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—Original Article—

The Effects of Calcitonin on the Development of and Ca^{2+} Levels in Heat-shocked Bovine Preimplantation Embryos *In Vitro*

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Abstract. Intracellular calcium homeostasis is essential for proper cell function. We investigated the effects of heat shock on the development of and the intracellular Ca^{2+} levels in bovine preimplantation embryos *in vitro* and the effects of calcitonin (CT), a receptor-mediated Ca^{2+} regulator, on heat shock-induced events. Heat shock (40.5 C for 10 h between 20 and 30 h postinsemination) of *in vitro*-produced bovine embryos did not affect the cleavage rate; however, it significantly decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the control cultured for the entire period at 38.5 C ($P < 0.05$). The relative intracellular Ca^{2+} levels at the 1-cell stage (5 h after the start of heat shock), as assessed by Fluo-8 AM, a fluorescent probe for Ca^{2+}, indicated that heat shock significantly lowered the Ca^{2+} level as compared with the control level. Semiquantitative reverse transcription PCR and western blot analyses revealed the expression of CT receptor in bovine preimplantation embryos. The addition of CT (10 nM) to the culture medium ameliorated the heat shock-induced impairment of embryonic development beyond the 5- to 8-cell stage. The Ca^{2+} level in the heat-shocked embryos cultured with CT was similar to that of the control embryos, suggesting that heat shock lowers the Ca^{2+} level in fertilized embryos *in vitro* and that a lower Ca^{2+} level is implicated in heat shock-induced impairment of embryonic development. Intracellular Ca^{2+}-mobilizing agents, e.g., CT, may effectively circumvent the detrimental effects of heat shock on early embryonic development.
Key words: Calcitonin, Calcitonin receptor, Calcium, Heat shock, Preimplantation embryo

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Mammalian preimplantation embryos sense environmental factors that can affect their developmental potential [1]. Among these factors, heat shock during oocyte maturation, fertilization, and early cleavage stage development of the embryo can directly impair preimplantation development [2, 3], which is thought to be partially responsible for reduced fertility in farm animals during hot seasons [4–6]. Efforts are underway to circumvent the detrimental effects of heat stress on early embryonic development [7–10].

Calcium (Ca^{2+}) is a versatile signaling messenger that regulates numerous cellular processes [11], including early embryonic development [12, 13]. For example, the increase in intracellular Ca^{2+} at fertilization is a pivotal signal for egg activation, thereby triggering early embryogenesis [12, 14]. In addition, Ca^{2+} signaling is involved in oocyte maturation and postfertilization embryonic development [13, 15].

Physiological stress can influence Ca^{2+} homeostasis in mammalian preimplantation embryos. For example, aged oocytes exhibit decreased intracellular Ca^{2+} stores and exhibit a lower amplitude and higher frequency of Ca^{2+} oscillation at fertilization, which are accompanied by poorer embryonic development [16, 17]. As in this example, too low intracellular Ca^{2+} levels are associated with impairment of cellular functions or cell death [18, 19], and so are too high Ca^{2+} [20]. However, the effects of heat shock on Ca^{2+} homeostasis in preimplantation embryos remain unknown.

Calcitonin (CT) is a 32-amino acid polypeptide hormone secreted in mammals primarily by the thyroid gland and in nonmammalian vertebrates by the ultimobranchial gland [21]. In addition, the mammalian uterus is also a CT producer [22, 23]. Furthermore, murine preimplantation embryos express a functional CT receptor (CTR), and CT has positive effects on the preimplantation development and implantation of these embryos [24–26]. These findings suggest that CT is of physiological importance with regard to the female reproductive tract, such that it affects mammalian preimplantation development. CT signaling is mediated by an increase in intracellular Ca^{2+} in many cell types including preimplantation embryos [24–26]. However, the expression profile of CTR and the effects of CT during bovine preimplantation development remain unknown.

In the present study, we report that (i) heat shock, which impairs the postfertilization development of bovine preimplantation embryos *in vitro*, decreases their intracellular Ca^{2+} levels, (ii) bovine preimplantation embryos exhibit a CTR expression pattern that is distinct from that of murine embryos, and (iii) CT treatment ameliorates the heat shock-induced developmental impairment of bovine embryos *in vitro* with the recovery of the intracellular Ca^{2+} levels.

Materials and Methods

Ethics statement

This study was carried out in accordance with the Regulation on Animal Experimentation at Kyoto University.

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Chemicals

All chemicals used were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified.

Culture media for in vitro production of bovine embryos

Culture media for *in vitro* maturation (IVM) of immature oocytes recovered from ovaries, *in vitro* fertilization (IVF) of matured oocytes, and *in vitro* culture (IVC) of zygotes up to the blastocyst stage were prepared based on synthetic oviduct fluid (SOF) containing amino acids [27] with some modifications: the concentration of sodium pyruvate was increased to 0.5 mM, and the media were modified for each of the applications. In brief, the medium for IVM (IVMM) was supplemented with 5.6 mM glucose, 10% (v/v) fetal calf serum (FCS), and 0.2 IU/ml follicular-stimulating hormone (Kyoritsu Seiyaku, Kawasaki, Japan), the medium for IVF (IVFM) was not supplemented with glucose, and the medium for IVC used from day 1 to day 3 (day of IVF = day 0) was supplemented with 1.5 mM glucose and designated as IVC1M, and that used from day 3 to day 8 was further supplemented with 5% (v/v) FCS and designated as IVC2M. All the media except for IVMM contained 3 mg/ml bovine serum albumin. The media were covered with mineral oil and used under 5% CO_2, 5% O_2 and 90% N_2 with high humidity.

In vitro production of bovine embryos

Bovine oocytes were recovered from abattoir-derived ovaries of Japanese Black or Japanese Black × Holstein F1 cows by aspirating 2- to 8-mm follicles with a needle attached to a syringe. Cumulus-enclosed oocytes (CEOs) with compact dense cumulus cell layers were selected. Groups of 10 CEOs were matured *in vitro* for 22 h at 38.5 C in 50 µl drops of IVMM. Matured oocytes were fertilized *in vitro* with frozen-thawed sperm from a single Japanese Black bull as described previously [28], except with regard to the medium used [29]. In brief, frozen-thawed bull semen was layered onto a discontinuous Percoll (Sigma, St. Louis, MO, USA) gradient solution (45% and 90% [v/v]) and centrifuged at 700 × *g* for 30 min. The pelleted spermatozoa were resuspended in IVFM and centrifuged again at 700 × *g* for 10 min. The spermatozoa in the pellet were again resuspended in IVFM at a concentration of 2×10^6 cells/ml. Immediately prior to insemination, groups of 10 CEOs after IVM were transferred to 50 µl drops of IVFM supplemented with 3.6 U/ml heparin (Sigma). Fifty microliters of the sperm suspension was added to each mineral oil-covered drop containing the CEOs. Thus, the final concentrations of spermatozoa and heparin in the drops were 1×10^6 cells/ml and 1.8 U/ml, respectively. The CEOs and spermatozoa were coincubated for 19 h at 38.5 C. The day of IVF and the beginning of insemination were designated as day 0 and 0 h postinsemination (hpi), respectively.

At 19 hpi, the putative zygotes were freed from cumulus cells by vortexing and subsequently cultured in 500 µl of IVC1M (30-45 embryos per group). At 72 hpi, only the embryos that had developed to the 5- to 8-cell stage or more were transferred into 50 µl drops of IVC2M in 8–12 embryos per drop and further cultured up to 192 hpi (day 8). The embryos were subjected to the following experiments in the course of culture.

The *in vitro* production of bovine embryos for each data was performed from July 2012 to March 2014 (Jan to Jun 2013 for Table 1, May to Oct 2013 for Table 2, Aug to Nov 2012 for Fig. 1, Jul 2012 to Aug 2013 for Fig. 2A, Oct to Dec 2013 for Fig. 2B, Aug to Sep 2013 for Fig. 3, Mar 2014 for Supplementary Table 1 and Fig. 1: on-line only).

Heat shock of bovine embryos

From 20 hpi, 1-cell embryos were cultured in IVC1M basically at 38.5 C (Normal). The heat-shocked embryos (Heat) were incubated at 40.5 C between 20 and 30 hpi and afterward at 38.5 C. All the embryos were at the 1-cell stage at the start (20 hpi) and midpoint (25 hpi) of heat shock, and 63 (152/241) and 37% (89/241) of embryos were at the 1-cell and 2-cell stages, respectively, at the end of the treatment (30 hpi). The heat shock condition roughly mimicked the daily change in the rectal temperature of cows exposed to high temperatures in the hot season [2, 30]. The cleavage rate and rate of development to the 5- to 8-cell stage for the Normal and Heat groups were compared at 72 hpi (the fastest developing embryos were at the 8- to 16-cell stage), and their progression to blastocysts was compared at 192 hpi. The cultures were replicated five times, and the number of embryos allocated to each treatment group was 31–42 per replicate.

Measurements of intracellular Ca^{2+} levels in bovine embryos

Intracellular cytosolic free Ca^{2+} levels were measured using Fluo-8 AM (AAT Bioquest, Sunnyvale, CA, USA). At 19 hpi, the 1-cell embryos were denuded from cumulus cells as described above and cultured for 1 h in IVC1M supplemented with 5 µM Fluo-8 AM and 0.02% (w/v) Pluronic F-127 (AnaSpec, Fremont, CA, USA). The Fluo-8-loaded embryos were then washed twice with IVC1M and allocated to each culture condition. As described above, 1-cell and 2-cell embryos coexisted at the end of heat shock (30 hpi). In addition, the 2-cell embryos exhibited higher fluorescence along with the cleavage plane compared with the cytosol, which hampered unbiased measurement of the fluorescence intensity due to the unequal orientation of the cleavage plane to the focal plane. Therefore, in order to equalize the developmental stage of the examined embryos (1-cell stage) and to avoid possible bias, measurement of Ca^{2+} levels was implemented at 25 hpi (5 h after the start of heat shock). At this time point, embryos were transferred into a 1 µl IVC1M drop covered with mineral oil in a well of a 4-well dish. Fluo-8 fluorescence was captured using a fluorescence microscope (FSX100, Olympus, Tokyo, Japan), and fluorescence intensities were measured using the ImageJ software (National Institute of Health, Bethesda, MD, USA). Embryos that fluoresced highly intensely (greater than three interquartile ranges, which were 4% of the total) were not used to evaluate the resting (basal) Ca^{2+} levels, because fertilized embryos generate Ca^{2+} transients at nuclear envelope breakdown and cleavage during the first mitosis [31, 32].

Semiquantitative RT-PCR of CTR transcripts

Bovine *in vitro*-produced embryos at the 1-cell, 2-cell, 8-cell, morula, and blastocyst stages (20, 36, 72, 144, and 192 hpi, respectively; n = 15 for each stage) were stored in small volumes of RNAlater (Sigma) at –20 C until RNA extraction. In addition, immature oocytes at the germinal vesicle stage immediately after recovery from the

Table 1. Effects of heat shock on the development of bovine embryos *in vitro*

Treatment	Number of embryos (replicates)	Rates of development (%)		
		Cleavage	5- to 8-cell	Blastocyst
Normal	188 (5)	79.2 ± 3.4	46.6 ± 2.2	24.3 ± 2.2
Heat	187 (5)	76.7 ± 3.4	27.4 ± 2.2**	9.8 ± 2.2*

Data are presented as least-squares means ± SEM. Asterisks denote significant differences from the Normal group: *P<0.05; **P<0.01.

Table 2. Effects of heat shock and calcitonin (CT) on the development of bovine embryos *in vitro*

Treatment	Number of embryos (replicates)	Rates of development (%)		
		Cleavage	5- to 8-cell	Blastocyst
Normal-None	266 (7)	74.0 ± 3.1	52.9 ± 2.6^{ab}	26.1 ± 2.5^{a}
Normal-CT	266 (7)	73.5 ± 3.1	55.8 ± 2.6^{a}	28.3 ± 2.5^{a}
Heat-None	266 (7)	71.2 ± 3.1	32.5 ± 2.6^{c}	10.7 ± 2.5^{b}
Heat-CT	267 (7)	65.0 ± 3.1	43.8 ± 2.6^{b}	18.3 ± 2.5^{ab}

Data are presented as least-squares means ± SEM. $^{a, b, c}$ Values without common superscripts differ significantly (P < 0.05).

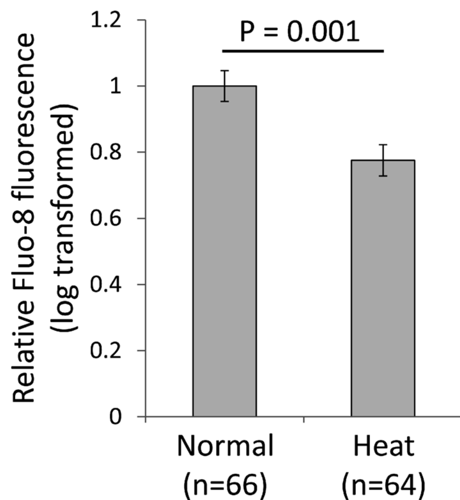

Fig. 1. Effects of heat shock on the intracellular Ca^{2+} levels of bovine embryos at 5 h after the start of heat shock (25 hpi). The Fluo-8 fluorescence intensities were obtained from the indicated number of embryos in 6 replicates. The least-squares means were normalized to that in normal thermal condition and presented with standard error.

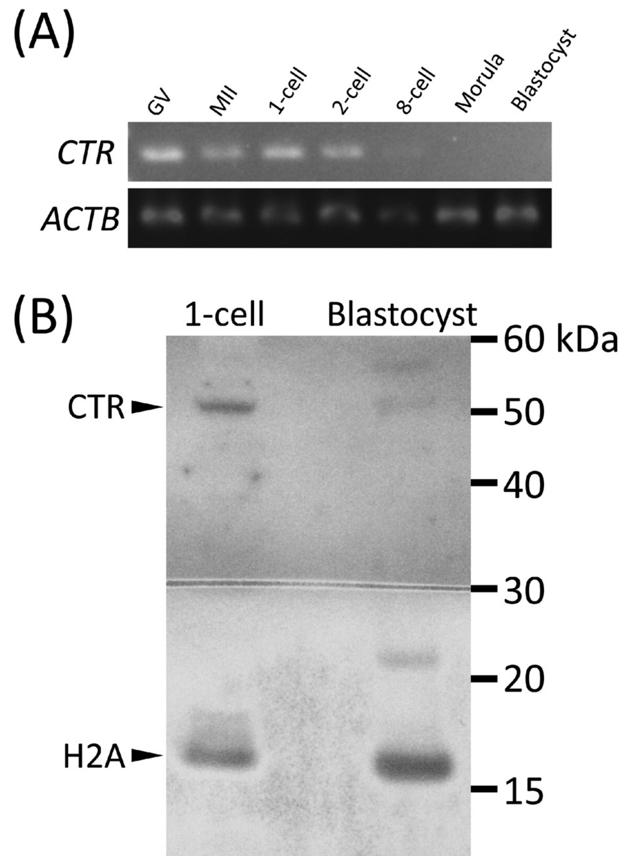

Fig. 2. (A) Semiquantitative RT-PCR of calcitonin receptor (*CTR*) mRNA in bovine oocytes and preimplantation embryos. Gel electrophoresis images of *CTR* and β-actin (*ACTB*, internal control) RT-PCR products are shown. GV, immature oocytes; MII, mature oocytes. (B) Western blot analysis of CTR protein in bovine preimplantation embryos at the 1-cell and blastocyst stages. Histone H2A (H2A) was examined as a loading control.

ovaries and mature oocytes with first polar bodies after IVM were also acquired. Total RNA was extracted from each sample using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and then subjected to first-strand cDNA synthesis in a 31.5 µl reaction volume using SuperScript III Reverse Transcriptase and Oligo-$(dT)_{20}$ primers (Invitrogen, Carlsbad, CA, USA). Transcripts of *CTR* and β-actin (*ACTB*) as an internal control were PCR amplified in a 25 µl reaction volume using Platinum PCR SuperMix (Invitrogen) from 1 µl of the cDNA solution with one of the following primer sets: AGCACTGCCCAGGCTATTTT (forward) and AGCTAAGGCTCCTAACACGC (reverse) for *CTR* and CCAAGGCCAACCGTGAGAAGAT (forward) and CCACGTTCCGTGAGGATCTTCA (reverse) for *ACTB* [33]. After an initial denaturation at 94 C for 1.5 min, 35 PCR cycles for *CTR* and 30 cycles for *ACTB*, which had been experimentally confirmed as the amplification stages, were performed as follows: 94 C for 30 sec, 58 C for 30 sec, and 72 C for 30 sec. The 10 µl PCR products of *CTR* (255 bp) and *ACTB* (256 bp) were subjected to electrophoresis through 2% (w/v) agarose gels and then stained with 0.5 µg/ml ethidium bromide. The stained gels were photographed by using an InGenius3 gel documentation system (Syngene, Cambridge, UK).

Western blot analysis of CTR protein

Bovine *in vitro*-produced embryos at the 1-cell (n=400) and blastocyst (n=10) stages were lysed in NuPAGE LDS Sample Buffer with Reducing Agent (Invitrogen) in a 20 µl volume and boiled

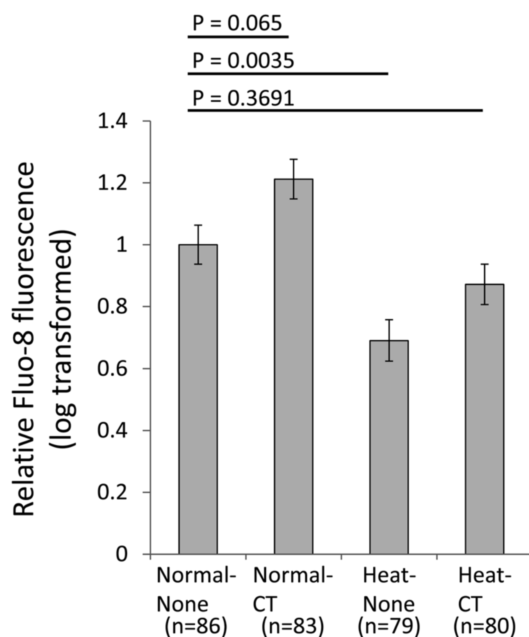

Fig. 3. Effects of calcitonin (CT) on the intracellular Ca^{2+} levels of bovine embryos under heat shock at 5 h after the start of heat shock (25 hpi). The Fluo-8 fluorescence intensities were obtained from the indicated number of embryos in 5 replicates. The least-squares means were normalized to that in Normal-None group and presented with standard error.

for 5 min. The samples were subjected to SDS-PAGE through a 4–12% Bis-Tris gel and transferred to a PVDF membrane using an iBlot Dry Blotting System (Invitrogen). The blotted membrane corresponding to 30–60 kDa was blocked with 10% (v/v) FCS in PBS containing 0.05% (v/v) Tween 20 (PBST) for 30 min. A rabbit polyclonal antibody to CTR (ab103422, Abcam, Cambridge, UK) was diluted 200 times with PBST containing 5% (v/v) FCS and mounted onto the membrane for 1 h. After extensive washing with PBST, the membrane was treated for 30 min with 2,000 times-diluted alkaline phosphatase-conjugated bovine anti-rabbit IgG (sc-2372, Santa Cruz Biotechnology, Dallas, TX, USA). After washing, the signal was developed for 5 min with Novex AP Chemiluminescent Substrate (Invitrogen) and exposed to FP-3000B film (Fujifilm, Tokyo, Japan). The same procedure except for the use of an anti-Histone H2A antibody (1,000-times diluted, ab88770, Abcam) was applied to the membrane corresponding to 10-30 kDa to assess the expression levels of Histone H2A as a loading control.

Exposure of heat-shocked bovine embryos to CT

Synthetic human CT was purchased from Peptide Institute, Inc. (Minoh, Japan), and reconstituted in IVC1M to obtain a 10 µM solution. The 10µM solution was further diluted with IVC1M (1:1000) to yield a final concentration of 10 nM. The preparation was performed 1 day before use. The concentration of CT (10 nM) in the culture medium was set according to an effective dose for the promotion of Ca^{2+} mobilization and preimplantation development of murine embryos [24, 25]. In addition, 10 nM CT was confirmed to be more effective compared with 100 nM in preliminary experiments (data not shown).

Beginning at 20 hpi, 1-cell embryos were cultured in 500 µl of IVC1M in the presence or absence of 10 nM CT until 72 hpi. Embryos were also allocated to the Normal and Heat thermal conditions as described above. Thus, the embryos were divided into four groups: (i) Normal in the absence of CT (Normal-None), (ii) Normal in the presence of CT (Normal-CT), (iii) Heat in the absence of CT (Heat-None), and (iv) Heat in the presence of CT (Heat-CT). The four groups were compared with respect to their cleavage rates and rates of development to the 5- to 8-cell stage at 72 hpi and with respect to their rates of development to the blastocyst stage at 192 hpi. The cultures were replicated seven times with 30-45 embryos allocated to each group per replicate.

Statistical analyses

The embryonic development expressed as a percentage and logarithmic-transformed Fluo-8 fluorescence intensities were subjected to a general linear model in which treatments and replicates were taken as fixed variables. All analyses were performed using SAS (SAS Institute, Cary, NC, USA). When multiple comparisons were made, Tukey's (for all pairwise comparisons) or Dunnett's (for pairwise comparisons with the control) test was used. Significance was accepted at $P<0.05$.

Results

Effects of heat shock on the development of and intracellular Ca^{2+} levels in bovine embryos

We investigated the effects of exposure to heat shock (40.5 C for 10 h) on day 1 post-IVF on postfertilization development of bovine embryos *in vitro* (Table 1). The heat shock did not affect the cleavage rate; however, it significantly decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the Normal group ($P < 0.05$). On the other hand, heat shock for only 5 h (between 20 and 25 h postinsemination) did not affect these developmental rates (Supplementary Table 1). The relative intracellular Ca^{2+} level for the two groups was determined using the fluorescence Ca^{2+} probe, Fluo-8 AM, at the midpoint (5 h) during heat shock, which corresponded to 25 hpi (Fig. 1). The heat shock significantly decreased the Ca^{2+} level as compared with that of the Normal group. In addition, embryos that remained at the 1-cell stage at the end point (10 h) of heat shock (30 hpi) also exhibited a lower Ca^{2+} level compared with 1-cell embryos of the Normal group (Supplementary Fig. 1).

Expression of CTR in bovine oocytes and preimplantation embryos

The results for the semiquantitative RT-PCR of *CTR* are shown in Fig. 2A. *CTR* transcripts were relatively more abundant in oocytes and the 1- and 2-cell embryos and decreased thereafter to undetectable levels in the morula and blastocyst stages. Western blot analysis of CTR protein in 1-cell and blastocyst stage embryos showed a band of approximately over 50 kDa corresponding to the published molecular weight of CTR on SDS-PAGE [34]. The signal of the band relative to that of the loading control (Histone H2A) was higher in the 1-cell

stage compared with the blastocyst stage (Fig. 2B).

Effects of CT on the development of and intracellular Ca^{2+} levels in the heat-shocked bovine embryos in vitro

The effects of CT treatment on embryonic development after heat shock were investigated (Table 2). As described above, heat shock decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the Normal-None group. However, the rates of development to the 5- to 8-cell and blastocyst stages in the heat-shocked and CT-treated group (Heat-CT) were not significantly different from those of the Normal-None group. Figure 3 shows the relative intracellular Ca^{2+} levels in bovine embryos cultured in the presence or absence of CT and with or without heat shock. When the normal temperature group was cultured in the presence of CT, the Ca^{2+} level tended to increase ($P=0.065$) in comparison with that of the normal temperature group without CT (Normal-None). The Ca^{2+} level at 25 hpi (5 h after the start of heat shock) in heat-shocked embryos cultured in the absence of CT was significantly lower ($P=0.0035$) than that in the Normal-None group. However, the heat-shocked embryos cultured in the presence of CT exhibited a Ca^{2+} level that was statistically indistinguishable from that of the Normal-None group ($P=0.37$).

Discussion

We examined the effects of heat shock (40.5 C for 10 h) on bovine embryos because this condition roughly mimics the body temperature of cattle exposed to high temperatures in the hot season [2, 30]. Heat shock on day 1 of embryo culture did not affect the cleavage rate; however, it significantly decreased the rate of development to the 5- to 8-cell and blastocyst stages.

Many studies have found direct inhibitory effects of heat shock on bovine preimplantation development *in vitro* [2, 8, 9]. The conditions for heat shock used in the present study can be considered to be slightly higher than the threshold necessary to induce impaired development: Rivera and Hansen reported that exposure of bovine 1-cell embryos to 40 C for 12 h had no effect on embryonic development; in contrast, exposure of embryos to 41 C for 9 h decreased their development to the blastocyst stage (similar to our finding), and heat shock at 41 C for 12 h decreased both cleavage and blastocyst rates [2]. Given our findings and previous results concerning the heat shock conditions under which embryonic development is affected, we wondered if heat shock affects intracellular Ca^{2+} levels of bovine embryos. As a result, heat shock significantly lowered the Ca^{2+} level as compared with the control level. Although 5 h of heat shock was not sufficient to impair development (Supplementary Table 1), this time point was chosen for the assessment of Ca^{2+} levels on account of the experimental reasonability described in the Materials and Method. In addition, 1-cell embryos after 10 h of heat shock also exhibited the lower Ca^{2+} levels compared with the control embryos (Supplementary Fig. 1). These results suggest that the persistent lower Ca^{2+} level (that persists more than 5 h) was implicated in the impaired development of heat-shocked embryos.

Deleterious stress can either increase [20] or decrease [18, 19, 35, 36] intracellular Ca^{2+} levels, and both types of Ca^{2+} changes are associated with the impairment of cellular functions and/or the promotion of cell death. In addition, certain stresses attenuate Ca^{2+} mobilization that is mediated by the G protein-coupled receptor [37, 38]. The stress-induced increase in the intracellular Ca^{2+} level has been attributed to the opening of various plasma membrane-localized channels [20]. The present result that heat shock decreased the intracellular Ca^{2+} level suggests that the used heat shock did not activate these plasma membrane-localized channels involved in the stress-induced rise in intracellular Ca^{2+} and/or that the embryos at the examined stage (1-cell) did not possess such stress-sensitive channels. For example, we could not detect the mRNA expression of a representative heat-sensitive plasma membrane-localized Ca^{2+} channel, transient receptor potential vanilloid type 1 (TRPV1), in bovine 1-cell embryos (unpublished result).

On the other hand, mechanisms by which stress decreases intracellular Ca^{2+} include dysfunction of the endoplasmic reticulum (ER)-resident Ca^{2+} channel, i.e., the inositol 1,4,5-trisphosphate receptor (IP3R) [37], and altered expression of genes involved in calcium homeostasis (e.g., those encoding calcium channels, calcium-binding proteins, and the plasma membrane calcium ATPase) [39, 40]. We measured Ca^{2+} levels at 25 hpi, which is long after the period of sperm-induced frequent Ca^{2+} oscillation [17, 32], and embryos that exhibited extremely high fluorescence (4% of total) possibly due to the Ca^{2+} transient during this period [31, 32] were discarded from the statistical analysis as outliers. Therefore, the presented data is considered to more reflect resting (basal) levels of Ca^{2+} rather than the transient Ca^{2+} increase. The resting intracellular Ca^{2+} could be lowered by the alteration of IP3R-dependent Ca^{2+} mobilization from intracellular stores [39, 41].

The finding that heat shock decreased the Ca^{2+} level in preimplantation embryos prompted us to explore the possibility that modulation of the intracellular Ca^{2+} level would ameliorate the detrimental effects of heat shock on preimplantation development. Although the physiological importance of CT in systemic Ca^{2+} homeostasis in mammals is controversial [42], the function of CT as a paracrine factor in the female reproductive tract has been suggested [24–26]. The expression of *CTR* mRNA in murine preimplantation embryos has been reported [24], and CT can increase intracellular Ca^{2+} levels by activating CTR [24, 25, 43, 44]. Therefore, we examined the expression of *CTR* mRNA in bovine preimplantation embryos and the effects of CT treatment on the *in vitro* development of embryos that had been heat shocked. Our RT-PCR study revealed a unique pattern of *CTR* expression in bovine embryos that is distinct from the pattern found for murine embryos. In the bovine embryos, *CTR* mRNA was relatively highly expressed in oocytes through the 2-cell stage and decreased afterward to undetectable levels in the morula and blastocyst stages (Fig. 2A), whereas murine oocytes and embryos exhibit relatively lower expression in oocytes and the early cleavage stage and higher expression in the later stage of preimplantation development [24]. Consistent with the transcript expression pattern, the western blot analysis showed that 1-cell embryos had higher expression of CTR than blastocysts on a per-cell basis (Fig. 2B), suggesting possible CT sensitivity in 1-cell stage embryos.

In accordance with the expression of CTR during the early cleavage stages, CT treatment during this time period tended to increase ($P = 0.065$) the intracellular Ca^{2+} levels in the bovine embryos (Fig. 3). Heat shock significantly decreased the Ca^{2+} levels in the embryos,

although CT treatment during heat shock resulted in a Ca^{2+} level similar to that of the Normal-None embryos. Interestingly, CT treatment also ameliorated the heat shock-induced developmental impairment (Table 2).

The rise in intracellular free Ca^{2+} caused by CT results from the release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+} [45]. Activation of phospholipase C pathways, which results in IP3R-mediated mobilization of Ca^{2+} from the ER is a well-known mechanism by which CT enhances the intracellular Ca^{2+} level [46, 47]. Constitutive IP3R-mediated release of Ca^{2+} from the ER and uptake of the released Ca^{2+} by mitochondria are required for efficient mitochondrial respiration [48, 49]. The proper mitochondrial bioenergetics is emerging as a correlation to developmental outcome in oocyte maturation, fertilization, and postfertilization development of the embryo [50–52]. Thus, CT may enhance embryonic development when an embryo is heat shocked through the ER-directed regulation of mitochondrial bioenergetics. When compared within the respective thermal conditions (38.5 and 40.5 C), CT treatment increased the Ca^{2+} levels ($P = 0.02$, *t*-test) compared with the no additive control at 38.5 C, while it did not ($P = 0.06$) at 40.5 C. This result also suggests that CT sensitivity differs between the thermal conditions; consequently, it suggests that IP3R-mediated mobilization of Ca^{2+} from the ER is one of the targets by which heat shock decreases intracellular Ca^{2+} in preimplantation embryos.

In addition, CT can induce a sustained Ca^{2+} elevation due to a Ca^{2+} release-activated capacitative Ca^{2+} influx from the extracellular environment [43]. Importantly, Wang *et al.* [25] reported that the P38MAPK pathway is partially involved in the enhancing effects of CT on murine preimplantation development *in vitro*. Therefore, it cannot be excluded that the heat shock-ameliorating effect of CT on bovine preimplantation embryos is also mediated by signal transduction pathways like this that are independent of Ca^{2+} signaling.

In summary, heat shock, which impaired the postfertilization development of bovine preimplantation embryos *in vitro*, decreased the intracellular Ca^{2+} level in the embryos. CT treatment ameliorated this heat shock-induced developmental impairment and recovered the normal intracellular Ca^{2+} level. Consequently, intracellular Ca^{2+} may be a new target for circumventing the detrimental effects of heat shock on preimplantation development.

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