—Research Note—

Heat Shock-derived Reactive Oxygen Species Induce Embryonic Mortality in *In Vitro* Early Stage Bovine Embryos

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Abstract. Heat shock is known to increase the mortality of early stage embryos, but the exact mechanism is unclear. In the present study, we investigated the possibility that the increased mortality is caused by heat shock-generated reactive oxygen species (ROS). The level of ROS was controlled by using β -mercaptoethanol (β -ME), a scavenger of ROS. *In vitro*-produced 8-cell stage embryos were cultured at 38.5 C or heat-shocked by exposure to 41 C for 6 h with 0, 10 and 50 μ M β -ME. Intracellular ROS levels were measured by a fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA), and intracellular reduced form of glutathione (GSH) contents were estimated by another fluorescent dye, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin. Total glutathione content was estimated by the glutathione recycling assay. On day 8 after insemination, heat shock decreased the percentage of embryos that developed to the blastocyst stage and increased intracellular ROS levels, but there was no significant effect on the GSH and total glutathione contents. In contrast, β -ME significantly decreased ROS levels in heat-shocked embryos and increased the GSH and total glutathione concentrations. Ten μ M β -ME significantly improved the viability of heat-shocked embryos. β -ME caused no detrimental effects when it was added at normal culture temperature (38.5 C). These results indicate that ROS is the primary cause of increased embryonic mortality in heat-shocked early stage embryos.

Key words: Bovine, Heat shock, β -Mercaptoethanol, Preimplantation embryo, Reactive oxygen species (ROS)

(J. Reprod. Dev. 54: 496–501, 2008)

Heat shock has various deleterious effects on cells. For example, heat shock increases the probability of DNA fragmentation [1], apoptosis [2, 3] and causes abnormal morphologies of the cell organelle and cell death [4]. Heat shock is reported to increase the concentration of intracellular reactive oxygen species (ROS) in cells [5, 6], and ROS have a role in heat-induced cell death [7, 8]. Thus, these findings indicate that heat shock induces oxidative stress and that heat shock-derived oxidative stress can inhibit some cell functions. Early embryonic development is also compromised by exposure to elevated temperatures. Maternal hyperthermia during early pregnancy leads to preimplantation cell death [9–12]. Exposure to high temperatures (40–41.5 C) also increases mortality of early cleavage stage (2 to 8-cell stages) embryos *in vitro* [13–17]. Moreover, even a short (<6 h) heat shock decreases embryo viability in early cleavage stage bovine embryos *in vitro* [18, 19]. The reason for this effect has not been fully determined although it has been demonstrated that heat shock increases the generation of intracellular ROS in preimplantation mouse and bovine embryos [11, 12, 19].

ROS damages preimplantation embryos by increasing lipid peroxidation [20, 21] and DNA fragmentation [22, 23] and leads to embryonic death [21, 24, 25]. Similarly, heat shock increases ROS generation and DNA fragmentation, but these changes are inhibited by antioxidant treatment [26]. Thus, heat shock may cause overproduction and decreased clearance of ROS by a scavenging mechanism and lead to embryonic death. Accordingly, it might be possible to reduce the mortality caused by heat shock in early-stage

embryos by reducing intracellular ROS levels.

β -mercaptoethanol (β -ME) is a strong reducing agent that can act as a biological antioxidant by scavenging hydroxyl radicals with its thiol (-SH) compound. Moreover, β -ME stimulates intracellular glutathione synthesis. Glutathione is a tripeptide thiol and an antioxidant synthesized by cysteine [27]. It maintains the intracellular redox status of cells and protects cells from toxins such as free radicals. β -ME includes thiols that stimulate the promotion of cystine uptake, the reaction that reduces cystine to cysteine and the synthesis of glutathione [28–30]. Moreover, β -ME is easy to add to culture medium because of its water solubility. The addition of β -ME during *in vitro* maturation and fertilization is effective in improving embryo viability [29, 31, 32]. In addition, addition of β -ME to *in vitro* culture medium reduces DNA fragmentation, ROS levels and embryonic mortalities under oxidative stress conditions (20% O₂) [30, 33–36].

Therefore, addition of β -ME to embryo culture medium likely reduces the ROS levels and increases the percentage of embryos that develop to the blastocyst stage under heat-shock conditions. In the present study, we used bovine 8-cell stages embryos exposed to 41 C for 6 h. This is the minimum heat shock required to increase ROS generation and decrease embryo viability according to previous reports [18, 19]. We also investigated the effects of β -ME-decreased ROS generation on the development of heat-shocked bovine embryos.

Materials and Methods

In vitro fertilization

Bovine ovaries were obtained from a local abattoir. The ovaries

were washed with prewarmed saline containing 100 IU/ml of penicillin (Nacalai Tesque, Kyoto, Japan) and 100 μ g/ml of streptomycin (Nacalai Tesque). Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles with a syringe connected to a 19-gauge needle. After washing with TCM-199 (Gibco, Grand Island, NY, USA), 50 COCs were cultured in 500 μ l TCM-199 containing FSH (0.02 AU/ml; Denka, Kawasaki, Japan), estradiol-17 β (1 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA), Gentamicin (10 μ g/ml, Sigma-Aldrich) and 5% (v/v) fetal calf serum (FCS; Gibco) for 22 h at 38.5 C in a humidified atmosphere of 5% CO₂ in air. After maturation culture, COCs were fertilized with sperm for 6 h at 38.5 C in 5% CO₂ in air and were washed and adjusted to 1×10^7 cells/ml as final concentration with BO solution as described previously [37].

Heat shock treatment

After insemination, the surrounding cumulus cells of putative zygotes were removed by pipetting and cultured in CR1aa [38] drops (20 embryos/50 μ l) containing 5% (v/v) FCS at 38.5 C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂. On day 2 (day 0=insemination), cleavage rates were evaluated, and 8-cell stage embryos were selected for use in the experiments. Twenty embryos were transferred to 50 μ l CR1aa drops containing 5% (v/v) FCS and supplemented with 0, 10 or 50 μ M β -ME (Nacalai Tesque), respectively. The culture dishes were then transferred to a 41 C setting incubator for 6 h in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂. After heat shock, the embryos were cultured at 38.5 C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂ until day 8. For the control group, 8-cell stage embryos were cultured with 0, 10 or 50 μ M β -ME at 38.5 C until day 8 without heat-shock. The percentage of embryos developing to the blastocyst stage and total cell numbers were evaluated on day 8.

Counting of blastocysts using differential staining

After observing embryo development, the blastocysts were used for differential staining [39] to evaluate the total cell number and ratio of inner cell mass (ICM) and trophectoderm cells (TE). The blastocysts were incubated for up to 60 sec in a solution of 0.1 mg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) in 2% (v/v) Triton X-100 in PBS(-), transferred into 99.5% EtOH containing 25 μ g/ml bisbenzimidazole (Hoechst 33342; Sigma-Aldrich) stored at 4 C for about 3 h. They were then washed in a drop of glycerol (Nacalai Tesque), mounted on a glass slide and flattened with a cover slip. The blastocysts were observed with a fluorescence microscope (TE-300; Nikon, Tokyo, Japan) with TE cells red and inner cell mass cells blue (460 nm excitation). The images of the embryos were recorded with a CCD camera (VB-7000; Keyence, Osaka, Japan). The cells were counted with the Lumina Vision software (Mitani, Fukui, Japan). Rates of TE divided by ICM (TE/ICM ratio) were calculated and compared among the different groups.

Detection of intracellular ROS and glutathione by fluorescence probes

After heat shock, intracellular ROS were immediately measured using fluorescence probes. Embryos were cultured with CR1aa

containing 5% (v/v) FCS and 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probe) for 20 min at 38 C in humidified atmosphere of 5% CO₂ in air and washed with PBS(-). The embryos were then placed on a slide glass with a 10 μ l drop of PBS(-), and the fluorescence emissions were detected with a fluorescence microscope (Nikon TE-300, 450–490 nm and 520 nm EX and BA filters, respectively) without a cover slip. The detected images of the embryos were taken by a CCD camera, and the fluorescence intensity was measured with the VZ analyzer software (Keyence).

Cell Tracker Blue CMF₂HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; Molecular Probes) was used to detect the intracellular reduced form of glutathione (GSH) levels of living cells as blue fluorescence [40]. Embryos were incubated for 30 min with CR1aa without FCS supplemented with 10 μ M Cell Tracker at 38 C in a humidified atmosphere of 5% CO₂ in air in the dark. They were then incubated for 30 min with CR1aa supplemented 5% FCS at 38 C in a humidified atmosphere of 5% CO₂ in air in the dark. After rinsing with PBS(-) containing 5% PVP, the embryos were placed on an 8-well chamber slide glass (Nunc, Rochester, NY, USA), and the fluorescence was observed by using a fluorescent microscope with a UV filter (370 nm). The fluorescence density was measured as described above.

Determination of intracellular total glutathione content

The intracellular total glutathione contents of embryos were determined using the 5, 5'-dithiobis (2-nitrobenzoic acid)-glutathione disulfide (DTNB-GSSG) reductase recycling assay according to Luciano *et al.* [41]. Briefly, after 6 h exposure to heat shock, ten or 20 of the 8-cell embryos in each group were washed with PBS(-). They were then transferred to 0.5-ml microfuge tubes with 5 μ l of distilled water (ddH₂O, Auto Still WG220; Yamato, Tokyo, Japan). The samples were subsequently frozen at -80 C until being assayed.

At the time of measurement, the embryos were thawed, and 45 μ l of ddH₂O were added to each tube. Each microtube was frozen in liquid nitrogen and thawed. The samples were then mixed by vortexing and centrifuged for 20 sec at 10,000 g. This procedure was repeated three times. Glutathione standards were diluted with ddH₂O at concentrations ranging from 0 to 200 pmol per 50 μ l. Each 50 μ l sample and standard was placed on a 96-well microtiter plate. A reaction mixture was prepared with final concentrations of 0.15 mM of DTNB, 0.2 mM of NADPH and 1.0 IU of glutathione reductase/ml in 0.1 M phosphate buffer supplemented with 1 mM of EDTA, pH 7.8. One hundred μ l of reaction mixture was added to each well, and the plate was analyzed at 410 nm in a microtiter plate reader (Microplate Manager PC/5.2.1.; Bio-Rad, Hercules, CA, USA) at 1 min intervals for 5 min.

Statistics

Statistical analyses of differences among the treatments were analyzed by ANOVA. Percentage data were subjected to arcsine and logarithmic transformation, respectively, before statistical analysis. When ANOVA indicated a treatment effect with a probability of less than 0.05, the data were analyzed by the Fisher post-hoc test to determine which means differed from each other.

Table 1. Effects of β -ME on embryo development

Treatment		Number of embryos	% developing to blastocysts	% developing to blastocysts (>expanded)
Temperature (C)	β -ME (μ M)			
38.5	0	111	48.40 $\pm$ 7.20 ^a	18.31 $\pm$ 3.77 ^{ab}
38.5	10	99	50.91 $\pm$ 8.61 ^a	21.16 $\pm$ 4.19 ^a
38.5	50	96	40.90 $\pm$ 3.14 ^{ab}	11.46 $\pm$ 2.37 ^{bc}
41.0	0	108	25.90 $\pm$ 2.98 ^b	7.60 $\pm$ 2.43 ^c
41.0	10	96	46.18 $\pm$ 4.53 ^a	10.56 $\pm$ 3.42 ^{bc}
41.0	50	97	34.86 $\pm$ 6.54 ^{ab}	7.08 $\pm$ 1.68 ^c

Experiments were replicated five times. Results were expressed means $\pm$ SEM. Values with different superscripts are significantly different ($P < 0.05$).

Table 2. Effects of β -ME for total cell number and TE/ICM ratio of blastocysts

Treatment		No. of embryos	Total cell numbers of blastocysts	TE / ICM ratio
Temperature (C)	β -ME (μ M)			
38.5	0	31	137.3 $\pm$ 8.53	2.90 $\pm$ 0.19
38.5	10	30	144.3 $\pm$ 9.76	2.95 $\pm$ 0.20
38.5	50	24	147.2 $\pm$ 11.66	3.19 $\pm$ 0.26
41.0	0	27	123.7 $\pm$ 6.88	3.12 $\pm$ 0.19
41.0	10	26	137.6 $\pm$ 10.37	3.54 $\pm$ 0.20
41.0	50	30	134.6 $\pm$ 9.70	2.61 $\pm$ 0.19

Experiments were replicated five times. Results were expressed means $\pm$ SEM.

Results

Effects of β -ME on development

Heat shock without β -ME significantly decreased the blastocyst rate (from 48 to 26%), while heat shock in the presence of 10 μ M β -ME did not significantly reduce the blastocyst rate (Table 1). Increasing the β -ME concentration to 50 μ M did not affect blastocyst rate.

Neither total cell number of blastocysts nor the TE/ICM ratio was affected by heat shock either with or without β -ME (Table 2). However, the total cell number of blastocysts tended to increase supplementation of 10 μ M β -ME in heat-shocked embryos compared with that of without β -ME ($P = 0.078$, Table 2).

Supplementation of β -ME to non heat-shocked embryos did not significantly improve embryo development.

Effects of β -ME on embryo redox status

Heat shock significantly increased intracellular ROS levels, but in the presence of 10 and 50 μ M β -ME, the ROS levels were no different between the heat shock and control groups (Fig. 1). At normal temperature, the ROS levels at 50 μ M β -ME were lower than those at 10 μ M β -ME (Fig. 1).

Judging from the results of both the fluorescence and enzyme recycling assays, the intracellular glutathione levels did not decrease due to heat shock (Fig. 2A, B). However, supplementation of β -ME increased the glutathione contents in a dose-dependent manner in both the control and heat-shocked groups. Supplementation of 50 μ M β -ME significantly increased the glutathione content in both the control and heat-shocked embryos (Fig. 2).

Fig. 1. Effect of heat shock and β -ME on intracellular ROS levels. Values are expressed as means $\pm$ SEM (pixels). Blank bars indicate non-heat-shocked embryos (38.5 C), and black bars indicate heat-shocked embryos (41.0 C). The number of replicates was five. Different superscripts denote significant differences ($P < 0.05$).

Discussion

Embryos exposed to heat shock without β -ME (0 μ M) showed significantly decreased development and increased intracellular ROS levels compared with non heat-shocked embryos, which was similar to the results of a previous study [19]. These results support the reports described above that an increase in intracellular ROS delays or blocks embryo development under heat-shocked conditions.

The addition of the GSH synthesis inhibitor buthionine sulfoximine (BSO) significantly decreases embryo development [33, 42].

Fig. 2. Effect of heat shock and β -ME on intracellular glutathione levels. Blank bars indicate non-heat-shocked embryos (38.5 C), and black bars indicate heat-shocked embryos (41.0 C). A shows intracellular glutathione generation evaluated by fluorescence dye (Cell Tracker Blue). Values are expressed as means $\pm$ SEM (pixels/embryo). B shows intracellular glutathione concentrations evaluated by glutathione recycling assay. Values are expressed as means $\pm$ SEM (pmol/embryo). The number of replicates was five. Different superscripts denote significant differences ($P < 0.05$).

Furthermore, embryos cultured in 20% O_2 showed a decrease in intracellular glutathione concentration and embryo development [33]. Glutathione synthesis has also been shown to be essential for embryo development [43, 44]. In this study, our hypothesis was that intracellular glutathione content is affected by heat-shock. However, the total glutathione content as evaluated by the glutathione recycling assay in the heat-shocked embryos did not decrease and remained at similar levels to the control embryos. This result was similar to the report of Arechiga *et al.* [42, 45].

Generally, more than 90% of intracellular glutathione exists as GSH and less than 10% is in the oxidized glutathione dimer form, glutathione disulphide (GSSG). Under oxidative stress, the reduced form of GSH scavenges ROS and is converted to GSSG by the enzyme glutathione peroxidase (GPx). The increase in ROS has been reported to decrease the intracellular GSH/GSSG ratio [44, 46]. Furthermore, hyperthermia increases GPx activity in erythrocytes and the liver [12, 47] and decreases the GSH/GSSG ratio in ovarian cells [48]. These reports indicate that ROS scavenging by GPx is activated and that the GSH/GSSG ratio is

decreased in heat-shocked embryos. However, GSH did not decrease in heat-shocked embryos without β -ME supplementation (0 μ M). In the present study, we used fluorescence detection to evaluate the GSH content. One problem with this method is that the fluorescence is enhanced by blastomere overlap. Therefore, we might not have evaluated the precise GSH content of each blastomere. Unfortunately, Cell Tracker was not suitable for analysis of the GSH contents of 8-cell stage embryos. However, it might be effective for oocytes or zygotes that are composed of one cell. Thus, fluorescence detection might not be appropriate for embryos.

Increased oxidative stress might exceed the reducing ability of the GSH reaction and GSSG converting reaction rate by glutathione reductase. A large amount of glutathione would be needed to scavenge the ROS generated by heat shock. Mitochondrial glutathione improves cell viability by eliminating ROS in the cell [49]. Heat shock is reported to destroy the morphology and function of embryonic mitochondria [4]. Therefore, if embryos under heat shock cannot eliminate the ROS, they would suffer the detrimental effects of the ROS.

The coexistence of embryos with β -ME under heat shock treatment significantly decreased the intracellular ROS levels compared with the heat-shocked embryos without β -ME supplementation. Moreover, the addition of β -ME increased the intracellular glutathione content. The effects of glutathione concentration on both the control and heat-shocked embryos were dependent on dosage. An increase in glutathione content enhanced the scavenging of ROS. Thus, supplementation of β -ME possibly decreased the ROS by scavenging it directly as well as increasing the glutathione levels. Therefore, in the presence of β -ME, the ROS content was decreased and the percentage of embryos developing into blastocysts increased under heat shock conditions similar to oxidative stress [30, 35, 36].

Coexistence of embryos with 10 μ M β -ME under heat shock treatment significantly improved the percentage of embryonic development to the blastocyst stage. Thus, β -ME might maintain the embryo developmental competence by improving the redox imbalance under heat shock conditions. On the other hand, coexistence of embryos with 50 μ M β -ME decreased the generation of intracellular ROS by dramatically increasing the glutathione levels. However, 50 μ M β -ME did not have a beneficial effect on the blastocyst formation rates in either the heat shock or non-heat shock groups. In fact, coexistence of embryos with 50 μ M β -ME significantly decreased the percentage of embryos developing into expanded or hatched blastocysts compared with that of 10 μ M β -ME supplemented non-heat-shocked embryos. A high concentration of antioxidant has been reported to be cytotoxic by inducing intracellular hypoxia [50, 51]. This evidence indicates that a certain level of ROS might be necessary for cell viability, but that ROS depletion would cause hypoxia and increase cell mortality. Thus, the results of the present study indicate that a high concentration of β -ME was not highly effective but was instead, toxic even when the ROS contents were reduced by adding glutathione under heat shock.

In conclusion, addition of 10 μ M β -ME to the culture medium improved the development of heat-shocked embryos by reducing intracellular ROS. Thus, these results indicate that the embryonic

death of heat-shocked early cleavage stage embryos is due to an increase in intracellular ROS generation.

Aknowledgements

The authors thank Ms. E Kawano and Ms. N Matsumoto for assistance with the experiments. We also thank to Ms. J Benke for assistance with looking over and correcting the paper. Special thanks are extended to the Kumamoto Meat Trading Center and the Kumamoto Prefecture Meat Inspection Office for providing ovaries. This work was supported by Grant-in-Aid for Scientific Research (16780209, Grant-in-Aid for Young Scientists (B)).

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