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Effects of heat stress on gene expression associated with antioxidant capacity of bovine early antral follicles

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ABSTRACT. Heat exposure at the physiological level of oocyte-cumulus-granulosa cell complexes (OCGCs) during *in vitro* growth (IVG) culture decreases the reduced glutathione (GSH) level of oocytes, an antioxidant, but its mechanism has been unclear. We subjected OCGCs to IVG for 4, 8, or 12 days and investigated the effects of heat exposure on the gene expression of cumulus-granulosa cells associated with antioxidant capacity. Heat exposure did not alter the expression of genes related to GSH synthesis or reuse. Conversely, heat exposure increased mRNA expression of superoxide dismutase 2 and decreased that of glutathione peroxidase 1, which are antioxidant enzymes. Ultimately, heat exposure alters the gene expression of antioxidant enzymes in growing follicles, which may indirectly decrease the GSH level of oocytes.

KEYWORDS: antioxidant capacity, bovine, early antral follicle, *in vitro* growth, oxidative stress

Due to global warming, the average temperature has increased by over 2°C between 1900 and 2020 in Japan [20]. Notably, dairy cows cannot maintain their body temperature when the outside temperature exceeds approximately 25°C [21], and an increase in body temperature is thought to decrease food intake and increase oxidative stress, resulting in reduced reproductive performance [3]. Oocytes matured *in vitro* and *in vivo*, as well as those derived from immature follicles (2–8 mm) commonly used in *in vitro* embryo production, have reduced developmental competence under heat stress [23]. Moreover, reduced oocyte developmental competence is observed in the subsequent cooler autumn, suggesting the continuous effect of summer heat stress [19]. It takes approximately 1–2 months for the reproductive performance of heat-stressed cows to recover [18, 25], and the development of early antral follicles (<1 mm) to ovulatory follicles also takes approximately 30–40 days [15]. These reports show that the oocytes in small antral follicles are damaged by heat stress in the summer, which causes the continuous reduction of oocyte developmental competence and fertility during autumn. We previously reported the effects of heat stress on the growth of bovine early antral follicles using an *in vitro* growth (IVG) culture system of bovine oocyte-cumulus-granulosa cell complexes (OCGCs) derived from early antral follicles (0.5–1.0 mm) [9]. OCGCs from slaughterhouse-derived ovaries were divided into a control group cultured at 38.5°C, close to the normal body temperature of cows, and a heat stress group cultured using a temperature cycle mimicking those of dairy cows in a hot environment (38.5°C: 5 hr, 39.5°C: 5 hr, 40.5°C: 5 hr, 39.5°C: 9 hr), and cultured for 12 days *in vitro* [9]. As a result, the blastocyst development rate was higher in the control group (27.7%) than in the heat stress group (0.0%) ($P<0.05$). In addition, the amount of reduced glutathione (GSH), which protects cells against oxidative stress, was higher in the control group than in the heat stress group ($P<0.05$). Furthermore, the addition of cysteine, which promotes the production of GSH, into the heat stress group conditions increased the GSH levels and improved the blastocyst rate (27.9%) compared with the same conditions without cysteine (6.1%, $P<0.05$). These results

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indicated that antioxidant capacity, such as the GSH level, of early antral follicles, is reduced by summer heat stress, resulting in the impaired developmental competence of oocytes and fertility during subsequent cooler autumn. However, the mechanism underlying the reduced antioxidant capacity of early antral follicles was not clarified.

GSH synthesis and accumulation in oocytes are regulated by surrounding somatic cells through gap junctions [2, 12] and controlled by several enzymes. Glutamate-cysteine ligase (GCL) is a rate-limiting enzyme that condensates glutamine and cysteine and is the first used for GSH synthesis [4]. Glutathione reductase (GR) is an enzyme that reuses GSH by reducing oxidized glutathione disulfide (GSSG) to GSH [1]. Additionally, some enzymes alleviate the oxidative stress in cells. Superoxide dismutase (SOD) changes superoxide into hydroxy peroxide, and SOD1 is distributed in the cytoplasm, whereas SOD2 is localized in mitochondria [5]. Glutathione peroxidase 1 (GPx1) and catalase (CAT) convert hydrogen peroxide into water [11]. However, GPx1 also uses GSH as a reducing substrate for hydrogen peroxide and produces GSSG, whereas CAT directly converts hydrogen peroxide into water without an additional substrate [11]. The exposure of OCGCs to heat would alter the gene expression of enzymes related to antioxidant capacity, directly or indirectly reducing the intracellular GSH level of oocytes. Therefore, we investigated the effects of heat exposure on the gene expression of enzymes related to GSH synthesis (*GCL*), GSH reuse (*GR*), and antioxidant enzymes (*SOD1*, *SOD2*, *GPx*, and *CAT*).

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Ethical approval for animal work was not necessary for this study because all the bovine ovaries were derived from cattle slaughtered at a local slaughterhouse for commercial food production purposes. OCGCs derived from early antral follicles (0.5–1 mm in diameter) were subjected to IVG culture, as previously described [9]. Briefly, ovaries were thinly sliced using a surgical blade (No. 11), and the ovarian cortical tissues were kept in isolation media [6]. The follicle diameter was judged using the 1-mm^2 grid scale on the 90-mm Petri dishes (FLAT Co., Ltd., Nagareyama, Japan). Then, early antral follicles were manually collected from the ovarian cortexes using fine forceps and a surgical blade (No. 20). Follicles were punctured to obtain OCGCs using fine forceps and an 18G needle. The growth medium was described in our previous study [8]. Morphologically healthy OCGCs with an evenly granulated ooplasm, completely enclosed by several layers of healthy cumulus and granulosa cells, were individually cultured in a 96-well culture plate (Primaria 353872, Corning Inc., Corning, NY, USA) with 200 µL of growth medium for 4, 8, or 12 days in humidified air with 5% CO_2. OCGCs that were cultured at 38.5°C for 24 hr, based on the normal body temperature of dairy cows, served as the control group [16]. For the heat stress group, a specific temperature cycle (38.5°C for 5 hr, 39.5°C for 5 hr, 40.5°C for 5 hr, and 39.5°C for 9 hr) was used for culturing OCGCs, to represent the change in body temperature of dairy cows under heat stress [17]. Half of the spent IVG medium (100 µL) was replaced with the same volume of fresh medium every 4 days of the culture (days 4, 8, and 12). The morphological appearance of the OCGCs was evaluated every 4 days during IVG culture using an inverted microscope (CK40, Olympus, Tokyo, Japan). Morphologically healthy OCGCs were used for the isolation of RNA. Cumulus-granulosa cells were collected from OCGCs after 4, 8, or 12 days of IVG culture because cumulus-granulosa cells regulate GSH synthesis and accumulation in oocytes [12]. To obtain enough RNA, cells from 10 OCGCs were pooled for the measurement on day 4, and cells from 5 OCGCs were pooled for days 8 and 12. Total RNA was extracted using a ReliaPrep RNA Cell Miniprep System (Promega Corp., Madison, WI, USA), following the manufacturer's instructions. Samples were stored at −80°C, and RNA extraction and cDNA synthesis were performed within 1 month from the start of storage. RNA concentration and purity were determined using a NanoDrop Spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). The purified total RNA (10 ng/µL) was treated as a template to synthesize cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). The cDNA samples were stored at −30°C until they were used in the PCR analysis. Real-time PCR was performed after preparing reaction mixtures in THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.). The primers were designed using Primer3 software and based on bovine sequences or a previous study [13]. The reaction efficiency ranged between 90% and 110%, with R^2 >0.991. The amplification program included preincubation at 95°C for 1 min, followed by 40 amplification cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec. All samples were run in triplicate in 48-well plates. Melting-curve analysis was performed at the end of amplification to confirm single-gene specificity. Relative mRNA abundance was calculated using the ΔΔCt method, with β-actin as the reference gene in each sample. All primer sequences, PCR product sizes, and GeneBank accession numbers are shown in Table 1. The mRNA expression was analyzed by two-way ANOVA followed by the Student's *t*-test using StatView 4.51 (AbacusConcepts, Calabasas, CA, USA). *P*-values ≤0.05 were considered significant.

As shown in Fig. 1, there were no differences in the mRNA expression of enzymes related to the metabolization of GSH (*GCL* and *GR*) at any time point of IVG culture. However, significant differences were observed in the mRNA expression of some antioxidant enzymes, as shown in Fig. 2, The mRNA expression of *SOD2* was higher in the heat stress group than in the control group on days 4 and 12 of IVG culture ($P \leq 0.05$), whereas that of GPx1 was lower in the heat stress group than in the control group on day 12 of IVG culture (Fig. 2, $P \leq 0.05$). No differences were observed in the expression of *SOD1* or *CAT* at any point of IVG culture (Fig. 2).

In the present study, the expression of *GCL* and *GR*, which are related to GSH synthesis and reuse, did not significantly differ between the two groups. Ho *et al.* [7] also reported that short-term acute heat exposure (41°C for 6 hr) did not significantly affect the expression of *GCL* or *GR* in bovine cumulus-granulosa cells. We previously reported that the heat exposure of OCGCs during 12 days of IVG did not affect the concentration of cystine, glycine, or glutamine, which are amino acids utilized for GSH synthesis, in culture media [8]. Therefore, GSH synthesis and reuse are not affected by acute or chronic heat exposure in the physiological range.

The expression of *SOD2* was increased and the expression of *SOD1* was not affected by heat exposure in the present study. Khan *et al*. [10] employed RNA sequencing to investigate the transcriptome profile of bovine granulosa cells cultured in four different temperature settings (38, 39, 40, or 41°C) for 2 hr. The findings suggested that the expression levels of *SOD1*, *SOD2*, and *CAT* increase in granulosa cells subjected to heat exposure of at least 40°C, compared with the control group (38°C) [10]. The discrepancy between the present and previous studies may be due to the accumulation of oxidative stress in mitochondria, where *SOD2* is localized, in

Table 1. Primers used in real-time PCR

Gene	Sequence (5′-3′)	Product length (bp)	Accession number	Reference
GCL	F: TGGAGCAGCTGTATCAGTGG R: GAATGTCAGGGATGCTCTCC	198	NM_001038143.1	[13]
GR	F: CCGCCTGAACACCATCTATC R: AGGATGTGAGGAGCGGTGTA	135	NM_001114190.2	
SOD1	F: GGAGATACAGTCGTGGTAACTGG R: AGGTCTCCAACATGCCTCTC	176	NM_174615.2	
SOD2	F: GGCCATATCAATCACAGCATC R: CTTGGACACCAACAGATACAGC	151	NM_201527.2	
GPx1	F: TGCTCATTGAGAACGTAGCATC R: ATTCAGGATCTCCTCGTTCTTG	164	NM_174076.3	
CAT	F: GTACAGCGCTTCAACAGTGC R: TCTCACACAGGCGTTTTCCTC	100	NM_001035386.2	
β-actin	F: TCCCTGGAGAAGAGCTACGA R: GGTAGTTTCGTGAATGCCGC	132	NM_173979.3	

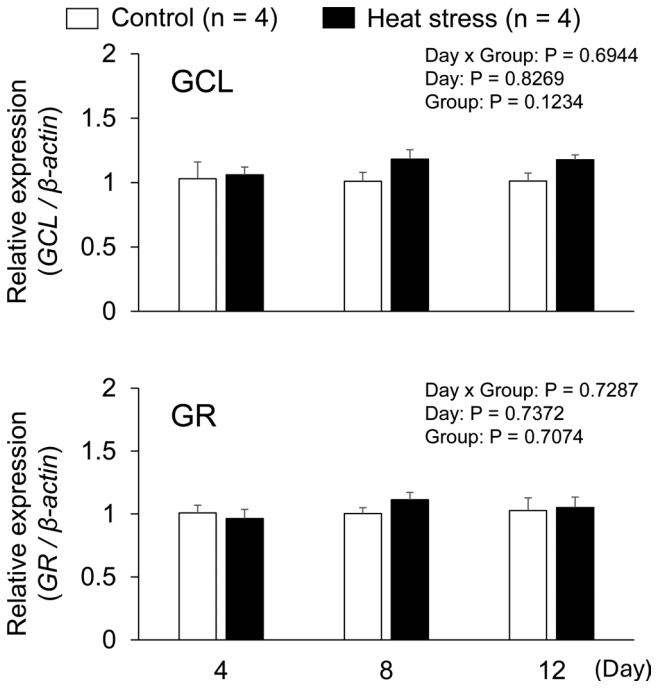

Fig. 1. Effects of heat exposure on the mRNA expression of enzymes related to the metabolization of reduced glutathione (GSH) in cumulus-granulosa cells after 4, 8, or 12 days of *in vitro* growth culture. The results of a factorial analysis by two-way ANOVA were shown above each panel. Each sample was derived from 10 oocyte-cumulus-granulosa cell complexes (OCGCs) for day 4, and 5 OCGCs for days 8 and 12. Error bars indicate the SEM.

cumulus-granulosa cells under long-term heat exposure (12 days). In addition to their different localization, SOD1 and SOD2 also require different cofactors. SOD1 mainly uses copper and zinc as cofactors, while SOD2 uses manganese [5]. The base medium in this study was medium 199 (12340-030, Thermo Fisher Scientific), which does not contain these cofactors as inorganic salts. However, fetal calf serum (FCS) was added to the medium before culturing. The cofactors present in FCS might explain the differences in how SOD1 and SOD2 are expressed under heat stress.

In our previous study [9], compared with the control group, the GSH level was not different in the heat stress group on day 4, whereas the GSH level became lower in the heat stress group between days 8 and 12. This result is consistent with the expression of *GPx1* in the present study, which was reduced by heat exposure on day 12. Considering that *GPx1* utilizes GSH to metabolize hydrogen peroxide, we speculate that the reduction of GSH concomitantly results in the lower expression of *GPx1*. Wang *et al.* [22] also suggested that the activities of GPx were reduced by exposure to 40°C for 2 hr. Similar to GPx1, CAT also converts hydrogen peroxide into water; however, no difference was observed in *CAT* expression between the groups. GPx requires GSH to reduce hydrogen peroxide, whereas CAT does not rely on any reducing agents [11]. The availability of GSH for both enzymes may explain

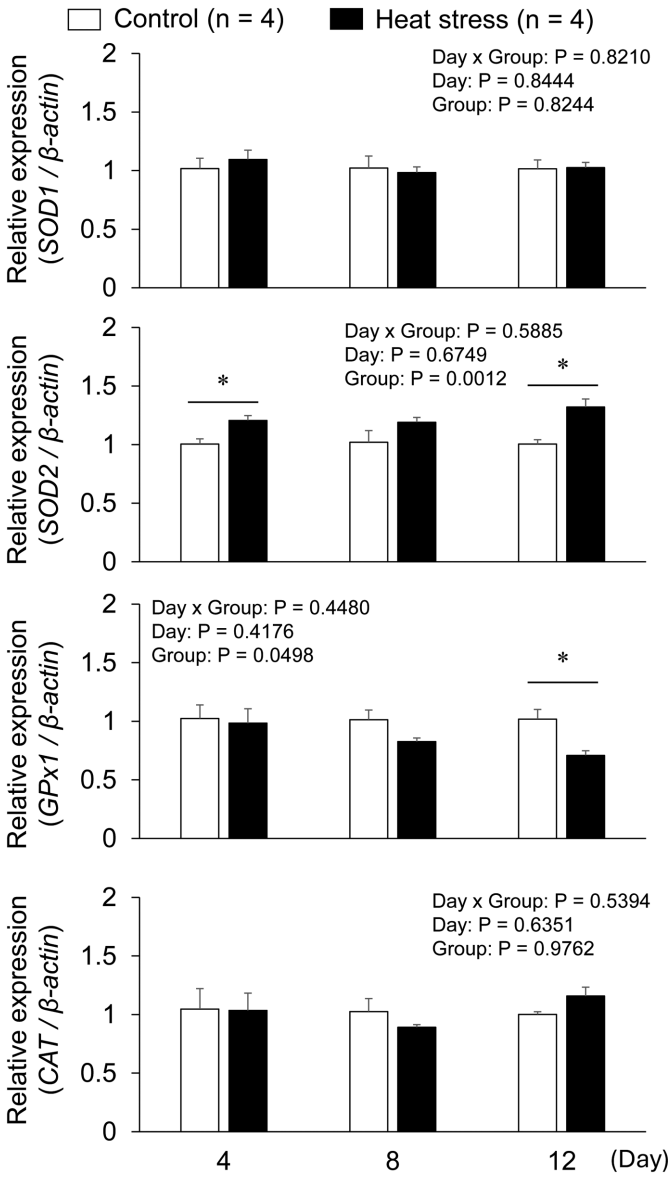

Fig. 2. Effects of heat exposure on the mRNA expression of antioxidant enzymes in cumulus-granulosa cells after 4, 8, or 12 days of *in vitro* growth culture. The results of a factorial analysis by two-way ANOVA were shown above each panel. Each sample was derived from 10 oocyte-cumulus-granulosa cell complexes (OCGCs) for day 4, and 5 OCGCs for days 8 and 12. Error bars indicate the SEM. * Significant difference between the two groups ($P \leq 0.05$).

the differences in the expression patterns of *GPx1* and *CAT*.

In addition to enzymes, there are several potential factors that may influence GSH levels in oocytes. GSH plays a crucial role in reducing reactive oxygen species (ROS) induced by heat stress [24]. However, we previously reported that heat exposure of OCGCs at the physiological level during IVG does not affect ROS levels in oocytes [9]. Another possible factor is a reduction in adenosine triphosphate (ATP) levels, as GCL, the rate-limiting enzyme in GSH synthesis, is ATP-dependent [14]. Further investigation into ATP content in oocytes, alongside enzyme activity, could provide valuable insights into the mechanisms behind the reduction of GSH levels under heat stress.

In the present study, we used the temperature cycle (38.5°C for 5 hr, 39.5°C for 5 hr, 40.5°C for 5 hr, and 39.5°C for 9 hr) to simulate the body temperature of dairy cows under heat stress, while the control group was kept at a constant temperature of 38.5°C. There are two possible reasons for differences in the gene expression of *SOD2* and *GPx1* between two groups: heat stress and/or stress from the changing temperatures. Since previous studies have shown that short term exposures to high temperatures can affect the expression of those genes in granulosa cells [10, 22], we cannot exclude the possibility that heat stress itself played a role, at least to some extent.

In conclusion, the exposure of OCGCs to heat affects the expression of *SOD2* and *GPx1*, which are antioxidant enzymes, but it does not alter the expression of the genes related to the synthesis or reuse of GSH (*GCL* and *GR*).

CONFLICT OF INTEREST. There are no conflicts of interest to declare.

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