embryos/vitrified embryos; survival rate: number of surviving embryos/retrieved embryos; developmental rate: number of embryos at each stage/surviving embryos; Offspring rate: number of offspring/transferred embryos; Implantation rate: number of implantation/transferred embryos. Offspring and implant rates were evaluated using the chi-squared test. Statistically significant differences between variables were determined at $P < 0.05$.

Table 3. Comparison of the developmental potential of *in vivo* and IVF 2-cell embryos after cryopreservation at −80ºC in the same tube

Embryo type	No. of embryos examined	No. (%) of embryos retrieved	No. (%) of embryos survived	No. (%) of embryos developed to		
				4–8 cell	Morula	Blastocyst
in vivo GFP (+)	50	49 (98)	47 (96) [a]	43 (91) [a]	43 (91) [a]	38 (81) [a]
IVF GFP (−)	50	49 (98)	27 (55) [b]	19 (70) [b]	18 (67) [b]	15 (56) [b]
in vivo GFP (−)	58	58 (100)	44 (76) [a]	43 (98) [a]	42 (95) [a]	40 (91) [a]
IVF GFP (+)	57	57 (100)	32 (56) [b]	30 (94) [a]	26 (81) [b]	20 (63) [b]

Retrieval rate: number of retrieved embryos/vitrified embryos; Survival rate: number of surviving embryos/retrieved embryos; developmental rate: number of embryos at each stage/surviving embryo. Survival and developmental rates were evaluated using the chi-squared test. Statistically significant differences between variables were determined at $P < 0.05$.

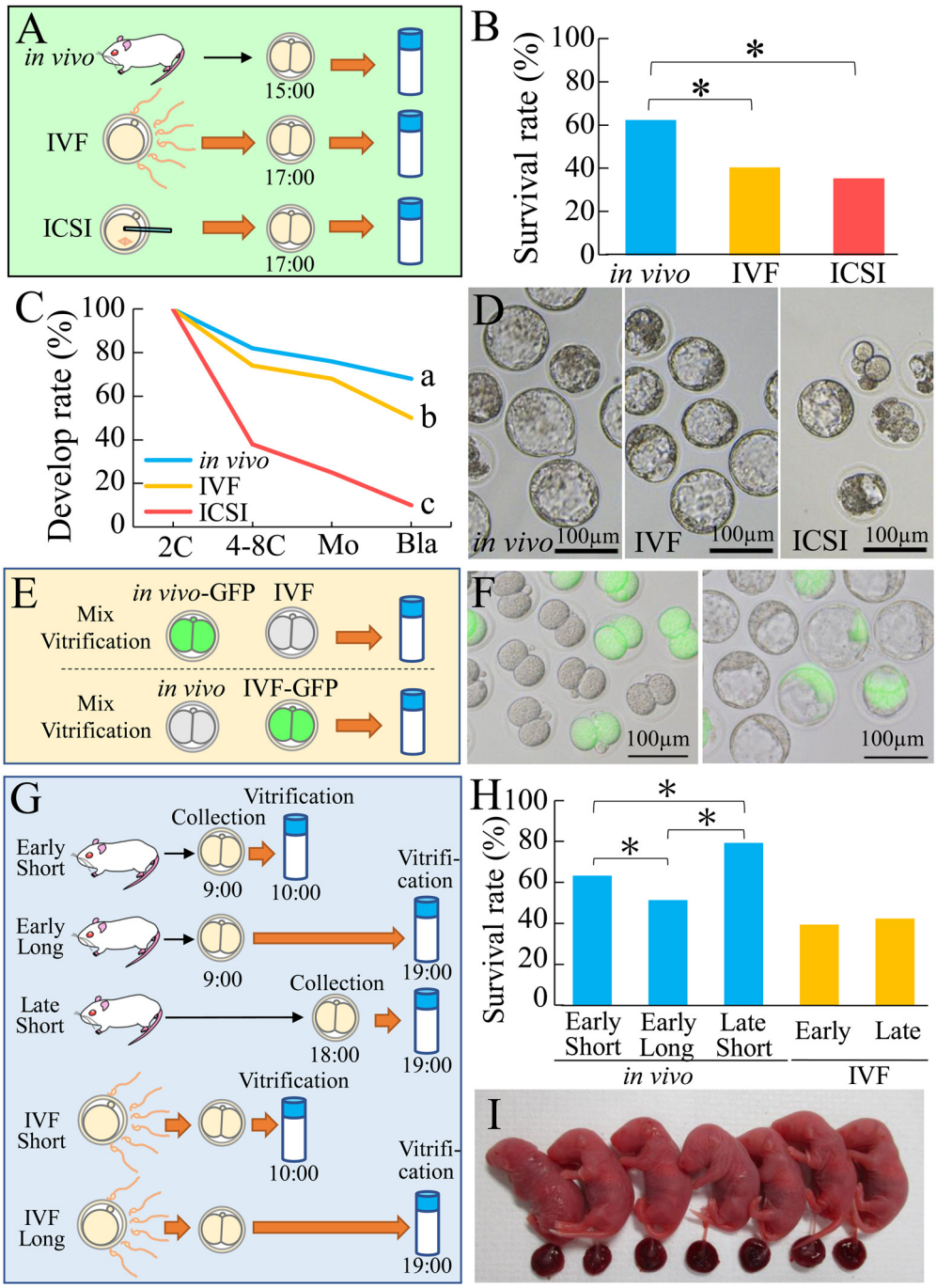

Fig. 1. Evaluation of the tolerance of different types of 2-cell embryos to preservation at −80°C. (A) Three fertilization methods were compared. *In vivo* fertilized embryos were collected from oviducts at the 2-cell stage and vitrified at approximately 1500 h. The IVF and ICSI embryos were vitrified at approximately 1700 h. (B, C) Survival rates after warming (B) and rate of development to the blastocyst stage (C) of embryos. The graphs are based on the original data in Table 1. (D) Blastocysts derived from vitrified-warmed 2-cell embryos after three days of culture. (E) *In vivo* and IVF 2-cell embryos and their counterpart 2-cell embryos derived from green fluorescent protein-expressing transgenic (eGFP-Tg) mice were mixed and vitrified in the same cryotubes. (F) Images of *in vivo* and IVF embryos mixed with their transgenic counterparts and differentiated based on GFP expression. Left: Immediately after warming. Right: Three days after warming. (G) Schematic for the determination of optimal collection timing and culture period of *in vivo* 2-cell embryos. (H) Survival rates of embryos after warming. This graph is based on the original data in Table 4. (I) Healthy offspring derived from vitrified-warmed *in vivo* late 2-cell embryos. The survival and developmental rates were analyzed using the Tukey-Kramer test ($P < 0.05$).

Effect of cryopreservation techniques on the survival and developmental rates of 2-cell embryos after preservation at −80°C

Cryopreservation of embryos by vitrification requires skilled techniques. When performed by an unskilled experimentalist, the survival rates of embryos preserved in each cryotube might differ, even when the same type of embryos are used. Therefore, we conducted an experiment using identifiable *in vivo* or IVF 2-cell embryos derived from eGFP-Tg mice and mixed them with corresponding IVF or *in vivo* wild-type embryos. The mixed embryos were then simultaneously vitrified in the same cryotubes (Fig. 1E, F). The survival rate and

developmental rate of blastocysts after warming were significantly higher in the mixed *in vivo* embryos than the mixed IVF embryos (Table 3). Therefore, the results of this study were not influenced by the technique, but by the embryo type.

Comparison of early and late 2-cell embryos, and short and long culture periods to identify the optimum conditions for the survival and developmental rates after cryopreservation at −80°C

The *in vivo* embryos were collected from the oviduct as early 2-cell embryos at 0900 h (40 h after hCG injection) or as late 2-cell embryos at 1800 h (49 h after hCG injection), cultured for 1 h (short) or 10 h (long), and then vitrified (Fig. 1G). As shown in Fig. 1H and Table 4, when the collection timing was compared, the survival rate of late 2-cell embryos after vitrification (79%) was better than that of early 2-cell embryos (51%–63%). The survival rate was also better when embryos were cultured for a short period than a long culture period, with survival rates of 63%–79% and 51%, respectively. Similarly, when the developmental rates to the blastocyst stage were compared, late 2-cell embryos had higher developmental rates to the blastocyst stage (71%) than early 2-cell embryos (54%–60%). Furthermore, short culture resulted in higher developmental rates to the blastocyst stage (60%–71%) than long culture (54%). After embryo transfer into recipient females, the highest birth rates were obtained from late collection-short culture 2-cell embryos (52%) relative to early collection-short culture (39%) and early collection-long culture 2-cell embryos (40%) (Fig. 1I, Table 5).

The survival and developmental rates of early vitrified or late vitrified IVF 2-cell embryos were assessed (Fig. 1G). The survival rates of early and late embryos were not significantly different at 39% and 42%, respectively (Fig. 1H) (similar to Table 1). However, the developmental rate to the blastocyst stage of early vitrified 2-cell embryos (70%) was higher than that of late vitrified 2-cell embryos (59%) (Table 4).

Assessment of the cell cycle of early and late 2-cell embryos

We hypothesized that the cell cycle of embryos may affect the tolerance to vitrification, particularly in the S-phase. Therefore, we examined the stage of the cell cycle in early and late 2-cell embryos using aphidicolin, in which cell division was prevented during the S-phase by inhibiting DNA polymerization [19]. As shown in Table 6, of the early 2-cell embryos treated with aphidicolin, 16 of 48

Table 4. Survival and *in vitro* developmental rates of early and late stage 2-cell embryos, and short-term and long-term cultured embryos after cryopreservation at −80ºC

Embryo type	Embryo Stage	Culture period	No. of embryos vitrified	No. (%) of embryos retrieved	No. (%) of embryos survived	No. (%) of embryos developed to		
						4–8 cell	Morula	Blastocyst
in vivo	Early	Short	189	185 (98)	117 (63) [a]	92 (79) [a]	91 (78) [a]	70 (60) [ab]
	Early	Long	217	211 (97)	108 (51) [b]	88 (81) [a]	71 (66) [a]	58 (54) [a]
	Late	Short	95	92 (97)	73 (79) [c]	62 (85) [a]	56 (77) [a]	52 (71) [b]
IVF	Early	24 h	51	51 (100)	20 (39) [a]	19 (95) [a]	16 (80) [a]	14 (70) [a]
	Late	34 h	53	52 (98)	22 (42) [a]	22 (100) [a]	18 (82) [a]	13 (59) [a]

Retrieval rate: number of retrieved embryos/vitrified embryos; Survival rate: number of surviving embryos/retrieved embryos; developmental rate: number of embryos at each stage/surviving embryo. Survival and blastocyst rates were evaluated using the chi-squared test and Tukey-Kramer test, respectively. Statistically significant differences between variables were determined at $P < 0.05$.

Table 5. Full-term development of early and late stage *in vivo* 2-cell embryos after cryopreservation at −80ºC

Embryo stage	Culture period	No. of embryos vitrified	No. (%) of embryos retrieved	No. (%) of embryos survived	No. of embryos transferred [no. of recipients]	No. (%) of offspring	No. (%) of implanted	Mean SD of offspring body weight (g)
Early	Short	65	63 (97)	38 (60)	33 [4]	13 (39) [a]	17 (52) [a]	1.71 ± 0.16
Early	Long	154	148 (96)	61 (41)	53 [6]	21 (40) [a]	27 (51) [a]	1.62 ± 0.26
Late	Short	87	87 (100)	64 (74)	56 [5]	29 (52) [a]	37 (66) [a]	1.67 ± 0.16

Retrieval rate: number of retrieved embryos/vitrified embryos; survival rate: number of surviving embryos/retrieved embryos; developmental rate: number of embryos at each stage/survived embryo; Offspring rate: number of offspring/transferred embryos; Implantation rate: number of implantation/transferred embryos. Offspring and implant rates were evaluated using the Tukey-Kramer test. Statistically significant differences between variables were determined at $P < 0.05$.

Table 6. *In vitro* development of early and late 2-cell embryos in CZB medium supplemented with aphidicolin

Embryo stage	Aphidicolin	No. of embryos	No. (%) of embryos arrested at 2-cell stage	No. (%) of embryos developed to 4-cell stage
Early	+	48	16 (33) [a]	32 (67) [a]
Late	+	67	5 (7) [b]	62 (93) [b]
Early	–	33	1 (3) [a]	32 (97) [a]
Late	–	35	0 (0) [a]	35 (100) [a]

Developmental rates were evaluated using the chi-squared test. Statistically significant differences between variables were determined at $P < 0.05$.

(33%) stopped at the 2-cell stage. Meanwhile, only 5 of the 67 late 2-cell embryos treated with aphidicolin stopped at the 2-cell stage (7%). Such finding indicates that some of the early 2-cell embryos were still in the S-phase, but almost all the late 2-cell embryos had already proceeded to the G2-phase (Table 6).

Discussion

The birth rates between ICSI or IVF embryos are thought to lack significant differences when normal spermatozoa are used [13, 16]. Therefore, ICSI has been widely used in fertility clinics, despite being a highly artificial method of fertilization compared to IVF. In the current study, when IVF and ICSI embryos were vitrified at the 2-cell stage, the survival rates after warming were lower than those of *in vivo* embryos, regardless of the storage temperature. For the ICSI embryos, not only the survival rates, but also the developmental rates were significantly reduced compared to those of the *in vivo* or IVF embryos. Interestingly, this damage was most likely a phenomenon observed only when the embryos were vitrified at the two-cell stage, as no differences were found with each fertilization method if vitrification was not performed.

Why did the different fertilization methods result in different survival and developmental rates after embryo vitrification and warming? ICSI embryos are produced via the injection of spermatozoa through a glass needle 5–7 μm in diameter [13], causing significant damage to the cell membrane of the oocytes. In addition, the PVP medium used in ICSI is injected simultaneously with the whole spermatozoa, including the acrosome membrane and some spermatozoa enzymes [13]. These materials do not enter the oocyte during natural fertilization and may be responsible for the lower developmental rates of ICSI 2-cell embryos. However, healthy babies were previously reported to be delivered from ICSI embryos, which were vitrified at the blastocyst stage before embryo transfer [20]. The damage derived from ICSI treatment may thus be recovered during development, at least at some points.

IVF and ICSI embryos underwent a longer period of *in vitro* culture than *in vivo* embryos collected at the 2-cell stage, which also suggested that the damage to IVF and ICSI embryos is due to this longer culture period *in vitro*. Previously, when embryos at different developmental stages were frozen and thawed, the survival and developmental rates of each stage of embryos obtained from *in vitro* culture decreased with increased culture periods [21], while embryos recovered from oviduct or uteri and frozen without *in vitro* culture had higher survival and developmental rates [22]. Therefore, we cannot exclude the possibility that the *in vivo* embryos might have had lower survival and developmental rates after warming when cultured *in vitro* from the 1-cell stage to the 2-cell stage before vitrification.

The current study revealed that embryos harvested at a late 2-cell stage (Late-short) had higher survival and developmental rates after warming than those harvested at an earlier 2-cell stage (Early-short). When early 2-cell embryos were vitrified at the late 2-cell stage after 10-h of *in vitro* culture (Early-long), their survival and developmental rates were lower than those of late 2-cell embryos collected 1 h before vitrification (Late-short) (Table 4). In general, the 1-cell to 2-cell stage of mouse embryos is well known as a critical period as maternally inherited RNA/protein degradation and zygotic genome activation (ZGA), which are essential events for normal embryo development, occur during this period [23]. When late-stage mouse 2-cell embryos were collected, ZGA was completed; however, when early-stage mouse 2-cell embryos were collected, ZGA was ongoing [24]. On the other hand, experiments using aphidicolin revealed that 33% of the early 2-cell stage embryos were still in the S-phase of DNA synthesis, whereas most of the late 2-cell embryos (93%) were in the G2 or M phase (Table 6). The M phase might be resistant to treatment of vitrification-warming of late-stage mouse 2-cell embryos as the chromosomes form a spindle and become compact. These results suggest that ZGA and the S-phase caused lower survival and developmental rates of early stage mouse 2-cell embryos (Early-short) after vitrification-warming (Fig. 1H). Although we did not examine the ZGA and cell cycle of *in vitro* cultured late 2-cell embryos (Early-long), it may cause a delay in these phenomena during the longer period of incubation compared to collected late 2-cell embryos (late-short).

The survival and developmental rates of IVF embryos did not improve when the 2-cell embryos were vitrified at an early stage compared the later stage. Such finding might be due to the IVF 2-cell embryos having already spent a longer period in the incubator (more than one day). Even if the culture period was reduced for several hours, it would no longer be effective. Thus, the period of *in vitro* culture had a negative impact on the survival rates of 2-cell embryos after warming. The culture medium was developed to enable embryo development *in vitro* and does not consider embryo vitrification tolerance. In the future, it will be necessary to develop an optimal culture medium for embryo culture as well as tolerance to vitrification.

When embryo culture experiments are performed on the ISS, the astronauts will be asked to perform the difficult operations of warming, washing, and culturing of the vitrified embryos. Therefore, sending the highest quality embryos is important to ensure the success of the experiments. In the current study, we found that the most appropriate embryos for use in the ISS experiments were late 2-cell embryos that had been vitrified without long culture. We believe that this method will enable the relationship between embryo development/cell differentiation and gravity to be clarified by astronauts on the ISS.

Conflict of interests: The authors declare no conflict of interests.

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