Cathepsin B activity has a crucial role in the developmental competence of bovine cumulus–oocyte complexes exposed to heat shock during *in vitro* maturation

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Abstract

Cathepsin B was found to be correlated inversely with the quality of bovine oocytes and embryos. The aims of this study were to evaluate i) the relationship between heat shock during *in vitro* maturation (IVM) of bovine cumulus–oocyte complexes (COCs) and cathepsin B activity in relation to apoptosis and ii) the effect of supplementation of cathepsin B inhibitor (E-64) during IVM of heat-shocked COCs on embryonic development. After IVM at 38.5 °C for 22 h (control group) or at 38.5 °C for 5 h followed by 41 °C for 17 h (heat shock group) either with or without 1 µM E-64, activities and protein expression of cathepsin B and caspase 3 were evaluated as well as TUNEL staining. After IVF, developmental rate, total cell number, and the percentage of apoptotic cells in blastocysts were evaluated on day 8 (day 0, IVF day). Heat-shocked IVM COCs showed significantly high activities and expressions of both cathepsin B, and caspase 3 accompanied by a significant increase in number of TUNEL-positive cells. Addition of E-64 significantly decreased the activities of cathepsin B and caspase 3, and TUNEL-positive cells in heat-shocked IVM COCs. Moreover, addition of 1 µM E-64 during IVM under heat shock conditions significantly improved both developmental competence and quality of the produced embryos. These results indicate that heat shock induction of cathepsin B is associated with apoptosis of COCs, and inhibition of cathepsin B activity can improve the developmental competence of heat-shocked COCs during IVM.

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Introduction

In tropical and subtropical areas, elevated ambient temperature in summer is considered one of the most important factors responsible for reduced fertility in farm animals (Ealy *et al.* 1993). Heat stress may reduce the fertility of dairy cows by the direct effects of elevated maternal temperatures that compromise the ovum as it matures in preparation for fertilization. Conception rates in dairy cattle were 10–20% in the hot summer vs 50–55% in cooler seasons in Florida (Badinga *et al.* 1985). In particular, elevated ambient temperatures sufficient to raise rectal temperatures ≥ 41.0 °C during estrus were previously shown to increase the proportion of degenerated or retarded embryos recovered after insemination of superovulated Holstein heifers (Putney *et al.* 1989). A similar reduction in embryonic

development has been reported when bovine cumulus–oocyte complexes (COCs) were exposed to heat shock at 41.0 °C during *in vitro* maturation (IVM) (Edwards & Hansen 1996, Lawrence *et al.* 2004, Roth & Hansen 2004a).

Although the precise mechanisms of reduced conception rates and low embryo viability during the hot season are not yet clear, the detrimental influence of heat stress might be partially responsible. Exposing bovine oocytes to heat shock during IVM impairs intracellular protein synthesis (Edwards & Hansen 1996), cortical granule translocation to the oolemma (Payton *et al.* 2004), and proper rearrangement of the cytoskeleton elements (Roth & Hansen 2005). Moreover, Ju *et al.* (2005) reported that heat shock affects polymerization and de-polymerization of meiotic spindle microtubules

in bovine oocytes leading to chromosomal dispersion and ultimately death of the cell may occur (Streffer 1984). However, the effect of heat shock on cumulus cells cannot be ignored. The cumulus cells are essential in the acquisition of developmental competence of mammalian oocytes through stimulating glutathione synthesis (de Matos *et al.* 1997, Gilchrist *et al.* 2004, Makabe *et al.* 2006) and protecting against the oxidative damage during IVM (Tatemoto *et al.* 2000). Exposing immature porcine COCs to heat shock disrupted the meiotic process and was accompanied by a significant increase in number of TUNEL-positive cells in the surrounding cumulus cells (Yuan *et al.* 2008). Thus, heat shock can disrupt the developmental competence of COCs by affecting both the oocyte itself and its surrounding cumulus cells.

These detrimental effects of heat shock on COCs during IVM could be controlled by different pathways (Ju & Tseng 2004, Roth & Hansen 2005, Wang *et al.* 2009). Oxidative stress is one of these pathways that is associated with an increase in reactive oxygen species either *in vivo* (Roth *et al.* 2008) or *in vitro* (Lawrence *et al.* 2004). Nevertheless, the administration of antioxidants did not fully rescue the developmental competence, suggesting a contribution of other factors (Roth & Hansen 2004a, Soto & Smith 2009).

It has been demonstrated that heat shock compromised the developmental competence of bovine oocytes through induction of apoptotic cascade (Roth & Hansen 2004a, Soto & Smith 2009). Heat shock during bovine oocyte maturation disrupts mitochondrial membrane permeability (Nabenishi *et al.* 2012) and stimulates group II caspase activation (Roth & Hansen 2004a), which is probably key to apoptosis induction as evidenced by TUNEL-positive cells. However, the complete mechanism of heat shock-induced apoptosis through mitochondrial membrane damage and caspase activation in COCs is still unrevealed.

Cathepsin B, a lysosomal cysteine protease, was found to induce the apoptotic pathway through activating initiator caspases rather than executioner caspases either directly or indirectly in mouse fibroblast L929 cells (Vancompernelle *et al.* 1998). Cathepsin B can activate caspases indirectly through induction of mitochondrial membrane degradation, leading to translocation of apoptosis inducing components from the mitochondria into the cytosol (Bossy-Wetzel *et al.* 1998). This translocation promoted the activation of caspase-3, leading to the completion of apoptosis (Krippner *et al.* 1996, Adachi *et al.* 1997, Scaffidi *et al.* 1998). Furthermore, cathepsin B increases in poor-quality bovine oocytes and embryos, which is associated with low developmental competence, than in good-quality ones. Supplementation of the IVM medium with cathepsin B inhibitor (E-64) improved the developmental competence of bovine COCs possibly by regulation of the apoptotic pathway (Bettegowda *et al.* 2008, Balboula *et al.* 2010a,

2010b). However, there is no available data about the possible role of cathepsin B during oocyte maturation and subsequent development under heat shock condition. In this study, we investigated the possible role of cathepsin B in heat shock-induced apoptosis during IVM of bovine COCs and the effect of its inhibition on the subsequent developmental competence of heat-shocked oocytes.

Materials and methods

Oocyte collection and IVM

This study was carried out during the cold seasons (November–June) where the average ambient temperature was 11.58 °C. Holstein ovaries were collected from a local abattoir. The ovaries were washed several times in a sterile saline containing 100 IU/ml penicillin and 100 µg/ml streptomycin (Nacalai Tesque, Kyoto, Japan).

COCs were aspirated from follicles (2–6 mm in diameter) using a 19-gauge needle attached to a 10 ml syringe and washed three times in TCM-199 (Gibco) containing 5% (v/v) FCS (Gibco). Fifty COCs were matured in a 500 µl drop of TCM-199 supplemented with 5% FCS, follicle-stimulating hormone (0.02 IU/ml; Denka, Kawasaki, Tokyo, Japan), estradiol-17β (1 µg/ml; Sigma–Aldrich), and gentamicin (10 µg/ml, Sigma–Aldrich) covered with mineral oil (Sigma–Aldrich) at 38.5 °C for 22 h (control group) or at 38.5 °C for 5 h followed by 41 °C for 17 h (heat shock group) in a humidified atmosphere of 5% CO₂ in air.

Cumulus cells were cultured in DMEM (Gibco) plus 5% FCS at 38.5 °C in humidified atmosphere of 5% CO₂ for 24 h according to Tani *et al.* (2000). The cumulus cells were detached, counted, and cultured on slide chambers (5000 cells/well) at 38.5 °C for 22 h (control group) or at 38.5 °C for 5 h followed by 41 °C for 17 h (heat shock group) in a humidified atmosphere of 5% CO₂ in air.

IVF

Frozen semen (from two fertility-proven bulls) was thawed by immersing the straw in warm water (37 °C) 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 IVF-100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and further centrifugation was applied at 800 g for 5 min. The spermatozoa pellet was diluted with IVF-100 to prepare the final sperm cell concentration of 5–10 × 10⁶/ml.

After maturation, COCs were washed three times with IVF-100 and then transferred into drops of sperm cell suspension (20 oocytes/100 µl drop) covered with mineral oil. Fertilization was carried out for 6 h at 38.5 °C in a humidified atmosphere of 5% CO₂ in air.

In vitro culture

After fertilization, cumulus cells were removed mechanically by pipetting in CR1aa medium (Rosenkrans *et al.* 1993)

containing 5% FCS. Presumptive zygotes were transferred to 50 μ l micro-drops (20–30 zygotes/drop) of CR1aa supplemented with 5% FCS covered with mineral oil. Embryos were then cultured in a humidified atmosphere of 5% O₂, 5% CO₂, and 90% N₂ at 38.5 °C for 8 days.

Detection of intracellular activity of cathepsin B and caspase 3

Detection of cathepsin B in oocytes, COCs, or cultured cumulus cells as well as caspase 3 activity in oocytes was carried out after IVM using Magic red detection kit for cathepsin B (MR-RR)₂ (Immunochemistry Technologies, LLC, P 6133, Bloomington, MN, USA) and caspase 3 (DEVD)₂ (Immunochemistry Technologies, LLC, kit no. 935) according to the manufacturer's protocols. In brief, 20–30 matured oocytes were stained in 500 μ l Dulbecco's PBS (DPBS) with 2 μ l reaction mix in a humidified atmosphere of 5% CO₂ at 38.5 °C for 20 min. To detect nuclei, bisbenzimidazole (Hoechst 33342; Sigma–Aldrich) was added in a concentration of 25 μ g/ml and incubated in the same culture condition for further 5–10 min. After rinsing in DPBS with 3 mg/ml polyvinylpyrrolidone (PVP, Sigma–Aldrich), the stained oocytes were mounted onto a glass slide and observed under the fluorescence microscope (TE-300, Nikon, Tokyo, Japan). An excitation filter of 365 nm was used for observing the nuclei of cumulus cells (blue), while an excitation filter of 590 nm was applied for detection of intracellular cathepsin B activity (red) as well as caspase 3 (red). The images were captured and fluorescence emission intensity (pixels) was analyzed by ImageJ Software (National Institutes of Health, Bethesda, MD, USA). In brief, the defined cells were selected and the red cytoplasmic background was excluded by setting the threshold above the background level. This threshold was maintained at the same level for all analyzed images. The average of total fluorescence emissions (pixels) was calculated.

Lysosomal localization and stability assessment

To label lysosomes, denuded oocytes after IVM were incubated with 75 nM LysoTracker red (Molecular Probes, L-7528, Eugene, OR, USA) for 30 min at 37 °C. After rinsing in PBS with 3 mg/ml PVP, the stained oocytes were mounted onto a glass slide and observed under the fluorescence microscope with a 590 nm excitation filter. The fluorescent images were captured and analyzed by ImageJ Software. In brief, the defined cells were selected and the red cytoplasmic background was excluded by setting the threshold above the background level. This threshold was maintained at the same level for all analyzed images. The average size of the lysosomal dots (red) was calculated.

TUNEL

A TUNEL assay kit was used to assess the presence of apoptotic cells (In Situ Cell Death Detection Kit, Roche Applied Science) in *in vitro* matured COCs and day 8 blastocysts. *In vitro* matured COCs or blastocysts were fixed in 4% (w/v) para-formaldehyde solution (pH 7.4) for 15–30 min, rinsed twice in PBS, and then permeabilized in PBS with 0.5% Triton-X for

20 min followed by twice washing for 10 min in PBS with 3 mg/ml PVP. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 37 °C. After incubation, the COCs and blastocysts were washed three times in PBS with 3 mg/ml PVP for 5 min each, followed by mounting onto glass slides using mounting solution containing DAPI, Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). The fluorescence of fragmented DNA ends was detected using an excitation filter of 488 nm by fluorescent microscope (Nikon TE-300). Total cell number was also recorded at the same time.

Western blot analysis

In vitro matured COCs (sets of five) were lysed in 1% SDS (Nacalai Tesuque), 1% β -mercaptoethanol (Nacalai Tesuque), 20% glycerol (Nacalai Tesuque), and 50 mM Tris–HCl (pH 6.8) and denatured at 95 °C for 5 min. Sample solutions were separated by electrophoresis in 5–20% gradient SDS–polyacrylamide precast gels (Wako Junyaku, Tokyo, Japan). Pre-stained marker proteins with known molecular mass (range: 14–200 kDa) were run simultaneously as standards. The electrophoretically separated proteins were transferred onto PVDF membrane using an iBlot Gel transfer System (Invitrogen). The membranes were incubated in PVDF blocking reagent (B1080501, Toyobo, Tokyo, Japan) for 1 h and then washed three times in Tris-buffered saline and Tween 20 (TBS-T) at room temperature. Membranes were incubated with primary antibodies for cathepsin B (1:1000 dilution; Mouse MAB to cathepsin B, cat no. ab58802, Abcam), caspase 3 (1:1000 dilution, Rabbit polyclonal antibody, cat no. AAP113, Stressgen, Ann Arbor, MI, USA), and β -actin (1:3000 dilution, cat no. G043, Applied Biological Materials, Inc., Richmond, BC, Canada) at 4 °C overnight. After three washes with TBS-T, membranes were incubated with a HRP-labeled anti-mouse IgG secondary antibody (1:25 000 dilution, cat no. NA931, GE Healthcare, Buckinghamshire, UK) or anti-rabbit IgG (1:25 000 dilution, cat no. RPN430, GE Healthcare) at room temperature for 1 h. Primary and secondary antibodies were diluted with immunoreaction enhancer, Can Get Signal (Toyobo). Membranes were washed extensively with TBS-T before detection of bound antibodies using the ECL-Plus Western Blotting Detection System (GE Healthcare) as per the manufacturer's instructions. Chemiluminescent signals were captured by ChemiDoc System (Bio-Rad) and analyzed using a software (Quantity One, Bio-Rad).

Experimental design

Experiment 1: the effect of heat shock during IVM on cathepsin B activity, caspase 3 activity, and lysosomal localization and destabilization in bovine COCs:

COCs and cultured cumulus cells were randomly allocated to three groups and matured at 38.5 °C for 22 h (control), 38.5 °C for 5 h followed by 41 °C for 17 h (heat shock), and 38.5 °C for 5 h followed by 41 °C for 17 h with 1 μ M E-64 (cathepsin B inhibitor, cat no. E3132, Sigma–Aldrich) (heat shock plus E-64). The effect of heat shock on the activity of cathepsin B was evaluated in denuded oocytes as well as in COCs and/or in

cumulus cells. Caspase 3 activity and lysosomal localization were also investigated in denuded oocytes. Denuded oocytes were obtained by removing the surrounded cumulus cells mechanically with 0.1% hyaluronidase.

Experiment 2: effect of heat shock during IVM on protein expression of cathepsin B and caspase 3 in bovine COCs:

To investigate whether the increase in cathepsin B and caspase 3 activities during heat shock is accompanied by increasing amounts of cathepsin B and caspase 3 proteins, both cathepsin B and caspase 3 protein expression were compared between *in vitro* matured COCs and heat-shocked COCs matured either with or without 1 μ M E-64 using western blot analysis.

Experiment 3: effect of heat shock during IVM on TUNEL apoptosis of bovine COCs:

To elucidate the apoptotic effect of heat shock and the protective effect of E-64, TUNEL staining as an apoptosis indicator was performed and compared between heat-shocked COCs and control ones. Moreover, the effect of cathepsin B inhibitor (1 μ M E-64) on heat shock apoptosis was also investigated.

Experiment 4: effect of E-64 on developmental competence of COCs compromised by heat shock during IVM:

Bovine COCs were randomly allocated to treatments in groups of 50 (control, heat shock, and heat shock plus E-64) and then cultured in the maturation medium. After 22 h of maturation, COCs were fertilized and cultured. Developmental competence was assessed by cleavage and blastocyst rates on days 2 and 8 respectively. Total cell number and TUNEL were also assessed on day 8 blastocysts.

Statistical analysis

Each experiment was replicated at least three times. The data were expressed as means $\pm$ S.E.M. The statistical significance of differences was analyzed by both Student's *t*-test and ANOVA using StatView, version 5.0 (Abacus Conceptus, Berkeley, CA, USA). When the ANOVA test yielded a *P* value < 0.05 , the data were analyzed using the Fisher *post-hoc* test.

Results

The effect of heat shock on cathepsin B activity, caspase 3 activity, and lysosome localization in *in vitro* matured oocytes and cultured cumulus cells

As shown in Fig. 1A and B, cathepsin B activity in bovine oocytes was significantly increased by heat shock ($P < 0.05$). On the other hand, addition of 1 μ M E-64 to the IVM medium during heat shock significantly diminished the increase in cathepsin B activity.

Moreover, control oocytes showed normal punctuate lysosomal staining (Fig. 1C and D). After exposure to heat shock conditions, lysosomal aggregation, and release of lysosomal contents into the cytosol increased

(Fig. 1C and D). The addition of E-64 to the IVM medium during heat shock could not rescue the abnormal localization and aggregation of the lysosomes (Fig. 1C and D).

Furthermore, cytoplasmic caspase 3 activity was clearly increased upon exposure to heat shock (Fig. 1E and F). We then investigated whether caspase 3 is involved in the cathepsin B pathway or not. Caspase 3 activity was significantly decreased by addition of E-64 during heat shock (Fig. 1E and F).

The same results for cathepsin B activity were obtained in COCs and cultured cumulus cells. Under heat shock condition, both cumulus cells of heat-shocked COCs (Fig. 2A) and cultured cumulus cells (Fig. 2B) showed higher cathepsin B activity than control COCs and such activity was decreased by addition of 1 μ M E-64 to the IVM medium.

The effect of heat shock during IVM on protein expression of cathepsin B and caspase 3

Western blot analysis clearly revealed a significant increase ($P < 0.05$) in both cathepsin B and caspase 3 expressions in heat-shocked COCs than in control COCs (Fig. 3). Moreover, the addition of 1 μ M E-64 to the IVM medium significantly decreased the expression of caspase 3 but not cathepsin B (Fig. 3).

The effect of heat shock during IVM on the TUNEL-positive cells of IVM COCs

To confirm the compromising effect of heat shock during IVM on the apoptotic status of bovine COCs, TUNEL staining was evaluated after incubation of bovine COCs at 41 °C for 17 h during IVM. The percentage of TUNEL-positive cells was obviously higher in heat-shocked COCs than in control COCs (Fig. 4). The addition of E-64 to the IVM medium significantly decreased ($P < 0.05$) the heat-shock-induced apoptosis of cumulus cells (Fig. 4).

Inhibition of cathepsin B activity improves the developmental competence of bovine COCs compromised by heat shock during IVM

Heat shock during IVM decreased the blastocyst rate significantly ($P < 0.05$). On the other hand, addition of 1 μ M E-64 to the maturation medium significantly ($P < 0.05$) improved the blastocyst rate under heat shock conditions (Table 1). In contrast the cleavage rate did not vary significantly among groups (Table 1). Exposure of COCs to heat shock during IVM decreased the quality of the embryos produced as evidenced by the significant decrease ($P < 0.05$) in total cell number and increase ($P < 0.05$) in number of TUNEL-positive cells (Table 2 and Fig. 5). Interestingly, addition of 1 μ M E-64 to IVM medium could rescue the quality yielding a significant

Figure 1 Effect of heat shock during IVM on intracellular activity of cathepsin B, and caspase 3, and lysosomal localization in bovine denuded oocytes. Bovine COCs were incubated at 38.5 °C for 22 h (control) or at 38.5 °C for 5 h followed by 41 °C for 17 h either with (heat shock plus E-64) or without (heat shock) 1 μ M E-64. IVM COCs were denuded and examined for cathepsin B activity, caspase 3 activity, and lysosome localization. (A) Intracellular localization of the active form of cathepsin B was detected as red fluorescent dots using a 590 nm excitation filter. (B) Quantification of the fluorescence intensity of cathepsin B activity. (C) Lysosomes were detected using a 590 nm excitation filter as red fluorescent dots. (D) Quantification of the lysosomal average size. (E) Intracellular localization of caspase 3 activity was detected as diffuse red fluorescence signal in oocytes' cytoplasm using a 590 nm excitation filter. (F) Quantification of the fluorescence intensity of caspase 3 activity. Values with different letters across treatments differ significantly, $P < 0.05$. $n = 3$ replicates, original magnification 200 $\times$.

Figure 2 Effect of heat shock during IVM on cathepsin B activity in bovine COCs and cultured cumulus cells. Bovine COCs and cultured cumulus cells were incubated at 38.5 °C for 22 h (control) or at 38.5 °C for 5 h followed by 41 °C for 17 h either with (heat shock plus E-64) or without (heat shock) 1 µM E-64. The nuclei of cells were stained with DAPI. Cathepsin B activity was detected as red fluorescent dots in COCs (A) and cultured cumulus cells (C) using a 590 nm excitation filter. (B and D) Quantification of the fluorescence intensity of cathepsin B activity in COCs and cumulus cells. Values with different letters across treatments differ significantly, $P < 0.05$. $n = 3$ replicates, original magnification 200× in cumulus cells and 100× in COCs.

($P < 0.05$) increase in total cell number and decrease in number of TUNEL-positive cells (Table 2 and Fig. 5).

Discussion

Our results showed that i) heat shock during IVM compromises oocyte developmental competence due to the increase in cathepsin B activity either in denuded oocytes or their surrounding cumulus cells and the increase in the number of TUNEL-positive cells in COCs

and ii) inhibition of cathepsin B activity during IVM with heat shock of bovine oocytes improves developmental competence and quality of the embryos produced by decreasing the number of TUNEL-positive cells in COCs and blastocysts.

Apoptosis was found to play a critical role in the harmful effect of thermal stress during mammalian oocyte maturation (Roth & Hansen 2004b, 2005, Yuan *et al.* 2008, Soto & Smith 2009). Our result showing a significant increase in the number of TUNEL-positive

Figure 3 Protein expression of cathepsin B and caspase 3 in bovine COCs by western blot. (A and B) Cathepsin B protein expression and relative expression levels (three replicates) in control, heat-shocked COCs either with or without E-64. (C and D) Caspase 3 protein expression and relative expression levels ($n=5$ replicates) respectively in relation to heat shock either with or without E-64. Values with different letters across treatments differ significantly, $P<0.05$.

cells in heat-shocked COCs is in agreement with previous reports (Roth & Hansen 2004a, Soto & Smith 2009). They found that heat shock during oocyte maturation was closely related to the apoptotic response, which, in turn, led to disruption of the oocyte's capacity to support early embryonic development following fertilization. These results clearly showed that apoptotic pathway can be considered to be an important factor for

impairing the developmental competence of bovine oocytes compromised by heat shock during IVF.

Cathepsin B is a lysosomal cysteine protease that plays an important role in the degradation of intracellular tissue proteins in lysosomes (Barrett & Kirschke 1981, Szpadarska & Frankfater 2001). Cathepsin B localizes in various types of cells including liver, ovaries, and COCs (Olstein & Liener 1983, Oksjoki *et al.* 2001,

Figure 4 Effect of heat shock during IVF on the apoptotic status of bovine COCs. Apoptotic status was compared between control COCs and heat-shocked COCs either with or without E-64. (A) TUNEL staining (green) was used to detect cumulus cell apoptosis while DAPI staining (blue) was used for DNA detection (B) Quantification of (A) showing the percentages of TUNEL-positive cells. Values with different letters across treatments differ significantly, $P<0.05$. $n=3$ replicates. Original magnification 100 $\times$.

Table 1 Effect of E-64 on developmental competence of COCs compromised by heat shock during *in vitro* maturation.

	No. of COCs	Cleavage rate (%)	Blastocyst rate (%)
Control	209	75.15 ± 7.9	37.34 ± 2.7
Heat shock	202	72.52 ± 6.1	23.11 ± 3.0*
Heat shock + E-64	204	72.99 ± 4.9	33.43 ± 3.8

Data pooled from six replicates. Values marked with asterisks differ significantly ($P < 0.05$) from other values in the same column.

Bettegowda *et al.* 2008), suggesting that cathepsin B has many cell functions. Bettegowda *et al.* (2008) first reported that cumulus cells of low developmental competence oocytes showed higher expression of cathepsin B mRNA than those of high developmental competence oocytes (Bettegowda *et al.* 2008). Moreover, our previous studies clearly showed that cathepsin B activity is inversely correlated with the quality of bovine COCs and embryos by the higher activity and protein expression of cathepsin B in poor-quality *in vitro* matured oocytes and embryos than in good-quality ones (Balboula *et al.* 2010a, 2010b). Furthermore, cathepsin B was found to impair the oocyte and embryo developmental competence by possible stimulation of the apoptotic pathway (Balboula *et al.* 2010a, 2010b). In this study, the significant decrease in TUNEL apoptosis in heat-shocked bovine COCs after the addition of cathepsin B inhibitor (E-64) to the IVM medium clearly showed that cathepsin B has one of the key roles in inducing apoptosis during heat shock of bovine COCs.

Cathepsin B purified from lysosomal protease has the ability to induce apoptosis in somatic cells by activating initiator caspases rather than executioner caspases (Vancompernelle *et al.* 1998). These observations are in agreement with our hypothesis that cathepsin B, in bovine COCs, plays an important role in heat shock-induced apoptosis and that caspase 3 is possibly one of its downstream targets. To test our hypothesis, both cathepsin B and caspase 3 along with lysosomal localization were compared after exposure of bovine COCs to heat shock during IVM either with or without E-64. Our results showing a significant increase in cathepsin B and caspase 3 during heat shock clearly revealed that cathepsin B and caspase 3 play a crucial role in the apoptotic cascade.

Cathepsin B can activate caspases through induction of mitochondrial membrane degradation, a condition known as permeability transition, which leads to the release of proapoptotic factors into the cytosol (Bossy-Wetzel *et al.* 1998, Boya *et al.* 2003). This finding is supported by the fact that mitochondria play a crucial role in regulation of cell death pathways (Wang 2001, Droga-Mazovec *et al.* 2008). The rate of apoptosis in mammalian oocytes was decreased after microinjection of mitochondria purified from non-apoptotic granulosa cells (Perez *et al.* 2000). Regardless of the effect of cathepsin B on caspases activation, cathepsin B itself

could induce nuclear apoptosis when added to a reconstituted system containing isolated nuclei in the absence of cytosolic caspase 3 (Ishisaka *et al.* 1998, Antunes *et al.* 2001).

Lysosomes are membrane-bound dynamic organelles that are indispensable for endocytosis, phagocytosis, and autophagy (Eskelinen *et al.* 2003). Exogenous stresses such as oxidative stress in somatic cells can induce lysosomal destabilization and leakage of its destructive components (Zdolsek *et al.* 1990, Brunk *et al.* 1995). Moreover, inhibiting general trafficking of the lysosomes in cancer cells resulted in their aggregation and destabilization of their membrane permeability with subsequent release of their contents to the cytosol, where they can trigger caspase-mediated apoptosis (Cardoso *et al.* 2009). Our results showed that control IVM oocytes have normal punctuate lysosomal staining with barely seen activities of either cathepsin B or caspase 3. On the other hand, heat shock exposure caused a defect in lysosomal membrane permeability followed by lysosomal aggregation and release of lysosomal cathepsin B into the cytosol. The addition of E-64 to the IVM medium during heat shock could not rescue the abnormal localization of the lysosomes but significantly decreased the activity of both cathepsin B and caspase 3. Moreover, addition of E-64 could decrease caspase 3 expression level but not cathepsin B protein expression. This stability of cathepsin B protein against E-64 could be explained by the fact that E-64 has the ability to inhibit the activity of cathepsin B but not the protein expression. These results support our hypothesis that increasing cathepsin B activity can induce apoptosis in bovine oocytes, at least in part, through stimulation of caspase 3. Moreover, these results are consistent with the findings of Guicciardi *et al.* (2000) who reported that hepatocytes from cathepsin B knockout mice are resistant to TNF α -induced apoptosis (Foghsgaard *et al.* 2001) and gives further explanation for the results of Roth and Hansen who concluded that heat shock induces apoptosis by stimulating group II caspases in bovine oocytes (Roth & Hansen 2004a).

Although cumulus cells have been known to be very resistant, our results clearly showed that bovine cumulus cells are highly affected by heat shock as evidenced by significant increase in both cathepsin B activity and TUNEL apoptosis. This finding is supported by the results

Table 2 Effect of E-64 treatment during *in vitro* maturation under heat shock condition on total cell number and apoptotic status of day 8 blastocysts.

	No. of blastocysts	Total cell no.	TUNEL-positive cells (%)
Control	28	162.30 ± 8.1	2.49 ± 0.3
Heat shock	24	127.11 ± 10.7*	8.24 ± 1.7*
Heat shock + E-64	26	158.26 ± 10.0	2.54 ± 0.6

Data pooled from four replicates. Values marked with asterisks differ significantly ($P < 0.05$) from other values in the same column.

Figure 5 Effect of E-64 treatment during IVM under heat shock condition on total cell number and apoptotic status of day 8 blastocysts after IVF and culture. TUNEL staining (green) was used to detect apoptotic cells while DAPI staining (blue) was used for DNA detection. $n=4$ replicates, number of blastocysts examined=78.

of Yuan *et al.* (2008) who concluded that heat shock causes apoptosis of porcine cumulus cells, which contribute to maturation failure of porcine oocytes. Bidirectional communication between the oocyte and cumulus cells occurs throughout follicular development (Sirard *et al.* 2006) and is essential for the acquisition of developmental competence of mammalian oocytes. Moreover, cumulus cells stimulate glutathione synthesis in bovine oocytes (de Matos *et al.* 1997, Gilchrist *et al.* 2004, Makabe *et al.* 2006) and help to protect against the damage induced by oxidative stress during IVM (Tatemoto *et al.* 2000). In addition, it is well known that cumulus cells play a crucial role in the follicular differentiation process, providing optimal conditions for oocyte growth, fertilization, and subsequent development (Adashi 1994). Thus, it is plausible that any disruption in the cumulus cells surrounding the oocyte can be responsible for a reduction in oocyte developmental competence under heat shock conditions. Exposure of bovine oocytes or cumulus cells to heat shock during IVM would stimulate cathepsin B to be leaked from lysosomes that were partially damaged. It is likely that this cathepsin B leakage together with its increased activity induces mitochondrial permeability transition followed by activation of caspases, thereby inducing the apoptotic pathway.

E-64 is an irreversible nontoxic cysteine protease inhibitor. The nontoxicity of E-64 makes it a good template for drugs to treat diseases where cysteine proteases are the primary cause, such as rheumatoid arthritis (Yoshifuji *et al.* 2005), muscular dystrophy (Tamai *et al.* 1986), and Alzheimer diseases (Trinchese *et al.* 2008). Moreover, E-64 has no compromising effect on embryo development, embryos that were produced from E-64-treated oocytes resulted in normal pregnancies (A Balboul & M Takahashi 2013, our unpublished data). According to the working concentrations

determined for E-64, 1 μ M E-64 was added to the IVM medium. We found that heat shock during IVM significantly reduced the blastocyst rate. On the other hand, maturation (A Balboul & M Takahashi 2013, our unpublished data) and cleavage rates did not vary significantly. Addition of 1 μ M E-64 significantly improved the blastocyst rate compared with the heat shock group. This result suggests that there is a strong relationship between cathepsin B activity during heat shock and the developmental competence of bovine oocytes and that inhibition of cathepsin B activity can be a means of improving developmental rates.

Average total cell number and the number of TUNEL apoptotic cells are considered valuable tools for evaluating the quality of pre-implantation embryos (Hardy 1997, Fouladi-Nashta *et al.* 2005, Matsuura *et al.* 2010, Su *et al.* 2011). In heat-shocked embryos, the apoptosis may arise in a wave as a result of the heat shock pulse during IVM leading to a decrease in total cell number of day 8 blastocysts. In this study, addition of E-64 during heat shock significantly increased total cell number of day 8 blastocysts compared with the heat shock group. Moreover, addition of E-64 significantly decreased the percentages of TUNEL-positive cells in day 8 blastocysts compared with the heat shock group. Our results clearly showed that controlling cathepsin B activity during heat shock can alter blastocyst quality by both increasing the total cell number and decreasing TUNEL-positive cells. Thus, these results clearly support a promising role of cathepsin B inhibitors under heat shock conditions in improving embryo development and quality.

Although E-64 inhibits some cysteine proteinases including cathepsin B, it does not inhibit cathepsins S, Z, and K (Barrett *et al.* 1982). Moreover, only cathepsins B, S, Z, and K showed negative association with bovine oocyte developmental competence in microarray experiments (Patel *et al.* 2007). Furthermore, addition of

CA-074, a specific inhibitor of cathepsin B, had the same improving effect on the developmental competence of bovine COCs compromised by heat shock (A Balboula & M Takahashi 2013, our unpublished data). Given the previous data, we suggest that E-64 improves developmental competence by inhibiting cathepsin B.

In conclusion, the present results implicate cathepsin B activity as one of the possible pathways that are responsible for heat shock-induced apoptosis in bovine COCs. Moreover, controlling the activity of cathepsin B can be a useful approach for blocking heat stress-induced apoptosis in mammalian oocytes with subsequent improvement in female fertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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