

—Original Article—

Heat shock decreases the embryonic quality of frozen-thawed bovine blastocysts produced *in vitro*

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Abstract. In this study, the effect of heat shock on frozen-thawed blastocysts was evaluated using *in vitro*-produced (IVP) bovine embryos. In experiment 1, the effects of 6 h of heat shock at 41.0 C on fresh blastocysts were evaluated. *HSPA1A* expression as a reflection of stress was increased by heat shock ($P < 0.05$), but the expressions of the quality markers *IFNT* and *POU5F1* were not affected. In experiment 2, frozen-thawed blastocysts were incubated at 38.5 C for 6 h (cryo-con) or exposed to heat shock at 41.0 C for 6 h (cryo-HS). Then, blastocysts were cultured at 38.5 C until 48 h after thawing (both conditions). Cryo-HS blastocysts exhibited a decreased recovery rate: *HSPA1A* expression was dramatically increased compared with that in fresh or cryo-con blastocysts at 6 h, and *IFNT* expression was decreased compared with that in cryo-con blastocysts at 6 h (both $P < 0.05$). Cryo-con blastocysts at 6 h also exhibited higher *HSPA1A* expression than fresh blastocysts ($P < 0.05$). At 48 h after thawing, the number of hatched blastocysts and blastocyst diameter were lower in cryo-HS blastocysts ($P < 0.05$). Cryo-con blastocysts showed lower *POU5F1* levels at 48 h than fresh, cryo-con or cryo-HS blastocysts at 6 h ($P < 0.05$), but their *POU5F1* levels were not different from those of cryo-HS blastocysts at 48 h. These results indicated that application of heat shock to frozen-thawed blastocysts was highly damaging. The increase in damage by the interaction of freezing-thawing and heat shock might be one reason for the low conception rate in frozen-thawed embryo transfer in summer.

Key words: Blastocysts, Cryopreservation, Heat shock, IVF

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A low conception rate in dairy cows in summer has been reported worldwide and is an important problem [1–4]. It has been suggested that the increase of maternal body temperature in hot climates induces embryonic death and leads to a low pregnancy rate [5, 6]. Some studies have suggested that a high body temperature on the days of insemination and early pregnancy would be associated with lower conception rates [3, 6, 7]. Both *in vivo* and *in vitro* experiments have revealed that early stage embryos are more susceptible to high temperature and more easily damaged by heat shock and decreased viability compared with later stage embryos such as morulae or blastocysts [6, 8–11]. Therefore, it has been suggested that embryo transfer that bypasses the heat-sensitive stage outside of the maternal body would be effective for improving the low conception rate in summer [12, 13]. Indeed, *in vivo* fresh embryo transfer has been shown to improve the conception rate of dairy cows under heat stress [14–16]. However, the hormonal reaction of superovulatory cows and the quality of embryos derived from superovulation were lower in summer [17, 18]. Thus, producing *in vivo* embryos under

heat stress conditions is not effective. Producing embryos *in vitro*, on the other hand, is effective in summer because the embryos are not affected by heat stress. Moreover, *in vitro* embryo production is less expensive than the corresponding *in vivo* process. In recent years, *in vitro*-derived embryo transfer has been increasingly used throughout the world (2012 International Embryo Transfer Society Reports). Therefore, it is thought that the use of *in vitro*-derived frozen embryos would contribute to an improvement in the summer conception rate in dairy cows. Freezing destroys the cell organelles and decreases viability. Cryopreservation of blastocysts also decreases embryo viability compared with fresh blastocysts [19, 20]. To enhance the cryotolerance of embryos, culture conditions and cryopreservation methods have been improved. However, the pregnancy rate of conventional frozen-thawed blastocysts has been shown to be lower than that of fresh embryo transfer not only in embryos cultured *in vitro* but also in those derived *in vivo* [21, 22]. In particular, the summer conception rate by *in vitro*-cryopreserved embryo transfer remains quite low [14, 23, 24].

In Japan, transferring beef cattle embryos to dairy cattle is a common means of augmenting income among farmers and also avoids accidents associated with delivery in heifers [25]. It is effective for producing thermotolerant embryos that can be implanted after embryo transfer even under high temperatures. However, there have been very few reports about the effect of heat shock after thawing on embryonic viability and proliferation in frozen *in vitro*-derived blastocysts. Therefore, in this study we focused our attention on the

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use of *in vitro*-derived beef cattle embryos and investigated the effect of heat shock on embryo viability, proliferation and gene expressions related to embryo quality in conventional frozen-thawed blastocysts.

Materials and Methods

Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich (Tokyo, Japan).

In vitro production of embryos

Experiment 1: Abattoir-derived cumulus oocyte complexes (COCs) from 2 to 6 mm follicles were matured with 5% fetal bovine serum (FBS; Life Technologies, Tokyo, Japan), 100 IU follicle-stimulating hormone (FSH, Antorin, Denka Pharmaceuticals, Tokyo, Japan) and 0.1 mg/ml gentamicin supplemented TCM-199 (Life Technologies) at 38.5 C in 5% CO₂ in humidified air for 20 h without mineral oil (50 COCs/500 µl). Then, oocytes were fertilized with frozen-thawed Japanese black bull sperm separated with 90% Percoll (GE Healthcare Japan, Tokyo, Japan) and diluted with IVF-100 (Research Institute for the Functional Peptides, Yamagata, Japan) to a final concentration of 5.0×10^6 sperm/ml for *in vitro* fertilization at 38.5 C in 5% CO₂ in humidified air for 6 h (20 oocytes/100 µl drop) [26]. Then, putative zygotes were cultured by two different methods to investigate the effect of *in vitro* production (IVP) methods on the gene expressions of blastocysts.

In the first method (M199), putative zygotes were cultured with cumulus cells in glucose-free modified medium 199 [27] containing 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin (1× PS) covered with mineral oil (Nacalai Tesque, Tokyo, Japan) at 38.5 C under 3% CO₂, 10% O₂ and 87% N₂ (50 zygotes/500 µl). On day 6 (day 0 = day of fertilization) of culture, the culture medium was changed from modified medium 199 to medium 199 containing 10% FBS and 1× PS at 38.5 C under 3% CO₂, 10% O₂ and 87% N₂.

In the second method (SOF), cumulus cells were removed by repeated mouth pipetting after fertilization. Then, putative zygotes were transferred to an SOF-BE1 drop [28] (30 zygotes/50 µl) covered with mineral oil and cultured at 38.5 C with 5% O₂, 5% CO₂ and 90% N₂ in a humidified atmosphere until day 7.

Experiment 2: The embryos cultured with cumulus cells (co-culture) in medium 199 showed higher cryotolerance than the embryos cultured without cumulus cells [29]. Therefore, we used the blastocysts derived from co-culture with M199 in experiment 2.

Approximately 50 abattoir-derived COCs were matured for 20 h in 500 µl IVMD101 medium (Research Institute for the Functional Peptides) covered with mineral oil. All cultures were maintained at 38.5 C in a humidified atmosphere of 3% CO₂, 10% O₂ and 87% N₂. Frozen semen from a Japanese black bull was used for *in vitro* fertilization, as previously described [30]. The embryos were then cultured with M199 as described in experiment 1.

Cryopreservation and thawing

Cryopreservation was performed as described in a previous report [30]. Briefly, blastocysts obtained at day 7 were transferred to a cryoprotective solution (5% [v/v] ethylene glycol, 6% [v/v] propylene glycol, 0.1 M sucrose and 4 mg/ml bovine serum albumin [BSA]

in Dulbecco's phosphate-buffered saline [D-PBS]), and then 1 or 2 blastocysts were introduced into a 0.25-ml straw (IMV Technologies, L'Aigle, France) at 23–25 C for 13 min of equilibration. Straws were directly set in a programmable freezer (Fujihira, Tokyo, Japan) at –7 C, and seeding was manually performed. Subsequently, straws were cooled at a rate of –0.3 C/min to –30 C and then directly transferred to liquid nitrogen for storage until analysis.

The straws were thawed in air for 10 sec and then immersed in a water bath at 30 C for 10 sec. Then, embryos were transferred to a culture drop including medium 199 containing 100 µM β-mercaptoethanol, 20% FBS and 1× PS.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Blastocysts were collected and washed with 0.1% (w/v) polyvinyl alcohol in D-PBS (0.1% PVA-PBS) and then transferred into a 0.5 ml sampling tube with a minimum volume of PVA-PBS and kept at –80 C until analysis. RNA extraction was performed with an RNeasy Plus Micro Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Then, the reverse transcription reaction was conducted with reverse transcriptase (SuperScript III; Life Technologies) according to the manufacturer's instructions. The qRT-PCR protocol consisted of 40 cycles of 95 C for 5 sec, 58 C for 10 sec and 72 C for 10 sec, followed by a single denaturing step of 95 C for 30 sec. qRT-PCR was performed using a Chromo4 Real-Time Thermal Cycler (Bio-Rad, Tokyo, Japan) with 20 µl reaction mix containing 10 µl of SsoFast EvaGreen Supermix (Bio-Rad), 7 µl of water, 2 µl of template cDNA and 0.5 µl each of the forward or reverse primers of 18s ribosomal RNA (*RN18S1*), heat shock protein 70 kDa (*HSPA1A* [31]), interferon tau (*IFNT* [31]) and POU domain class 5 transcription factor 1 (Oct4, *POU5F1* [32]) (Table 1).

The cycle thresholds of each gene were calculated. Then, the fold changes of target genes were evaluated by the $\Delta\Delta CT$ method using the reference gene (*RN18S1*) expression.

Experimental design

Experiment 1 – Effect of heat shock on *in vitro*-derived blastocysts: Embryos were derived using two different methods to evaluate the effect of the culture system on gene expressions.

On day 7 at 168 hpi (hours post insemination), blastocysts were exposed at 41.0 C in 5% O₂, 5% CO₂ and 90% N₂ for 6 h (M199-HS, SOF-HS). Control blastocysts were incubated at 38.5 C in 5% O₂, 5% CO₂ and 90% N₂ until 174 hpi (M199-con, SOF-con). Then, five expanding or expanded blastocysts from each treatment were collected for real-time qPCR analysis.

Experiment 2 – Effect of heat shock on *in vitro*-derived frozen-thawed blastocysts: On day 7 (at 168 hpi), blastocysts derived from M199 were cryopreserved and kept in liquid nitrogen until thawing.

On the day of the experiment, straws were thawed and blastocysts were washed with medium 199 containing 100 µM of β-mercaptoethanol, 20% FBS and 1×PS. Then, the thawed embryos were incubated for 6 h at 38.5 C (cryo-con) or 41.0 C (cryo-HS) with medium 199 containing β-mercaptoethanol, 20% FBS, and 1×PS in a 2.0 ml microtube (3 to 5 thawed embryos/500 µl) with a closed lid in a thermostatic chamber (MD-MINI dry bath; Major Science, Pan-chiao, Taiwan). The culture medium was equilibrated

Table 1. Primer information

Gene	Sequence (5'–3')	Product length	Accession number	References
<i>RN18S1</i>	F: AAACGGCTACCACATCCAAGG R: GCGGAAGGATTTAAAGTGGACTC	138	DQ066896	
<i>HSPA1A</i>	F: GACAAGTGCCAGGAGGTGATTT R: CAGTCTGCTGATGATGGGGTTA	117	U09861	[31]
<i>IFNT</i>	F: TCCATGAGATGCTCCAGCAGT R: TGTTGGAGCCAGTGCAGA	103	X65539	[31]
<i>POU5F1</i>	F: TGCAGCAAATTAGCCACATC R: AATCCTCACGTTGGGAGTTG	123	NM_174580.2	[32]

Table 2. Effect of heat shock on blastocysts without cryopreservation

Treatment	n (replicates)	Number of blastocysts at 6 h after heat shock (%)	
		Shrunken	Normal (developing)
M199-con	38 (5)	10 (26.3)	28 (73.7)
M199-HS	38 (5)	4 (10.5)	34 (89.5)
SOF-con	40 (5)	7 (17.5)	33 (82.5)
SOF-HS	40 (5)	4 (10.0)	36 (90.0)

at 38.5 C in a humidified atmosphere of 3% CO₂, 10% O₂ and 87% N₂ prior to incubation.

Blastocyst morphologies were observed after 6 h of incubation. Then, five re-expanding or re-expanded blastocysts (Cryo-HS includes shrunken blastocysts because of a very low recovery rate) per replicate were collected for gene expression analysis. The rest of the heat-shocked blastocysts were moved to the incubator at 38.5 C in a humidified atmosphere of 5% CO₂ in air, and then the recovery culture was continued until 48 h after thawing. The viability and diameters of blastocysts were analyzed, and three of the hatched-blastocysts from each replicate were collected for gene expression analysis.

Statistical analysis

Experiments were replicated at least five times in experiment 1 and three times in experiment 2. One-way analysis of variance (ANOVA) was used to determine significant differences between groups for the gene expression analysis, followed by the Tukey-Kramer test (StatView program Ver. 5.0; Abacus Concepts, Berkeley, CA, USA). Data for embryo viability at 6 h after heat shock or thawing were analyzed using chi-square analysis and residual analysis (StatView). Data for the embryos hatched at 48 h after thawing were analyzed by Fisher's exact test (StatView). The diameters of surviving blastocysts at 48 h after thawing were analyzed by Student's *t*-test (StatView). A *P* value of < 0.05 was considered statistically significant.

Results

Experiment 1

There were no significant differences in blastocysts rate between the two culture methods (data not shown). The numbers of shrunken

embryos and normal developed embryos after heat shock are shown in Table 2. Most blastocysts were developed after 6 h of heat shock without cryopreservation, some blastocysts had shrunken blastocoels. No effects of heat shock were observed on the numbers of shrunken blastocysts and surviving embryos (Table 2).

A total of 25 blastocysts that had developed beyond the expanding or expanded stage in each of the five replicates for each treatment (five blastocysts/replicate) were analyzed for gene expression. Gene expressions after heat shock are shown in Fig. 1. Data are shown as the gene expression relative to M199-con (M199-con = 1). There were no significant differences in any genes between media. However, after 6 h of heat shock, the *HSPA1A* expression was dramatically increased in heat-shocked blastocysts compared with control blastocysts (*P* < 0.05, Fig. 1A). The *IFNT* and *POU5F1* expressions were not affected by heat shock (Figs. 1B and C).

Experiment 2

Embryonic viability: A total of 34 blastocysts in each of the four replicates for each treatment were evaluated with regard to recovery after thawing. The numbers of embryos recovered and images of blastocysts at 6 h after thawing are shown in Table 2 and Fig. 2, respectively. More than 90% of all blastocysts were re-expanding or re-expanded under the cryo-con conditions at 6 h (Table 3, Fig. 2). However, 44% of the blastocysts under the cryo-HS conditions at 6 h were not recovered (*P* < 0.01, Table 3), and fewer blastocysts from the heat shock treatment were re-expanded than under the control conditions (*P* < 0.05, Table 3, Fig. 2).

A total of 14 blastocysts in each of the three replicates for each treatment were cultured until 48 h and evaluated for embryo recovery and morphology. The numbers of embryos recovered after 48 h of thawing are shown in Table 3 and Fig. 2, respectively. Cryo-HS blastocysts at 48 h showed a lower hatching rate (14.3%) compared with the cryo-con blastocysts at 48 h (64.3%; *P* < 0.05, Table 4). Even among the blastocysts that survived the heat shock, the diameter of the cryo-HS blastocysts was significantly lower than that of the cryo-con blastocysts at 48 h (*P* < 0.05, Table 4, Fig. 2).

Gene expression: Five blastocysts from each of the 4 replicates from each treatment group at 6 h after thawing and 3 recovered blastocysts from each of the 3 replicates from each treatment group at 48 h were used for gene expression analysis. Each synthesized cDNA was diluted with RNase- and DNase-free water depending on the number of blastocysts to adjust the volume per blastocyst (10 µl/

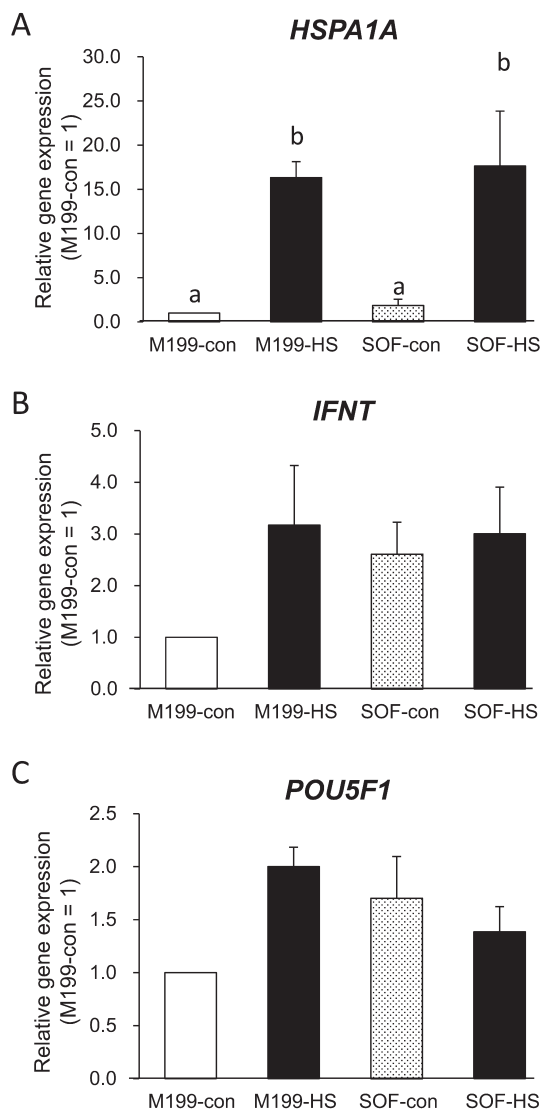


Fig. 1. Effect of heat shock applied to fresh blastocysts on gene expression. Gene expressions of blastocysts at 174 hpi. Heat shock protein 70 kDa (*HSPA1A*, A), interferon tau (*IFNT*, B) and POU domain, class 5, transcription factor 1 (*POU5F1*, C). Results are means \pm SEM of fold changes relative to M199 culture at 38.5 C (M199-con = 1). White bars and polka-dot bars represent blastocysts cultured at 38.5 C (M199-con and SOF-con, respectively), and black bars represent blastocysts exposed to 41.0 C for 6 h as heat shock (M199-HS, SOF-HS). Different letters above bars indicate $P < 0.05$.

blastocyst). The fold changes of real-time qPCR results are shown in Figure 3. Data were compared with fresh blastocysts (collected at 174 hpi without cryopreservation and heat shock, fold change of 1 = fresh). Interestingly, cryopreservation without heat shock (cryo-con at 6 h) showed higher *HSPA1A* expression compared with fresh blastocysts ($P < 0.05$). Moreover, *HSPA1A* expression was dramatically increased by heat shock (cryo HS at 6 h, $P < 0.05$). However, the *HSPA1A* levels returned to the levels in fresh blastocysts after 48 h

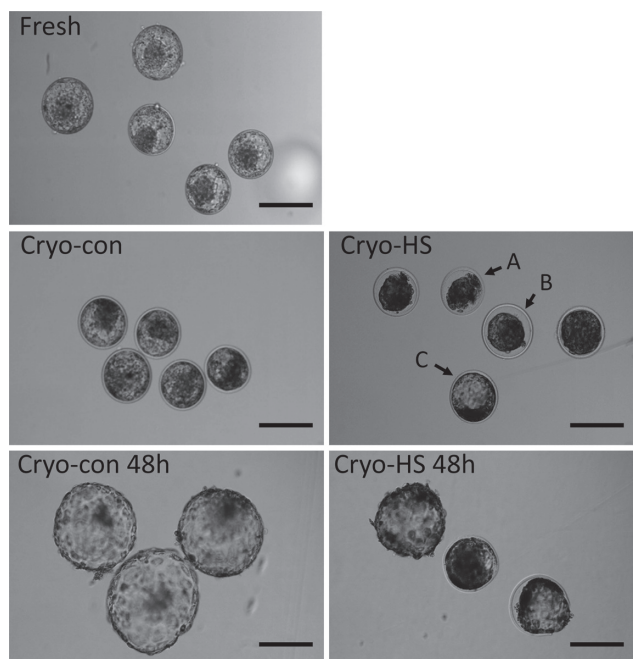


Fig. 2. Representative images of embryos exposed to heat shock in experiment 2. Fresh: blastocysts on day 7 (at 168 hpi) derived from the second method (M199). Cryo-con: blastocysts incubated at 38.5 C for 6 h after thawing. Cryo-HS: blastocysts cultured at 41.0 C for 6 h after thawing. Cryo-con 48 h: blastocysts incubated at 38.5 C for 48 h after thawing. Cryo-HS 48 h: blastocysts exposed at 41.0 C for 6 h and then cultured at 42 h at 38.5 C (magnification, $\times 100$; scale bar, 200 μ m). A: Shrunken (no re-expansion of blastocoel but not completely filling the blastocyst and reaching the zona pellucida). B: Expanding (re-expanding blastocoel but not completely filling the blastocyst and reaching the zona pellucida). C: Expanded (recovered to expanded blastocyst).

Table 3. Effect of heat shock on blastocyst survival at 6 h after thawing (after heat shock)

Treatment	n (replicates)	Number of blastocysts at 6 h after thawing (%)		
		Shrunken	Expanding	Expanded
Cryo-con	34 (4)	3 (8.9) ^a	14 (41.2)	17 (50.0) ^a
Cryo-HS	34 (4)	15 (44.1) ^b	12 (35.3)	7 (20.6) ^c

Values with different symbols are significantly different (^{a, b} $P < 0.01$; ^{a, c} $P < 0.05$).

Table 4. Effect of heat shock on blastocyst survival and size at 48 h after thawing

Treatment	n (replicates)	Number of hatched blastocysts at 48 h after thawing (%)	Diameter of blastocysts (μ m)*
Cryo-con	14 (3)	9 (64.3) ^a	279.4 \pm 79.7 ^a
Cryo-HS	14 (3)	2 (14.3) ^b	215.3 \pm 63.0 ^b

* Excluding degenerated blastocysts (Cryo-con, average of 13 blastocysts; Cryo-HS, average of 10 blastocysts). Values with different symbols are significantly different (^{a, b} $P < 0.05$).

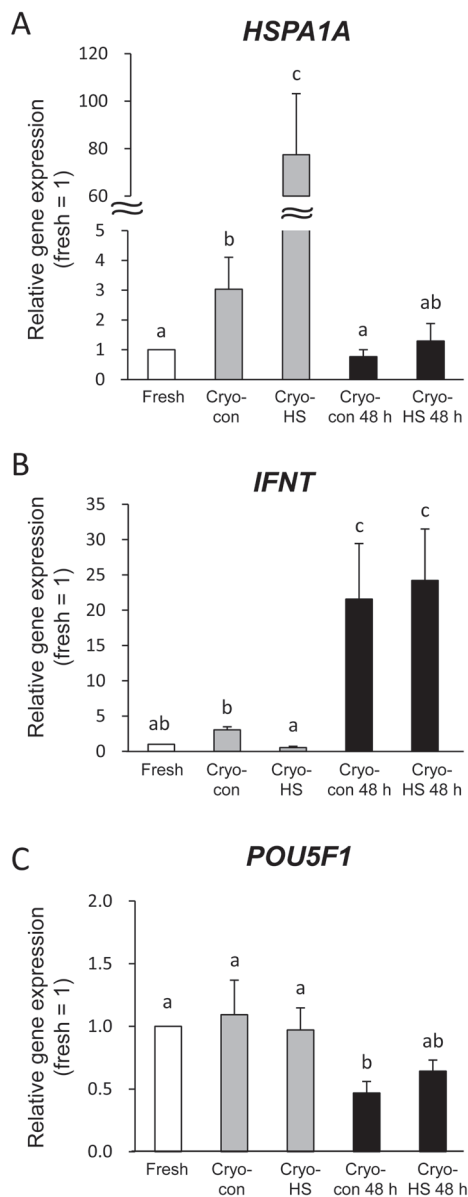


Fig. 3. Effect of heat shock applied to frozen-thawed blastocysts on gene expression. Results are means \pm SEM of the fold change relative to fresh blastocysts. Heat shock protein 70 kDa (*HSPA1A*, A), interferon tau (*IFNT*, B) and POU domain, class 5, transcription factor 1 (*POU5F1*, C). Cryo-con: blastocysts incubated at 38.5 C for 6 h after thawing. Cryo-HS: blastocysts incubated at 41.0 C for 6 h after thawing. Cryo-con 48 h: blastocysts incubated at 38.5 C for 48 h after thawing. Cryo-HS at 48 h: blastocysts exposed at 41.0 C for 6 h and then cultured at 38.5 C for 42 h. Different letters above bars indicate $P < 0.05$.

of thawing both in the cryo-con blastocysts at 48 h and the cryo-HS blastocysts at 48 h (Fig. 3A).

The *IFNT* level was not different between the fresh and cryo-con blastocysts at 6 h. But cryo-HS blastocysts at 6 h showed lower *IFNT* expression than cryo-con blastocysts at 6 h ($P < 0.05$, Fig. 3B). Recovered blastocysts at 48 h after thawing showed significantly

higher *IFNT* levels than those collected at 174 hpi (fresh, cryo-con at 6 h and cryo-HS at 6 h, $P < 0.05$, Fig. 3B). There was no difference between cryo-con and cryo-HS blastocysts at 48 h.

POU5F1 levels were significantly lower in cryo-con blastocysts at 48 h compared with those collected at 174 hpi (fresh, cryo-con at 6 h and cryo-HS at 6 h, $P < 0.05$, Fig. 3C). But the *POU5F1* levels of the cryo-HS blastocysts at 48 h were no different from those of other groups (fresh, cryo-con at 6 h and cryo-HS at 6 h, Fig. 3C).

Discussion

This study aimed to understand the reasons for conception failure after the transfer of frozen bovine embryos in summer. To this end, we evaluated the effects of heat shock on frozen-thawed IVP blastocyst viability and gene expressions related to stress, embryo quality and pregnancy recognition. A temperature of 41 C for 6 h was used as the heat shock treatment, since it reflects the daytime body temperature of Holstein cows in summer [3, 33].

In the present study, no differences in embryo development and gene expressions were observed between the culture media in experiment 1. Heat shock induced *HSPA1A* expression in both fresh (M199-HS and SOF-HS) and frozen-thawed (cryo-HS) blastocysts (Figs. 1A and 3A). *HSPA1A* is one of the important stress markers of cells. Many studies have demonstrated that heat shock induces *HSPA1A* expression in bovine embryos including blastocysts [34–37]. In experiment 2, *HSPA1A* expression in frozen-thawed blastocysts without heat shock (cryo-con at 6 h) was increased compared with that in fresh blastocysts. It has been reported that cell organelles were damaged and viability was decreased in frozen-thawed blastocysts compared with fresh blastocysts [19, 20]. DNA fragmentation, apoptosis and higher *HSPA1A* expression were also observed in frozen-thawed blastocysts [38]. Our present finding of an increase in *HSPA1A* in cryo-con blastocysts at 6 h was in good agreement with these previous reports and suggested that the freezing and thawing process increased the damage to the blastocysts. The remarkable increase of *HSPA1A* in cryo-HS blastocysts at 6 h is likely due to the interaction between the freezing-thawing process and heat shock. Although no effect of heat shock was observed on the rate of shrunken blastocysts in experiment 1, our viability data at 6 h after thawing showed that about 40% of cryo-HS blastocysts were still shrunken and that the number of re-expanded blastocysts in the cryo-HS group was significantly lower than that in the cryo-con group. In addition, the cryo-HS conditions resulted in a lower number of hatched blastocysts and lower diameter of blastocysts than the cryo-con conditions even after 48 h of recovery culture. These results indicate that the heat shock after the freezing-thawing process caused severe impairment of blastocysts. In contrast, *HSPA1A* expression returned to the same levels as in fresh or cryo-con blastocysts after 48 h in the blastocysts that survived heat shock and were re-hatching or already re-hatched at 48 h after thawing. This suggests that *HSPA1A* expression was transiently elevated just after heat shock and decreased continuously with time.

IFN tau is a type I interferon and acts as an important signaling molecule during pregnancy in ruminants [39, 40]. IFN tau secretion is increased notably at more than 12 days after pregnancy in cattle, but the IFN tau gene is already expressed in day 7 blastocysts and

thus is used as a marker of embryo quality [40–42]. A decrease in the IFN tau concentration leads to failure of pregnancy, and dairy cows with a pregnancy rate is low in summer show lower IFN tau secretion [43].

Hickman *et al.* reported that *IFNT* expression in heat-shocked blastocysts was increased [37]. In the present study, however, *IFNT* expression was not affected by heat shock in blastocysts without cryopreservation (experiment 1), and *IFNT* expression was significantly decreased in the cryo-HS group at 6 h post thawing. It is considered that *IFNT* expression or secretion would be directly affected by the cell number or damage to the trophectoderm because the trophectoderm mainly secretes IFN tau in fetuses [40]. In experiment 1 of the present study, the heat shock treatment consisted of 41 C for 6 h, which was milder than that in Hickman's experiment (42 C for 4 h). Also, the sampling of blastocyst stages did not differ between the treatments in experiment 1, and no significant differences in embryonic morphologies were observed in heat-shocked blastocysts. Therefore, *IFNT* expression was not changed in experiment 1. Correa *et al.* reported that no *IFNT* increase was observed in embryos cultured under a high oxygen concentration. This evidence also supported the results of our experiment 1 [41].

On the other hand, more than 40% of cryo-HS blastocysts at 6 h were still shrinking in experiment 2, and there were very few re-expanded blastocysts compared with under the cryo-con conditions at 6 h (Table 3). Moreover, the collected samples of cryo-HS blastocysts at 6 h included some shrunken blastocysts. This may have indicated that the cryo-HS samples had low numbers of living cells and that heat shock after freeze-thawing damaged the trophectoderm. Therefore, it is possible that the *IFNT* expression in cryo-HS blastocysts at 6 h was decreased because of the low quality and the number of living cells in the trophectoderm. *IFNT* expressions at 48 h after thawing were dramatically increased both in cryo-con blastocysts at 48 h and cryo-HS blastocysts at 48 h. This indicated that most of the collected blastocysts at 48 h were hatching or hatched blastocysts, and these blastocysts had a much higher number of trophectoderm cells contributing to IFN tau secretion than those at 6 h after thawing. In this study, we did not count the number of cells in blastocysts. However, Leidenfrost *et al.* compared the cell numbers of bovine non-expanded, expanded and hatched blastocysts and reported that the total number of cells nad number of cells in the trophectoderm increased depending on the blastocysts stage but that the number of cells in the ICM was not affected by the blastocysts stage [44]. Another study reported a correlation between total cell number and the diameter of blastocysts [45]. This indicated that the total cell numbers of blastocysts were inferable from the diameter of the blastocysts. Therefore, the blastocysts collected at 48 h after thawing would have a higher number of cells in the trophectoderm and increased *IFNT* expression.

POU5F1, which is mainly expressed in the ICM of blastocysts, is used not only as a pluripotency marker but also as a marker of embryo quality. *POU5F1* expressions were not changed at 6 h of heat shock in experiments 1 and 2. Although cryo-con blastocysts at 48 h after thawing exhibited significantly decreased *POU5F1* expression compared with fresh, cryo-con and cryo-HS blastocysts at 6 h, *POU5F1* expression in cryo-HS blastocysts at 48 h was not different from other groups (fresh, cryo-con at 6 h and cryo-HS at 6

h). As mentioned above, blastocysts collected at 48 h after thawing were mostly hatching or hatched. The number of trophectoderm or primitive endoderm cells increased continuously with blastocyst growth [44]. As a consequence, the ratio of the ICM to total cell number decreased. The fold changes in *POU5F1* expression were calculated relative to the expression of inner standard genes (*RN18S1*) by the $\Delta\Delta CT$ method. Therefore, it is likely that *POU5F1* expression in cryo-con blastocysts at 48 h would decrease along with a decrease in the ratio of the ICM to total cell number. The number of blastocysts hatched and the diameter of cryo-HS blastocysts at 48 h were smaller than those of cryo-con blastocysts at 48 h (Table 3 and 4). This suggested that proliferation of the trophectoderm or primitive endoderm was inhibited or delayed under heat shock conditions, and the ICM to total cell number ratio was higher than that of cryo-con blastocysts at 48 h. Therefore, it is possible that *POU5F1* expression in cryo-HS blastocysts at 48 h was not decreased as much as in cryo-con blastocysts at 48 h.

In conclusion, the present study suggested that application of heat shock to frozen-thawed blastocysts induced higher levels of cell stress, damage and greater decreases in viability and embryonic quality via an interaction between the freezing-thawing process and heat shock. Even among the blastocysts that survived the heat shock, recovery at 48 h was delayed, and growth was inhibited. These results indicated that heat-shock damage would cause a low conception rate in blastocysts subjected to frozen-thawed embryo transfer during summer. Further research will be needed before frozen-thawed embryo transfer can be applied to improve the low conception rate of cows in summer, such as by producing thermotolerant frozen embryos.

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