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—Original Article—

Time-lapse monitoring reveals that vitrification increases the frequency of contraction during the pre-hatching stage in mouse embryos

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Abstract. Contraction during the blastocyst stage is observed during embryonic development of various mammals, including humans, but the physiological role of this process is not well understood. Using time-lapse monitoring (TLM), we studied the influence of vitrification and contractions on embryonic development in mice. Mouse embryos were cultured at the 2-cell stage. At the 8-cell stage, embryos were randomly divided into a fresh group (FG) and vitrified group (VG) and observed for up to 144 h. Strong contractions (i.e., contractions causing a decrease in volume of more than 20% and expansion of the perivitelline space) occurred significantly more often in unhatched embryos than hatching embryos in both groups. Regarding hatching embryos, contractions in the pre-hatching stage were significantly more frequent in the VG than the FG. Furthermore, mRNA expression levels of genes related to contractions were determined at three time points, the 8-cell stage, early blastocyst stage, and 20 h after blastocoel formation, with quantitative reverse transcription-polymerase chain reaction. There was no significant difference in *Hspa1a* expression between the FG and VG, but *Hspa1a* overexpression was observed just after thawing and tended to decrease gradually thereafter in some blastocysts. Furthermore, in the VG, *Atp1a1* tended to show higher expression in the strong contraction group than in the weak contraction group. Overall, vitrification is an excellent method for cryopreservation but could increase contractions in the pre-hatching stage and may increase energy demands of the embryo. Observation of contraction by TLM may improve the evaluation of embryo quality.
Key words: Blastocyst, Contraction, Hatching, Time-lapse, Vitrification

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Since the first reported pregnancy from a frozen-thawed human embryo [1], which resulted in a live birth [2], assisted reproductive technology has progressed rapidly. The number of successful pregnancies resulting from the transfer of vitrified and thawed embryos increases annually. However, the influence of the vitrification and thawing procedures on embryo quality in a variety of mammals remains unclear. For example, there are many reports showing alterations in gene expression [3–6] and zona hardening [7–9] following vitrification and thawing. In recent years, technical improvements in time-lapse monitoring (TLM) have enabled consecutive observations of embryonic development, revealing novel features of embryonic behavior [10–12].

Contraction of the blastocyst was first reported by Lewis and Gregory in 1929 [13]. In 2003, Niimura alluded to the mechanism of contraction in the mouse blastocyst [14], but since then, there have been few studies of the physiological role of blastocyst contraction. Maezawa *et al*. reported the utility of TLM in the selection of human shrunken blastocysts by observing contractile behavior [15]. They performed vitrification and thawing in blastocyst-stage embryos, and divided the expanded blastocysts and shrunken blastocysts after 5 h. They confirmed that many shrunken blastocysts showed full implantation potential after they were warmed for at least 5 h more. We considered that more detailed observations of embryonic developmental stages and blastocyst contraction using TLM, might contribute to improvement of embryo quality during the sorting procedure. However, evaluating this possibility in human embryos requires long-term culture without transplantation, which is associated with ethical limitations. Therefore, we carried out these observations using mouse embryos to provide a preliminary assessment of the potential of this method for assisted reproduction in humans.

Vitrification has been proposed to affect embryonic gene expression [3–6]. However, there has been no report confirming a difference in gene expression according to the presence of contractile behavior. Therefore, we evaluated the mRNA expression of genes encoding proteins that are considered to play an important role in blastocyst contraction and compared the level of expression between embryos collected at different developmental stages (8-cell stage, early blastocyst stage and 20 h after blastocoel formation). Specifically, the expression levels of *Hspa1a*, which has been reported as an indicator of cell stress [16, 17], *Mylk,* which is thought to play a direct role in contractions and blastocoel formation [14, 18, 19], and *Atp1a1*, which plays an important role in re-expansion of the blastocyst [14, 20–23], were evaluated using quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Furthermore, the embryos were divided

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into a vitrified group (VG) and fresh group (FG) and characterized by the degree of observed contractions using TLM.

The aims of this study were to examine the influences of vitrification and contractions on embryo quality by using TLM and mRNA analysis and to determine the feasibility of using contractile observation by TLM as a new noninvasive embryo sorting method.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee (Permission number: a-1-2642) and carried out according to Akita University Animal Experimentation Regulations. The experimental design and timing schedule are shown in Fig. 1.

Collection of mouse embryos

Mice of strain C57BL/6J (CLEA Japan, Tokyo, Japan, and Japan SLC, Shizuoka, Japan) were reared in a climate-controlled environment (temperature, 21 ± 2ºC; humidity, 50 ± 10%; daily light period, 0700–1900 h) and allowed *ad libitum* access to food and water.

Mouse embryos were collected at the 2-cell stage according to a previously described method [24] with slight modifications. To induce ovulation, female mice aged 9–11 weeks received an intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (Pergogreen, Serono Laboratories, Geneva, Switzerland) and then 7.5 IU of human chorionic gonadotropin (Mochida Pharmaceutical, Tokyo, Japan) 48 h later. The mice were mated, and the presence of a vaginal plug was confirmed in each female the next morning. Twenty-four hours later, the mice were euthanized by cervical dislocation, and the fallopian tubes and (in some cases) uteri were resected. Preimplantation embryos at the 2-cell stage were obtained by perfusing the fallopian tubes with human tubal fluid medium (NK Systems, Osaka, Japan) including 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA).

Culture environment and observation period

Embryos were cultured with CZB medium (5000 mg/l bovine serum albumin, 251 mg/l $CaCl_2$-$2H_2O$, 1000 mg/l D-glucose, 360 mg/l KCl, 160 mg/l KH_2PO_4, 146 mg/l L-glutamine, 290 mg/l $MgSO_4$•$7H_2O$, 4770 mg/l NaCl, 2110 mg/l $NaHCO_3$, 5.848 mg/l sodium lactate, 29 mg/l sodium pyruvate, 100000 U/l penicillin G, 10 mg/l phenol red, and 100 mg/l streptomycin sulfate) under mineral oil to prevent concentration by evaporation. Cells were cultured in 5% O_2/5% CO_2 at 37ºC in a shaded environment in a time-lapse incubator.

At the 8-cell stage, 108 embryos were vitrified using a Cryotop Safety Kit (Kitazato Biopharma, Shizuoka, Japan) [25]. Three to eight embryos were vitrified in each Cryotop.

The FG was observed for 120 h from the 2-cell stage, and the VG was observed for 96 h after thawing. Observation was terminated when hatching was complete or after degeneration had clearly occurred. When neither of these events occurred, the embryos were observed for 144 h.

Observation by TLM

Preimplantation embryos were observed with two TLM systems (CCM-M1.4, Astec, Fukuoka, Japan, and Primo Vision, Vitrolife, Vastra Frolunda, Sweden) at 3–5-min intervals. Morphological evaluation was performed using continuous photographs and still images.

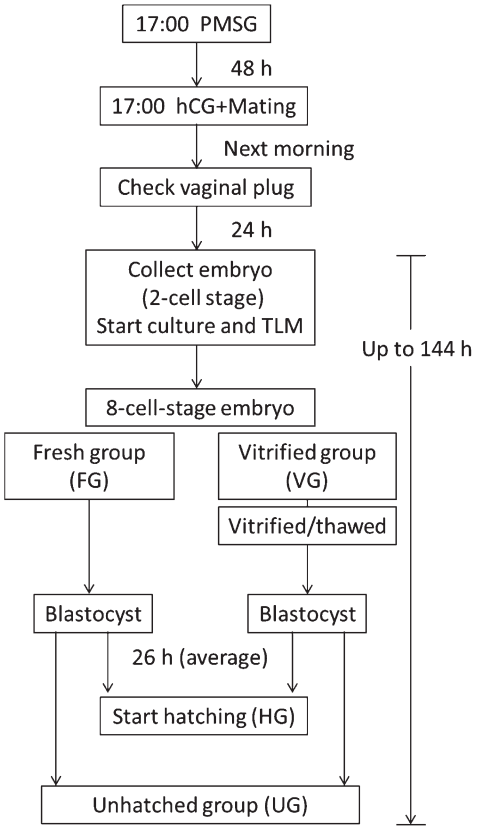

Fig. 1. Timetable of the experimental design. See text for details.

The number of contractions in the pre-hatching stage, blastocyst rate and hatching rate were measured in the FG and VG. Contraction movement was also compared between the hatching group (HG) and unhatched group (UG). In accordance with a previous study [14], we defined strong contractions as those when the blastocyst volume decreased by more than 20% and expansion of the ovum circumference cavity was observed, and all other contractions were defined as weak contractions (Fig. 2).

RNA extraction and RT-qPCR

We divided the embryos into the following groups: 8-cell stage, early blastocyst stage (FG and VG), and 20 h after blastocoel formation; the VG was further divided into groups with and without strong contractions at 20 h after blastocoel formation (SC+ and SC–, respectively). We collected 10 embryos from each group for genetic analysis. RNA was extracted from the embryos using the NucleoSpin RNA XS kit (Macherey-Nagel, Duren, Germany). Total RNA was subjected to RT using PrimeScript RT Master Mix (TaKaRa Bio, Otsu, Japan).

RT-qPCR was performed in a Thermal Cycler Dice® Real-time System (TP850, TaKaRa Bio) using SYBR Premix Ex Taq II (TaKaRa Bio). The target and reference primers used for PCR are listed in Table 1. Thermocycling was carried out in a final volume of 25 µl containing 2.0 µl of cDNA, 0.4 µM of each of the forward and

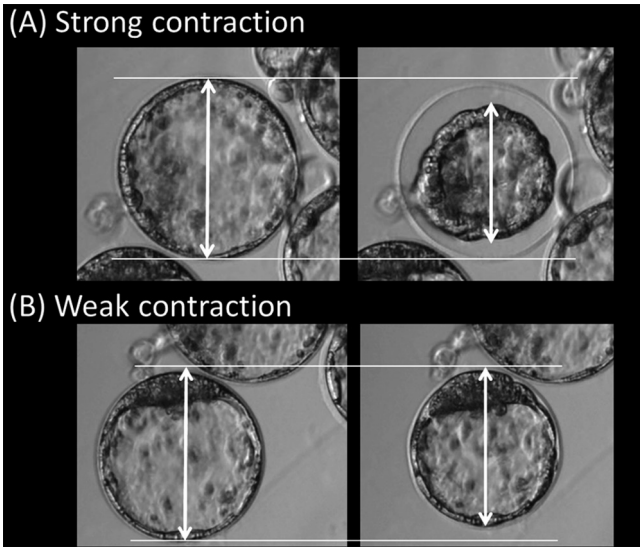

Fig. 2. Definition of contraction types. All images were taken at the same magnification and in the same field of view. The images show contraction from the anteroposterior view of the blastocyst. Arrows indicate the diameter of each blastocyst. (A) An example of strong contraction: the volume reduction is greater than 20%, and is associated with expansion of the perivitelline space. This example shows a 41% volume reduction. (B) Weak contraction: the volume reduction is less than 20%, and is not associated with expansion of the perivitelline space. This example shows a 17% volume reduction.

reverse primer and 12.5 µl of SYBR Premix Ex Taq II. The PCR conditions were as follows: initial denaturation step of 95ºC for 30 sec, followed by 40 cycles of 95ºC for 5 sec and 60ºC for 30 sec. Gene expression levels were determined from at least three replicates.

Statistical analysis

All statistical analyses were performed using Statistical Package for the Biosciences software (Version 9.67, Akita University, Akita, Japan). Fisher's exact test was used to detect differences in the blastocyst rates and hatching rates between the FG and VG. The same test was used to detect differences in hatching initiation rate between the VG (SC–) and VG (SC+) groups. The Wilcoxon rank-sum test was used to detect differences between groups with respect to the number of contractions and the time to hatching from blastocoel formation. The paired t-test was used to detect differences in gene expression levels between groups. P-values less than 0.05 were considered statistically significant. The mRNA levels determined by RT-qPCR are presented as means ± SEM. The numbers of contractions are presented as means (25th percentile, median, 75th percentile).

Results

Influence of vitrification on embryonic development

The survival rate after vitrification in the 8-cell-stage was 100%. There was no significant difference in the blastocyst rate and hatching rate between the FG and VG (Table 2).

The average time (25th percentile, median and 75th percentile) from blastocyst formation to the start of hatching was 24.34 h (15.75 h, 23.75 h, 32.25 h) in the FG and 26.05 h (17 h, 26.5 h, 30.75 h) in the VG, with no significant difference. Therefore, this time range was chosen for performing the contractile measurements.

Numbers of contractions related to vitrification and hatching

We observed the number of contractions for the mean durations from blastocoel formation to hatching (24.5 h in FG and 26.5 h in VG) using TLM. The contractions were classified as strong or weak (Fig. 2) and compared in the FG and VG as well as in the HG and UG.

Within the FG, there were significantly more total contractions and strong contractions in the UG than in the HG. There was no significant difference in the number of weak contractions between the HG and UG. Within the VG, there were significantly more strong contractions in the UG than in the HG, and significantly fewer weak contractions, but there was no significant difference in the total number of contractions between the HG and UG (Table 3). Furthermore, when a strong contraction occurred, more time was required for re-expansion. Therefore, fewer weak contractions were observed in the UG compared with the HG.

Within the HG, there were significantly more total contractions, strong contractions, and weak contractions in the VG than in the FG. The same trend was observed within the UG, but there was no significant difference between the number of strong or weak contractions in the FG and VG (Table 4).

Table 1. Primers used in RT-qPCR

Gene			Sequence (5'–3')	GenBank accession no.	bp
Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)	Reference	For	TGTGTCCGTCGTGGATCTGA	NM_008084.3	150
		Rev	TTGCTGTTGAAGTCGCAGGAG		
Heat shock protein 1A *(Hspa1a*)	Target	For	GTGGTTGCACTGTAGGACTTGTTTC	NM_010479.2	102
		Rev	GACCCGAGTTCAGGATGGTTG		
Myosin, light polypeptide kinase (*Mylk*)	Target	For	CTGCTGTAGCTCAACCCAGATCC	NM_139300.3	109
		Rev	ACCGCTTCCAGAATGAGACACC		
ATPase, Na+/K+ transporting, alpha 1 polypeptide (*Atp1a1*)	Target	For	CTGATCAGCATGGCCTATGGAC	NM_144900.2	87
		Rev	ACCGTTCTCAGCCAGAATCACA		

For, forward; Rev, reverse.

Table 2. Comparison of the rates of *in vitro* development to blastocysts and hatching between the fresh group (FG) and vitrified group (VG)

Condition of embryos	% (no.) of embryos		
	Survived	Developed to blastocyst	Hatching
Fresh	–	97.1 (100/103)	72 (72/100)
Vitrified	100 (111/111)	97.3 (108/111)	66.7 (72/108)

There was no significant difference in blastocyst rate and hatching rate between the FG and VG. P-values were analyzed with Fisher's exact test.

Influence of strong contractions on hatching

We assessed the association of strong contractions, with hatching rate in the FG and VG. Overall, a strong contraction was associated with a low hatching rate. Among the SC– embryos, 96% (46/48) of the FG and 94% (31/33) of the VG started hatching. On the other hand, among the SC+ embryos, only 50% (26/52) of the FG and 55% (41/75) of the VG could start hatching. Overall, strong contraction was found to have a negative influence on hatching, in both the FG and VG, as the embryo had a significantly harder time to hatch when strong contraction occurred (FG, odds ratio, 23.00, 95% CI, 5.05–104.80; VG, odds ratio, 12.85, 95% CI, 2.87–57.63; $P < 0.01$).

RT-qPCR

We analyzed the mRNA expression levels of contraction-related genes at three different stages: 8-cell stage, early blastocyst stage (FG and VG), and 20 h after blastocoel formation (FG and VG [SC+/–]). No significant difference between the FG and VG was observed at any stage. Furthermore, there was no significant difference between the SC+ and SC– groups of the VG. However, *Hspa1a* and *Mylk* showed lower expression just after thawing and were overexpressed in some early blastocyst-stage embryos compared with the FG, and the levels of these genes gradually reduced at later stages. In the VG (SC+), mRNA expression in some embryos tended to be lower than that of embryos of the VG (SC–), but *Atp1a1* was overexpressed in the group with stronger contractions (Fig. 3).

Discussion

In this study, we assessed the influence of vitrification on hatching and contractions without using assisted hatching to examine its effects from a noninvasive point of view. Among the embryos that start hatching, we observed significantly more contractions in the VG than in the FG. Zona hardening has been shown to influence embryonic growth [9] and might cause an increase in contractions; however, this factor does not appear to explain the phenomenon of contractions alone. The procedures involved in vitrification are thought to cause cell damage due to handling and exposure to very low temperature, and the precipitants involved in this process may increase contractions. Previously, we showed that inhibition of gap junctions could delay growth to the blastocyst stage and cause frequent contractions in the blastocyst [24]. Gap junctions are crucial for intercellular communication and play an important role in the process of embryonic development [26]. In addition, expression of gap junction components changes in response to the culture environment [27]. We could not directly demonstrate that dysfunction of gap junctions is the primary cause of the strong contractions, but our findings suggest a connection between these phenomena.

The physiological role of blastocyst contraction is not clear;

Table 3. Numbers of contractions in the pre-hatching stage in the hatching group (HG) and unhatched group (UG)

Condition of embryos	Group (n)	Average no. of contractions (25th percentile-median-75th percentile)		
		Total	Strong	Weak
Fresh	HG (72)	2.375 (0-1-4)*[b]	0.569 (0-0-1)**[b]	1.806 (0-1-3)
	UG (28)	2.786 (3-2.5-4)[a]	1.714 (1-2-2.5)[a]	1.071 (0-1-1)
Vitrified	HG (72)	3.875 (1-3-6)	1.167 (0-1-2)**[b]	2.736 (1-2-4.5)*[a]
	UG (36)	3.722 (3-3.5-5)	2.333 (1-2-3)[a]	1.389 (1-1-2)[b]

The number of contraction was counted for the mean durations from blastocoel formation to the start of hatching. Values with different superscripts in the same column are significantly different (*$P < 0.05$; **$P < 0.01$). P-values were analyzed with the Wilcoxon rank-sum test.

Table 4. Numbers of contractions in the pre-hatching stage in the fresh group (FG) and vitrified group (VG)

Condition of embryos	Group (n)	Average no. of contractions (25th percentile-median-75th percentile)		
		Total	Strong	Weak
Hatching	FG (72)	2.375 (0-1-4)**[b]	0.569 (0-0-1)**[b]	1.806 (0-1-3)**[b]
	VG (72)	3.875 (1-3-6)[a]	1.167 (0-1-2)[a]	2.736 (1-2-4.5)[a]
Unhatched	FG (28)	2.786 (3-2.5-4)*[a]	1.714 (1-2-2.5)	1.071 (0-1-1)
	VG (36)	3.722 (3-3.5-5)[b]	2.333 (1-2-3)	1.389 (1-1-2)

The number of contraction was counted for the mean durations from blastocoel formation to the start of hatching. Values with different superscripts in the same column are significantly different (*$P < 0.05$; **$P < 0.01$). P-values were analyzed with the Wilcoxon rank-sum test.

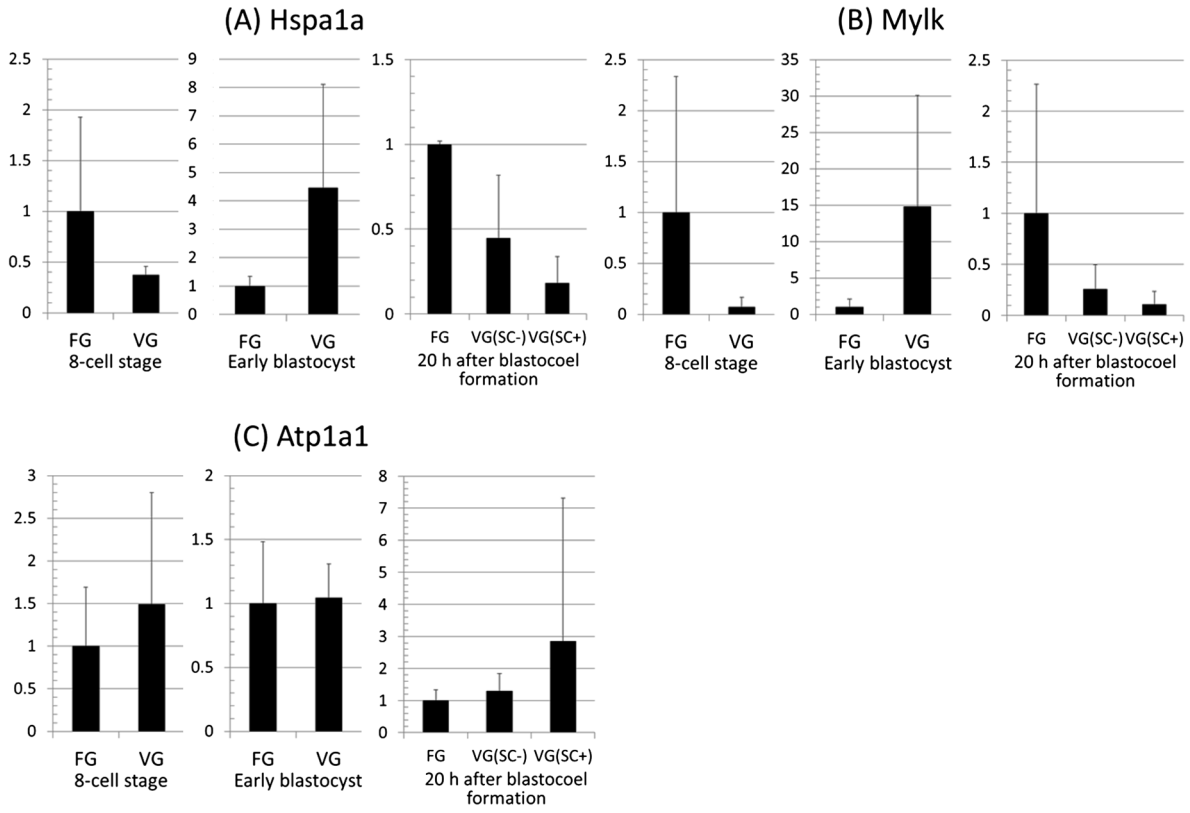

Fig. 3. Relative expression (fold change) of mRNA levels of three genes (*Hspa1a*, *Mylk* and *Atp1a1*) in mouse embryos of different stages. At each stage, we extracted RNA from 10 embryos. FG, fresh group (control); VG, vitrified group; SC–, without strong contractions within 20 h after blastocoel formation; SC+, strong contractions occurred within 20 h after blastocoel formation. Data are means ± SEM for 10 embryos per stage from three to five experiments. The relative mRNA expression of *Hspa1a* (A), *Mylk* (B) and *Atp1a1* (C) was determined at the 8-cell stage, early blastocyst stage and 20 h after blastocoel formation. There was no significant difference between the FG and VG at any stage. There was also no significant difference between the VG (SC–) and VG (SC+) at any stage. P-values were determined with the paired Student's *t*-test.

however, it is known that strong contractions in the blastocyst stage have a negative influence on hatching and that weak contractions have a positive influence [14]. In this study, consistent with a previous report [28], we observed many strong contractions in the UG. Thus, when strong contractions occurred in the pre-hatching stage, the hatching rates were low. These results lend support to the idea that strong contractions have a negative effect on hatching. On the other hand, there was no significant difference in the number of strong contractions between the FG and VG within the UG. This may be because contractions more easily occur in blastocysts with low hatching ability, which may have masked the difference in the number of strong contractions between the FG and VG.

Using TLM, we classified embryos by the presence of strong contractions during the 20 h following blastocoel formation and investigated the effect of this behavior on expression of specific mRNAs. This is the first study in which the contractile behavior of blastocysts was correlated with mRNA expression. No statistically significant difference in gene expression levels was observed, but *Atp1a1* was overexpressed in some embryos in the VG (SC+) (Fig. 3 [C]). We speculate that the present results were caused by extracting mRNA from 10 blastocysts in each group at a fixed time. Embryo contraction might not occur with the same timing, even if the culture environment and sampling time are the same. Furthermore, we could not prevent the possibility that a degenerated embryo might have been mixed in the VG (SC+). *Atp1a1* encodes the α1-subunit of the Na/K-ATPase protein [21], which has been histochemically observed to be more highly expressed in contracted blastocysts than in expanded blastocysts. Niimura inferred that re-expansion of contracted blastocysts occurs by active transport and accumulation of Na^+ from trophectoderm cells into the blastocoelic fluid because of the action of Na/K-ATPase that is activated in the membrane of trophectoderm cells [14]. Our result also suggests that the demand for Na/K-ATPase increased after strong contractions occurred. Similarly, the overexpression of *Atp1a1* in some embryos implies that the ATP demand increases during the recovery process after a strong contraction. We speculate that this phenomenon may be one of the causes of embryo disorders after the vitrification and thawing processes. Furthermore, the overexpression of *Hspa1a* and *Mylk* observed in some early blastocyst-stage embryos may be related to the increase in contractions and resultant cell damage.

As the field of assisted reproductive technology progresses, the rate of pregnancies resulting from frozen and thawed embryos is

increasing. To reduce the risk of iatrogenic multiple births, single embryo transfer (SET) has recently attracted attention [29], but the risk of monozygotic twinning remains to be addressed [30]. Furthermore, the elective single embryo transfer (eSET) method, in which a fertilized egg is cultured to the blastocyst stage, is expected to improve the pregnancy rate [31]. In order to choose a suitable embryo for transplantation, it is necessary to evaluate embryos at the individual level. The Gardner classification [32], a rating system that uses morphological findings of the blastocyst obtained at a fixed point, has commonly been used for this evaluation; however, as TLM has become more widely adopted and dynamic observations of blastocysts are more common, several new indices have been proposed [32, 33]. Examples include pronuclear dynamics and morphology, duration of first cytokinesis and reappearance of nuclei after cleavage, and time to various cleavage stages; however, a single index that can predict the likelihood of a successful transplant has not yet been established [34, 35]. In this study, we evaluated embryos at a stage before transplantation; specifically, we conducted vitrification and thawing at the 8-cell stage and observed the results at the pre-hatching stage. We found that the total number of contractions of the pre-hatching stage blastocyst increased because of vitrification at the 8-cell stage, although vitrification did not affect the hatching rate. Furthermore, we confirmed that there were significantly more strong contractions in blastocysts in the UG. In addition, the probability of an embryo starting to hatch decreased when a strong contraction occurred in the pre-hatching stage. These results suggest that the start of hatching could be predicted by observing the trends in contractile movement during the pre-hatching stage. For example, the presence of a strong contraction for the blastocyst stage may be used as an index for embryo sorting when there are 2–3 candidate embryos for transplantation, which can aid in the decision of whether or not assisted hatching is required. Moreover, this observation could serve as a useful evaluation of a cryopreservation method and the culture medium.

Based on our findings, we propose that observations of contraction at the pre-hatching stage should be used as an index for blastocyst evaluation. However, given that this study was conducted in mouse embryos, the direct application in humans is not yet clear. Because of the ethical limitations in using human embryos for this type of study, it is necessary to develop a theoretical model for better translation between clinical studies in humans and fundamental research in animal models.

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