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**Original Article**

# 5-Aminolevulinic acid combined with sodium ferrous citrate mitigates effects of heat stress on bovine oocyte developmental competence

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Abstract. High summer temperatures have deleterious effects on oocyte developmental competence. The antioxidant and autophagy-related properties of 5-aminolevulinic acid (5-ALA) gives the compound a broad range of biological activities. This study aimed to evaluate the effects of: 1) a high temperature-humidity index (THI) on the developmental competence of bovine oocytes, and 2) 5-ALA administration in combination with sodium ferrous citrate (SFC) during *in vitro* maturation (IVM) on bovine oocyte developmental competence evaluated at high THI. Bovine ovaries were collected from a local slaughterhouse at moderate environmental temperature (MT; THI of 56.2) and high environmental temperature (HT; THI of 76.7) periods; cumulus-oocyte complexes (COCs) were aspirated from medium-sized follicles, matured *in vitro* for 22 h, fertilized, and cultured for 10 days. For COCs collected during the HT period, 0 (control), 0.01, 0.1, 0.5, or 1 µM 5-ALA was added to the maturation medium in combination with SFC at a molar ratio of 1:0.125. The results showed that HT adversely affected blastocyst and hatching rates compared with MT. Adding 5-ALA/SFC (1 µM/0.125 µM) to the maturation medium of oocytes collected during the HT period improved cumulus cell expansion and blastocyst rates compared with the no-addition control. In conclusion, this study showed that high THI can disrupt bovine oocyte developmental competence. Adding 5-ALA to SFC ameliorates this negative effect of heat stress and improves subsequent embryo development.

Key words: 5-Aminolevulinic acid, Bovine, Heat stress, *In vitro* maturation, Oocyte

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Heat stress has a detrimental effect on hormonal production, estrous expression, ovarian function, oocyte quality, and embryonic development in cattle [1, 2]. Beef cattle (*Bos taurus*) pregnancy rate decreases by 3.2% when the temperature-humidity index (THI) is above 70 [3]. In addition, beef cows exposed to THI 77.4 showed increased oxidative stress and a prolonged estrous cycle [4]. Moreover, high environmental temperature affects their age at first calving, particularly if the animals are exposed to such temperatures for three days before and after insemination [5]. Oocyte maturation is a crucial developmental period for progression to subsequent embryonic stages [6]. Bidirectional crosstalk occurs between oocytes and their surrounding cumulus cells. Cumulus cells facilitate the transfer of metabolic substrates, elimination of toxic substances, and modulation of environmental influences to support oocyte growth and development [7]. Heat stress impairs the function of cumulus-oocyte complexes (COCs), specifically their nuclear and cytoplasmic maturation, through inducing mitochondrial dysfunction and accumulation of reactive oxygen species (ROS) [8–10]. High ROS levels have deleterious effects on both oocyte maturation and fertilization rate, limiting oocyte penetration and embryonic development through decreasing blastocyst rate and inducing embryo cell block [11]. Therefore, supplementation of maturation medium with antioxidants such as cysteine, cysteamine, melatonin, and resveratrol may be effective against oxidative stress [12].

Recently, 5-aminolevulinic acid (5-ALA; $C_5H_{12}NO_7P$) has attracted considerable attention as a potential antioxidant. 5-ALA is a natural non-alpha amino acid found in vegetables, fruits, and fermented products. As a tetrapyrrole, 5-ALA is part of the same class as heme, vitamin $B_{12}$, and chlorophyll [13]. It is formed from a combination of glycine and succinyl-CoA in the presence of 5-ALA synthetase (5-ALAS) [14]. 5-ALA exerts anti-inflammatory effects through inhibiting TNFα and iNOS expression [13]. Moreover, 5-ALA in combination with sodium ferrous citrate (SFC) induces heme oxygenase-1 (HO-1) expression, thus protecting cells both *in vivo* and *in vitro* against oxidative stress, as well as against other conditions such as hydrogen peroxide-induced cardiomyocyte hypertrophy, cisplatin-induced nephrotoxicity, and ischemia-reperfusion-induced renal injury [15–19]. Recent studies indicate that 5-ALA treatment rescues heat-and metabolic stress-exposed bovine mammary epithelial cells via inhibiting the expression of unfolded protein response components and thereby relieving strain on the endoplasmic reticulum [20, 21]. Notably, 5-ALA dietary supplementation improves milk protein composition, iron status, and immunity in pigs and dairy cattle [22, 23]. Supplementation also increases egg yolk quality and egg production in laying hens [24].

Combining 5-ALA with SFC (5-ALA/SFC) has a greater positive effect than either 5-ALA or SFC alone because oxidative phosphorylation protein and gene levels are further increased and HO-1 is upregulated [23]. Despite the beneficial effects of 5-ALA/SFC in various cell lines, to the best of our knowledge, no studies have

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investigated the impact of 5-ALA/SFC on oocyte developmental competence *in vitro*. Therefore, this study aimed to elucidate *in vitro* developmental competence of oocytes derived from beef cattle exposed to moderate or high THI. Additionally, we aimed to clarify 5-ALA/SFC effects on the maturation and subsequent development of heat-stressed oocytes.

## Materials and Methods

*Animal care and use*

This study was approved by the Institutional Animal Care and Use Committee, University of Miyazaki (2021-015).

*Meteorological data*

All meteorological data were obtained from the Japan Meteorological Agency at the Miyakonojo City observation point (131°4.9' E, 31°43.8' N, 154 m above sea level). Daily average THI was calculated according to the following formula: THI = (0.8 × T) + [(RH × 100) × (T − 14.4)] + 46.4 [25], where T is the daily average temperature (°C) and RH is the daily average relative humidity (%). Table 1 lists the parameters related to the environmental parameters.

Early antral follicles of approximately 0.5 to 1.0 mm in diameter are sensitive to heat stress [26]. A 0.5 mm follicle takes approximately 15 days to reach a diameter of 6 mm [27]. Therefore, THI was calculated 15 days before each trial under the moderate conditions of spring (March to May) (characterized by mild temperature and humidity with a daily average THI = 56.2 [3]; moderate temperature or MT) and more extreme conditions of summer (June to August) (characterized by high temperature and humidity with a daily average THI = 76.7; high temperature or HT) (Figs. 1A and B). Table 1 lists THI values during MT and HT.

*Experimental design*

Experiment 1: *In vitro* oocyte developmental competence was evaluated in ovaries of Japanese Black cattle under MT and HT conditions (Fig. 2).

Experiment 2: Oocytes collected under high summer temperatures were treated with different 5-ALA/SFC concentrations; effects on oocyte maturation and subsequent embryo development were determined (Fig. 2).

**Table 1.** Comparison of metrological data during spring (March to May; moderate temperature) and summer (June to August; high temperature) at the study site (Miyakonojo City) where ovaries were collected

| | Daily maximum temperature (°C) | Daily minimum temperature (°C) | Daily average temperature (°C) | Daily average relative humidity | Daily average THI |
|---|---|---|---|---|---|
| Moderate temperature [a] | 19.5 ± 3.2 | 7.7 ± 3.2 | 13.3 ± 2.4 | 69.5 ± 13.8 | 56.2 ± 3.5 |
| High temperature [b] | 30.9 ± 4 | 22.3 ± 2.5 | 26. ± 2.7 | 82.7 ± 8.2 | 76.7 ± 3.9 |

Results are shown as mean ± SD. a/b: all metrological data in each column are significantly different at P < 0.0001.

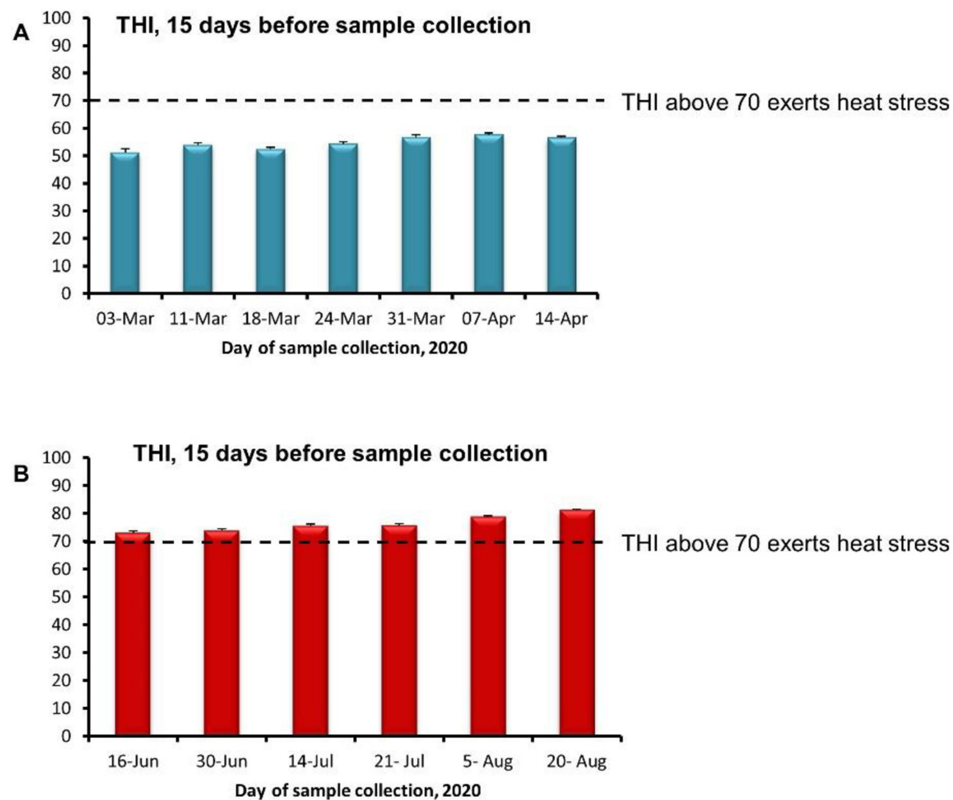


**Fig. 1.** THI 15 days before each batch of sample collection in moderate (MT; A) and high (HT; B) environmental temperatures. Values are expressed as mean ± SEM.

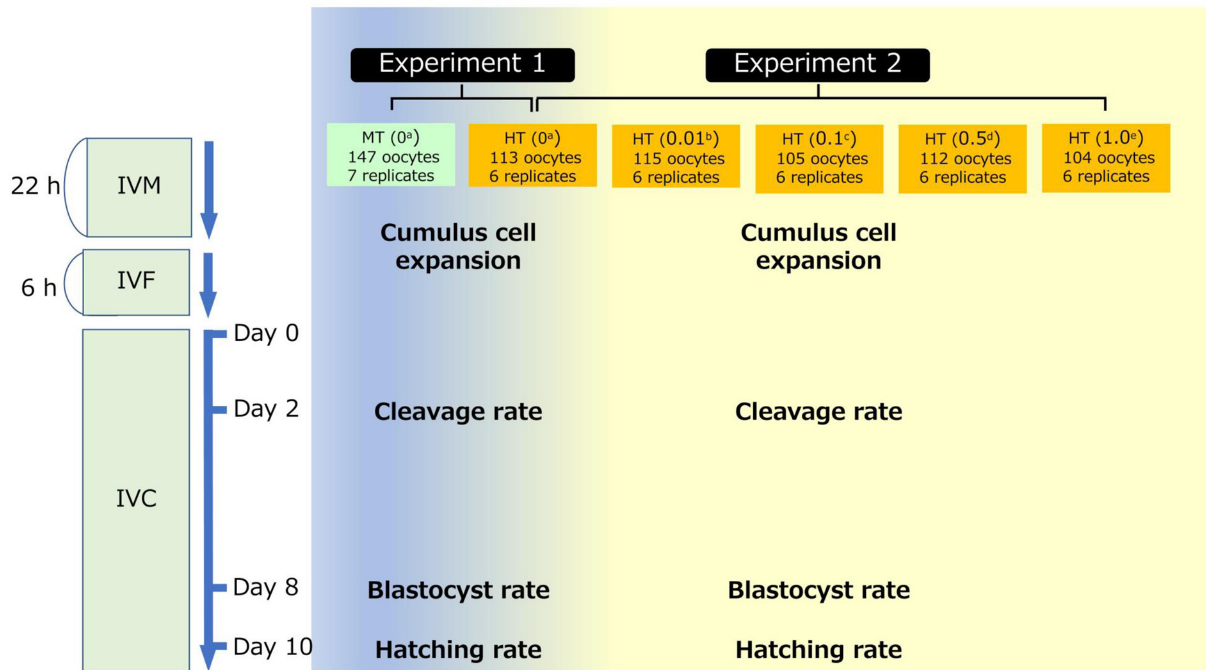


**Fig. 2.** Schematic representation of Experiments 1 and 2. Experiment 1: COCs were aspirated from ovaries collected in moderate temperature (MT) during spring (March to May) and high temperature (HT) during summer (June to August); aspirated COCs were then subjected to *in vitro* maturation, fertilization, and culture (IVM, IVF, and IVC) at 38.5°C. Experiment 2: COCs were aspirated from ovaries collected under HT and treated with different 5-ALA/SFC concentrations during IVM. Oocytes were then subjected to IVM, IVF, and IVC. Images of COCs were recorded before and after maturation to calculate cumulus expansion ratios. Embryonic development was recorded on days 2 (cleavage rate, %), 8 (blastocyst rate, %), and 10 (hatching rate, %) post-IVF. IVM, *in vitro* maturation; IVF, *in vitro* fertilization; IVC, *in vitro* culture; 5-ALA, 5-aminolevulinic acid; SFC, sodium ferrous citrate. Experiments were conducted in the dark. a: 5-ALA/SFC (0 µM / 0 µM), b: 5-ALA/SFC (0.01 µM / 0.00125 µM), c: 5-ALA/SFC (0.1 µM / 0.0125 µM), d: 5-ALA/SFC (0.5 µM / 0.0625 µM), e: 5-ALA/SFC (1 µM / 0.125 µM).

*Ovary and oocyte collection*

Ovaries of Japanese Black cattle were collected from a local slaughterhouse in Miyakonojo City, Japan and transported within 2 h to the laboratory in 0.9% saline (Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan), supplemented with a 100 µg/ml penicillin-100 U/ml streptomycin antibiotic mixture (Nacalai Tesque, Inc., Kyoto, Japan), at 37°C. Ringer's solution supplemented with 5% heat-inactivated bovine serum (Life Technologies New Zealand Ltd., Auckland, New Zealand) and a 10-ml syringe attached to a 19-G needle (Terumo, Tokyo, Japan) were used for oocyte extraction from medium-sized follicles (3–6 mm in diameter). Oocytes were selected if they had homogeneous cytoplasm surrounded by three or more layers of intact cumulus cells [28].

*In vitro maturation (IVM)*

Selected oocytes were cultured in TCM-199 (Gibco, Grand Island, NY, USA) supplemented with 5% standard fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 0.2 AU/ml follicle stimulating hormone (FSH; Kyoritsu Pharmaceutical Co., Ltd. Tokyo, Japan), and a combination of 100 µg/ml penicillin and 100 U/ml streptomycin. For IVM, selected COCs were randomly divided into five groups: control and four 5-ALA/SFC combinations in a molar ratio of 1:0.125 (0.01 µM/0.00125 µM, 0.1 µM/0.0125 µM, 0.5 µM/0.0625 µM, and 1 µM/0.125 µM). These concentrations were selected based on a patent study [29]. 5-ALA and SFC were provided by Neopharma Japan Co. Ltd. (Tokyo, Japan). Approximately 20 oocytes per group were placed in a four-well culture dish (Nunc, Nalge Nunc International, Roskilde, Denmark) containing 1mL of maturation medium and cultivated at 38.5°C and 5% $CO_2$ for 22 h.

*Assessment of cumulus expansion ratio*

Cumulus expansion of COCs was assessed before and after IVM, as previously reported [30]. A digital image of each group was captured using the same magnification and parameters under a stereomicroscope (Meiji Techno Co., Ltd., Tokyo, Japan). The total area of each COC was measured and calculated in ImageJ (version 1.47v; NIH, Bethesda, MD, USA). To calculate the cumulus expansion ratio, mean total area of post-IVM COCs was divided by the mean total area of pre-IVM COCs for each group.

*In vitro fertilization (IVF)*

After IVM, most expanded cumulus cells were removed to facilitate sperm penetration. Oocytes were placed in a 35 mm Petri dish (AS ONE Corporation, Osaka, Japan) containing 50 µl of IVF medium (IVF100, Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) and covered with mineral oil (Fujifilm Irvine Scientific, Inc., Santa Ana, CA, USA). Fertilization was performed with frozen-thawed semen from a fertile Japanese Black bull; a 0.5 ml straw from the same lot was thawed for 40 sec in a 38.5°C water bath. Semen was placed in a falcon tube containing 4 ml of IVF100 medium and centrifuged at 624 × *g* for 5 min. The pellet was reconstituted with 4 ml of IVF100 medium and centrifuged again at 624 × *g* for 5 min. After removing the supernatant, sperm concentration and motility were assessed. The sperm suspension was diluted to 10 million spermatozoa/ml, and a 50 µl aliquot was added to a 50-µl fertilization drop, resulting in a final sperm concentration of five million spermatozoa/mL. IVF was performed at 38.5°C in a humidified atmosphere with 5% $CO_2$ for 6 h. The day of fertilization was considered day 0.

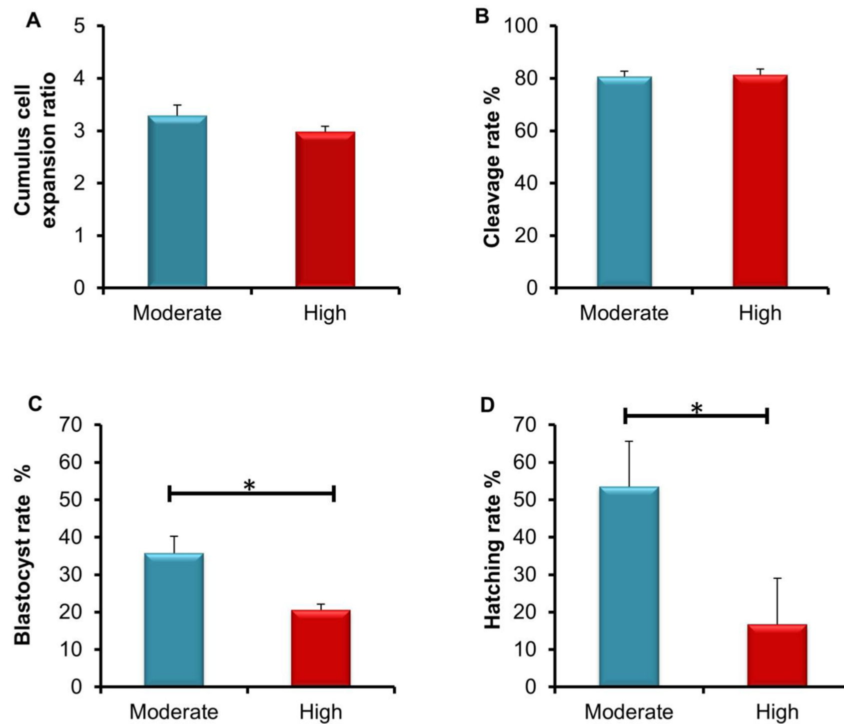


**Fig. 3.** Effects of different environmental temperatures on cumulus cell expansion ratio (A), cleavage rate (B), blastocyst rate (C), and hatching rate (D). * P < 0.05. Moderate: moderate temperature, high: high temperature. Cumulus cell expansion ratio = mean of total COC area after maturation/mean of total COC area before maturation in each group. Ceavage rate: (number of cleaved embryos/ number of inseminated oocytes) × 100. Blastocyst rate: (number of blastocysts/number of cleaved embryos) × 100. Hatching rate: (number of hatched embryos/number of blastocysts) × 100.

*In vitro culture (IVC)*

After IVF, presumptive zygotes were washed several times in the culture medium (IVMD101, Research Institute for the Functional Peptides Co., Ltd.), placed in four-well culture dishes containing 600 µL of IVMD101 medium, and incubated in a humidified atmosphere with 5% $CO_2$ for 24 h. Cumulus cells were completely removed through gentle pipetting, and embryos were washed in culture medium (IVD101, Research Institute for the Functional Peptides Co., Ltd.) before incubation in a collagen type 1-coated 6-well plate (Research Institute for the Functional Peptides Co., Ltd.), with each well containing 200 µl of IVD101 supplemented with 5% FBS. Embryos were covered with mineral oil for 9 days (from day 2 to day 10). Cleavage rate (number of cleaved embryos/number of inseminated oocytes) was recorded on day 2, blastocyst rate (number of blastocysts/number of cleaved embryos) on day 8, and hatching rate (number of hatched embryos/number of blastocysts) on day 10.

*Statistical analyses*

Statistical analyses were performed in SigmaStat (SPSS Inc., Chicago, IL, USA). Assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variance (F test) were tested in cumulus cell expansion ratio, cleavage rate, blastocyst rate, and hatching rate of MT and HT samples. T-tests were used to determine between-group differences in cleavage and blastocyst rates, while Mann-Whitney U tests were used for differences in hatching rate. Control and treatment groups were analyzed with one-way ANOVA, followed by the least significant Fisher's test (LSD) to separate means. Data are presented as mean ± SEM. Significance was set at P < 0.05.

## Results

*Experiment 1*

Cumulus expansion ratios did not differ significantly between the MT (n = 147; 3.29 ± 0.20) and HT (n = 113; 2.98 ± 0.11) groups (Fig. 3A). Cleavage rate also did not differ significantly between the MT (n = 119; 80.6 ± 2.2%) and HT (n = 92; 81.2% ± 2.3%) groups (Fig. 3B), whereas blastocyst rate was lower in the HT (n = 19; 20.6% ± 1.6%) group than in the MT group (n = 43; 35.7% ± 4.5%) (P < 0.05) (Fig. 3C). Moreover, the HT group (n = 4; 16.7 ± 12.4%) had a lower hatching rate than the MT group (n = 27; 53.5 ± 12.1%) (P < 0.05; Fig. 3D).

*Experiment 2*

We determined the effect of different 5-ALA/SFC concentrations on oocytes collected under HT (0.0 µM/0.0 µM, total COC number = 113; 0.01 µM/0.00125 µM, total COC number = 115; 0.1 µM/0.0125 µM, total COC number = 105; 0.5 µM/0.0625 µM, total COC number = 112; and 1 µM/0.125 µM, total COC number = 104). The 1 µM/ 0.125 µM 5-ALA/SFC group (3.6 ± 0.3) had a significantly higher cumulus cell expansion ratio than control (2.9 ± 0.1) and 0.01 µM/0.00125 µM (3 ± 0.2) groups (P < 0.05, Fig. 4A). The 1 µM/ 0.125 µM 5-ALA/SFC group had the highest cleavage rate (n = 93; 89.8 ± 2.3%), although the difference was not statistically significant from control (n = 92; 81.2 ± 2.3%) or the other 5-ALA/SFC groups (0.01 µM/0.00125 µM: n = 93, 80.6 ± 3.7%; 0.1 µM/0.0125 µM: n = 91, 87.3 ± 2.9%; 0.5 µM/0.0625 µM: n = 92, 81.1 ± 4.4%; 0.05 < P < 0.1, Fig. 4B). Similarly, the 1 µM/0.125 µM group had a significantly higher blastocyst rate (n = 30; 32.4 ± 2%) than the control (n = 19; 20.6 ± 1.6%, P < 0.05) and trended toward having

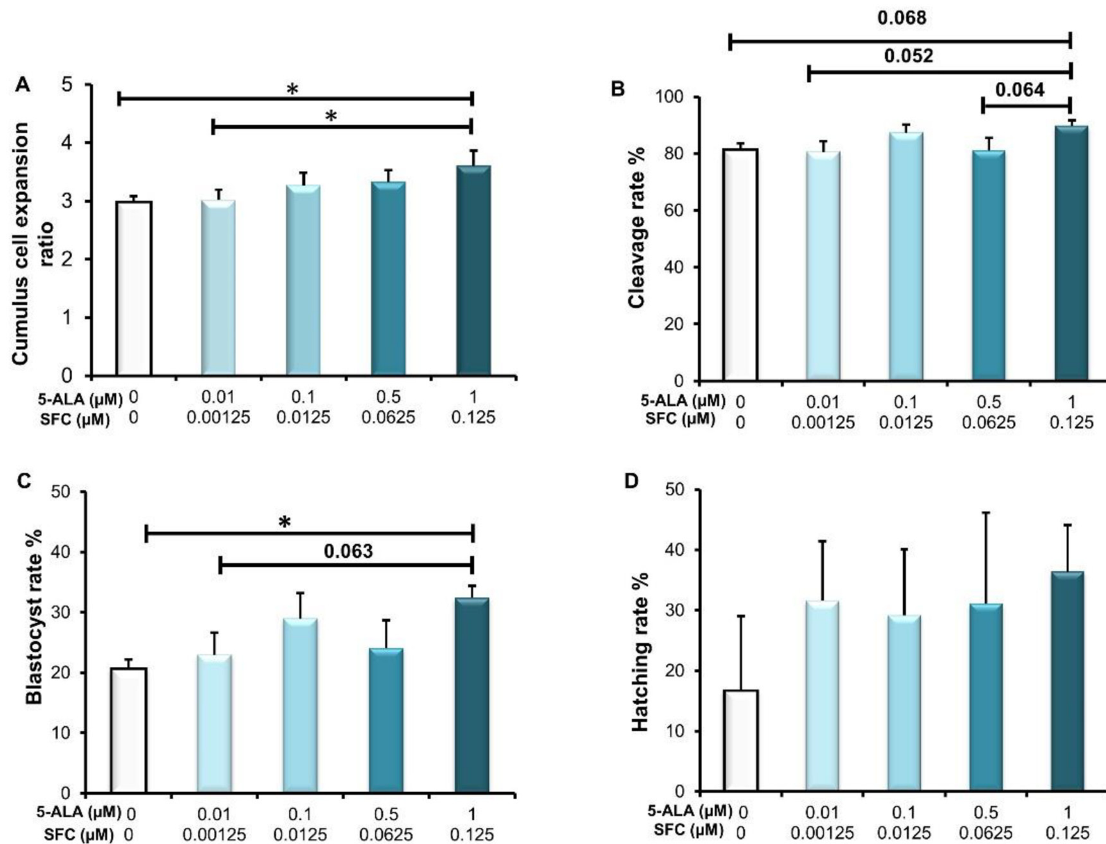


**Fig. 4.** Effects of different 5-ALA/ SFC concentrations on cumulus cell expansion ratio (A), cleavage rate (B), blastocyst rate (C), and hatching rate (D). * $P < 0.05$. Cumulus cell expansion ratio = mean of total COC area after maturation/mean of total COC area before maturation in each group. Cleavage rate: (number of cleaved embryos/ number of inseminated oocytes) × 100. Blastocyst rate: (number of blastocysts/numbers of cleaved embryos) × 100. Hatching rate: (number of hatched embryos/number of blastocysts) × 100.

a higher rate than the 0.01 μM/0.00125 μM group (n = 22; 23 ± 3.7%, P = 0.063; Fig. 4C). Hatching rates did not differ significantly between the control and 5-ALA/SFC treatment groups (Fig. 4D).

## Discussion

As THI increases from winter to summer, reproductive performance of Japanese Black cattle tends to decrease [4, 5, 31, 32]. However, one study showed that hot (summer) or cool (autumn) conditions did not affect the developmental competence of oocytes collected via ovum pick-up from Japanese Black cattle [33]. In the context of this report, and recognizing that limited details are available regarding HT effects on Japanese Black oocytes, we aimed to clarify variation in oocyte developmental competence across HT and MT conditions. We also tested the ability of 5-ALA/SFC to mitigate heat stress effects and to restore oocyte developmental competence *in vitro*.

This study did not find a significant difference in cumulus cell expansion ratio between HT (THI: 76.7) and MT (THI: 56.2, a condition that does not exert heat stress on beef cows). Indeed, cumulus cells are somatic cells with high antioxidant activity and tolerance to stressful conditions [34, 35]. However, a previous study indicates that summer thermal stress increased the cumulus cell apoptotic index [10]. We also did not observe significant differences between HT and MT in cleavage rate, in agreement with previous studies showing that heat shock had no effect on cleavage rate in bovine oocytes [34]. This outcome is likely because early cleavage is linked to autophagy, a process that acts as a pro-survival response against heat stress [34]. Autophagy is upregulated after fertilization, peaks at the 2–4 cell stage, and then decreases gradually from the morula to blastocyst stage; research has shown that inducing autophagy improves preimplantation embryo development [36, 37]. We did note that blastocyst and hatching rates were significantly lower under HT than under MT. Heat-induced adverse effects on oocyte quality and embryo development may be mediated through retarding nuclear maturation by arresting oocytes at metaphase I [38], or hastening nuclear maturation (thus aging oocytes) by disrupting gap junctions and intracellular cAMP [6, 39]. Moreover, excessive heat disrupts oocyte cytoplasmic maturation via stressing the endoplasmic reticulum and impairing mitochondrial function. Additionally, heat stress altering oxidative phosphorylation complex-associated genes [9, 40, 41] causes an imbalance between ROS production and elimination, consequently inducing intracellular ROS accumulation and GSH decline [9].

Here, we demonstrated that 5-ALA/SFC treatment improves the cumulus cell expansion ratio, corroborating previous studies using different antioxidants [42, 43]. A known biological activity of 5-ALA/SFC is activating MAPK subunits (ERK1/2) [44]. At the cumulus cell level, this activation upregulates the expression of cumulus-cell-expansion genes, including hyaluronic acid synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), pentraxin-3 (*PTX3*), and TNF-stimulated gene-6 (*TNFAIP6*) [45]. However, additional experiments are required to corroborate this hypothesis.

Similarly, we observed that 5-ALA/SFC restored the developmental competence of heat-stressed bovine oocytes through improving blastocyst rates. This result is in line with a previous study showing that 5-ALA protects against heat stress in bovine mammary epithelial cells, specifically exerting antioxidant effects and relieving endoplasmic reticulum stress [21]. 5-ALA/SFC acts via the activation of MAPK subunits that in turn stimulates the nuclear translocation of a redox-sensitive transcription factor called nuclear factor (erythroid-derived 2)-like 2 (Nrf-2). The binding of Nrf-2 to antioxidant response elements (ARE) then activates ROS scavengers [44]. Nrf-2 and its downstream antioxidants are important to bovine granulosa cell and preimplantation bovine embryo survival *in vitro* under oxidative stress [46, 47]. Moreover, Nrf-2 translocation to the nucleus upregulates HO-1 expression [17, 44]. HO-1 is critical for iron homeostasis, apoptosis prevention, autophagy, and antioxidant defense systems [48, 49]. Furthermore, 5-ALA with SFC upregulates aerobic energy metabolism through enhancing cytochrome c, cytochrome P450, and oxidative phosphorylation complex III/IV/V expression, thus elevating ATP production and improving mitochondrial function [50]. ATP is needed for oocyte maturation and reorganization of cytoplasmic organelles, so its increase improves oocyte developmental competence [51].

Although 5-ALA/SFC significantly enhanced blastocyst rate in a dose-dependent manner, HR was not significantly higher than control levels. This result is in line with other studies that used different antioxidants [52, 53]. Therefore, 5-ALA/SFC appears to rescue embryos that would otherwise have failed to develop, rather than stimulating embryo developmental kinetics. The lower 5-ALA/SFC concentrations used in this study may be another reason why we did not observe a significant difference in hatching blastocysts on day 10. Further studies are needed to determine whether higher 5-ALA/SFC doses will improve HR.

To the best of our knowledge, this is the first study to show the impact of 5-ALA/SFC on bovine oocyte maturation and progression to subsequent embryonic stages under heat stress, as well as the first to analyze the combination treatment's mitigation capacity on HT-associated decrease in oocyte developmental competence. We hypothesized that 5-ALA/SFC supports the autophagic response to heat stress and the antioxidant function of oocytes via the MAPK-Nrf-2-HO-1 signaling pathway. However, we still do not know the exact mechanism underlying 5-ALA/SFC restoration of oocyte quality.

Further research is needed to clarify whether higher doses of 5-ALA will be useful or toxic. Moreover, experiments should be conducted to elucidate 5-ALA/SFC's cellular and molecular mechanism of action on oocytes and their surrounding cells after IVM. Research investigating nuclear maturation, ROS, and antioxidant-related gene expression should provide a better understanding of how 5-ALA/SFC improves oocyte quality. Another limitation of our study was that we collected the ovaries from a slaughterhouse. Therefore, we did not have data on nutrition, health, transportation, and follicular wave stages in subject animals. We attempted to address potential confounding effects by dividing collected oocytes randomly into experimental groups. We recommend that future studies aim to use oocytes with characteristics known to the greatest extent possible.

In conclusion, a high temperature-humidity index of 76.7 disrupted bovine oocyte developmental competence through reducing blastocyst and hatching rates. Moreover, 5-ALA/SFC improved the quality of heat-stressed oocytes via increasing cumulus cell expansion and blastocyst rate, thereby spurring progression to subsequent embryonic stages.

**Conflict of interests:** The authors declare no conflict of interest.

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