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Heat-shock-induced cathepsin B activity during IVF and culture compromises the developmental competence of bovine embryos

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Abstract

Heat stress can cause significant reproductive dysfunction in mammals and previous studies report that expression and activity of cathepsin B (CTSB), a lysosomal cysteine protease, is negatively correlated with the developmental competence of bovine oocytes and embryos. However, the relationship between heat shock (HS) and CTSB remains largely unknown. Here, we investigated the effects of HS during IVF and early embryonic stages of IVC on CTSB activity and developmental competence in bovine embryos. HS (40°C for 6 h during IVF and 20 h during IVC) caused a significant increase in CTSB activity irrespective of the developmental stage or duration of HS. The developmental rate to the blastocyst stage was also significantly decreased by HS. Additionally, HS during IVC significantly increased the number of apoptotic cells in blastocysts. Notably, these HS-induced changes in blastocyst development and quality were significantly improved by inhibition of CTSB activity, indicating a key role for CTSB. These results showed that CTSB activity plays an essential role in HS-induced dysfunction in bovine embryo development, and that inhibition of this activity could enhance the developmental competence of heat-shocked embryos.

Keywords: cathepsin B, heat shock, embryonic development, bovine

1. Introduction

Reproductive processes in mammals are very sensitive to hyperthermia, which can cause significant decreases in fertility in females and sperm quality in males. Hyperthermia occurs when high ambient temperatures (typically >27°C) cause body temperature to rise to between 40°C and 42°C [1, 2]. Notably, exposing cows to hyperthermic heat stress reduces their conception rate and affects subsequent embryonic development [3]. *In vivo* studies indicate that the observed reduction in conception rate can occur quickly and potentially be affected by elevated body temperature on the day of insemination [1, 4, 5]. Furthermore, multiple *in vitro* studies reported that high temperatures during the period from oocyte maturation to preimplantation embryo development degrade oocyte quality and developmental competence by promoting oxidative stress and DNA damage [6-12]. Similarly, oocyte competency with regard to fertilization and blastocyst formation was also reduced by high temperature during fertilization *in vitro* [11]. Together, these data suggest that the mechanism by which heat stress affects fertility and embryo viability is multifactorial and likely involves many aspects of reproductive physiology. Because heat stress effects reproductive health, many attempts have been made to improve the developmental competence of heat-shocked oocytes and embryos via changes in media composition or supplementation. Interestingly, the addition of chemicals or antioxidants, such as insulin-like growth factor-1 [13], 2-mercaptoethanol [12, 14], astaxanthin [15], anthocyanins [16], melatonin [17, 18], and coagulansin-A [19], into the culture medium appears to enhance oocyte and embryo quality, as well as improve developmental competence during heat shock (HS).

Cathepsin B (CTSB) is a lysosomal cysteine protease that participates in the degradation of intracellular proteins in lysosomes [20]. CTSB is synthesized as an enzyme precursor in an inactive form prior to post-translational processing into its active form after passing through the endoplasmic reticulum and subsequent incorporation into the acidic environment of the lysosome [21, 22]. The active form of CTSB has disulfide-linked heavy and light-chain subunits and a molecular weight of 30 kDa [23]. CTSB is also involved in many physiological and pathological

processes, including initiation of the apoptosis pathway, remodeling of extracellular matrix, autophagy, cancer differentiation, and inflammation [24, 25]. CTSB expression is detected in many types of cells, including ovaries and cumulus cells [26, 27]. Bettegowda et al. [26] reported that higher expression of *CTSB* mRNA was observed in cumulus cells from prepubertal cows, which are a model with poor oocyte competence, relative to levels observed in adult cows. We also reported that higher CTSB activity was observed in low-quality bovine cumulus-oocyte complexes (COCs) and embryos as compared with that observed in those of better quality. Additionally, CTSB also plays a role in apoptosis, which is associated with low developmental competence in both COCs and embryos [28, 29]. Importantly, addition of a CTSB inhibitor (E-64) to the culture medium improved developmental competence in low-quality oocytes and embryos. Moreover, CTSB inhibition also improved blastocyst quality via regulation of the apoptosis pathway [28, 29]. Although bovine oocytes exposed to HS during *in vitro* maturation also exhibited increased CTSB activity, low developmental competence, and increased apoptosis, these changes could be counteracted by CTSB inhibition [30]. Despite these findings, our understanding of how CTSB inhibitors function under HS conditions during fertilization and early embryo development is limited.

In this study, we clarified the relationship between CTSB activity and low developmental competence caused by HS during fertilization and early embryonic development using *in vitro* culture techniques. Furthermore, we also investigated the effect of CTSB inhibition on the developmental competence of HS-treated embryos. To our knowledge, this is the first study investigating the role of CTSB and the benefits of its inhibition during HS-induced changes at these particular stages of embryogenesis.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless noted otherwise.

2.2. Oocyte collection and in vitro maturation

Bovine ovaries were collected from different breeds at the local abattoir. COCs were aspirated from follicles (2–6 mm in diameter) using an 18-gauge needle attached to a 10-mL syringe and washed three times in TCM-199 (Thermo Fisher Scientific, Waltham, MA, USA, USA) containing 5% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific). A total of 50 COCs were matured in a 500-μL drop of TCM-199 supplemented with 5% FBS, FSH (0.02 IU/mL; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (10 mg/mL; Nacalai Tesque, Kyoto, Japan), covered with liquid paraffin (Nacalai Tesque), and incubated at 38.5ºC for 22 h in a humidified atmosphere of 5% CO_2 in air.

2.3. IVF and IVC

Frozen semen was thawed by immersing the straw in warm water (37ºC) for 20 s. Spermatozoa were washed by centrifugation (800*g* for 10 min) in 90% (v/v) percoll solution (GE Healthcare Bio-Sciences AB, Stockholm, Sweden). After removing the supernatant, the pellet was diluted with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and centrifuged at 600*g* for 5 min. The spermatozoa pellet was then diluted with IVF100 to prepare a final sperm-cell concentration of 5.0×10^6 sperm/mL. IVF was performed at 38.5ºC (control group) or at 40ºC (HS group) in 5% CO_2 in air under humidified conditions for 6 h (25 oocytes per 100 μL sperm drop covered with liquid paraffin).

After fertilization, cumulus cells were removed mechanically by pipetting in CR1aa medium [31] containing 5% FBS, and putative zygotes with polar bodies were placed into micro-drops (20–25 zygotes per 50 μL drop) of CR1aa medium supplemented with 5% (v/v) FBS. The drops were then covered with liquid paraffin and cultured at 38.5°C (control group) or at 40.0ºC for 20 h after fertilization, followed by incubation at 38.5ºC (HS group) in a humidified atmosphere of 5% O_2, 5% CO_2, and balanced with N_2 through day 8 (day 0 represented the day of insemination).

2.4. Detection of intracellular CTSB activity

Detection of embryonic CTSB activity was performed using a Magic red CTSB detection kit (MR-RR)2 (Immunochemistry Technologies LLC, Bloomington, MN, USA) according to manufacturer instructions. Briefly, *in vitro* embryos were incubated in 500 μL PBS along with 2 μL of reaction mix in a humidified atmosphere of 5% CO_2 at 38.5ºC for 20 min. To detect nuclei, 25 μg/mL Hoechst 33342 was added and incubated in the same culture conditions for an additional 5 min. After rinsing with PBS containing 0.5 mg/mL polyvinylpyrrolidone (PVP; Nacalai Tesque), the stained embryos were mounted onto a glass slide and observed under a fluorescence microscope (EVOS FL; Thermo Fisher Scientific). Nuclei were observed following excitation at 375 nm, and intracellular CTSB activity was detected at 585 nm excitation. Images of CTSB activity were captured under the same exposure time and analyzed with ImageJ software (v1.55; National Institutes of Health, Bethesda, MD, USA).

2.5. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)

A TUNEL assay kit (*in situ* cell death detection kit; Roche Applied Science, Indianapolis, IN, USA) was used to assess the presence of apoptotic cells in day 8 blastocysts. Blastocysts were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 40 min, rinsed three times in 0.05% (w/v) PVP-PBS, and then permeabilized in 0.05% (w/v) PVP-PBS containing 0.5% TritonX-100 for 20 min, followed by three washes with 0.05% (w/v) PVP-PBS for 5 min. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 38.5°C. After incubation, blastocysts were washed three times in 0.05% (w/v) PVP-PBS for 5 min each, followed by mounting onto glass slides using mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI; Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). The fluorescence of the fragmented DNA ends was detected using a~~by~~ fluorescence microscope (EVOS® FL, Thermo Fisher Scientific). The apoptotic index was calculated for each embryo as follows: apoptotic index = (number of TUNEL-positive nuclei/total number of nuclei in blastocyst) × 100.

147 *2.6. Experimental design*

148 We conducted three experiments described as follows and illustrated in Figure 1.

149

150 *2.6.1. Experiment 1: effect of CTSB inhibitor (E-64) on the development of embryos exposed to*
151 *HS during IVF or early IVC*

152 To assess the effects of E-64 supplementation on HS-mediated changes in embryonic
153 development, E-64 at different concentrations (0, 0.1, 0.5, and 1.0 μM) was added to the medium
154 during HS treatment. To examine the effect of HS treatment at different times on developmental
155 competence, HS treatment under two different conditions was conducted as follows: 1) at 40.0ºC
156 for 6 h during IVF and 2) at 40.0ºC for 20 h after the beginning of IVC, during which almost all
157 viable embryos complete the first cleavage [32]. After HS treatment, embryos were cultured at
158 38.5ºC in E-64-free medium until day 8 (day 0 represents the day of insemination).
159 Developmental competence was assessed by cleavage and blastocyst rates on days 2 and 8,
160 respectively.

161

162 *2.6.2. Experiment 2: effect of HS and CTSB inhibitor on CTSB activity in HS-exposed embryos*

163 We then examined weather HS increass CTSB activity, and whether this change could be
164 inhibited by E-64. Matured oocytes and/or zygotes were cultured with 0.5 μM E-64, followed by
165 exposure to HS at 40.0ºC for 6 h during IVF or for 20 h after the beginning of IVC. The number
166 of fluorescent dots highlighting the active form of CTSB was analyzed immediately following
167 HS treatment.

168

169 *2.6.3. Experiment 3: effect of CTSB inhibitor on the quality of blastocysts derived from*
170 *HS-exposed embryos*

171 To examine the effects of HS and E64 inhibition on blastocyst quality, matured oocytes or
172 zygotes were cultured with 0.5 μM E-64 and exposed to HS at 40.0ºC for 6 h during IVF or for
173 20 h after the beginning of IVC, followed by incubation at 38.5ºC until day 8 of IVC.

Developmental rate (cleavage rate on day 2 and blastocyst rate on day 8), total cell number, and apoptotic status of day 8 blastocysts were analyzed.

2.7. Statistical analysis

Each experiment was repeated at least five times. Data are expressed as the mean ± standard error. All data were analyzed using analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test. All percentage data were arcsine transformed prior to statistical analysis. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of CTSB activity improves the developmental competence of bovine embryos exposed to HS during IVF or early IVC

HS during IVF decreased the developmental rate of embryos as compared with that observed in the control group ($P < 0.05$). However, embryos cultured with E-64 (0.5 μM) under the same HS conditions increased the developmental rate of embryos to a rate similar to that observed in the control group (Table 1). As shown in Table 2, HS during early IVC decreased the developmental rate to blastocyst stage as compared with control group ($P < 0.05$); however, this rate was increased by addition of 0.5 μM E-64 to the culture medium under HS conditions ($P < 0.05$), although the rate was lower than that in the control groups ($P < 0.05$). Based on these results, we selected the E-64 concentration that had the greatest effect on the developmental competence of HS-treated embryos. Therefore, all subsequent experiments were performed using 0.5 μM E-64.

3.2. HS increases intracellular CTSB activity in bovine embryos

To investigate the relationship between elevated temperature during culture and CTSB activity in embryos, HS treatment was applied during IVF (6 h) or early IVC (20 h). CTSB activity was detected immediately following HS treatment. As shown in Figure 1A, the active form of CTSB

was clearly detected in the cytoplasm, with the number of fluorescent dots counted and used as an indicator of CTSB activity. Notably, the average number of fluorescent dots was higher in the HS-treated group relative to that observed in the control and HS+E-64 groups under both HS-treatment conditions (6 h during IVF: $P < 0.01$, Fig. 1B; 20 h in early IVC: $P < 0.01$, Fig. 1C). Additionally, the average number of fluorescent dots in embryos exposed to HS during early IVC was higher as compared with that in embryos exposed to HS during IVF (74.1 ± 12.2 vs. 46.6 ± 6.1, respectively; $P < 0.05$). By contrast, addition of E-64 to the culture medium reduced the number of fluorescent dots induced by HS ($P < 0.01$; Fig. 1B and C).

3.3. Inhibition of CTSB activity improves the quality of HS-treated embryos by decreasing apoptosis in the blastocyst stage

Incubation of embryos at elevated temperatures during IVF and early IVC reduced the developmental rate in the blastocyst stage ($P < 0.05$), whereas the addition of E-64 increased the developmental rate as compared with embryos cultured without E-64 under HS conditions ($P < 0.05$; Figs. 2A and 3A). HS during IVF also reduced the cleavage rate, which was again increased by treatment with E-64 (Fig. 2A). The total cell number and apoptotic status of the blastocysts were also assessed to investigate embryo quality. HS during IVF had no significant effect on the total cell number or the percentage of apoptotic cells in the blastocyst stage (Fig. 2B and C). By contrast, although no difference was found in the total cell number among groups, the percentage of apoptotic cells in the blastocyst stage increased when embryos were exposed to HS during early IVC ($P < 0.01$; Fig. 3C). Furthermore, E-64 treatment decreased the percentage of apoptotic cells in these HS-affected blastocysts ($P < 0.01$) to a level similar to that in the control group (Fig. 3C).

4. Discussion

Multiple studies reported that HS during IVF and the early stage of IVC reduces the success of subsequent embryonic development [11, 33, 34]. The mechanism by which HS affects fertility

and embryo viability appears to be multifactorial, and various supplements have been investigated *in vitro* to determine their possible anti-HS effects. However, the efficacy and mechanisms of these and other treatments have not been fully elucidated, and HS remains an issue affecting mammalian reproductive health. CTSB is a lysosomal cysteine protease that plays an important role in the degradation of intracellular-tissue proteins in the lysosome [35]. Its activity has also been linked to changes in embryonic competency based on its high activity causing significant decreased in *in vitro* developmental rates and increases in apoptosis [28, 29]. However, the relationship between CTSB activity and low developmental competence in HS embryos remains largely unknown. To better understand the role of CTSB during HS-induced reproductive changes, we used a bovine embryo *in vitro* model. We observed that CTSB activity was elevated by HS during IVF and early IVC in bovine embryos. Moreover, supplementing the media with a CTSB inhibitor (E-64) improved the developmental competence of HS-treated embryos. These results suggested a role for CTSB in compromising the developmental competence of bovine embryos under HS conditions.

Previous study reported that higher expression of *CTSB* mRNA in cumulus cells surrounding oocytes with low developmental competence as compared with those with high developmental competence [26]. Furthermore, we also demonstrated that CTSB activity was higher in poor-quality oocytes and embryos than in good-quality ones, and that inhibition of this activity improved subsequent developmental competence [28, 29]. However, these previous studies did not specifically account for temperature-related stress. A previous study of the effects of HS investigated with regard to CTSB activity in cumulus cells and oocytes during IVM revealed that CTSB activity was elevated following HS and compromised subsequent developmental competence [30]. Moreover, administering a CTSB inhibitor neutralized these HS-mediated detrimental effects. Although the present study focused on IVF and early IVC, these data support our findings that CTSB plays a significant role in HS-induced changes during embryonic development. Similarly, we also found that addition of 0.5 μM E-64 to the culture medium under HS conditions increased the developmental competence of treated embryos. These results are

again supported by previous studies showing that E-64 improves the subsequent development of oocytes and embryos [26, 28-30, 36].

One particularly interesting result in the present study indicated that HS during IVF reduced the cleavage rate, but that the same effect was not observed during early IVC. We previously showed that HS during IVF decreases the cleavage rate by compromising the anti-polyspermy systems of oocytes [11]. Oxidative stress also plays a role in these effects, as it can damage sperm-cell structures and decreases subsequent embryonic development when the damaged sperm are applied for IVF [37]. These findings indicate that HS during IVF could impair processes in both the oocyte and sperm, resulting in decreased cleavage. On the other hand, the inhibition of CTSB activity increased the cleavage rate of embryos fertilized under HS. Collectively, increased CTSB activity in response to any form of cellular stress might be the key to understanding the observed changes in fertilization rates and embryo competency.

The mechanism of CTSB during the observed HS-induced changes in embryo viability is likely related, at least in part, to its cellular localization in the lysosome. Lysosomes are membrane-enclosed organelles that are essential for macromolecule degradation and many other cellular functions [38]. In addition to CTSB, lysosomes also contain many other hydrolytic enzymes and play pivotal roles in endocytosis, phagocytosis, and autophagy. Previous studies demonstrated that lysosome function is significantly related to early embryonic development in mice [39-41] and cattle [42]. Furthermore, exogenous stress, such as HS and oxidative stress, in somatic cells can induce lysosomal destabilization and leakage of their components into the cytosol [43, 44]. We previously showed that lysosomal aggregation and possible release of lysosomal contents into the cytosol occur in bovine oocytes after HS treatment [30]. Therefore, leakage of lysosomal CTSB into the cytoplasm is likely the initiating step in the observed increase in CTSB activity, leading to unnecessary proteolysis and subsequent arrested embryonic development.

Leakage of lysosomal enzymes also induces apoptosis. Apoptosis is a normal physiological process often used as a quality marker in preimplantation embryo development both *in vivo* and

in vitro [45-48]. Previous studies report that HS induces apoptosis in preimplantation embryos [49, 50]. Furthermore, in the present study, we also showed that HS during early IVC increased the number of apoptotic cells in the blastocyst stage. The mechanism underlying these effects appeared to be related to mitochondrial function, whereby HS-induced increases in reactive-oxygen-species production triggers mitochondrial-permeability transition pore opening, followed by release of cytochrome c to the cytosol. These changes cause apoptosis in somatic cells [51, 52], as well as bovine embryos [6, 12, 14, 53]. More importantly, previous studies report that CTSB can promote this process via induction of mitochondrial-membrane degradation [54, 55]. These studies are supported by our present observation that CTSB inhibition significantly decreased apoptosis in HS-treated embryos. Interestingly, HS during IVF had no significant effect on apoptosis at the blastocyst stage, even though increased CTSB activity was observed after HS treatment. This discrepancy might be related to the difference in the duration of HS treatment, which was shorter during IVF (6 h) as compared with early IVC (20 h). Moreover, CTSB activity in embryos exposed to HS during IVF was also lower than that in embryos exposed to HS during early IVC, indicating that this treatment time was not long enough to induce the level of CTSB activity needed for activation of apoptosis-signaling pathways. Although we observed variable responses depending on the *in vitro* stage (IVF vs. early IVC) and the duration of HS treatment (6 h vs. 20 h), the mechanism underlying the effects of HS likely involves lysosome leakage leading to CTSB activation, which causes a subsequent increase in apoptosis and, ultimately, dysfunctional embryo development.

In conclusion, our results showed that CTSB activity was elevated by HS during IVF and early IVC. Moreover, inhibition of CTSB enhanced the developmental competence and quality of HS-treated bovine embryos, suggesting that excess CTSB activity was indeed involved in the observed HS-induced arrested development. Although further research is required to provide additional details concerning the intermediate signaling cascades involved and to confirm the in vivo development of HS embryos treated with E-64 through embryo transfer, the present study is useful for elucidating the comprehensive mechanism underlying HS-induced developmental

changes in bovine embryos and provides a framework for future studies, as well as insight into developmental processes and treatment for HS-affected embryogenesis in mammals.

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Figure legends

Fig. 1. Schematic diagrams of the experimental design. In all experiments, two types of HS treatment with a different condition was conducted as follows. HS during IVF: mature oocytes were cultured at 40.0ºC for 6 h during IVF; HS during early IVC: putative zygotes were cultured at 40.0ºC for 20 h after the beginning of IVC. Experiment 1: CTSB inhibitor (E-64) with different concentrations (0, 0.1, 0.5, and 1.0 μM) was added to the medium during HS treatment. After HS treatment, zygotes/oocytes were cultured at 38.5ºC in E-64-free medium until day 8. Developmental competence was assessed by cleavage and BL rates on days 2 and 8, respectively. Experiment 2: mature oocytes or putative zygotes were cultured with or without E-64 (0.5 μM) during each HS treatment. After HS treatment, CTSB activity in their respective cytoplasm was analyzed immediately. Experiment 3: mature oocytes or putative zygotes were cultured with or without E-64 (0.5 μM) during each HS treatment. After HS treatment, they were cultured at 38.5ºC in E-64-free medium until day 8. BL quality was evaluated by counting total cell number and TUNEL-positive cells in BLs.

BL, blastocyst; CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

Fig. 2. Effect of HS on intracellular CTSB activity. Intracellular localization of the active form of CTSB was detected (red fluorescent dots) (A). Average number of dots showing the active form of CTSB in embryos exposed to HS (40.0°C) for 6 h during IVF (B) and for 20 h during early IVC (C). HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment, respectively. In the control group, IVF and IVC were performed without HS and E-64 supplementation. Data represent the mean ± standard error. Bars labeled with different letters represent significant differences ($P < 0.01$).

CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock.

Fig. 3. Effect of HS and E-64 administration during IVF on subsequent embryonic development, total cell number, and apoptotic index of blastocysts. Mature oocytes were exposed to HS (40.0°C) for 6 h during IVF with or without the addition of E-64. Developmental rates (A), total cell number (B), and apoptotic index (C) of the blastocysts were analyzed. HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment, respectively. In the control group, IVF and IVC were performed without HS and E-64 supplementation. Data represent the mean ± standard error. Bars labeled with different letters indicate significant differences ($P < 0.01$).

CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock.

Fig. 4. Effect of HS and CTSB administered during early IVC on subsequent embryonic development, total cell number, and apoptotic index of blastocysts. Putative zygotes were exposed to HS (40.0°C) for 20 h during early IVC with or without the addition of E-64. Developmental rates (A), total cell number (B), and apoptotic index (C) of the blastocysts were analyzed. HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment, respectively. In the control group, IVF and IVC were performed without HS and E-64 supplementation. Data represent the mean ± standard error. Bars labeled with different letters indicate significant differences ($P < 0.01$).

CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock.

514 **Table 1** Effect of E-64 on *in vitro* development of bovine embryos exposed to HS (40.0°C) for 6
515 h during IVF.

Treatment	No. embryos	No. (%) cleaved embryos	No. (%) blastocysts
Control	148	126 (85.1 ± 2.3)[a]	46 (31.1 ± 3.0)[a]
HS	155	104 (67.1 ± 1.5)[b]	17 (11.0 ± 1.1)[b]
HS+E-64 (0.1 μM)	142	118 (83.1 ± 3.0)[a]	26 (18.3 ± 1.7)[bc]
HS+E-64 (0.5 μM)	138	112 (81.8 ± 1.0)[ac]	33 (23.9 ± 2.0)[ac]
HS+E-64 (1.0 μM)	141	103 (73.0 ± 1.7)[bc]	24 (17.0 ± 1.0)[bc]

516 HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment,
517 respectively. In the control group, IVF and IVC were performed without HS and E-64
518 supplementation. Data represent the mean ± standard error from five replicates. The data are
519 presented as the means ± standard error from 5 replicates. [a–c] Values with different superscripts
520 within the same column are significantly different ($P < 0.05$).
521 E-64, cathepsin inhibitor; HS, heat shock.

Table 2 Effect of E-64 on *in vitro* development of bovine embryos exposed to HS (40.0°C) for 20 h during early IVC.

Treatment	No. embryos	No. (%) cleaved embryos	No. (%) blastocysts
Control	153	123 (80.4 ± 2.0)	47 (30.7 ± 2.3)[a]
HS	164	135 (82.3 ± 1.4)	19 (11.6 ± 0.8)[b]
HS+E-64 (0.1 μM)	152	120 (78.9 ± 3.1)	23 (15.1 ± 1.4)[bc]
HS+E-64 (0.5 μM)	159	127 (79.9 ± 2.8)	35 (22.0 ± 1.5)[c]
HS+E-64 (1.0 μM)	139	114 (82.0 ± 2.1)	24 (17.3 ± 1.3)[bc]

HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment, respectively. In the control group, IVF and IVC were performed without HS and E-64 supplementation. Data represent the mean ± standard error from five replicates.

[a–c] Values with different superscripts within the same column are significantly different ($P < 0.05$).

E-64, cathepsin inhibitor; HS, heat shock.

Fig.1

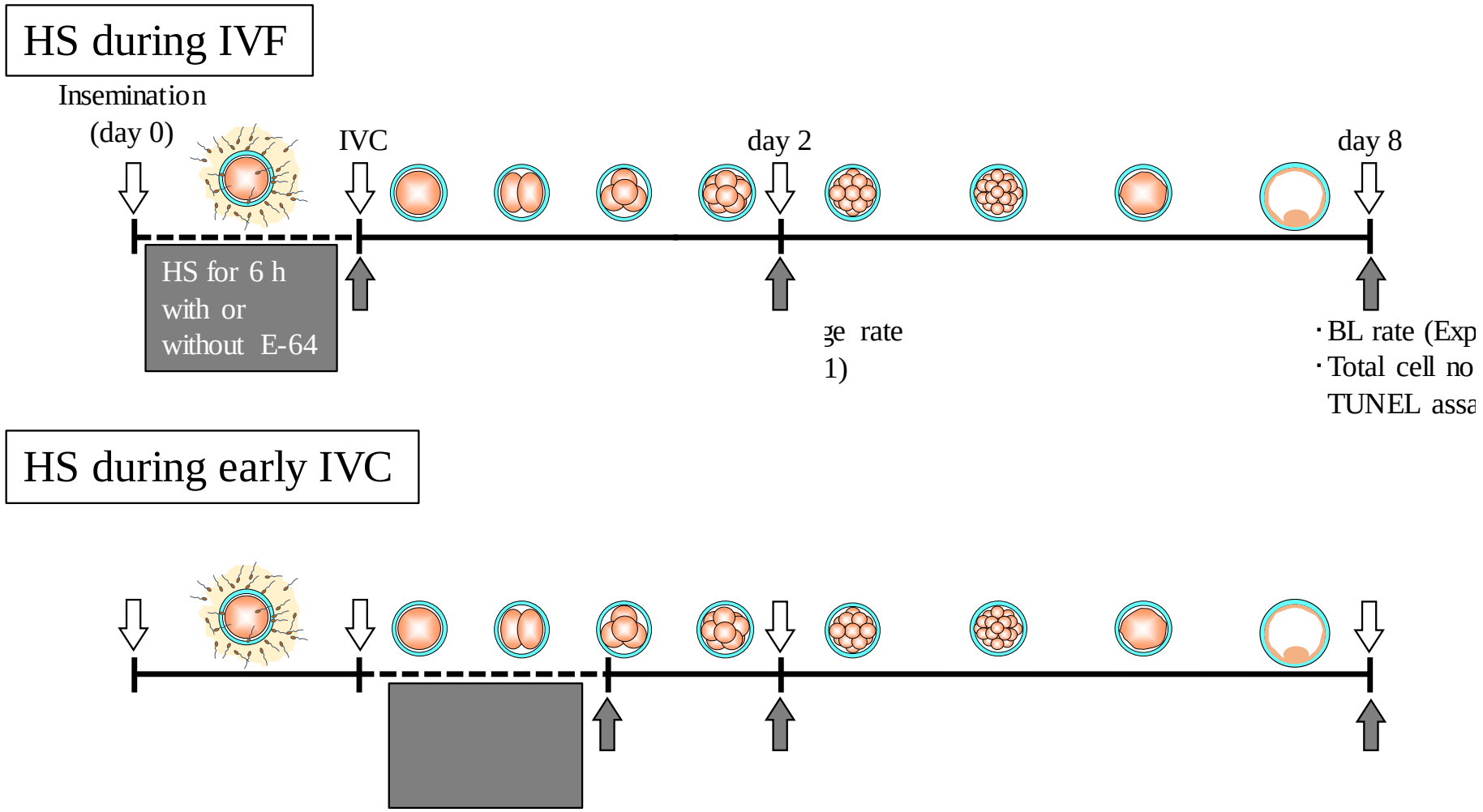

HS during IVF
Insemination
(day 0)
IVC
day 2
day 8
HS for 6 h
with or
without E-64
ge rate
1)
· BL rate (Exp
· Total cell no
TUNEL assa
HS during early IVC

Fig. 2

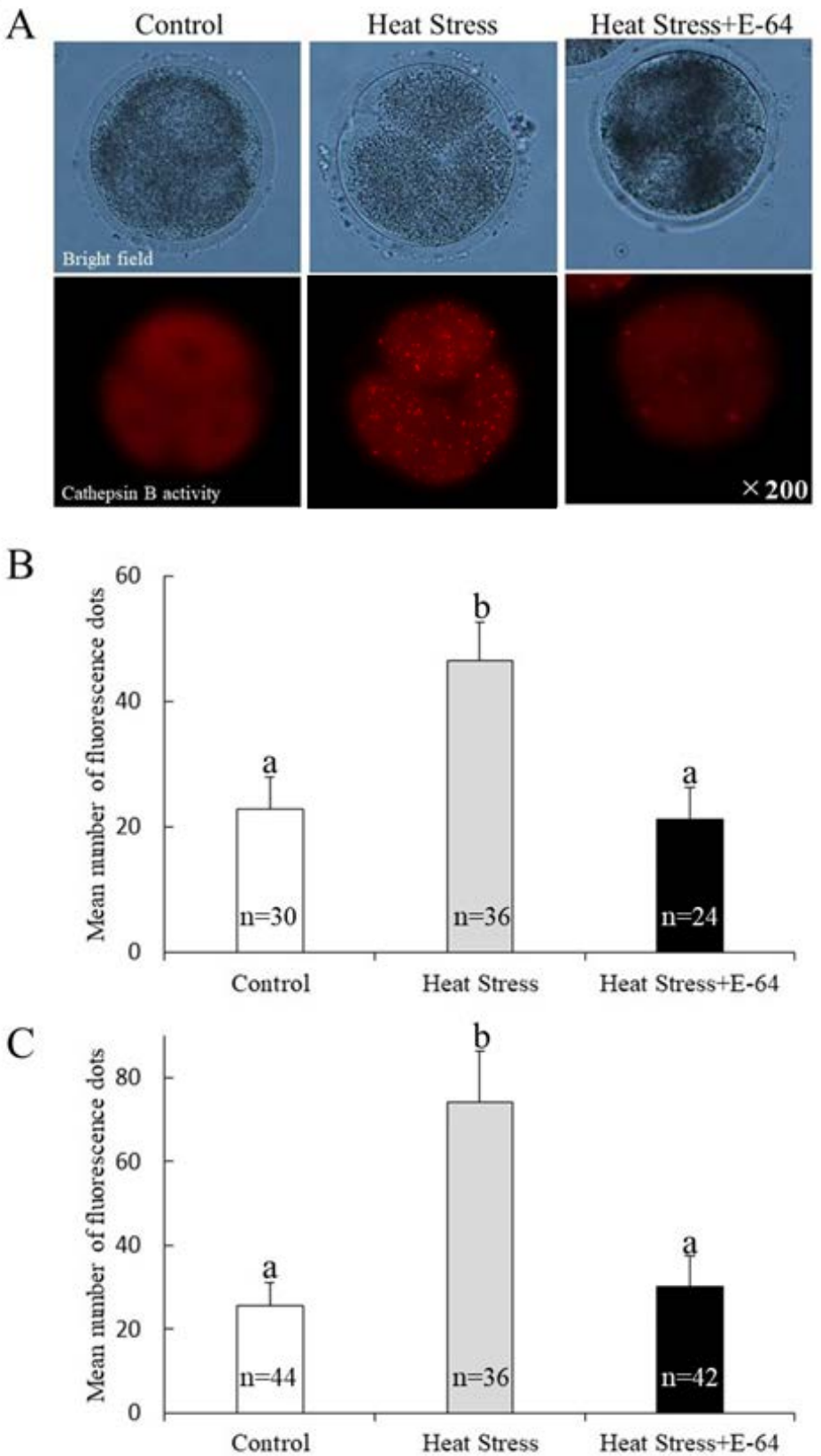

A
Control
Heat Stress
Heat Stress+E-64
Bright field
Cathepsin B activity
×200
B
Mean number of fluorescence dots
60
40
20
0
a
b
a
n=30
n=36
n=24
Control
Heat Stress
Heat Stress+E-64
C
Mean number of fluorescence dots
80
60
40
20
0
a
b
a
n=44
n=36
n=42
Control
Heat Stress
Heat Stress+E-64

Fig. 3

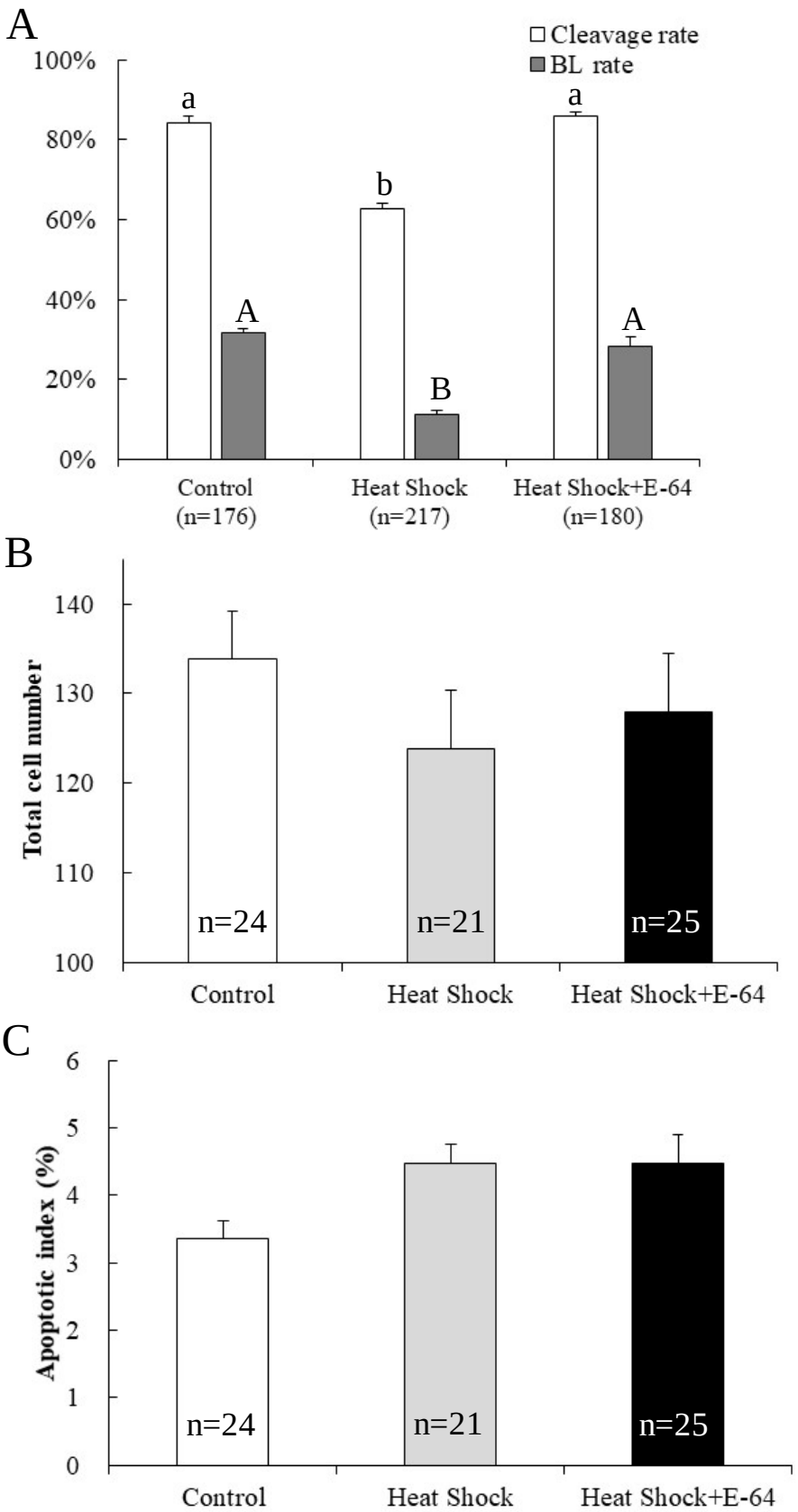
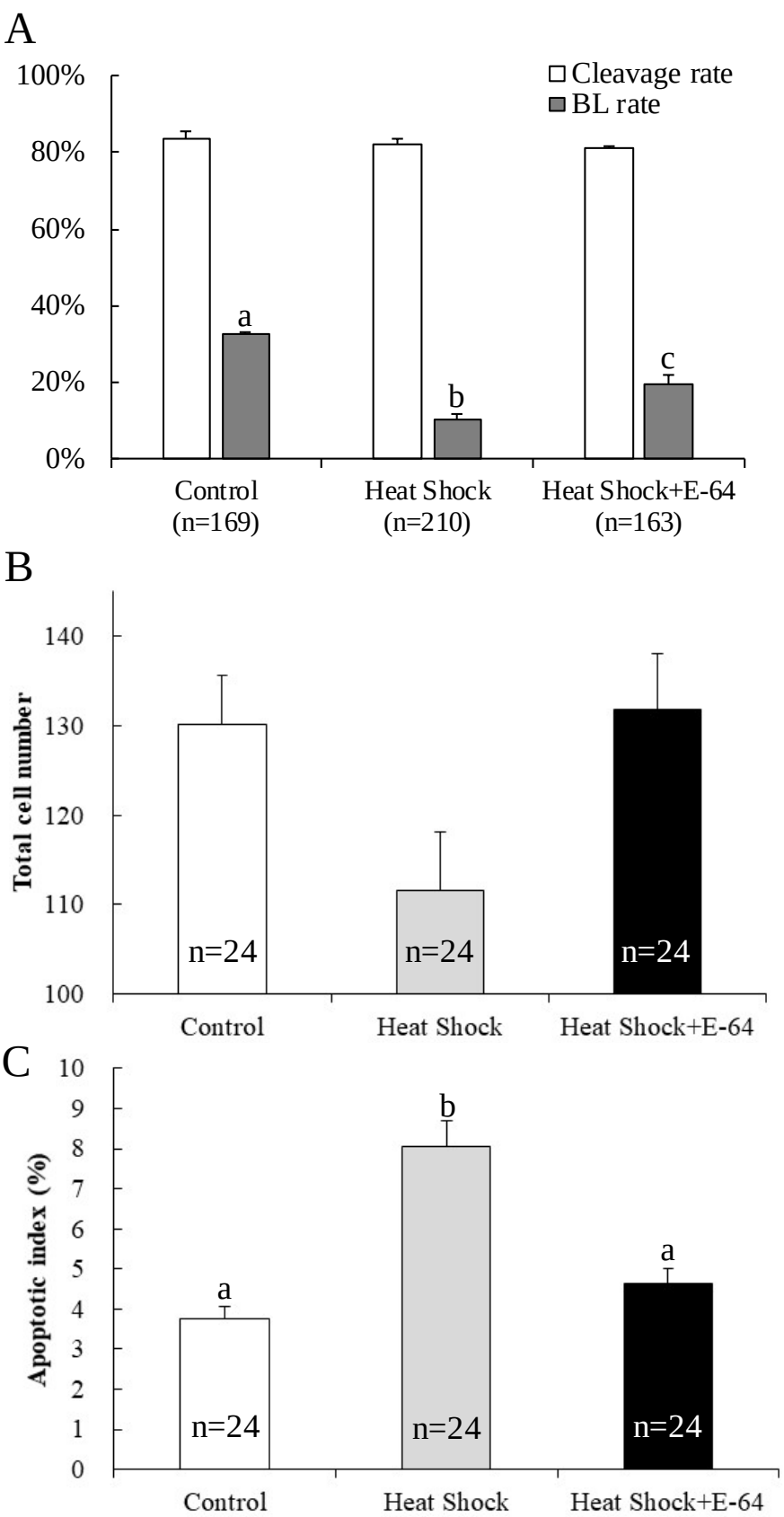

A
Cleavage rate
BL rate
100%
80%
60%
40%
20%
0%
a
A
b
B
a
A
Control
(n=176)
Heat Shock
(n=217)
Heat Shock+E-64
(n=180)
B
Total cell number
140
130
120
110
100
n=24
n=21
n=25
Control
Heat Shock
Heat Shock+E-64
C
Apoptotic index (%)
6
5
4
3
2
1
0
n=24
n=21
n=25
Control
Heat Shock
Heat Shock+E-64

Fig. 4


A
100%
80%
60%
40%
20%
0%
Cleavage rate
BL rate
a
b
c
Control
(n=169)
Heat Shock
(n=210)
Heat Shock+E-64
(n=163)
B
Total cell number
140
130
120
110
100
n=24
n=24
n=24
Control
Heat Shock
Heat Shock+E-64
C
Apoptotic index (%)
10
9
8
7
6
5
4
3
2
1
0
a
b
a
n=24
n=24
n=24
Control
Heat Shock
Heat Shock+E-64