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FULL PAPER

Theriogenology

# The effect of heat and cold stress on oxidative stress of follicular microenvironment in beef cattle

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**ABSTRACT.** This study hypothesized that heat and cold stress affect the balance between oxidants and antioxidants (oxidative stress) of oocytes and follicular fluid (FF) in beef cattle and the oxidative stress between blood and FF is correlated. Thirty-two Japanese Black cattle that were divided into four seasonal groups [fall, winter (WG), spring (SpG), and summer (SmG) group]. Plasma and FF were measured diacron-reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and oxidative stress index (OSI). Reactive oxygen species (ROS) were measured in oocytes. Temperature-humidity index was lower in WG and higher in SmG than in the other three groups ($P<0.05$). d-ROMs value was lower in FF than in plasma in all groups, BAP value was lower in FF than in plasma in WG, SpG, and SmG, and OSI was lower in FF than in plasma in WG and SpG ($P<0.05$). In FF, d-ROMs and BAP values were positively correlated in WG, SpG, and heifer groups (HG, $P<0.05$). d-ROMs or OSI values between plasma and FF were positively correlated in all animals and cow group (CG), SpG, CG, and HG ($P<0.05$). ROS levels in oocytes were higher in WG and SpG than in SmG ($P<0.05$). In conclusion, oxidative stress in the blood and FF are linked, and oxidative stress of oocytes increases in winter and spring in beef cattle that are kept on pasture, except in winter.

**KEYWORDS:** cattle, diacron-reactive oxygen metabolite, follicular fluid, oocyte, oxidative stress index

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## INTRODUCTION

Temperature and humidity fluctuations are stressors in bovines. The thermoneutral zones were 10–20°C for beef cattle and 4–20°C for dairy cattle [36]. However, especially, the minimum degree of these thermoneutral zones is defined by immediate reactions in laboratory experiments, and long-term exposure to cold environments is a stressor for bovine livestock production, even if they do not deviate from the thermoneutral zone [49]. In Korean beef cattle, exposure to hot and cold environments increased standing time and cortisol concentration in the blood [22, 23]. Bovine reproductive performance is reduced in hot and cold environments. For instance, the conception rate by first artificial insemination in lactating cows decreased in summer (July–September) when exposed to heat stress, compared with winter (January–March) [48]. In Japanese Black cows, the first-service conception rate decreased in winter, increasing cold stress compared with summer and fall [39].

The balance between oxidants and antioxidants (oxidative stress) is defined as a condition that impairs the transmission and regulation of redox signals and is caused by an excess of oxidants owing to an imbalance between oxidants and antioxidants [41]. Homeotherms try to maintain a constant internal environment in response to changes in temperature and humidity in the external environment; however, excessive changes increase internal oxidative stress. In Japanese Black cattle, oxidative substances in the blood increase during the hot season [37], and antioxidant enzymes in the blood increase during winter grazing [32]. In pigs, increasing

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oxidative stress in summer causes poor quality oocytes and infertility [4]. However, few studies have reported the effects of heat and cold stress on oxidative stress in the follicular microenvironment, including oocytes and follicular fluid (FF). Additionally, the relationship of oxidative stress between systemic circulation and follicular microenvironment remains unclear.

In animal reproduction, the developmental status of oocytes affects embryo quality and fertility. The amount of reactive oxygen species (ROS) in granulosa cells was increased by culturing at 41°C *in vitro* [1]. When the level of ROS in bovine oocytes was high, the concentration of antioxidant in FF was low, and the blastocyst formation rate decreased [26]. In mice, adding $H_2O_2$ to an *in vitro* maturation (IVM) medium decreases the fertilization rate [43], and the amount of ATP decreases in the cytoplasm of oocytes exposed to $H_2O_2$ [52]. FF surrounding the oocyte also contains several substances that affect oocyte maturation. Studies have shown a relationship between oxidative substances and embryonic development or conception. In FF from obese women, malondialdehyde levels, which are biomarkers of lipid peroxidation, increased, and the conception rate after intracytoplasmic sperm injection was decreased [40]. High oxidative stress in FF is associated with abnormal fertilization, abnormal cleavage, and poor-quality blastocysts after intracytoplasmic sperm injection in humans [46]. Therefore, FF is a useful measurement material for biomarkers to assess oxidative stress in oocytes.

The diacron-reactive oxygen metabolite (d-ROMs) test, biological antioxidant potential (BAP) test, and oxidative stress index (OSI) calculated from d-ROMs and BAP are some of the ways to easily quantify oxidative stress [51]. d-ROMs test and BAP test are useful for evaluating the balance between oxygen and antioxidants because these can quantify the total amount of ROS and antioxidants, respectively. In humans, a previous report used d-ROMs, BAP, and OSI in the blood as biomarkers of the anti-inflammatory effect of probiotics [3], whereas others showed a relationship between the value of these tests using follicular fluid and embryo development [46]. In the veterinary field, blood tests have been used to evaluate the oxidative stress caused by prolonged submaximal exercise in equines [5] and left displacement of the abomasum in dairy cows [10]. However, few studies have focused on measuring the oxidative stress of FF by d-ROMs and BAP value in bovine.

Furthermore, the proportion of reaching blastocyst was higher in beef cow than in beef heifer [35]. On the other hand, the cleavage rates and blastocyst rates were higher in 12 months old heifers than 7–8 years and 15 years old cows in Murray Grey × Brahman crossbreed cattle [42]. However, the differences in the thermal stress, especially cold stress tolerance due to parity are unclear in beef cattle, although heat stress tolerance differs between cow and heifer in dairy cattle [38]. Dairy cows' body temperature was more sensitive than that of dairy heifers to external temperature, and the summer-produced embryo quality was poorer in cows than in heifers [34]. Therefore, when the oocytes and FF quality are assessed, it needs to take into account the effect of age or parity.

From the above, we hypothesized that heat and cold environments affect oxidative stress in the systemic circulation and follicular microenvironment and that oxidative stress in the follicular microenvironment is related to that in the systemic circulation. In addition, we focused on the differences in oxidative stress between the different parities. Thus, we measured the oxidative stress of blood and FF using the d-ROMs and BAP tests, quantified the amount of ROS in oocytes, and compared the results between seasonal groups. In addition, an analysis was performed to compare parity.

## MATERIALS AND METHODS

### *Animal care and use*

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

Thirty-two Japanese Black cattle bred in Sumiyoshi Livestock Science Station, an experimental farm of the University of Miyazaki (latitude 31° 59' north, longitude 131° 28' east), were enrolled in this study. All animals were assessed in nine classes of body condition score (BCS) when Day 8 (the day after the second gonadotropin releasing hormone (GnRH) administration was defined as Day 1 in synchronization of ovulation) following a previous study [47].

The study was conducted between October 2022 and August 2023. By date of sampling, the cattle were classified from October to November in the fall group (FG) [n=8 (four cows, four heifers), age in months: cows; 107.5 ± 23.54, heifers; 17.25 ± 1.11, BCS: cows; 5.5 ± 0.65, heifers; 7.25 ± 0.25], from January to February in winter group (WG) [n=8 (four cows, four heifers), age in months: cows; 133.75 ± 31.99, heifers; 15.25 ± 1.31, BCS: cows; 5.75 ± 0.22, heifers; 6.25 ± 0], from April to May in spring group (SpG) [n=9 (six cows, three heifers), age in months: cows; 145.26 ± 15.55, heifers; 13.33 ± 0.33, BCS: cows; 5.43 ± 0.22, heifers; 6.33 ± 0.33], and from July to August in summer group (SmG) [n=7 (four cows, three heifers), age in months: cows; 100.75 ± 28.15, heifers; 14.33 ± 0.33, BCS: cows; 5.25 ± 0.25, heifers; 7 ± 0]. Additionally, the cow group (CG) (n=18, age in months: 123.5 ± 12.2, parity: 7.67 ± 0.83, BCS: 5.50 ± 0.17) and the heifer group (HG) (n=14, age in months: 15.2 ± 0.6, parity: 0, BCS: 6.71 ± 0.16) were divided. Before starting sampling, all cows calved at least four weeks prior, and all heifers were confirmed to be in cycled estrus.

All cattle were maintained in a free barn and fed intermittent grasses (Italian ryegrass, Guinea grass, and Rhodes grass) and corn silage. All heifers were maintained in a free barn and fed grass (Italian ryegrass, Guinea grass, and Rhodes grass) and concentrated feed, including barley, corn, fu, soybean meal, shochu lees, and hay cubes. The cattle grazed for 7–10 days per month in March, April, and November, 14 days per month in May, and every day per month from June to October. In December to February, the cattle did not graze. The grass varieties in the grassland were Italian ryegrass and Bahia grass from March to April and Bahia grass from May to November. The nutritional conditions of all cattle met the Japanese Feeding Standard for Beef Cattle for fiscal year 2022 [31].

### *Determination of temperature-humidity index (THI)*

During each period, we measured the temperature and relative humidity every three hours, every day in the barn, using a data logger (Hygrochron; KN Laboratories Co., Ltd., Osaka, Japan). The barn was open with no walls around it, and the temperature and relative humidity were measured at a point 1 m above the ground, outside of direct sunlight, windows, and rain. The THI was calculated from

the temperature and relative humidity data according to a previous study [11].

THI=0.8 × T + (RH/100) × (T−14.4) + 46.4

*T*: temperature [°C], RH: relative humidity [%]

*Synchronization of ovulation*

At arbitrary estrus cycle, intravaginal progesterone releasers (CIDR 1900; Zoetis Japan Co., Ltd., Tokyo, Japan) were implanted, and GnRH (100 µg of fertirelin acetate, Fertirelin Injection Fujita; Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intramuscularly (IM). Seven days later, all cattle were removed from CIDR 1900 and injected with prostaglandin (PG) $F_{2\alpha}$ (250 µg of cloprostenol, cloprostenol C; Fujita Pharmaceutical. Co., Ltd.), and then injected GnRH at 56 hr after $PGF_{2\alpha}$ IM, respectively. The day after the second GnRH administration was defined as Day 1 (Fig. 1).

*Collection of FF and oocytes*

The FF and oocytes were collected using an aspirator (FV-6; FHK Fujihira Industry Co., Ltd., Tokyo, Japan) and a 54-cm 17-gauge needle (COVA Needle, Misawa Medical Industry, Tokyo, Japan) under ultrasound guidance using a B-mode ultrasound scanner connected micro-convex type 8 MHz ovum pick up (OPU) probe (MyLabOneVET; Esaote, Genova, Italy).

The cattle were retained in the stall, and lidocaine hydrochloride (xylocaine injection 2% with epinephrine; Sandoz, Tokyo, Japan) was injected into the caudal epidural space, 60 mg in cows and 40 mg in heifers. Prifinium bromide (Padrinium Injection; Riken Vets Pharma, Tokyo, Japan) was injected intravenously to prevent rectal straining (75 mg to cows and 60 mg to heifers). When the treatment of sedation was required during operation, 0.4–0.8 mg of xylazine hydrochloride (Xylazine Injection 2% [Fujita], Fujita Pharmaceutical Co., Ltd.) was administered IM. After injection, the vulva was washed with 0.02% mono, bis (trimethylammonium methylene chloride)-alkyl toluene and disinfected with 70% ethanol. The OPU probe was then inserted into the vagina. On Day 8, FF was collected from an ovarian follicle with a diameter of 8.5 mm or more to avoid contamination of blood, then the FF was defined as typical for each cattle. When multiple follicles were 8.5 mm or larger in a heifer or cow, the follicular fluid from each follicle was pooled and collected. On Days 8 and 15, the oocytes were collected from the follicles with a diameter of 2–6 mm to pick up immature oocytes, as they are used in OPU−*in vitro* embryo production [8]. Ringer's solution (500 mL) supplemented with 5 mL of heat-inactivated bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 5 mL of heparin (Heparin Sodium Injection 5,000 units/5 mL MOCHIDA; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) were mixed to collect the solution. All operations were performed by the same technician (KK).

*Blood sampling*

On Day 8, blood was collected from the jugular vein using a 21 G blood-collection needle and vacutainer tube with heparin added (Venoject II vacuum blood-collecting vessel; Terumo Corp., Tokyo, Japan) and brought to the laboratory within 3 hr while kept at 4°C. In the laboratory, blood was centrifuged at 1,550 × *g* for 15 min to separate the plasma and blood cells. Plasma samples were stored at −30°C until analysis. Blood for serum was collected using a plain vacutainer tube (Venoject II vacuum blood-collecting vessel; Terumo Corp.) and brought to the laboratory within three hours while kept at 4°C. In the laboratory, blood was centrifuged at 1,550 × *g* for 15 min to separate the serum, and blood biochemical tests were performed on serum samples (Fig. 1).

*Samples of FF*

FF was brought to the laboratory within three hours while maintained at 4°C and centrifuged at 1,550 × *g* for 15 min to separate the supernatant. Samples were stored at −30°C until analysis.

*Biochemical tests of blood*

Blood biochemical parameters were measured to confirm the metabolic conditions of each animal. The following parameters were evaluated: total protein (TP) level using the Biuret method, blood urea nitrogen (BUN) using the Urease-GLDH method, total

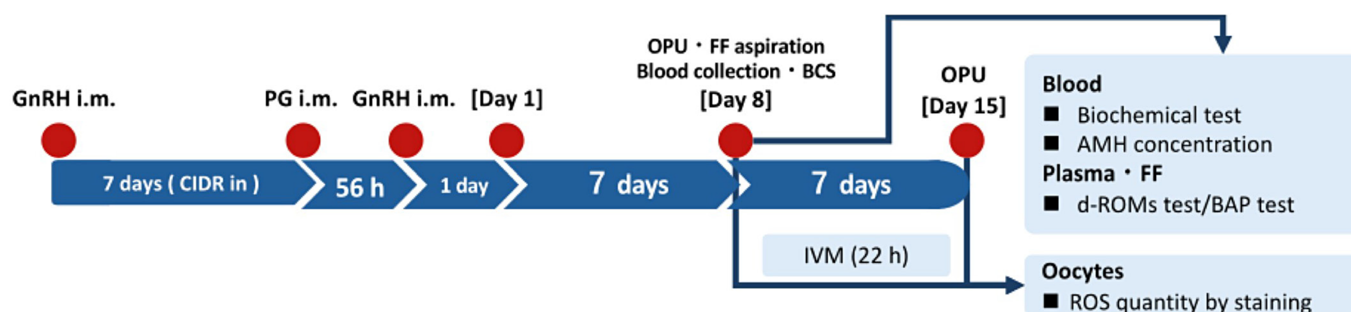


**Fig. 1.** Experimental design. GnRH, gonadotropin-releasing hormone; CIDR, controlled intravaginal drug releasing device; PG, prostaglandin $F_{2\alpha}$; IM, injection intramuscularly; OPU, ovum pick up; FF, follicular fluid; BCS, body condition score; ROS, reactive oxygen species; IVN, *in vitro* maturation; AMH, anti-Müllerian hormone; d-ROMs, diacron-reactive oxygen metabolites; BAP, biological antioxidant potential.

cholesterol (T-Cho), free fatty acid (FFA), β-hydroxybutyric acid (BHBA) and glucose levels (Glu) using enzymatic analyses, and aspartate aminotransferase (AST) using Japanese Society of Clinical Chemistry transferable method.

### *Measurement of blood anti-Müllerian hormone (AMH) concentration*

Heparinized plasma samples were used to measure AMH concentration using a commercial enzyme-linked immunosorbent assay kit (AL-144; Ansh Lab, Webster, TX, USA). Assay sensitivity and intra- and inter-assay coefficients of variation were 11 pg/mL, 8%, and 8%, respectively.

### *Measurement of d-ROMs and BAP values*

Oxidative stress in the plasma and FF was assessed using d-ROMs and BAP values (Fig. 1). Both values were measured using a free-radical analyzer system (FREE Carpe Diem, Wismerll Co., Ltd., Tokyo, Japan). The d-ROMs value was determined as the quantity of hydroperoxides (ROOH), which are metabolites of reactive oxygen and free radicals. The unit of measurement was "CARR U" (1 CARR U=0.08 mg $H_2O_2$/dL), and the assay sensitivity was 11 CARR U. The BAP test uses the reductive change from $Fe^{3+}$ to $Fe^{2+}$. OSI was measured using the following formula:

OSI=(d-ROMs/BAP) × 100

### *IVM*

Due to the inability to culture bovine oocytes individually, we conducted group culture. After arriving at the laboratory, the solution containing the pooled oocytes without distinguishing between heifers and cows was washed and filtered using a collecting solution, and oocytes were selected for analysis if they had a homogeneous cytoplasm surrounded by closely packed cumulus cells [13]. Oocytes were cultivated in the 200 µL of IVM medium (IVMD 101; Research Institute for the Functional Peptides, Yamagata, Japan) at 38.5°C and 5% $CO_2$ for 22 hr using a cultivating incubator (STERI-CYCLE; Thermo Fisher Scientific). Group cultures were performed to ensure an even number of oocytes per well (20 oocytes maximum). An operator handled all the oocytes (KK).

### *Measurement of oocytes ROS*

After IVM, cumulus cells surrounding the oocytes were removed by pipetting in DPBS (DPBS [10x]; Thermo Fisher Scientific) with 0.1% polyvinyl pyrrolidone (polyvinyl pyrrolidone K 30; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), which is PvP-DPBS. Oocytes were cultivated in 500 µL of cell-cultivating medium (TCM-199; Thermo Fisher Scientific) at 38.5°C and 5% $CO_2$ for 15 min. After cultivating, oocytes were dyed using 500 µL of ROS staining solution (CellROX DeepRed; Thermo Fisher Scientific) diluted 50x in cell-cultivating medium and washed with 500 µL of PvP-DPBS two times. A fluorescence microscope (EVOS 7000 Imaging System; Thermo Fisher Scientific) was used to image the stained oocytes with 721 nm of the fluorescence wavelength.

In analyzing ROS, the relative fluorescence of oocytes was measured using image analysis software (ImageJ 1.53k; National Institutes of Health, Bethesda, MD, USA). Relative fluorescence was calculated using the following formula, and the values were logarithmically transformed and normalized.

Relative fluorescence=integrated density (IntDen)−(area of selected oocyte [px] × mean fluorescence of background readings [256 gradations])

### *Statistical analysis*

Statistical analyses were performed using EZR (Jichi Medical University Saitama Medical Center, Saitama, Japan) [19], a graphical user interface software for statistical analysis (R ver. 4. 3. 1).

In comparing THI, the Bonferroni correction was applied for multiple comparisons.

In comparing all values except ROS and THI, Shapiro-Wilk analysis was conducted to confirm the normality of data. The values of AMH, BHBA and FFA were log transformation so that the data were normal distribution. Afterwards, multiway analysis of variance was conducted by including season, parity, and the type of sample (FF or plasma), along with the interaction term between season and parity as explementary variables. After that, when performing pairwise comparisons as part of a *post-hoc* test, the Bonferroni correction was applied to control for multiple comparisons. The correlation in each season between d-ROMs and BAP levels in plasma or FF, or between plasma and FF in each d-ROMs, BAP, and OSI levels was assessed using partial correlation coefficients, with parity considered as a confounding factor. The other correlations were analyzed by Pearson product-moment correlation coefficient.

In the value of ROS, Kruskal-Wallis test was conducted by season. After that, when performing pairwise comparisons by season as part of a *post-hoc* test, the Bonferroni correction was applied to control for multiple comparisons.

Data are presented as mean ± SEM. Differences were considered statistically significant at $P<0.05$.

## RESULTS

### *THI*

The THI was 64.90 ± 0.28 in FG, 49.95 ± 0.35 in WG, 64.18 ± 0.27 in SpG, and 79.54 ± 0.13 in SmG (Fig. 2). The THI in WG was lower, and that in SmG was higher than those in the other three groups ($P<0.05$).

### *Biochemical levels in blood*

The biochemical and AMH values in blood did not differ significantly among the seasonal groups. In contrast, the values of BUN,

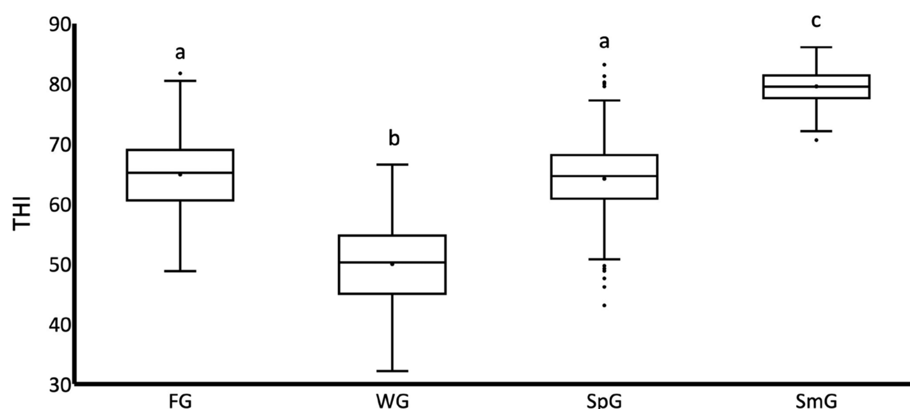


**Fig. 2.** Comparison of temperature-humidity index (THI) between seasonal groups. FG, fall group; WG, winter group; SpG, spring group; SmG, summer group. Different lowercase letters indicate significant differences (*P*<0.05).

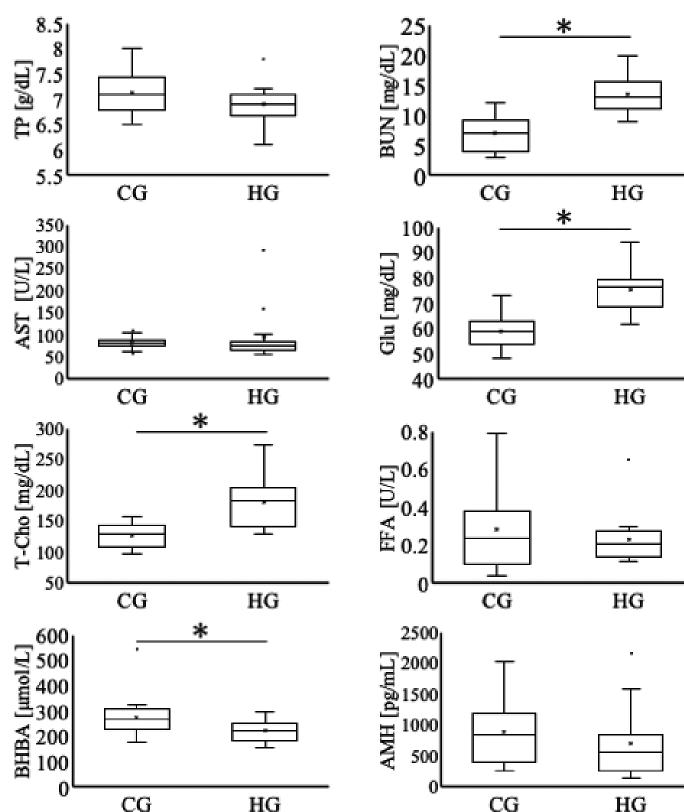


**Fig. 3.** The parity difference in blood biochemical tests. CG, cow group; HG, heifer group; TP, total protein; BUN, blood urea nitrogen; T-Cho, total cholesterol; FFA, free fatty acid; AST, aspartate aminotransferase; Glu, glucose; BHBA, β-hydroxybutyric acid: AHM. anti-Müllerian hormone. *; *P*<0.05.

T-Cho, and Glu in the HG were higher than those in the CG (*P*<0.05), and the value of BHBA in the CG was higher than that in the HG (*P*<0.05) (Fig. 3). No interaction was observed between the season and parity in all biochemical levels in blood.

### *d-ROMs and BAP values in FF and plasma*

No significant interactions were found for any d-ROMs, BAP, and OSI (Supplementary Table 1) in plasma and FF with the season and the parity as factors. Therefore, in subsequent analyses, the data in heifers and cows were combined when comparing between seasons. There were no significant differences between seasons for the d-ROMs, BAP, and OSI values in plasma or FF (Fig. 4). The d-ROMs values of FF were significantly lower than those of plasma in all seasonal groups (*P*<0.05). The BAP value of FF was significantly lower than that of plasma in the WG, SpG, and SmG. The OSI of FF was significantly lower than plasma in WG and

SpG ($P$<0.05).

d-ROMs value of FF was significantly lower than that of the plasma in both parity groups ($P$<0.05, Fig. 5). The BAP values of plasma and FF did not differ significantly between the parity groups; however, the BAP value of FF was significantly lower than that of plasma in both parity groups. The OSI of plasma and FF was significantly higher in the HG than in the CG, and the OSI of FF was significantly lower than that of plasma in both parity groups ($P$<0.05).

There was a significant positive correlation between the d-ROMs and BAP values of the FF in the seasonal groups (WG: r=0.777, SpG: r=0.914, $P$<0.05, Table 1), and the parity group (HG: r=0.663, $P$<0.05, Table 1). In the plasma, these values did not show a significant correlation.

For d-ROMs values, there was a significant positive correlation between plasma and FF in the seasonal group (all animals: r=0.787, $P$<0.05, Table 2) and the parity group (CG: r=0.724, $P$<0.05, Table 2). For the BAP value, there was no significant correlation between plasma and FF. The OSI between plasma and FF showed a significant positive correlation in the seasonal group (all animals: r=0.759, SpG: r=0.850, $P$<0.05, Table 2), and the parity group (CG: r=0.566, HG: r=0.602, $P$<0.05, Table 2).

### *Expression of oocytes ROS*

In this study, IVM was performed without distinguishing cows and heifers. The relative fluorescence of oocytes was significantly higher in WG and SpG than in FG and SmG and significantly lower in SmG than in the other three seasonal groups ($P$<0.05, Fig. 6).

## DISCUSSION

We hypothesized that hot and cold environments affect oxidative stress in the systemic circulation and follicular microenvironment and that oxidative stress in the follicular microenvironment is related to that in the systemic circulation. To the best of our knowledge, the present study is the first report in bovines that evaluates oxidative stress in FF and shows the relationship between oxidative stress in blood and FF. Additionally, we revealed that the redox balance in FF and the differences in oxidative stress between the blood and FF changed seasonally. In the WG and SpG, d-ROMs values and BAP values of FF were correlated, the OSI of FF was less than that of plasma, and the amount of ROS in oocytes was increased. There are two important points in this study: the oxidative stress in FF in the SpG was similar to that in the WG, and the level of ROS in oocytes was not increased in the SmG.

The biochemical, hormonal, and oxidative stress values of blood and the oxidative stress in FF showed no interaction between season and parity. However, there was a report indicating that the sensitivity to ambient temperature differs between nulliparous and multiparous dairy cows [34]. Therefore, since the sample size used in this study may have been a limiting factor, further follow-up studies are necessary in the future.

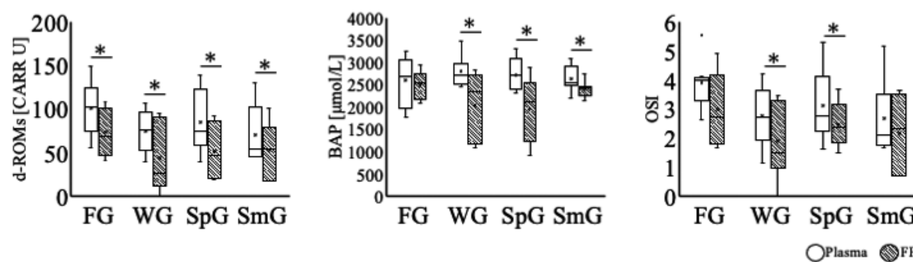


**Fig. 4.** Within-seasonal group and between-seasonal group comparison of oxidative stress in plasma and follicular fluid (FF). FG, fall group; WG, winter group; SpG, spring group; SmG, summer group. *; $P$<0.05.

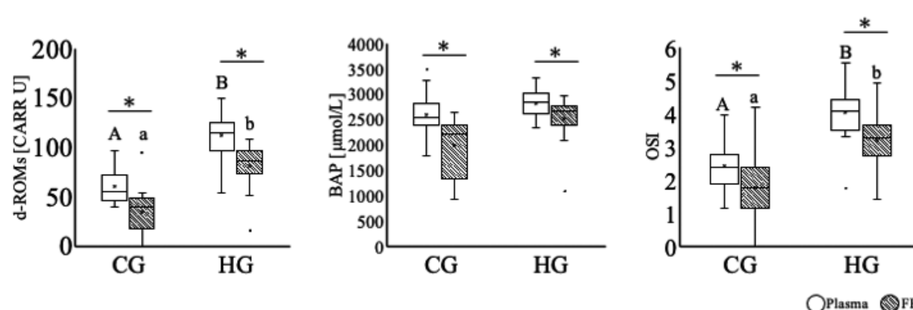


**Fig. 5.** Within-parity group and between-parity group comparison of oxidative stress in plasma and follicular fluid (FF). CG, cow group; HG, heifer group. Different capital letters indicate significant differences ($P$<0.05). Different lowercase letters indicate significant differences ($P$<0.05). *; $P$<0.05.

**Table 1.** The correlation between diacron-reactive oxygen metabolites and biological antioxidant potential of plasma and follicular fluid (FF)

| Sample | Group | | n | r |
|---|---|---|---|---|
| Plasma | Season | FG | 8 | 0.498 |
| | | WG | 8 | −0.739 |
| | | SpG | 9 | 0.572 |
| | | SmG | 7 | −0.673 |
| | Parity | CG | 18 | 0.098 |
| | | HG | 14 | −0.093 |
| FF | Season | FG | 8 | −0.426 |
| | | WG | 8 | **0.777*** |
| | | SpG | 9 | **0.914*** |
| | | SmG | 7 | −0.056 |
| | Parity | CG | 18 | 0.412 |
| | | HG | 14 | **0.663*** |

FG, fall group; WG, winter group; SpG, spring group; SmG, summer group; CG, cow group; HG, heifer group. *; *P*<0.05.

**Table 2.** The correlation between plasma and follicular fluid (FF) of diacron-reactive oxygen metabolites, biological antioxidant potential, and oxidative stress index

| Parameter | Group | | n | r |
|---|---|---|---|---|
| d-ROMs | | All animals | 32 | **0.787*** |
| | Season | FG | 8 | 0.262 |
| | | WG | 8 | 0.488 |
| | | SpG | 9 | 0.593 |
| | | SmG | 7 | 0.615 |
| | Parity | CG | 18 | **0.724*** |
| | | HG | 14 | 0.366 |
| BAP | | All animals | 32 | 0.136 |
| | Season | FG | 8 | −0.211 |
| | | WG | 8 | 0.302 |
| | | SpG | 9 | 0.286 |
| | | SmG | 7 | 0.512 |
| | Parity | CG | 18 | −0.055 |
| | | HG | 14 | 0.129 |
| OSI | | All animals | 32 | **0.759*** |
| | Season | FG | 8 | 0.137 |
| | | WG | 8 | 0.685 |
| | | SpG | 9 | **0.850*** |
| | | SmG | 7 | 0.686 |
| | Parity | CG | 18 | **0.566*** |
| | | HG | 14 | **0.602*** |

FG, fall group; WG, winter group; SpG, spring group; SmG, summer group; CG, cow group; HG, heifer group. *; *P*<0.05.

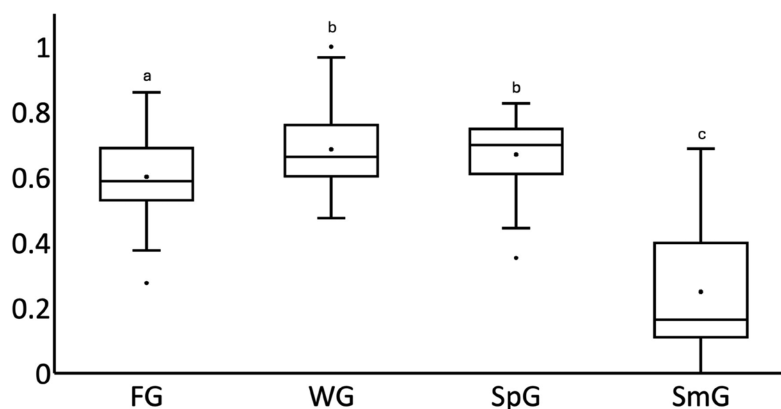


**Fig. 6.** Reactive oxygen species (ROS) of oocytes in each seasonal group. FG, fall group; WG, winter group; SpG, spring group; SmG, summer group. Different lowercase letters indicate significant differences (*P*<0.05).

In this study, THI increased in the SmG and decreased in the WG. The THI of SmG was 79.54 ± 0.13, which is a stressor to reproductive function for beef cattle because the threshold of THI that affected the conception rate of beef cattle was 73 in a previous study [2]. In contrast, the THI of WG was 49.95 ± 0.35. Exposure to THI <50 decreases the subsequent conception rate [30]. In addition, beef cattle may experience cold stress even if the temperature does not fall below the lower critical temperature [49]. Therefore, WG is considered to be exposed to cold stress, which can affect reproduction.

We measured BCS, blood biochemistry parameters, and blood concentration of AMH in all animals. The number of transferable embryos obtained from fat or lean cows was lower than that from cows under normal body conditions during *in vivo* embryo production [20]. The blastocyst rate of dairy cows decreased when the IVM medium was supplemented with stearic acid at a concentration similar to that of FF in a negative energy balance [24]. When OPU-IVP was performed, low blood AMH concentration (<0.1 ng/mL) decreased the blastocyst rate in Korean beef cows [12], and blood concentrations of BHBA and Glu had a negative correlation with the number of aspirated follicles and oocytes in Holstein cows [28]. The previous study *in vitro,* ROS in granulosa cells was increased by direct exposure to heat stress [1]. However, in the present study, none of the measured items differed significantly among the seasonal

groups; thus, it is thought that there is no effect on nutrition and AMH levels and external change of environment directly affected follicle and oocyte competence *in vivo*. By contrast, between the parity group, BUN, T-Cho, and Glu levels were increased in HG, and BHBA levels were increased in CG. This may be attributed to nutritional differences in that heifers were fed high-protein and high-fat feeds, and cows required more energy because of lactation. In dairy cows, during the summer season, serum concentration of glucose was decreased both in first and second parity, but the variance of some metabolites was different between first and second parity [17]. As such, under heat stress, metabolic conditions may vary depending on the parity. However, in this study, no significant differences in metabolic conditions were observed between parities under heat and cold stress. It is possible that the small number of cows provided for each parity prevented the detection of differences, and it will be necessary to increase the number of cows provided for each parity and reanalyze the data in the future.

The d-ROMs and BAP values in FF were correlated in the WG and SpG in this study. In peripartum cows that are considered as increasing oxidative stress, the concentration of peroxide marker and antioxidants in blood transited in parallel [6]. Therefore, in the present study, this follicular fluid correlation may show increasing antioxidants in response to increasing ROS in WG and SpG. Hence, in WG, antioxidant capacity is likely to increase in response to environmental cold stress. In contrast, the THI of SpG did not reach the level of cold stress in the present study, but cold stress in Japanese Black cows lowered fertility over 80 days after exposure [29]. Therefore, the correlation between d-ROMs and BAP values in the SpG is considered to depend on the carryover effect of winter cold stress. Among the parity groups, d-ROMs and BAP values showed a positive correlation only in the HG group. The lipid peroxidation marker malondialdehyde concentration was increased in the mammary gland cells of dairy cows fed concentrated feed [25], so heifers that were fed a high-protein and high-fat diet may have increased oxidative stress and antioxidants in this study.

In CG, the d-ROMs value correlated with plasma and FF, as was the OSI in this study. In mares, OSI was correlated with serum and FF [16], and high d-ROMs values in the blood caused a decrease in the rate of blastocyst development [15]. In humans, when an oocyte undergoes unfertilization, the d-ROMs value of the FF that contains the oocyte increased [46]. The BAP value was not correlated between plasma and FF in the present study, which is similar to previous reports on equines [16] and humans [7]. Therefore, although we could not reveal the relationship between the developmental ability of oocytes and d-ROMs value in FF in the present study, the d-ROMs value may be one of the parameters for evaluating the oocyte competence of not only equine or humans but also bovine oocytes. Transferring a substance from serum to FF is easy [35]. However, how exactly oxidants and antioxidants are transferred from the blood to the follicular fluid is not known and should be further investigated, including the mechanism of seasonal changes. The results of this study suggest that measuring d-ROMs and BAP in blood may be able to predict the degree of oxidative stress in the follicular fluid of beef cows

In this study, the OSI of FF was less than that of plasma, which was the same as in a previous equine study [16]. Additionally, in the WG and SpG, the OSI of FF decreased more than that of the plasma. A human report suggested that preovulatory follicles have a unique antioxidant mechanism [18]. For example, melatonin is a radical scavenger, and its concentration in preovulatory follicles is higher than that in blood [45]. In OPU-IVP of dairy cattle, the blastocyst rate was increased by adding melatonin to the IVM medium [14]. For these reasons, increasing oxidative stress due to environmental cold stress is considered to have caused a decrease in OSI with the effect of antioxidants, such as melatonin, in the follicle.

The d-ROMs values and OSI in the HG were lower than those in the CG. Extensive feeding of concentrate feed causes increased oxidative stress [25]; thus, feeding high-protein and high-fat feed to the HG can affect the results.

In this study, the amount of ROS increased in the WG and SpG. In contrast, the d-ROMs values in the FF did not differ significantly between the seasonal groups. When performing IVM with a pathological concentration of palmitic acid as a model of metabolic disorder in dairy cows, the gene expression levels of catalase and superoxide dismutase increased in the embryo; however, when performing IVC with an antioxidant that targets mitochondria, gene expression decreased [27]. Our previous research revealed that the addition of antioxidant to IVM medium improved oocyte quality by increasing the mitochondrial distribution pattern [9]. The results of the present study are similar to the previous study, and the ROS observed in oocytes may be due to the increased ROS produced by the mitochondria rather than transferred from the FF. However, it needs to evaluate the seasonal changes in mitochondrial function of oocyte picked up by OPU in the future. In this study, we analyzed COCs from cows and heifers without distinction. The amount of ROS in oocytes from aged cow is higher than that from young cows [26]. In the future we should confirm the differences by parity in effect of thermal to the quality of oocytes or embryos.

SmG may have been exposed to heat stress in this study; however, there were no results showing increased oxidative stress in the FF and oocytes. Morphological differences between the cumulus-oocyte complex that assessed the layer of cumulus cells and the homogeneous cytoplasm were not observed between summer and fall in Japanese Black cows [44]. In contrast, the blastocyst rate and the hatching rate were decreased when performing IVP in high THI season [8]. The reduction in oxidative stress during summer may be due to differences in feeding. The animals in the present study grazed and were able to eat green grass. In a previous study performed at the same farm, the serum concentration of β-carotene increased from spring to summer [29]. Feeding β-carotene to beef calves decreased d-ROMs value and increased BAP value in the serum [33], and to tropical dairy cows increased the conception rate [21]. Additionally, in a previous study of mice, adding β-carotene increased the releasing rate of the first polar body compared with the additive-free group when performing IVM with a high concentration of ROS [50]. In the present study, we could not measure the concentration of vitamins in the blood. Eating green grass that has a lot of vitamins, including β-carotene, may reduce oxidative stress in the summer season.

In the present study, we could not investigate embryonic development and did not measure antioxidants in the oocyte cytoplasm. We previously revealed that the blastocyst rate decreased during the summer season using oocytes from slaughtered ovaries [8]. Further studies will be needed *in vivo* to clarify the effect of oxidative stress in the blood and FF on embryo development and to elucidate the

antioxidant mechanism of the oocyte by measuring the expression of genes related to oxidative stress in the oocyte.

In conclusion, we revealed that oxidative and antioxidative dynamics are linked during winter and spring. The level of oxidative stress decreased more in FF than in the blood, especially in winter. In addition, environmental cold stress may decrease oocyte quality by changing the balance of ROS and antioxidants in FF and increasing ROS in the oocyte cytoplasm. Cold stress is carried over to spring and causes oxidative stress in oocytes. In contrast, there was no effect of oxidative stress on FF and oocytes in the summer season, but it may be that the effects of the rearing environment, such as grazing, differed depending on the season. In beef cows, the d-ROMs value of blood could be used as an indicator of redox damage to follicular fluid.

CONFLICT OF INTEREST. The authors declare that they have no conflicts of interest.

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