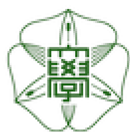

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Revised

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.

93

94 *2.2. Oocyte collection and in vitro maturation*

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

103

104 *2.3. IVF and IVC*

105 Frozen semen was thawed by immersing the straw in warm water (37ºC) for 20 s. Spermatozoa
106 were washed by centrifugation (800*g* for 10 min) in 90% (v/v) percoll solution (GE Healthcare
107 Bio-Sciences AB, Stockholm, Sweden). After removing the supernatant, the pellet was diluted
108 with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and
109 centrifuged at 600*g* for 5 min. The spermatozoa pellet was then diluted with IVF100 to prepare a
110 final sperm-cell concentration of 5.0×10^6 sperm/mL. IVF was performed at 38.5ºC (control
111 group) or at 40ºC (HS group) in 5% CO_2 in air under humidified conditions for 6 h (25 oocytes per
112 100 μL sperm drop covered with liquid paraffin).

113 After fertilization, cumulus cells were removed mechanically by pipetting in CR1aa medium
114 [31] containing 5% FBS, and putative zygotes with polar bodies were placed into micro-drops
115 (20–25 zygotes per 50 μL drop) of CR1aa medium supplemented with 5% (v/v) FBS. The drops
116 were then covered with liquid paraffin and cultured at 38.5°C (control group) or at 40.0ºC for 20 h
117 after fertilization, followed by incubation at 38.5ºC (HS group) in a humidified atmosphere of 5%
118 O_2, 5% CO_2, and balanced with N_2 through day 8 (day 0 represented the day of insemination).

119

120 *2.4. Detection of intracellular CTSB activity*

121 Detection of embryonic CTSB activity was performed using a Magic red CTSB detection kit
122 (MR-RR)2 (Immunochemistry Technologies LLC, Bloomington, MN, USA) according to
123 manufacturer instructions. Briefly, *in vitro* embryos were incubated in 500 μL PBS along with 2
124 μL of reaction mix in a humidified atmosphere of 5% CO_2 at 38.5ºC for 20 min. To detect nuclei,
125 25 μg/mL Hoechst 33342 was added and incubated in the same culture conditions for an
126 additional 5 min. After rinsing with PBS containing 0.5 mg/mL polyvinylpyrrolidone (PVP;
127 Nacalai Tesque), the stained embryos were mounted onto a glass slide and observed under a
128 fluorescence microscope (EVOS FL; Thermo Fisher Scientific). Nuclei were observed following
129 excitation at 375 nm, and intracellular CTSB activity was detected at 585 nm excitation. Images
130 of CTSB activity were captured under the same exposure time and analyzed with ImageJ
131 software (v1.55; National Institutes of Health, Bethesda, MD, USA).

132

133 *2.5. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)*

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

146

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.

174 Developmental rate (cleavage rate on day 2 and blastocyst rate on day 8), total cell number, and
175 apoptotic status of day 8 blastocysts were analyzed.
176
177 *2.7. Statistical analysis*
178 Each experiment was repeated at least five times. Data are expressed as the mean ± standard
179 error. All data were analyzed using analysis of variance (ANOVA), followed by the
180 Tukey-Kramer multiple comparison test. All percentage data were arcsine transformed prior to
181 statistical analysis. A $P < 0.05$ was considered statistically significant.
182
183
184 **3. Results**
185 *3.1. Inhibition of CTSB activity improves the developmental competence of bovine embryos*
186 *exposed to HS during IVF or early IVC*
187 HS during IVF decreased the developmental rate of embryos as compared with that observed in
188 the control group ($P < 0.05$). However, embryos cultured with E-64 (0.5 μM) under the same HS
189 conditions increased the developmental rate of embryos to a rate similar to that observed in the
190 control group (Table 1). As shown in Table 2, HS during early IVC decreased the developmental
191 rate to blastocyst stage as compared with control group ($P < 0.05$); however, this rate was
192 increased by addition of 0.5 μM E-64 to the culture medium under HS conditions ($P < 0.05$),
193 although the rate was lower than that in the control groups ($P < 0.05$). Based on these results, we
194 selected the E-64 concentration that had the greatest effect on the developmental competence of
195 HS-treated embryos. Therefore, all subsequent experiments were performed using 0.5 μM E-64.
196
197 *3.2. HS increases intracellular CTSB activity in bovine embryos*
198 To investigate the relationship between elevated temperature during culture and CTSB activity in
199 embryos, HS treatment was applied during IVF (6 h) or early IVC (20 h). CTSB activity was
200 detected immediately following HS treatment. As shown in Figure 1A, the active form of CTSB

201 was clearly detected in the cytoplasm, with the number of fluorescent dots counted and used as
202 an indicator of CTSB activity. Notably, the average number of fluorescent dots was higher in the
203 HS-treated group relative to that observed in the control and HS+E-64 groups under both
204 HS-treatment conditions (6 h during IVF: $P < 0.01$, Fig. 1B; 20 h in early IVC: $P < 0.01$, Fig.
205 1C). Additionally, the average number of fluorescent dots in embryos exposed to HS during
206 early IVC was higher as compared with that in embryos exposed to HS during IVF (74.1 ± 12.2
207 vs. 46.6 ± 6.1, respectively; $P < 0.05$). By contrast, addition of E-64 to the culture medium
208 reduced the number of fluorescent dots induced by HS ($P < 0.01$; Fig. 1B and C).
209

210 *3.3. Inhibition of CTSB activity improves the quality of HS-treated embryos by decreasing*
211 *apoptosis in the blastocyst stage*

212 Incubation of embryos at elevated temperatures during IVF and early IVC reduced the
213 developmental rate in the blastocyst stage ($P < 0.05$), whereas the addition of E-64 increased the
214 developmental rate as compared with embryos cultured without E-64 under HS conditions ($P <$
215 0.05; Figs. 2A and 3A). HS during IVF also reduced the cleavage rate, which was again
216 increased by treatment with E-64 (Fig. 2A). The total cell number and apoptotic status of the
217 blastocysts were also assessed to investigate embryo quality. HS during IVF had no significant
218 effect on the total cell number or the percentage of apoptotic cells in the blastocyst stage (Fig. 2B
219 and C). By contrast, although no difference was found in the total cell number among groups, the
220 percentage of apoptotic cells in the blastocyst stage increased when embryos were exposed to HS
221 during early IVC ($P < 0.01$; Fig. 3C). Furthermore, E-64 treatment decreased the percentage of
222 apoptotic cells in these HS-affected blastocysts ($P < 0.01$) to a level similar to that in the control
223 group (Fig. 3C).
224

225 **4. Discussion**

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

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

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

255 again supported by previous studies showing that E-64 improves the subsequent development of
256 oocytes and embryos [26, 28-30, 36].
257 One particularly interesting result in the present study indicated that HS during IVF reduced
258 the cleavage rate, but that the same effect was not observed during early IVC. We previously
259 showed that HS during IVF decreases the cleavage rate by compromising the anti-polyspermy
260 systems of oocytes [11]. Oxidative stress also plays a role in these effects, as it can damage
261 sperm-cell structures and decreases subsequent embryonic development when the damaged
262 sperm are applied for IVF [37]. These findings indicate that HS during IVF could impair
263 processes in both the oocyte and sperm, resulting in decreased cleavage. On the other hand, the
264 inhibition of CTSB activity increased the cleavage rate of embryos fertilized under HS.
265 Collectively, increased CTSB activity in response to any form of cellular stress might be the key
266 to understanding the observed changes in fertilization rates and embryo competency.
267 The mechanism of CTSB during the observed HS-induced changes in embryo viability is
268 likely related, at least in part, to its cellular localization in the lysosome. Lysosomes are
269 membrane-enclosed organelles that are essential for macromolecule degradation and many other
270 cellular functions [38]. In addition to CTSB, lysosomes also contain many other hydrolytic
271 enzymes and play pivotal roles in endocytosis, phagocytosis, and autophagy. Previous studies
272 demonstrated that lysosome function is significantly related to early embryonic development in
273 mice [39-41] and cattle [42]. Furthermore, exogenous stress, such as HS and oxidative stress, in
274 somatic cells can induce lysosomal destabilization and leakage of their components into the
275 cytosol [43, 44]. We previously showed that lysosomal aggregation and possible release of
276 lysosomal contents into the cytosol occur in bovine oocytes after HS treatment [30]. Therefore,
277 leakage of lysosomal CTSB into the cytoplasm is likely the initiating step in the observed
278 increase in CTSB activity, leading to unnecessary proteolysis and subsequent arrested embryonic
279 development.
280 Leakage of lysosomal enzymes also induces apoptosis. Apoptosis is a normal physiological
281 process often used as a quality marker in preimplantation embryo development both *in vivo* and

282 *in vitro* [45-48]. Previous studies report that HS induces apoptosis in preimplantation embryos
283 [49, 50]. Furthermore, in the present study, we also showed that HS during early IVC increased
284 the number of apoptotic cells in the blastocyst stage. The mechanism underlying these effects
285 appeared to be related to mitochondrial function, whereby HS-induced increases in
286 reactive-oxygen-species production triggers mitochondrial-permeability transition pore opening,
287 followed by release of cytochrome c to the cytosol. These changes cause apoptosis in somatic
288 cells [51, 52], as well as bovine embryos [6, 12, 14, 53]. More importantly, previous studies
289 report that CTSB can promote this process via induction of mitochondrial-membrane
290 degradation [54, 55]. These studies are supported by our present observation that CTSB
291 inhibition significantly decreased apoptosis in HS-treated embryos. Interestingly, HS during IVF
292 had no significant effect on apoptosis at the blastocyst stage, even though increased CTSB
293 activity was observed after HS treatment. This discrepancy might be related to the difference in
294 the duration of HS treatment, which was shorter during IVF (6 h) as compared with early IVC
295 (20 h). Moreover, CTSB activity in embryos exposed to HS during IVF was also lower than that
296 in embryos exposed to HS during early IVC, indicating that this treatment time was not long
297 enough to induce the level of CTSB activity needed for activation of apoptosis-signaling
298 pathways. Although we observed variable responses depending on the *in vitro* stage (IVF vs.
299 early IVC) and the duration of HS treatment (6 h vs. 20 h), the mechanism underlying the effects
300 of HS likely involves lysosome leakage leading to CTSB activation, which causes a subsequent
301 increase in apoptosis and, ultimately, dysfunctional embryo development.
302 In conclusion, our results showed that CTSB activity was elevated by HS during IVF and
303 early IVC. Moreover, inhibition of CTSB enhanced the developmental competence and quality
304 of HS-treated bovine embryos, suggesting that excess CTSB activity was indeed involved in the
305 observed HS-induced arrested development. Although further research is required to provide
306 additional details concerning the intermediate signaling cascades involved and to confirm the in
307 vivo development of HS embryos treated with E-64 through embryo transfer, the present study is
308 useful for elucidating the comprehensive mechanism underlying HS-induced developmental

309 changes in bovine embryos and provides a framework for future studies, as well as insight into
310 developmental processes and treatment for HS-affected embryogenesis in mammals.
311
312
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463
464

465 **Figure legends**

466 **Fig. 1. Schematic diagrams of the experimental design.** In all experiments, two types of HS
467 treatment with a different condition was conducted as follows. HS during IVF: mature oocytes
468 were cultured at 40.0ºC for 6 h during IVF; HS during early IVC: putative zygotes were cultured
469 at 40.0ºC for 20 h after the beginning of IVC. Experiment 1: CTSB inhibitor (E-64) with
470 different concentrations (0, 0.1, 0.5, and 1.0 μM) was added to the medium during HS treatment.
471 After HS treatment, zygotes/oocytes were cultured at 38.5ºC in E-64-free medium until day 8.
472 Developmental competence was assessed by cleavage and BL rates on days 2 and 8, respectively.
473 Experiment 2: mature oocytes or putative zygotes were cultured with or without E-64 (0.5 μM)
474 during each HS treatment. After HS treatment, CTSB activity in their respective cytoplasm was
475 analyzed immediately. Experiment 3: mature oocytes or putative zygotes were cultured with or
476 without E-64 (0.5 μM) during each HS treatment. After HS treatment, they were cultured at
477 38.5ºC in E-64-free medium until day 8. BL quality was evaluated by counting total cell number
478 and TUNEL-positive cells in BLs.

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

481

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

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

490

491 **Fig. 3. Effect of HS and E-64 administration during IVF on subsequent embryonic
492 development, total cell number, and apoptotic index of blastocysts.** Mature oocytes were
493 exposed to HS (40.0°C) for 6 h during IVF with or without the addition of E-64. Developmental
494 rates (A), total cell number (B), and apoptotic index (C) of the blastocysts were analyzed. HS
495 and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment,
496 respectively. In the control group, IVF and IVC were performed without HS and E-64
497 supplementation. Data represent the mean ± standard error. Bars labeled with different letters
498 indicate significant differences ($P < 0.01$).
499 CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock.
500
501 **Fig. 4. Effect of HS and CTSB administered during early IVC on subsequent embryonic
502 development, total cell number, and apoptotic index of blastocysts.** Putative zygotes were
503 exposed to HS (40.0°C) for 20 h during early IVC with or without the addition of E-64.
504 Developmental rates (A), total cell number (B), and apoptotic index (C) of the blastocysts were
505 analyzed. HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS
506 treatment, respectively. In the control group, IVF and IVC were performed without HS and E-64
507 supplementation. Data represent the mean ± standard error. Bars labeled with different letters
508 indicate significant differences ($P < 0.01$).
509 CTSB, cathepsin B; E-64, cathepsin inhibitor; HS, heat shock.
510
511
512
513

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.

522 **Table 2** Effect of E-64 on *in vitro* development of bovine embryos exposed to HS (40.0°C) for

523 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]

524 HS and HS+E-64 groups were supplemented with and without 0.5 μM E-64 during HS treatment,

525 respectively. In the control group, IVF and IVC were performed without HS and E-64

526 supplementation. Data represent the mean ± standard error from five replicates.

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

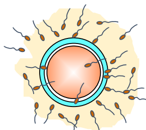
528 0.05).

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

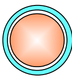
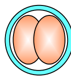
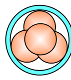
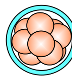
Fig.1

HS during IVF

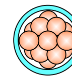
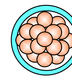
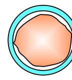
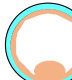
Insemination
(day 0)



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

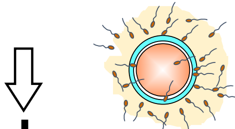
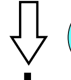
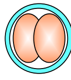
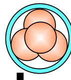
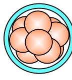
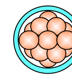
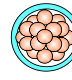
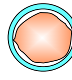
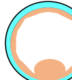
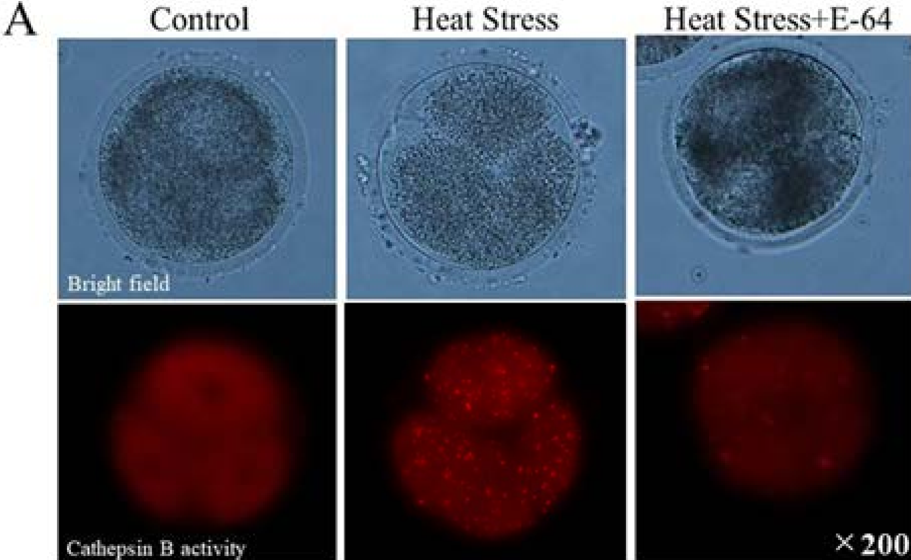
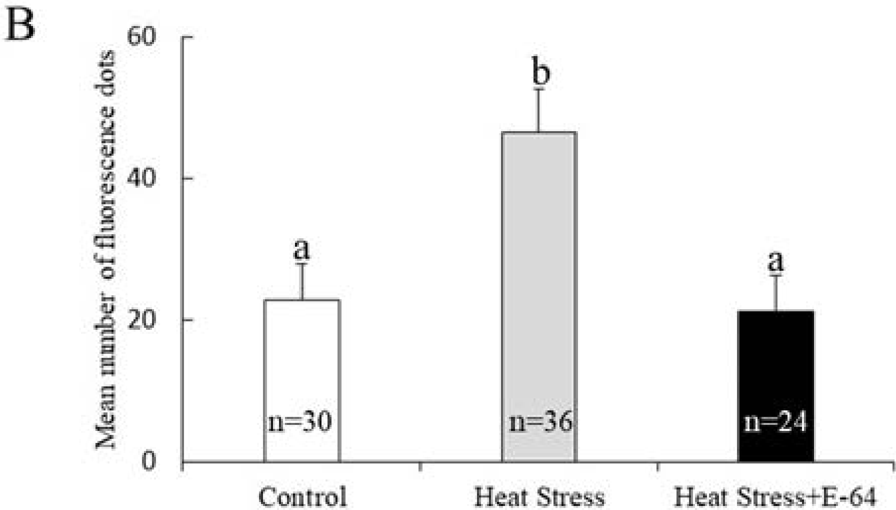










Fig. 2





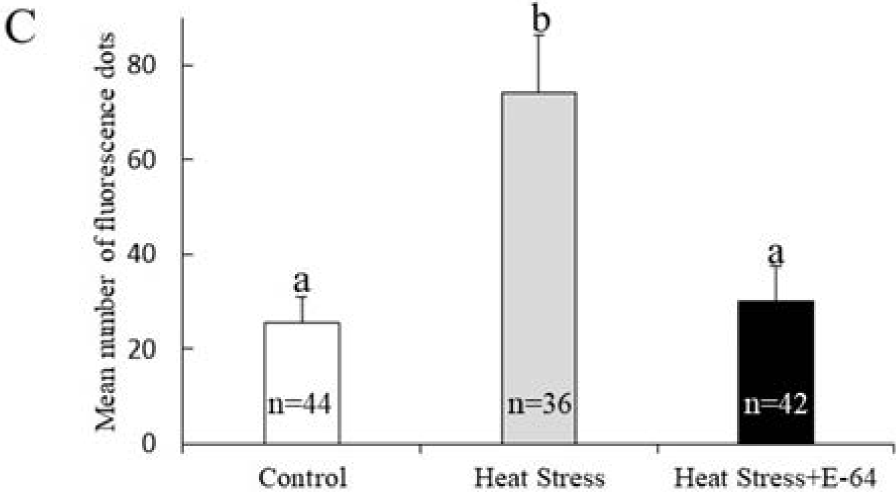


Fig. 3

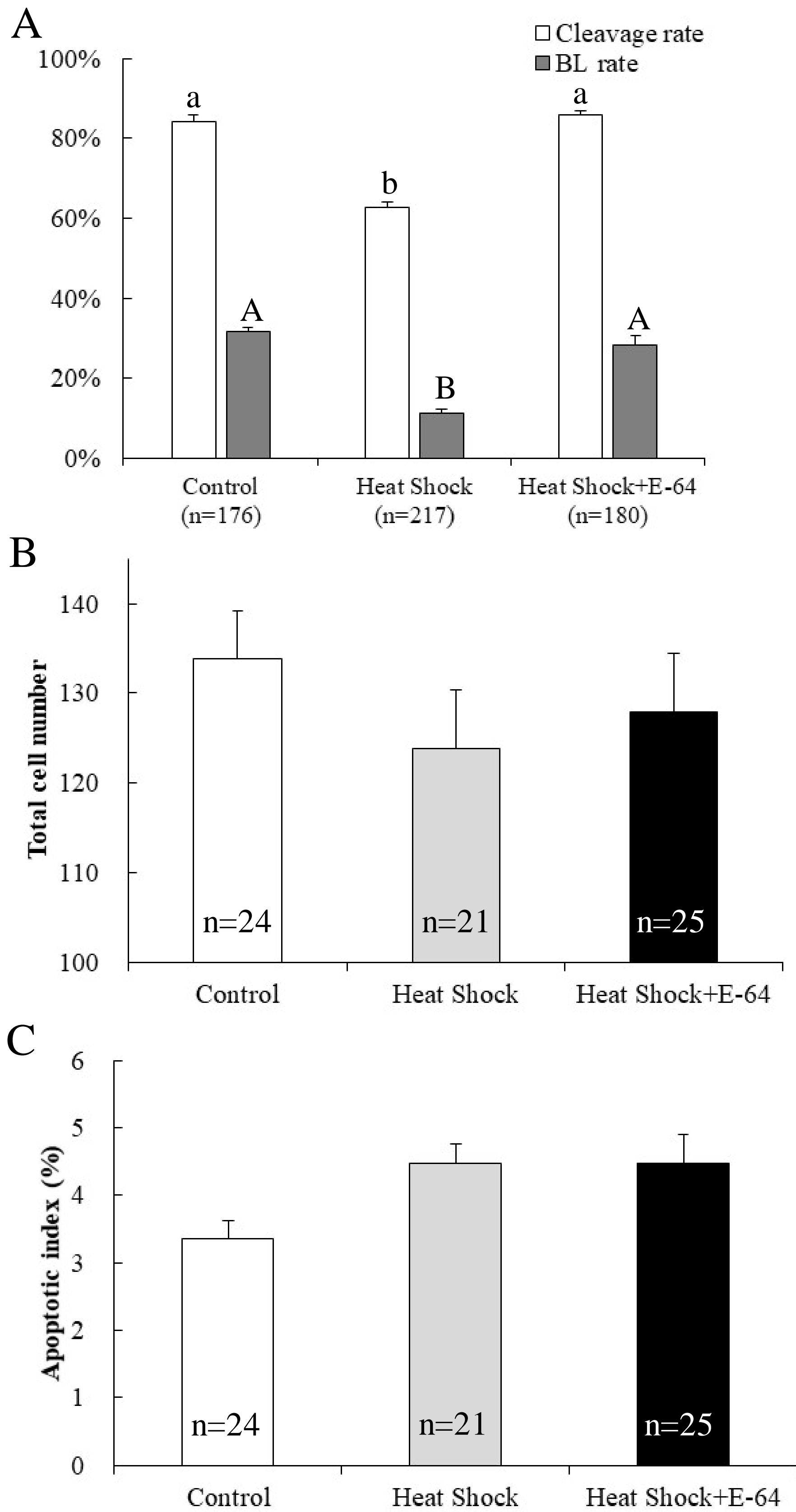


Fig. 4