Mechanisms causing reduced fertility under heat stress in relation to uterine environment and oocyte developmental competence in dairy cows

(乳牛の子宮環境と卵母細胞の発生能に関連した暑熱ストレス下での受胎性低下のメカニズム)

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Abbreviations

A_4: androstenedione

AI: artificial insemination

ANOVA: analysis of variance

AT: ambient temperature

BSA: bovine serum albumin

CL: corpus luteum

CMO: carboxymethiloxime

CV: coefficient of variation

DCHFDA: 2',7'-dichlorodihydrofluorescein diacetate

DMSO: dimethyl sulfoxide

DPBS: Dulbecco's phosphate-buffered saline

E_2: estradiol-17β

EGF: epidermal growth factor

ER: estrogen receptor

ET: embryo transfer

FCS: fetal calf serum

FSH: follicle stimulating hormone

FTAI: fixed-time artificial insemination

GnRH: gonadotropin releasing hormone

GSH: reduced glutathione

GV: germinal vesicle

HEPES: 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid

HSP: heat shock protein

IETS: international embryo technology society

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVG: *in vitro* growth

IVM: *in vitro* maturation

LH: luteinizing hormone

OCGCs: oocyte-cumulus-granulosa complexes

P_4: progesterone

PGE_2: prostaglandin E_2

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$

PVA: polyvinyl alcohol

PVP: polyvinylpyrrolidone

RB: repeat breeder
RH: relative humidity
ROS: reactive oxygen species
SD: standard deviation
SEM: standard error of the mean
THI: temperature humidity index
TMB: 3,30,5,50-tetramethylbenzidine
TZPs: transzonal projections
UHP: urea hydrogen peroxide

Notes

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Contents

Preface

Heat stress can be defined as forces external to the animal that act to elevate body temperature from set-point temperature [1]. Body temperature is closely regulated by matching heat production with heat loss to the environment via conduction, convection, radiation and evaporation. Ambient temperature, relative humidity, wind speed, and solar radiation are the environmental factors that can elevates body temperature. Heat stress during the hot climate significantly reduces the productivity of livestock animals, resulting in a large economic loss which is estimated between 1.7 and 2.4 billion USD per year in USA alone [2]. Currently, the human population continues to increase, especially in the tropical and subtropical areas of the planet [3]. Livestock industry in these warm areas will need to expand to keep up with the demand for livestock product, such as meat and milk. Given the global population growth especially in warm regions and the progression of global warming, the problem caused by the influence of heat stress on livestock industry is an urgent issue.

Among the livestock animals, lactating dairy cows are highly sensitive to summer heat stress. In the last several decades, dairy cattle have been genetically selected for milk production. To maintain high levels of milk production, lactating dairy cows needs an increased amount of feed, and produce more metabolic heat compared to non-lactating cows [4]. It is thought that their body temperature apparently begins to elevate when the ambient temperature exceeds 25℃ [5]. However, a recent study showed that lactating dairy cows are heat-stressed even at the ambient temperature of 21℃, if the relative humidity is higher than 75% [6]. Heat stress significantly reduces productivity of dairy cows through negatively impacting milk yield and composition, growth and reproduction, and increasing the incidence of disease and death. Thus, the economic loss in dairy industry account for a significant proportion of the overall economic loss in the livestock industry [2].

The most common and basic strategy to alleviate the effect of heat stress on production in dairy cows is providing shade and evaporative cooling, based on sprinkling and ventilation. This cooling system helps cows to maintain normal body temperature under the hot environment. A study using a large-scale data in Israel reported the effects of cooling management on the milk production and conception rate of artificial insemination (AI) during summer [7]. The summer-to-winter milk production ratio in Israel was 0.985 under the intensive cooling management (a combination of sprinkler and forced ventilation for a total of 7.5 cumulative hour/day), indicating that decline in milk production during summer can be prevented in most part. However, the summer-to-winter conception rate was 0.607 (34/56%) even the intensive cooling was utilized, suggesting the difficulty in reproductive management in lactating dairy cows during summer.

Summer heat stress decreases the reproductive performance in dairy cows through multifactorial causes, such as disturbed follicular growth [8] and ovulation [9], suppressed

expression of estrus [10], and the increased incidence of embryonic loss [10]. The overall reproductive performance of a dairy herd is often estimated by calculating pregnancy rate, *i.e.,* the multiplication of estrus detection rate (how many cows in estrus are detected in estrus by farm personnel) and conception rate (how many cows that are inseminated are diagnosed as pregnant). Therefore, the reduced estrus detection rate and increased embryonic loss are the causes of reduced fertility. As a solution for the suppressed estrus behavior, the fixed-time AI (FTAI) using hormonal drugs has been indicated [11]. The insemination can be implemented at a fixed time without the estrus detection, namely, cows are inseminated more frequently in summer. Therefore, the use of FTAI protocols during summer can improve the overall pregnancy rate and pregnancy rates at specific timing of postpartum [12–16], although it generally does not improve conception rate. In addition, the administration of more active analogs of gonadotropin releasing hormone (GnRH) can reduce the incidence of ovulation failure in summer [17]. Thus, effective solutions are shown for the suppressed estrus expression and the impairment of ovulation caused by heat stress.

As a solution for improving summer fertility through reducing the incidence of embryonic loss, many studies consistently have shown that embryo transfer (ET) is effective; conception rates of ET are higher than those of AI in the hot season [18–22]. The efficacy of ET is based on the ideas that 1) most negative effects of heat stress on fertility are considered to involve actions on oocyte growth and maturation, fertilization, and early embryos before 4-8 cell stage and 2) the embryo has acquired resistance to elevated body temperature by the time it is transferred at the morula or blastocyst stage [23]. However, a previous study reported a decreased conception rate after ET during the hot season [24]. On the other hand, other studies found no or only slight differences in conception rates between the hot and cool seasons [25–28]. Thus, it is unclear whether heat stress reduces conception rate after ET or not, and if so, how much the degree of its influences. Heat stress alters the production and circulating levels of ovarian steroid hormones [29], which regulates the expression of uterine growth factors and cytokines [30]. In addition, it also changes the synthesis and secretion of proteins [31] and prostaglandins [32,33] in the uterine endometrium. Therefore, it has been pointed out that heat stress may reduce fertility through uterine dysfunction [8,34]. Altered uterine function induced by heat stress possibly increase the incidence of embryonic loss and decrease the conception rate even after ET.

In cattle, the epidermal growth factor (EGF) profile in the uterine endometrium has been identified as an indicator of endometrial function and fertility [35,36]. Endometrial EGF concentrations exhibit a cyclic change with two peaks on Days 2–4 and 13–14 during the estrous cycle [35,37]. The loss of these peaks reduces fertility with an increase in embryonic loss in repeat breeder (RB) and high-yielding dairy cows [36,38,39]. The normalization of the EGF

profile by treatments with hormonal drugs [40] and seminal proteins [41] restored fertility in RB cows. Furthermore, the pregnancy rate was lower in apparently normal recipient cows with low EGF concentrations on Day 3 (< 4.70 ng/g tissue weight) than in those with EGF concentrations within the normal range (33.3 vs. 76.9%) [42]. Seasonal heat stress reduces the circulating levels of ovarian steroid hormones [29], which primarily regulates the expression of endometrial growth factors and cytokines network including EGF [30,36]. Therefore, heat stress possibly induces the embryonic loss through the altered expressions of EGF in the endometrial tissue. However, the effects of heat stress on the endometrial EGF profile in lactating dairy cows have not been investigated yet.

Low conception rate caused by heat stress is not only limited to summer season, but also continues into the subsequent cooler autumn season [43,44]. As well as the data of conception rate, the reduced developmental competence of oocytes collected from middle-sized antral follicles (3-8 mm in diameter) is found in the autumn season [43,45]. The delayed effect of heat stress on fertility would be related to the damage of oocytes enclosed in small follicles during summer [46]. The period of 1-2 months after the end of summer is necessary for recovering the fertility caused by heat stress [43,44]. Given the duration of development from small antral follicles to dominant follicles [47], it is estimated that heat stress during summer damages follicles between the secondary and early antral stages (0.3-1.0 mm in diameter) and results in the continued low fertility in the subsequent cooler autumn. Although, the early antral follicle stage is the critical for oocytes to acquire the developmental competence [48], the underlying mechanisms by which heat stress disrupts the developmental competence of oocytes during the growing phase is still unclear.

Heat stress would impair the developmental competence of oocyte at growing phase through multifactorial mechanisms. Although steroid hormones are important factors for acquiring the developmental competence of oocytes derived from early antral follicles during follicular growth [48–50], heat stress disrupts the steroidogenic capacity of antral follicles of various sizes [51–53]. In addition, studies using an *in vitro* maturation (IVM) system showed that heat exposure reduced the developmental competence of oocytes by inducing the oxidative stress, *i.e.,* increasing the intracellular reactive oxygen species (ROS) and decreasing the reduced glutathione (GSH) levels [54]. GSH works to scavenge ROS and maintain the cellular redox status, and GSH is supplied to oocyte from surrounding cumulus cells via transzonal projections (TZPs) [55]. Thus, various changes induced by heat stress could reduce the developmental competence of developing oocytes. However, the effects of the alterations in steroidogenesis, oxidative stress, and TZPs between oocyte and cumulus cells in growing follicles under the heat stress on developmental competence of oocytes has not been investigated.

In order to investigate mechanisms how heat stress impairs the developmental

competence of oocytes in the small follicles, I utilized the *in vitro* growth (IVG) culture of oocyte-cumulus-granulosa complexes (OCGCs) derived from early antral follicles (0.5-1 mm in diameter). IVG culture system enables oocytes without maturational competence from early antral follicles to grow and acquire maturational and developmental competence to develop into the blastocyst stage [56,57]. If I can develop the experimental model mimicking the growth of small follicles under the heat stress, it would be easy to screen effects of factors which may improve the developmental competence of oocytes at growing phase. The effective factors found in this experimental model may contribute to a better management of dairy cows during summer to improve the fertility in the autumn season.

In Chapter I, I examined the relationship between decreased fertility during the heat stress period and uterine dysfunction caused by an altered endometrial EGF profile in dairy cows. I initially investigated the effects of seasons and regions (Hokkaido and Kyushu) on the EGF profile. Then, I examined the effects of an elevated body temperature on Day 0 (estrus) and Day 3 on the EGF profile and pregnancy rate after ET. In Chapter II, first, I examined the effect of heat exposure during IVG culture on growth, maturation, and subsequent developmental competence to the blastocyst stage. Second, to investigate the mechanisms by which heat exposure reduces the developmental competence of oocytes during growing phase, I evaluated E_2 and progesterone (P_4) production from granulosa cells, the intracellular ROS and GSH levels of oocytes, and the number of TZPs between oocytes and cumulus cells. Third, I investigated the effect of cysteine supplementation, which stimulates GSH synthesis, to IVG medium. The GSH level, the growth and developmental competence of oocytes exposed to high temperature during IVG were examined.

Chapter I: Effects of heat stress on the endometrial epidermal growth factor profile and fertility in dairy cows

Introduction

Heat stress can be defined as an environment that increases body temperature above the set-point temperature [1], and is one of the major contributing factors to low fertility in dairy cows. The conception rate of AI begins to decline when uterine temperature at insemination increases by approximately 0.5°C above the normal range (38.3-38.6°C) [58]. The temperature-humidity index (THI), which is calculated from ambient temperature (AT) and relative humidity (RH), has been widely used as an indicator of heat stress in dairy cows. The following equation is an example to calculate THI; THI = (1.8 × AT + 32) – (0.55 – 0.0055 × RH) × (1.8 × AT – 26) [59]. The typical stress threshold of THI is 72 [29]. THI higher than 72 may be reached in tropical and subtropical zones, and recently in temperate and some cold zones [60]. The effect of heat stress on fertility was not examined in detail in the latter zones approximately 3-4 decades ago, however, it is now becoming a major contributing factor for low fertility in high-yielding cows.

Summer heat stress decreases fertility through multifactorial causes, such as disturbed follicular growth and ovulation [9], the suppressed expression of estrus [10], and embryonic loss [61]. The detrimental effects of heat stress on oocytes and early embryos are considered to be the main cause of increased embryonic loss [23]. The exposure of cattle to heat stress between the follicular phase and within 3 days after AI at estrus in the natural cycle as well as after superovulatory treatment was found to decrease fertility [62–65]. Studies using *in vitro* embryo production systems also demonstrated that a high temperature (41.0°C) during *in vitro* maturation cultures [66,67] reduced the developmental competence of oocytes. Furthermore, the developmental competence of zygotes and two-cell stage embryos was reduced in an *in vitro* culture at a high temperature (41.0°C) [68,69].

Early bovine embryos after Day 3 were found to be less sensitive to heat stress [63,68,69]. Consequently, heat stress decreases the pregnancy rate of AI more than that of ET [20,21] and, thus, ET has been used to compensate for low fertility during the hot season [10]. However, the effects of heat stress on pregnancy after ET has not been well understood. A previous study reported a decreased pregnancy rate after ET in the hot season in comparison to the cool seasons [24], while other studies found no or only slight differences in pregnancy rates [25–28]. Nevertheless, changes in the production and circulating levels of ovarian steroid hormones [29], and the synthesis and secretion of proteins [31] and prostaglandins [32,33] in the endometrium by heat stress may increase the incidence of embryonic loss even after ET due to an improper endocrine environment [61] or uterine dysfunction [8,34].

In cattle, the EGF profile in the uterine endometrium has been identified as an indicator of endometrial function and fertility [35,36]. Endometrial EGF concentrations exhibit a cyclic change with two peaks on Days 2-4 and 13-14 during the estrous cycle [35,37]. The loss of these peaks reduces fertility with an increase in embryonic loss in RB and high-yielding dairy cows [36,38,39]. Alterations in the endometrial EGF profile have been linked to changes in circulating E_2 and P_4 concentrations in RB and high-yielding cows [36]. In dairy cows, a high feed intake supporting a large amount of milk production increases liver blood flow and, in turn, the clearance of E_2 and P_4 from the circulation [70]. This may cause a slower increase and lower peaks in E_2 and P_4 concentrations in the circulation [71]. Although RB cows may not necessarily be high producers, they show similar alterations in ovarian steroid hormone profiles to those in high-yielding cows [36]. Since the expression of EGF in the endometrium is primarily regulated by E_2 and P_4 [30,72], changes in circulating E_2 and P_4 concentrations may be amplified in the endometrium as an altered EGF profile [36]. Seasonal heat stress was also found to suppress the production and circulating concentrations of E_2 and P_4 in dairy cows [29]; therefore, reduced fertility during the heat stress period may be attributed, at least in part, to uterine dysfunction caused by the alterations in the endometrial EGF profile.

The study in this chapter examined the relationship between decreased fertility during the heat stress period and uterine dysfunction caused by an altered endometrial EGF profile in dairy cows. I initially investigated the effects of seasons and regions on the EGF profile on Day 3. I then examined the effects of an elevated body temperature on Days 0 and 3 on the EGF profile on Day 3 and pregnancy rate after ET.

Materials and Methods

Animals

A total of 444 Holstein cows (8,500-12,000 kg of 305-day fat-corrected milk) between 2 and 5 in parity in commercial farms in the Hokkaido (central area: 42-44°N, 141-142°E) and Kyushu (north-west area: 32-34°N, 130-131°E) regions in Japan were used. All cows were observed for estrus at least twice a day or estrus was detected using an automated activity monitor. All cows showed a normal inter-estrus interval (18-23 days) and ovulated within 48 h of the onset of estrus. In cows exhibiting weak signs of estrus, particularly during the heat stress period, estrus was confirmed by ovulation within 48 h and blood concentrations of E_2 ($\geq$ 5 pg/mL) and P_4 (< 1 ng/mL). All experimental procedures were approved by the Hokkaido University Animal Care and Use Committee (No. 16-0071).

Biopsy of endometrial tissues

Uterine endometrial tissues were obtained using a biopsy instrument (3050100, Fujihira Industry, Tokyo, Japan) under caudal epidural anesthesia with 3 ml of 2% lidocaine (2% xylocaine, AstraZeneca, Osaka, Japan) as previously described [38]. Two pieces of uterine endometrial tissues from the inter-caruncle region (25-50 mg) were obtained from the middle of 3 sections in the uterine horns, which were equally divided along the longitudinal axis. The caruncle region was distinguished from the inter-caruncle region as fluffy cut surface due to rich blood vessels. If the caruncle was greater than one-third of the tissue, another biopsy was collected. However, if the caruncle was approximately one-third or less of the biopsy, the caruncle was dissected out and the rest of the tissue was used [35]. All tissue samples were obtained from the uterine horns on the contralateral side to corpus luteum (CL). Tissues were immediately frozen in liquid nitrogen and stored at -30°C for the EGF assay.

Measurement of EGF concentrations and judgement of the EGF profile

Uterine endometrial tissue samples were processed as previously described [38,73] with a modification of changing the concentration of acetic acid (01021-70, Kanto Chemical Co., Inc., Tokyo, Japan) for extraction solution from 1 M to 0.1 M. EGF concentrations in uterine endometrial tissue extracts were assessed using double-antibody sandwich EIA with 96-well microtiter plates (Costar 3590, Corning, NY, USA) [38]. An anti-human EGF mouse monoclonal antibody (MAB636, R & D Systems, Inc., Minneapolis, MN, USA) was used as the solid-phase antibody and anti-human EGF rabbit antiserum (5022-100, Biogenesis, Poole, UK) for detection with a peroxidase-conjugated anti-rabbit IgG goat antibody (270335, Seikagaku, Tokyo, Japan). Neither of these antibodies showed significant cross-reactivity with other cytokines tested by the manufacturers. The assay system was verified using increasing concentrations of recombinant bovine EGF. A linear regression analysis of recombinant bovine EGF concentrations and assay results gave $y = 0.96x + 0.39$, $r = 0.97$ [41]. The sensitivity of the assay was 10 pg/well. Intra- and inter-assay coefficient of variations (CV) at 50 pg/well were 4.2 and 5.3%, respectively. The EGF profile was determined by the endometrial EGF concentration on Day 3; EGF concentration between 4.70 and 13.50 ng/g tissue weight (normal range) was considered to be normal, whereas that of lower than 4.70 and higher than 13.50 ng/g tissue weight was considered to be altered based on previous findings [37,38].

Measurement of rectal temperature

Rectal temperature was measured using a clinical thermometer once a day between 13:00 and 17:00 on the day of estrus (Day 0) and Day 3.

Measurement of plasma E_2 and P_4 concentrations

Plasma E_2 and P_4 concentrations were determined using competitive double-antibody enzyme immunoassays, as described previously [74]. For the E_2 assay, 2 mL of plasma was extracted with 6 mL of diethyl ether (Kanto chemical, Tokyo, Japan). For the P_4 assay, 200 μL of plasma were extracted with 2 mL of diethyl ether. Then the diethyl ether was decanted into a new tube after freezing the plasma. After evaporating the diethyl ether, 0.5 mL of acetonitrile (Kanto chemical) and 1 mL of hexane (Kanto chemical) were added and mixed well in the extracted samples for E_2 assays for delipidation. Thereafter, 1 mL of hexane was added again, and all the hexane was discarded using an aspirator. The acetonitrile was evaporated after repeating delipidation by hexane three times. Samples for E_2 assays were reconstituted with 100 μL of assay buffer (145 mM NaCl, 40 mM Na_2HPO_4, and 0.1% BSA (w/v), pH 7.2). The extracted samples for P_4 reconstituted without delipidation using acetonitrile and hexane. Extracted samples from plasma were assayed without dilution or subjected to 11-fold dilution. Samples (20 mL) were incubated with the primary antisera, and horseradish peroxidase-labeled hormone (100 mL each) in the wells of a 96-well microplate (Costar 3590, Corning, NY, USA) coated with the secondary antiserum at 4°C for 16 h. The primary antisera used for the E_2 and P_4 assays were anti-estradiol-17β-6-carboxymethyloxime (CMO)-BSA (FKA204; Cosmo Bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13; Cosmo Bio), respectively. Goat anti-rabbit serum (111-005-003; Jackson ImmunoResearch, West Grave, PA, USA) was used as the secondary antiserum. After washing all wells four times with 300 mL of washing buffer (0.05% Tween 80), 150 mL of 3,30,5,50-tetramethylbenzidine (TMB) solution (5 mM citric acid, 50 mM Na_2HPO_4, 500 mM UHP, 1 mM TMB, and 2% DMSO) was added to each well and incubated at 37°C for 40 min. The absorbances of the solution in the wells were measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan) after stopping the chromogenic reaction with 50 mL of 4 N H_2SO_4. All samples were assayed in triplicate. The assay sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and intra-assay CVs were 9.7 and 3.5% for E_2, and 4.7 and 6.5% for P_4, respectively.

AT, RH, and THI

Data on hourly AT and RH during the study period (3 years; 2015 - 2017) were obtained from the local meteorological observatory in the Hokkaido region (Sapporo and Tomakomai) and Kyushu region (Fukuoka and Kumamoto), in which the commercial farms used in the present study are located. The following equation was used to calculate THI [59].

$$THI = (1.8 \times AT + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26)$$

Monthly THI in the Kyushu region ranged from 44.3 to 79.8, whereas that in the Hokkaido region was from 31.0 to 70.4 (Fig. I-1). Mean AT and THI between June and September and between October and January in each of the four areas are summarized in Table I-

1. In the four areas, mean THI between June and September ranged from 63.7 to 76.0, while that between October and January was from 39.6 to 55.5. The number of days when daily maximum THI exceeded 72 between June and September was from 26 to 118, and that that between October and January was from 0 to 15.

Embryo transfer (ET)

ET was performed by one technician and two veterinarians. A frozen *in vivo* produced embryo (IETS standards; Codes 1-2) was transferred into the uterine horn ipsilateral to CL on Day 7.

Study design

Study 1: Effects of seasons and regions on the incidence of cows with altered EGF profile in dairy cows

Study 1 was conducted between 2015 and 2017. Lactating Holstein cows (n = 365) between 60 and 90 days postpartum in the Hokkaido and Kyushu regions were used to examine the effects of seasons and regions on the proportion of cows with an altered EGF profile and the endometrial EGF concentration on Day 3. Hokkaido is located in the northeastern region of Japan and has a cool and dry climate, whereas Kyushu is in the southwestern region and has a hot and humid climate (Table I-1 and Fig. I-1). During the heat stress period (between June and September), endometrial tissues were obtained for the EGF assay on Day 3 of the estrous cycle from 211 cows (90 cows in the Hokkaido region and 121 cows in the Kyushu region). During the control (cool) period (between October and January), endometrial tissues were obtained from 154 cows (86 cows in the Hokkaido region and 68 cows in the Kyushu region).

Study 2: Effects of rectal temperature on Days 0 (estrus) and 3 on the EGF profile and conception rate after ET

Study 2 was performed between June and September in 2017. Lactating Holstein cows (n = 79) between 60 and 90 days postpartum in the Kyushu region were used to examine the effects of rectal temperature on Days 0 and 3 on the proportion of cows with an altered EGF profile and the pregnancy rate after ET. Rectal temperature on Days 0 and 3 and the endometrial EGF concentration on Day 3 were measured in all cows. ET was performed on Day 7 of the same estrous cycle (n = 67). Pregnancy was diagnosed by palpation of the uterine tract per rectum between Days 56 and 60.

Data analysis

In study 1, the proportion of cows with an altered EGF profile was compared between the different regions and seasons using the chi-squared test. The effects of seasons (June-September and October-January), regions (Hokkaido and Kyushu) and EGF profile (normal and altered) on endometrial EGF concentrations were evaluated by the three-way analysis of variance (ANOVA). In study 2, cows were divided into four groups based in combination of rectal temperature; 39.5°C or higher (≥ 39.5°C) and lower than 39.5°C (< 39.5°C), on Days 0 and 3. The effects of rectal temperature on Days 0 and 3 on the proportion of cows with an altered EGF profile and the pregnancy rate after ET were evaluated using Fisher's exact test. Endometrial EGF concentrations were not normally distributed based on the Shapiro-Wilk test, and, thus, were transformed to ranks. The effects of the rectal temperature category (≥ 39.5°C and < 39.5°C) and days of heat stress (Days 0 and 3) on endometrial EGF concentrations were evaluated by a nonparametric two-way ANOVA. EGF concentrations were compared between cows with a rectal temperature ≥ 39.5°C and < 39.5°C on Day 0 using the Mann-Whitney U test. Pregnancy rates were compared between cows with normal and altered EGF profiles by Fisher's exact test. All statistical analyses were performed using JMP software version 14.0.0 (SAS Institute Japan, Tokyo, Japan) or SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Study 1: Effects of seasons and regions on the incidence of cows with altered EGF profile in dairy cows

In the present study, the endometrial EGF concentrations on Day 3 were within or lower than the lower limit of the normal range (4.70 ng/g tissue weight) and, thus, all altered EGF profiles were characterized with a suppressed EGF peak [37,38]. The proportion of cows with an altered EGF profile was higher between June and September than between October and January in both regions ($P < 0.05$) (Table I-2). The proportion of cows with an altered EGF profile increased by approximately 2- and 3-fold in the Hokkaido and Kyushu regions, respectively, during the heat stress period. No significant differences were observed in the proportion of cows with an altered EGF profile between the two regions in each seasonal period; however, the proportion of cows with an altered EGF profile was slightly higher in the Kyushu region than in the Hokkaido region throughout the study period ($P = 0.07$). The three-way ANOVA for seasons (June-September and October-January), regions (Hokkaido and Kyushu) and EGF profile (normal and altered) indicated only main effect of EGF profile for the endometrial EGF concentrations ($P < 0.01$). EGF concentrations in cows with normal and altered EGF profiles did not differ between the seasons in both regions. On the other hand, EGF concentrations in all cows (subtotal) were lower between June and September than between

October to January in both regions ($P < 0.05$), reflecting the higher proportion of cows showing an altered EGF profile with low EGF concentrations in June to September than October to January.

Study 2: Effects of rectal temperature on Days 0 (estrus) and 3 on the EGF profile and conception rate after ET

Rectal temperature between Days 0 and 3 were similar in both rectal temperature groups (Table I-3). Regardless of rectal temperature on Day 3, the proportion of cows with an altered EGF profile was higher in the cows with a rectal temperature ≥ 39.5°C on Day 0 than in the cows with a rectal temperature < 39.5°C on Day 0 ($P < 0.05$) (Table I-4). EGF concentrations in all cows, and in cows with normal and altered EGF profiles indicated the significant main effects of rectal temperature on Day 0 ($P < 0.05$). EGF concentrations were lower in the cows with a rectal temperature ≥ 39.5°C on Day 0 than in the cows with a rectal temperature < 39.5°C ($P < 0.05$), regardless of the EGF profile. Regardless of rectal temperature on Day 3, pregnancy rates after ET were lower in the cows with a rectal temperature ≥ 39.5°C on Day 0 than in the cows with a rectal temperature < 39.5°C on Day 0 ($P < 0.05$) (Table I-5). EGF concentrations in all recipient cows and in recipient cows with a normal EGF profile indicated the significant main effects of rectal temperature on Day 0 ($P < 0.05$). EGF concentrations in cows with a normal EGF profile were lower in the cows with a rectal temperature ≥ 39.5°C on Day 0 than in the cows with a rectal temperature < 39.5°C on Day 0 ($P < 0.05$). EGF concentrations in cows with an altered EGF profile tended to low in the cows with a rectal temperature ≥ 39.5°C on Day 0 than in the cows with a rectal temperature < 39.5°C on Day 0 ($P = 0.09$). However, in cows with the normal EGF profile, no significant differences were observed in the pregnancy rate after ET between the two rectal temperature groups on Day 0. In cows with an altered EGF profile, no difference was observed in the pregnancy rate after ET between the cows with a rectal temperature ≥ 39.5°C and < 39.5°C on Day 0. The pregnancy rate after ET was markedly lower in cows with an altered EGF profile (6.3%, n = 32) than in those with a normal EGF profile (71.4%, n = 35) ($P < 0.05$). The overall conception rate of all recipient cows throughout the present study (n = 67) was 40.3%.

Discussion

The present results demonstrated that an elevated body temperature on the day of estrus caused by heat stress increased the incidence of abnormalities in the uterine endometrial EGF profile and reduced fertility. This may be one of the mechanisms contributing to reduced fertility in summer.

The proportion of cows with an altered EGF profile (i.e., lowered EGF peak on Day 3) was similar in both regions (approximately 16%) during the control period and increased by

approximately 2- and 3-fold in the Hokkaido and Kyushu regions, respectively, during the heat stress period. An altered endometrial EGF profile has been linked to reduced fertility [36]; therefore, greater alterations in endometrial EGF profiles may explain, at least partly, the reductions observed in conception rates in summer. The degree of summer heat stress is milder in the Hokkaido region than in the Kyushu region. Kyushu is classified as a temperate zone. The number of days on which daily maximum THI exceeded 72 in this region was more than 115 and the monthly average of daily maximum THI ranged from 75.4 to 83.4 between June and September during the study period. Hokkaido is classified as a cold zone. The number of days on which daily maximum THI exceeded 72 in this region was approximately 40 and the monthly average of daily maximum THI ranged from 61.5 to 74.5 during the same period. The present results indicate that even the milder heat stress in Hokkaido was sufficient to alter the EGF profile. This result might be simply due to differences in susceptibility to heat stress in dairy cows. Dairy cows in Hokkaido region would be more sensitive to heat stress than those in Kyushu region. In addition, this may be attributed to differences in the cooling management of herds. In the Kyushu region, the majority of farms use intensive cooling management typically involving a combination of fan cooling and intermittent sprinklers, while cooling management in the Hokkaido region is limited to a less intensive fan cooling system.

The present study revealed that a rectal temperature of 39.5°C and higher on the day of estrus (Day 0), regardless of that on Day 3, resulted in the suppression of EGF concentrations on Day 3. The underlying mechanisms by which heat stress on Day 0 impairs the endometrial EGF profile may be multifactorial. Most importantly, an elevated body temperature on Day 0 may induce similar changes in E_2 and P_4 concentrations to those found in RB and high-yielding cows [36]. The alterations in plasma steroid hormones suppresses the expression of EGF in the uterus since E_2 and P_4 are the primary regulators of EGF in the endometrium [30,72]. Heat stress suppresses ovarian steroid hormone production by inhibiting the systemic endocrine system and ovarian cell activity [29]. Heat stress was previously shown to reduce the number of luteinizing hormone (LH) pulses in lactating dairy cows [75]. This may lead to a decline in E_2 secretion by granulosa cells. The exposure of cultured follicle tissues from dominant follicles to a high temperature (41.0°C) decreased E_2 production by approximately 30% from that in the control (37°C) [76]. Therefore, plasma concentrations of E_2 at the time of luteolysis [77] and estrus [78] decrease under heat stress conditions. Furthermore, heat stress was found to suppress the LH surge during the natural estrous cycle in Guernsey heifers [79] and its release in response to gonadotropin-releasing hormone administration in dairy cows [80]. The suppressed LH surge may delay the time of ovulation and CL formation; therefore, increases in the plasma concentration of P_4 may be delayed.

A reduced blood flow to the uterus may also be one of the mechanisms by which heat stress decreased the endometrial EGF concentrations on Day 3. Blood flow to the uterus increases, particularly on the day of estrus (Day 0) with positive correlations to the increased estrogen concentration and the ratio of plasma E_2/P_4 concentrations [81,82]. However, the redistribution of blood flow from visceral organs, including the ovary and uterus, to the periphery occurs for thermoregulation during heat stress [34]. An elevated uterine blood flow in response to treatment with E_2 in ovariectomized cows decreased under the heat stressed condition [83]. Decreased blood flow to the uterus under the heat stressed condition at estrus may reduce the supply of hormones including E_2 to the endometrial tissues and alter the endometrial EGF profile.

Elevations induced in body temperature by heat stress may have a direct adverse effect on the expression of EGF. A high body temperature has been suggested to exert detrimental effects on uterine cell functions [34,84]. The exposure of the cultured bovine endometrium to a high temperature increased the synthesis of heat shock protein (HSP) 70 and HSP90 [31]. Since HSPs are part of the complex of proteins that associate with P_4 and estrogen receptors [85–87], changes in HSP synthesis may alter the assembly, transport, or binding activities of steroid receptors. Therefore, heat stress may inhibit the effects of ovarian steroid hormones on EGF production in the uterus. A previous study demonstrated that an increase in the cellular levels of HSP90 at an elevated temperature negatively interfered with estrogen receptor (ER) -dependent transcription [87].

The rectal temperature showed a relatively wide range from 38.2℃ to 40.8℃ in the present study. This may be due to the differences in cooling management of farms or the daily variation of ambient temperature and relative humidity. Difference in rectal temperature can also be attributed to the difference in susceptibility to heat stress of individual cows associated with the levels of milk yield [88]. Further, genetic variation for tolerance of heat stress in dairy cows [89] could be a potential cause since the specific single nucleotide polymorphisms of genes for tolerance to heat stress has been identified [90,91].

The pregnancy rate of ET recipients showing an altered EGF profile during the summer months in the present study (6.3%, n = 32) was lower than that in a previous study that reported year-round ET results (33.3%, n=87) [42]. However, the pregnancy rate of recipients with a normal EGF profile in the present study was similar to that the previously reported (71.4 vs. 76.9%, respectively). Differences in pregnancy rates in recipients with an altered EGF profile may be associated with a combination of potential role of EGF in the regulation of luteal function via prostaglandin synthesis in the endometrium and heat stress-induced enhancements in luteolytic effects. The EGF peak on Days 13-14 (the second peak) appeared to be important for the maintenance of CL. Although the EGF concentration at the second peak was not examined in the present study, the absence and recovery of the first and second peaks coincided in

approximately 90% of cows [38]. The absence of EGF peaks would be associated with enhanced luteolytic effects because EGF increases the production ratio of prostaglandin E_2 /prostaglandin $F_{2\alpha}$ ($PGE_2/PGF_{2\alpha}$) [92] in the cultured endometrium and PGE_2 functions as a luteotropic agent [93]. Moreover, the adverse effect of the absence of EGF peak on luteolysis may become apparent since an elevated temperature enhances the secretion of $PGF_{2\alpha}$ (i.e., luteolytic factor) from a cultured bovine endometrium collected on Day 17 of the estrous cycle [94,95].

The pregnancy rate after ET between June and September (the summer period) in the present study (40.3%) was similar to that in a previous study, which was performed during the same season in the same region (43.7%, n = 197) [96]. The pregnancy rate in the present study was within the range of previously reported pregnancy rates after ET during the hot season (14.3-55.4%) [18–22,24–28,97], and higher than that of AI during the summer period in the commercial farms used in the present study (24.5%, n = 3863, data were obtained between 2016 and 2017) (unpublished data). Therefore, the present pregnancy rate after ET may be acceptable in summer trials. However, the present results indicated that heat stress, particularly on the day of estrus, decreased the pregnancy rate after ET through improper uterine functions that may be attributed to alterations in EGF profile. The pregnancy rate of ET in summer may be further improved by treatment targeting the uterine EGF expression [40,41] or an intensive cooling around the day of estrus, which has been shown to increase the conception rate of AI during the summer period [98].

In conclusion, the present results indicate that impaired fertility under heat stress conditions is associated with an increase in the proportion of cows with an altered endometrial EGF profile. The pregnancy rate after ET was reduced in cows with a high body temperature on Day 0. This result cannot be explained by the direct effects of a high body temperature on periovulatory oocytes, sperm, and zygotes [34]. It suggests that heat stress causing an elevated body temperature (≥ 39.5°C) on Day 0, but not on Day 3, disturbed the endometrial EGF profile and increased embryonic loss.

Table I-1. Ambient temperature (AT), temperature humidity index (THI), and number of days when daily maximum THI exceeded 72 between June and September and between October and January in two study regions, Hokkaido and Kyushu.

Regions		Seasons	AT (°C)	THI	Number of days when daily maximum THI exceeded 72
Hokkaido	Sapporo	Jun-Sep	19.8 ± 4.3	66.0 ± 6.2	50
		Oct-Jan	2.7 ± 6.8	40.7 ± 10.1	0
	Tomakomai	Jun-Sep	17.9 ± 3.8	63.7 ± 6.2	26
		Oct-Jan	2.4 ± 6.9	39.6 ± 10.8	0
Kyushu	Fukuoka	Jun-Sep	26.2 ± 3.7	76.0 ± 5.1	116
		Oct-Jan	12.7 ± 6.2	55.5 ± 9.3	9
	Kumamoto	Jun-Sep	26.0 ± 3.9	75.8 ± 5.1	118
		Oct-Jan	12.0 ± 7.2	51.1 ± 11.1	15

Values are presented as means ± standard deviations (SD).

Table I-2. Proportion of dairy cows showing the normal and altered epidermal growth factor (EGF) profile and their EGF concentrations on Day 3 in Hokkaido and Kyusyu regions

Region	EGF profile	June - September		October - January		Total	
		No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)	No. (%) of cows showing indicated profile	EGF concentrations (ng/g tissue weight)
Hokkaido	Normal	58 (64.4)[a]	6.71 ± 0.97	72 (83.7)[b]	6.71 ± 1.04	130 (73.9)	6.71 ± 0.99
	Altered	32 (35.6)[a]	1.66 ± 0.73	14 (16.3)[b]	1.42 ± 0.70	46 (26.1)[A]	1.59 ± 0.71
	Subtotal	90 (100)	4.92 ± 2.59[a]	86 (100)	5.81 ± 1.83[b]	176 (100)	5.37 ± 2.11
Kyushu	Normal	66 (54.5)[a]	7.01 ± 1.32	57 (83.8)[b]	6.76 ± 0.62	123 (65.1)	6.89 ± 0.44
	Altered	55 (45.5)[a]	1.12 ± 0.48	11 (16.2)[b]	1.68 ± 0.66	66 (34.9)[B]	1.21 ± 0.53
	Subtotal	121 (100)	4.33 ± 2.61[a]	68 (100)	5.97 ± 2.34[b]	189 (100)	5.01 ± 2.42
Total	Normal	124 (58.8)[a]	6.87 ± 1.12	129 (83.8)[b]	6.73 ± 0.92	253 (69.3)	6.80 ± 0.99
	Altered	87 (41.2)[a]	1.32 ± 0.68	25 (16.2)[b]	1.53 ± 0.68	112 (30.7)	1.37 ± 0.68
	Subtotal	211 (100)	4.58 ± 2.60[a]	154 (100)	5.88 ± 2.06[b]	365 (100)	5.13 ± 2.32

[a, b] Values with different letters within the same row significantly differ ($P < 0.05$).

[A, B] Values with different letters within the same column slightly differ ($P = 0.07$).

The three-way ANOVA for seasons (June-September and October-January), regions (Hokkaido and Kyushu) and EGF profile (normal and altered) indicated only main effect of EGF profile on the endometrial EGF concentrations ($P < 0.01$). None of the interactions were significant.

EGF concentrations are presented as means ± SDs.

EGF profile was determined by the value of endometrial EGF concentration on Day 3; EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal, whereas that of < 4.70 and 13.5 < ng/g tissue weight was considered to be altered [37,38].

Table I-3. Rectal temperature on Days 0 and 3 in cows with a rectal temperature ≥ 39.5ºC and < 39.5ºC in Study 2

	Rectal temperature ≥ 39.5ºC	(n)	Rectal temperature < 39.5ºC	(n)	Total	(n)
Day 0	40.0 ± 0.32 (39.6–40.8)	39	39.0 ± 0.27 (38.2–39.4)	40	39.5 ± 0.59 (38.2–40.8)	79
Day 3	40.0 ± 0.26 (39.5–40.4)	34	39.0 ± 0.28 (38.3–39.4)	45	39.4 ± 0.54 (38.3–40.4)	79

Rectal temperatures are presented as means ± SDs.

Numbers in parentheses show the ranges of rectal temperature.

Table I-4. Effects of rectal temperature on Days 0 and 3 on endometrial epidermal growth factor (EGF) concentrations in dairy cows

Rectal temperature		No. of cows	Proportion of cows with an altered EGF profile†	EGF concentrations		
Day 0	Day 3			Altered	Normal	All
≥ 39.5ºC	≥ 39.5ºC	18	66.7	2.48 ± 1.03 (12)	6.62 ± 1.45 (6)	3.86 ± 2.28 (18)
≥ 39.5ºC	< 39.5ºC	21	61.9	2.09 ± 1.01 (13)	6.82 ± 1.42 (8)	3.89 ± 2.58 (21)
Sub total		39	64.1[a]	2.28 ± 1.04[a] (25)	6.73 ± 1.44[a] (14)	3.88 ± 2.45[a] (39)
< 39.5ºC	≥ 39.5ºC	16	31.3	3.63 ± 0.40 (5)	7.74 ± 1.38[a] (11)	6.46 ± 2.23 (16)
< 39.5ºC	< 39.5ºC	24	29.2	2.86 ± 1.30 (7)	8.02 ± 1.15[a] (17)	6.51 ± 2.63 (24)
Sub total		40	30.0[b]	3.18 ± 1.10[b] (12)	7.91 ± 1.25[b] (28)	6.49 ± 2.48[b] (40)

[a, b] Values with different letters significantly differ between cows with a rectal temperature of 39.5°C or higher (≥ 39.5°C) and lower than 39.5°C (< 39.5°C) on Day 0 ($P < 0.05$).

EGF concentrations are presented as means ± SDs.

Numbers in parentheses show the number of cows.

†EGF profile was determined by the value of endometrial EGF concentration on Day 3; EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal, whereas that of < 4.70 and 13.5 < ng/g tissue weight was considered to be altered based on previous findings [37,38].

Table I-5. Effects of rectal temperature on Days 0 and 3 on endometrial epidermal growth factor (EGF) concentrations and conception rates after embryo transfer (ET) in dairy cows

Rectal temperature		EGF profile† (n)	EGF conc.	Conception (%)
Day 0	Day 3			
≥ 39.5ºC	≥ 39.5ºC	Normal (3)	6.05 ± 0.54	2/3 (66.7)
		Altered (9)	2.76 ± 0.98	1/9 (11.1)
≥ 39.5ºC	< 39.5ºC	Normal (7)	6.68 ± 0.55	5/7 (71.4)
		Altered (11)	2.22 ± 1.03	0/11 (0.0)
Sub total		Normal (10)	6.49 ± 1.35[a]	7/10 (70.0)
		Altered (20)	2.47 ± 1.05[A]	1/20 (5.0)
		All (30)	3.81 ± 2.22[a]	8/30 (26.7[a])
< 39.5ºC	≥ 39.5ºC	Normal (10)	7.95 ± 1.28	6/10 (60.0)
		Altered (5)	3.63 ± 0.40	1/5 (20.0)
< 39.5ºC	< 39.5ºC	Normal (15)	7.93 ± 1.18	12/15 (80.0)
		Altered (7)	2.86 ± 1.30	0/7 (0.0)
Sub total		Normal (25)	7.93 ± 1.18[b]	18/25 (72.0)
		Altered (12)	3.18 ± 1.10[B]	1/12 (8.3)
		All (37)	6.39 ± 2.52[b]	19/37 (51.4[b])
Total		Normal (35)	7.52 ± 1.42	25/35 (71.4[x])
		Altered (32)	2.73 ± 1.12	2/32 (6.3[y])
		All (67)	5.23 ± 2.71	27/67 (40.3)

[a, b] Values with different letters significantly differ between cows with a rectal temperature of 39.5°C or higher (≥ 39.5°C) and lower than 39.5°C (< 39.5°C) on Day 0 ($P < 0.05$).

[A, B] Values with different letters within the same column slightly differ between cows with a rectal temperature ≥ 39.5°C and < 39.5°C on Day 0 ($P = 0.09$).

[x, y] Values with different letters significantly differ between cows with normal and altered EGF profiles ($P < 0.05$).

EGF concentrations are presented as means ± SDs.

†EGF profile was determined by the value of endometrial EGF concentration on Day 3; EGF concentration of between 4.7 and 13.5 ng/g tissue weight (normal range) was considered to be normal, whereas that of < 4.70 and 13.5 < ng/g tissue weight was considered to be altered based on previous findings [37,38].

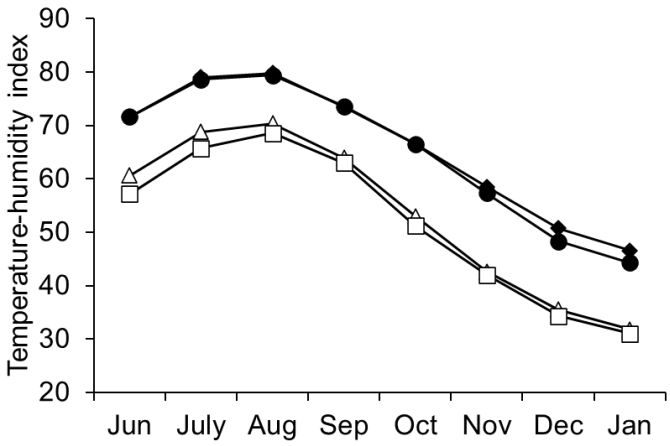

Figure I-1. Mean monthly temperature-humidity index (THI) of the study period (2015 - 2017) in two regions (Hokkaido: Sapporo △ and Tomakomai □; Kyushu: Fukuoka ◆ and Kumamoto ●).

Summary

The endometrial epidermal growth factor (EGF) profile is an indicator of uterine function and fertility in cattle. The study of this chapter aimed to investigate the effects of heat stress on the endometrial EGF profile and fertility in lactating Holstein cows. The endometrial EGF profiles of 365 cows in the Hokkaido (cold zone) and Kyushu (temperate zone) regions were examined between June and September (heat stress period, n = 211) and between October and January (control period, n = 154). EGF profiles were investigated using uterine endometrial tissues obtained by biopsy 3 days after estrus (Day 3). The proportion of cows with an altered EGF profile was higher in the heat stress period than in the control period (41.2 vs. 16.2%, $P < 0.05$). This proportion in the heat stress period increased by 2- and 3-fold in the Hokkaido and Kyushu regions, respectively. The effects of rectal temperature on Days 0 and 3 on the endometrial EGF profile were also assessed in cows (n = 79) between June and September in the Kyushu region. A single embryo was transferred to cow on Day 7 to evaluate fertility (n = 67). Regardless of the rectal temperature on Day 3, the proportion of cows with an altered EGF profile was higher (64.1 vs. 30.0%, $P < 0.05$) and the pregnancy rate after embryo transfer (ET) was lower (26.7 vs. 51.4%, $P < 0.05$) in cows with a rectal temperature ≥ 39.5°C on Day 0 than in cows with a rectal temperature < 39.5°C on Day 0. The results of this chapter indicate that alterations in the endometrial EGF profile induced by an elevated body temperature on Day 0 contributed to reductions in fertility in lactating dairy cows during the heat stress period.

Chapter II: Effect of heat exposure on the growth and developmental competence of bovine oocytes derived from early antral follicles

Introduction

Low fertility caused by summer heat stress continues into the subsequent cooler autumn season [43,44]. Consistent with the data of conception rate after AI, the developmental competence of oocytes derived from antral follicles (3-8 mm in diameter) also remains low in the cooler autumn [43,45]. This carry-over effects of summer heat stress on fertility would be caused by the damage of oocytes enclosed in the small follicles during summer [46]. The negative effects of heat stress on fertility continue for one or two months after summer heat stress ends [43,44]. Considering the estimated duration of development from small antral follicles to dominant follicles [47], it is hypothesized that summer heat stress damages between the secondary and early antral follicles (0.3-1.0 mm in diameter), then resulting in the impaired developmental competence of oocytes from dominant follicles and the continued low fertility in the cooler autumn. Especially, the early antral follicle stage is critical for oocytes to acquire the developmental competence [48], however, there are no reports focusing on the effect of heat stress on the growth of early antral follicles.

Low developmental competence of heat stressed developing germinal vesicle (GV) stage oocytes derived from antral follicles (3-8 mm) is associated with an increase in the variation in fatty acid profiles of the membrane [44], altered transcriptional levels of genes involved in oogenesis, folliculogenesis, and embryonic development [99]. The GV stage oocytes exposed to high temperature *in vitro* or collected in summer also exhibit disrupted nuclear and cytoplasmic events, including translocation of the cortical granule to the oolemma [100], and impaired mitochondrial distribution and polarization in matured oocytes [45]. However, the mechanism underlying the disruption in the developmental competence of GV stage oocytes in the process of growth is still not clear.

Altered steroidogenesis in growing follicles may be associated with reduced oocyte developmental competence during summer. Steroid hormones are important factors needed to acquire oocyte developmental competence during follicular growth. In *in vitro*, E_2 is essential for oocytes to acquire maturational and developmental competence during IVG culture of bovine OCGCs derived from early antral follicles (0.4-0.7 mm in diameter) [48–50]. However, *in vivo* studies demonstrated that the E_2 concentration in the follicular fluid of dominant follicles was lower during the hot season than the cool season [51]. Furthermore, the steroidogenic capacity of antral follicles of various sizes is also disrupted by heat stress [53]. The E_2 and androstenedione (A_4) production levels were reduced in the cultured granulosa and theca cells

obtained from medium-sized follicles (6-9 mm in diameter) three weeks after acute heat stress [52], suggesting that early antral follicles are sensitive to heat stress. However, the relationship between the steroidogenesis in growing follicles and developmental competence of oocytes under the effects of heat stress has not been investigated directly.

Hyperthermia-induced oxidative stress is suggested to be another underlying mechanism by which heat stress impairs the developmental competence of oocytes [101]. Studies using an IVM system revealed that heat exposure increases the intracellular ROS levels in bovine oocytes and reduces the percentage of oocytes developing to blastocysts [54,102]. Glutathione is the most abundant non-protein thiol in mammalian cells [103] and GSH maintains the cellular redox status, and protects the cell from ROS [104]. Granulosa cells have a critical role in supplying GSH to oocytes by cell-to-cell communication via TZPs [55]. The decrease in the intracellular GSH level in oocytes occurs along with an increase in the intracellular ROS level caused by heat exposure during IVM [54]. The supplementation of cysteine to the IVM medium, which stimulates the GSH synthesis [105], reduced the ROS level in oocytes and mitigated the negative effects of heat exposure on oocyte developmental competence [54]. If I can develop an experimental model mimicking the follicular growth under heat stress by using an IVG culture system, it may be easier to evaluate the effects of the supplementation of some substances (i.e., antioxidants) on the developmental competence of oocytes exposed to heat stress during the growth phase. The effective substances selected in the experimental model could be useful to manage dairy cows during summer to collect better quality oocytes for IVM and improve fertility in the subsequent cooler autumn.

In *in vivo*, seasonal heat stress is likely to impair the developmental competence of oocytes at growing phase not only through high body temperature, but also through other physiological changes under the heat stressed environment (i. e., impaired gonadotropin secretion, altered metabolic system due to reduced feed intake, and so on) [29]. However, in this study, I aimed to investigate the effects of high temperature within the physiological range on GV stage oocytes during the growth phase using an IVG culture system of bovine oocytes. IVG culture enables oocytes in early antral follicles (0.5-1 mm in diameter) to grow and acquire maturational and developmental competence to develop into the blastocyst stage [56,57]. Therefore, in the study of this chapter, first, I examined the effect of heat exposure during IVG culture on the competence of growth, maturation, and subsequent developmental competence to the blastocyst stage. Second, to investigate the mechanisms by which heat exposure reduces the developmental competence of GV stage oocytes, I evaluated E_2 and P_4 production from granulosa cells, the intracellular ROS and GSH levels of oocytes, and the number of TZPs between oocytes and cumulus cells. Third, I investigated the rescue effect of cysteine supplementation to IVG medium on the GSH level, the growth and developmental competence of oocytes exposed to high

temperature during IVG.

Materials and Methods

All the chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Collection of OCGCs and IVG culture

Ethical approval for animal work was not required for this study as all the bovine ovaries were derived from cattle slaughtered at two local slaughterhouses for commercial food production purposes only.

OCGCs were collected from the early antral follicles (0.5-1 mm in diameter) of bovine ovaries obtained from two slaughterhouses. Sliced ovarian cortex tissues were prepared using a surgical blade (No. 11), and follicles were dissected from cortical strips using a No. 20 blade under a stereomicroscope in an isolation medium; TCM-199 (31100-035, Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol (PVA), 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium, pH 7.4) at 37°C, as described elsewhere [106]. Early antral follicles were punctured to release OCGCs using a pair of fine forceps as described previously [107]. The growth medium was HEPES (25 mM)-buffered TCM-199 (12340-030, Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen, Waltham, MA, USA), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (PVP) (MW 360,000), 50 μg/mL gentamicin sulfate, and 10 ng/mL A_4 as a precursor for E_2. OCGCs with oocytes surrounded by a cumulus investment and attached mural granulosa-cell layer were cultured individually in a 96-well culture plate (Primaria 353872, Corning Incorporated, Corning, NY, USA) with 200 μL of growth medium for 12 days in humidified air with 5% CO_2. OCGCs in the control group were cultured at 38.5°C for 24 h, mimicking the body temperature in non-heat-stressed dairy cows (Fig. II-1) [65]. OCGCs in the heat shock group were cultured using a temperature cycle of 38.5°C for 5 h, 39.5°C for 5 h, 40.5°C for 5 h, and 39.5°C for 9 h, which is similar to the body temperature of heat-stressed dairy cows [108]. In each culture session, the temperature in the incubator was monitored using a data logger (GL10-TH, Graphtec, Kanagawa, Japan) at one-hour intervals for 12 days, and averages of the temperature were calculated (Fig. II-1). In addition, increases in temperature *in vitro* cause decreased CO_2 solubility in the medium and an increase in medium pH, therefore I also confirmed the effects of elevated culture temperature on the pH value of the IVG medium. Half (100 μL) of the IVG medium without OCGCs was replaced every four days (days 4, 8, and 12).

The pH values in the spent IVG medium were evaluated by the i-STAT system ($G3^+$ cartridge, Abbot Point of Care Inc., Princeton, NJ, USA). Every measurement was performed at 22:00 (after 5h incubation at 40.5℃ in the heat shock group). I found that the pH values in both the groups were in the range of 7.30 to 7.40, and were higher in the heat shock group than in the control group ($P < 0.05$). However, the difference in the pH values between the groups was less than 0.02. In a previous study, there were no significant differences between pH 7.2 and pH 7.4 in the meiotic arrest of oocytes cultured with a meiotic inhibitor (dibutyryl cyclic adenosine monophosphate or hypoxanthine) and follicle-stimulating hormone (FSH)-stimulated meiotic resumption of oocytes [109]. The difference in pH between the control and heat shock groups is thought to be a slight difference throughout the 12 days of IVG culture and it is unlikely that the increase in the pH value caused by elevated culture temperature adversely affected the developmental competence of oocytes in the present study. (Fig. II-2). At the onset of the IVG culture, OCGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan). The diameters of the oocytes on day 0 were assessed using software (Motic Images Plus 2.2s, Shimadzu). In the 12-day IVG culture, half (100 μL) of the medium was replaced every four days. The spent medium was stored at -30°C for a hormone assay.

Evaluation of OCGC morphology

The morphological appearance of the OCGCs was evaluated every four days during IVG culture under an inverted microscope. OCGCs with an evenly granulated ooplasm and completely enclosed by several layers of healthy cumulus and granulosa cells with or without cavities (Fig. II-3b, c, respectively) were defined as normal. OCGCs with scattered cumulus and granulosa cells or denuded oocytes were defined as abnormal (Fig. II-3d).

Evaluation of growth and nuclear maturation

After IVG culture, OCGCs were subjected to IVM as previously described [110]. Briefly, OCGCs were individually cultured in microwell plates (Mini Trays 163 118; NUNC, Roskilde, Denmark) containing 6 mL of IVM medium at 38.5℃ under 5% CO_2 in air for 22 h. The IVM medium consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 20 μg/mL FSH (from porcine pituitary), 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate. After IVM, the oocytes were denuded by pipetting, photographed, and their diameters were measured. Oocyte nuclear maturation was assessed using 1% (w/v) aceto-orcein staining, as described elsewhere [111]. Oocytes with a polar body were defined as mature.

Evaluation of developmental competence

After IVM, some OCGCs were subjected to *in vitro* fertilization (IVF) as previously described [112]. Briefly, after thawing frozen semen from a Holstein bull in a 37°C water bath for 40 sec, motile sperm (2×10^6 sperm/mL) were separated by centrifuging twice (300×g) for 5 min in a semen preparation medium (BO-SemenPrep; IVF Bioscience, Cornwall, UK). OCGCs were cultured in a group with separated sperm in 500-μL of fertilization medium (BO-IVF; IVF Bioscience) in 4-well dishes (9-15 zygotes/well) for 18 h at 38.5℃ in 5% CO_2 in air. After IVF, the presumptive zygotes were cultured in a 30-μL droplet (9-15 zygotes/ droplet) of culture medium (BO-IVC; IVF Bioscience) with an overlay of paraffin oil at 5% O_2, 5% CO_2, and 90% N_2 at 39℃ for 150 h. The cleavage and blastocyst rates were examined at 48 h and 168 h after IVF, respectively. Cell numbers in the blastocysts were counted using an air-drying method [113].

E_2 and P_4 assays of IVG culture medium

Spent medium from IVG was assayed for the E_2 and P_4 concentrations using a competitive double-antibody enzyme immunoassay as described in Chapter I. Samples were subjected to 2 to 200-fold serial dilutions with assay buffer. The assay sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and intra- coefficients of variation were 5.6 and 4.0% for E_2 and 3.4 and 3.9% for P_4, respectively. The steroid hormone production during each period (days 0-4, 4-8, and 8-12) was calculated using the following formula:
Steroid hormone production (ng) = 0.2 (mL) × Concentration at the end of the period (ng/mL) – 0.1 (mL) × Concentration at the start of the period (ng/mL)

Detection of ROS and GSH in oocytes

Briefly, denuded oocytes were washed with Dulbecco's PBS (DPBS) (Nissui Pharmaceutical CO., LTD. Tokyo, Japan) supplemented with 0.2% PVP (DPBS-PVP) and treated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) for ROS staining, or 10 μM CellTracker Blue (Thermo Fisher Scientific Inc. Waltham, USA) for GSH staining at 38.0℃ for 30 min. The denuded oocytes were then washed three times with 0.2% DPBS-PVP, and placed on a glass slide. Images of the oocytes were acquired using a digital fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The fluorescence intensity (arbitrary unit) was calculated using Image J software. The mean fluorescence intensity of the control group (experiment 2) or the cysteine untreated group (experiment 3) in each replicate was set at 1.0, and that of the heat shock group or the cysteine treated group was expressed as a relative value, as previously described [114].

Evaluation of the transzonal projection (TZP) number

The OCGCs were washed in DPBS containing 0.1% PVA (DPBS-PVA), then fixed in 4% paraformaldehyde in DPBS-PVA for 60 minutes. Fixed OCGCs were washed in DPBS-PVA, and the oocytes were denuded with a fine pipette. The denuded oocytes were then stored in DPBS-PVA containing 1 mg/mL bovine serum albumin (DPBS-PVA-BSA) at 4℃ overnight. The oocytes were treated with fluorescein isothiocyanate-labeled Phalloidin (2 μg/mL in DPBS–PVA–BSA; Phalloidin–fluorescein isothiocyanate) at 38.5℃ for 90 minutes. The oocytes were washed in DPBS–PVA–BSA before being mounted on glass slides and observed under a confocal laser scanning microscope (LSM800) with ZEN software (Carl Zeiss, Jena, Germany). The pictures of oocytes were visualized using software (NIS-Elements D 4.10.00, Nikon, Tokyo, Japan). The pictures of the widest cross-section of each oocyte were selected for counting TZPs. In each picture of an oocyte, the TZPs completely crossed/traversing the zona pellucida from the cumulus cells to the oocytes were confirmed visually and counted one by one using a counter tool of the software manually.

Experimental design

A schematic drawing of the experimental design is shown in Fig. II-4. The present study was conducted from June 2020 to February 2022 in Hokkaido, Japan. Bovine ovaries were collected from a slaughter house: slaughterhouse 1 (Hokkaido Hayakita meat inspection center, Abira, Hokkaido; 42°43'30"N, 141°46'40"E) for experiment 1 and 2, and from two slaughterhouses: slaughterhouse 1 and slaughterhouse 2 (NICHIRO CHIKUSAN CO., LTD., Nayoro Plant, Nayoro, Hokkaido; 44°22'29"N, 142°27'39"E) for experiment 3. The mean monthly AT ranged from -7.5 to 21.6℃ in the location of slaughterhouse 1, and -9.6 to 23.2℃ in that of slaughterhouse 2, respectively. To exclude bias among the sampling timings and between each slaughter house, I examined two groups every time in each replicate. In each experiment, OCGCs were evenly distributed to the control and the heat shock groups (experiment 1 and 2), or the cysteine untreated and the cysteine treated groups (experiment 3) according to the oocyte diameter and the thickness of the mural granulosa-cell layer.

During IVG culture, OCGCs in the heat shock group (experiment 1 and 2) and in both groups (experiment 3) were cultured at a range of temperatures (38.5°C for 5 h, 39.5°C for 5 h, 40.5°C for 5 h, and 39.5°C for 9 h) that were similar to those experienced by heat-stressed cows (Fig. II-1) [108]. To obtain the temperature condition, I changed the temperature setting of the incubator. OCGCs in the control group were cultured at a constant temperature of 38.5°C for 24 h, mimicking the body temperature of cows without heat stress [65].

Study 1: Effects of heat exposure during IVG culture on OCGC morphology, oocyte growth, and maturational and oocyte developmental competence

A total of 1182 OCGCs were cultured for study 1 and 2 with the following sample distribution: 590 OCGCs in the control group and 592 OCGCs in the heat shock group. The viability of OCGCs was calculated based on 689 OCGCs that were cultured for 12 days (345 from the control group and 344 from the heat shock group). The percentage of antrum formation in the granulosa cell layer was calculated based on 406 OCGCs surviving on day 12 (208 from the control group and 198 from the heat shock group). Both the viability and antrum formation of the OCGCs were evaluated at days 4, 8 and 12 of IVG culture. Some surviving OCGCs (58 from the control group and 54 from the heat shock group) after 12 days of IVG culture were subjected to IVM culture to check the nuclear status of the oocytes. The diameters of these oocytes before IVG culture and after IVG and IVM cultures were also measured. Some surviving OCGCs (47 from the control group and 48 from the heat shock group) were subjected to IVM, IVF, and *in vitro* culture (IVC) to investigate their developmental competence. OCGCs cultured until IVM (evaluation of growth and nuclear status) or IVC (evaluation of developmental competence) were derived from different culture sessions. The blastocyst rates were calculated based on the number of inseminated oocytes.

Study 2: Effects of heat exposure during IVG culture on the steroidogenesis of granulosa cells, the ROS and GSH levels in oocytes, and the number of TZPs in oocytes

The production levels of E_2 and P_4 in the culture medium were assessed every four days (days 4, 8, and 12). Spent medium was derived from the IVG culture of 33 OCGCs in the control group and 31 OCGCs in the heat shock group. The intracellular ROS and GSH levels of the oocytes were evaluated every four days during IVG culture. For the ROS assay, 141 OCGCs were used (71 from the control group and 70 from the heat shock group). For the GSH assay, 177 OCGCs were used (85 from the control group and 92 from the heat shock group). The number of TZPs was evaluated at days 0, 4, 8, and 12 of IVG culture. To evaluate the number of TZPs, 174 OCGCs were used (76 from the control group, 73 from the heat shock group and the remaining 25 OCGCs were used to evaluate the number of TZPs at day 0).

Study 3: Effects of cysteine supplementation on the growth, developmental competence, and GSH levels of oocytes exposed to high temperature during IVG culture

A total of 367 OCGCs were cultured for study 3 with the following sample distribution: 184 OCGCs in the cysteine untreated group (IVG medium without cysteine supplementation) and 183 OCGCs in the cysteine treated group (IVG medium supplemented with 1.2 mM cysteine). The cysteine concentration of medium in the cysteine untreated group was not 0.0 mM exactly, since the basal medium (HEPES-buffered TCM-199) contains about 0.6 μM cysteine. OCGCs in both groups were cultured in a range of temperatures (38.5°C for 5 h, 39.5°C for 5 h, 40.5°C

for 5 h, and 39.5°C for 9 h) that were similar to those experienced by heat-stressed cows [108]. Some surviving OCGCs (27 from the cysteine untreated group and 36 from the cysteine treated group) after 12 days of IVG culture were subjected to IVM culture to check the nuclear status of the oocytes. The diameters of these oocytes before IVG culture and after IVG and IVM cultures were also measured. Some surviving OCGCs (49 from the cysteine untreated group and 43 from the cysteine treated group) were subjected to IVM, IVF and IVC to investigate their developmental competence. Other surviving OCGCs (18 from the cysteine untreated group and 19 from the cysteine treated group) after 12 days of IVG culture were used to evaluate the GSH levels in oocytes.

Statistical analysis

All statistical analyses were performed using software (JMP version 14.0.0, SAS Institute, Cary, USA). The normality of distribution was analyzed by the Shapiro-Wilk W test, and the homogeneity of variance was analyzed by the Levene test for all data. The growth of the oocytes before and after culture was analyzed by the Student's *t*-test. The viability and antrum formation of OCGCs, and the nuclear maturation and cleavage rates were analyzed by the chi-squared test, while the blastocyst rate was analyzed by Fisher's exact test. As some data for the E_2 and P_4 production and the number of TZPs were non-parametric, the E_2 and P_4 production levels and the number of TZPs were analyzed by the Kruskal-Wallis test followed by the Steel-Dwass test. The pH value of the IVG medium between the groups on the same culture day were analyzed by the Student's *t*-test. The cell numbers in the blastocysts between the groups in study 3 were analyzed by the Mann-Whitney U test. P- values of less than 0.05 were considered significant.

Results

Study 1: Effects of heat exposure during IVG culture on OCGC morphology, oocyte growth, and the maturational and developmental competence of oocytes

During IVG culture, I observed the morphological appearance of OCGCs in the two groups, but there was no significant difference in the viability or antrum formation rates of OCGCs between the control and heat shock groups (Fig. II-5). Changes in oocytes diameter before and after culture were smaller in the heat shock group (8.0 ± 0.7 μm; mean ± standard error of the mean (SEM)) than in the control group (12.1 ± 0.7 μm; mean ± SEM) ($P < 0.05$) (Fig. II-6). The nuclear maturation and cleavage rates were similar between the control group (62.1% and 55.3%, respectively) and the heat shock group (51.9% and 45.8%, respectively) (Fig. II-6 and Table II-1). However, no oocytes developed to blastocysts in the heat shock group (0.0%), while

27.7% of oocytes developed to blastocysts in the control group ($P < 0.05$) (Table II-1).

Study 2: Effects of heat exposure during IVG culture on the steroidogenesis of granulosa cells, ROS and GSH levels in oocytes, and number of TZPs in oocytes

The E_2 and P_4 production levels were similar between the control and heat shock groups (Fig. II-7). In both groups, the E_2 production increase from days 0-4 to 4-8 was maintained until the end of the IVG culture (days 8-12). On the other hand, P_4 production continuously increased during the culture period. The E_2/P_4 ratio did not differ between the two groups. Furthermore, the intracellular ROS levels in the oocytes were similar between the two groups (Fig. II-8); however, the GSH levels in the oocytes were lower in the heat shock group than in the control group at days 8 and 12 (Fig. II-9). At day 12, the GSH levels in the heat shock group (0.57 ± 0.06; mean ± SEM) were about half of those in the control group (1.00 ± 0.00; mean ± SEM). The number of TZPs between the oocytes and the surrounding cumulus cells did not differ between the two groups (Fig. II-10). In both groups, the number of TZPs decreased until day 8 of IVG culture.

Study 3: Effects of cysteine supplementation on the growth, developmental competence and GSH levels of oocytes exposed to high temperature during IVG culture

The nuclear maturation and cleavage rates did not significantly differ between the cysteine treated group (66.7% and 67.4%, respectively) and the cysteine untreated group (55.6% and 57.1%, respectively) (Fig. II- 11, Table II-2). However, the changes in oocytes diameter before and after culture were greater in the cysteine treated group (11.4 ± 1.0 μm; mean ±SEM) than in the cysteine untreated group (8.8 ± 1.0 μm; mean ± SEM) ($P < 0.05$) (Fig. II-11). The blastocyst rate was significantly higher in the cysteine treated group (27.9%) than in the cysteine untreated group (6.1%) ($P < 0.05$) (Table II-2). In addition, the cell number in the blastocysts was slightly higher in the cysteine treated group (122.1 ± 10.8; mean ± SEM) than in the cysteine untreated group (74.3 ± 6.8; mean ± SEM) ($P = 0.06$). The intracellular GSH levels in oocytes after 12 days of IVG culture were higher in the cysteine treated group (1.91 ± 0.22; mean ± SEM) than in the cysteine untreated group (1.00 ± 0.00; mean ± SEM) (Fig. II-12).

Discussion

To the best of our knowledge, I have for the first time demonstrated that heat exposure during IVG culture impairs the growth and developmental competence of oocytes derived from early antral follicles (0.5-1 mm). The intracellular GSH depletion in oocytes can be one cause of the impaired growth and developmental competence of oocytes induced by heat exposure

during oocyte growth from early antral follicles.

Although the cleavage rate was similar between the control and heat shock groups, the blastocyst rate was significantly lower in the heat shock group than in the control group. This result is similar to previous studies that compared the developmental competence of oocytes derived from 3-8 mm follicles in summer and winter [99,115]. These studies also showed that the cleavage rates were similar between the groups or slightly lower in summer than in winter, whereas the blastocyst rates were significantly lower in summer than in winter. However, they compared the results between two different experimental terms and using different oocyte sources. Our experimental model can be used to investigate the mechanisms by which summer heat stress impairs the developmental competence of oocytes using the same oocyte sources at the same time between the control and heat shock groups.

The changes in oocytes diameters during culture was significantly smaller in the heat shock group than in the control group. Impaired oocyte growth can be one of the characteristics associated with the reduced developmental competence of oocytes caused by heat exposure. It is known that the full competence for the meiotic maturation and subsequent embryonic development is acquired at an oocyte diameter of about 110 μm [116,117]. Therefore, an oocyte diameter ≥110 μm could be an important indicator to determine whether or not an oocyte will acquire subsequent developmental competence. Consistent with the previous study, the percentage of oocytes ≥110 μm in diameter was higher in the control group (75.9%) than in the heat shock group (46.3%) ($P < 0.01$) in the present study.

The E_2 and P_4 production levels did not differ between the control and heat shock groups. Consistent with this finding, the rates of antrum formation, which can be an indicator of healthy steroidogenesis in OCGCs [49,106], did not differ between the two groups. A previous study showed that OCGCs that formed an antrum produce more E_2 and less P_4 than OCGCs without an antrum in bovine IVG [106]. On the other hand, summer heat stress reduces the peripheral E_2 concentration [77,78] and the E_2 concentration in the follicular fluid of the dominant follicle in dairy cows in the late lactation period [51]. The P_4 concentration in the follicular fluid of the dominant follicle is not affected by seasonal heat stress in lactating dairy cows [51]. One possible reason I could not find any difference in E_2 production compared to the *in vivo* situation may have been the absence of theca cells in the IVG culture system in the present study. Heat stress may suppress E_2 production by inhibiting the systemic endocrine system or the function of theca cells *in vivo*. Heat stress was previously shown to reduce the number of LH pulses in lactating dairy cows [75]. In addition, theca cells are highly susceptible to heat stress; seasonal heat stress drastically reduces A_4 production by theca cells [51], and this reduction seems to continue for a long period after the end of summer heat stress [51,52]. These changes may lead to a decline in E_2 secretion by granulosa cells *in vivo*. Our results clearly suggest that granulosa

cells from early antral follicles have relatively higher resistance against high temperature to maintain steroidogenesis, which is the main physiological function of granulosa cells during follicular growth.

Although the intracellular ROS levels in the oocytes did not differ between the control and heat shock groups, the GSH levels in the oocytes were lower in the heat shock group than in the control group. GSH is one of the most important scavengers of ROS [104]. GSH consumption may have been higher in the heat shock group than in the control group to alleviate the increased oxidative stress in the oocyte cytoplasm. In the present study, a GSH level decline in the heat shock group was observed at days 8 and 12. The diameter of oocytes becomes significantly larger even during the late culture period (between days 10 and 12) in the bovine IVG of OCGCs derived from early antral follicles [107]. In addition, the number of granulosa cells during IVG culture of OCGCs markedly increases between days 4 and 12 [56]. Therefore, the demand for amino acids for GSH synthesis (cysteine, glutamic acid, glycine) may increase to support the growth of oocytes, granulosa cell proliferation, and to maintain the oxidative stress in OCGCs as the culture period becomes longer. Therefore, the GSH supply from cumulus-granulosa cells to oocytes during this period may have been reduced in the heat shock group. Although I hypothesized that GSH depletion in the heat shock group may also be caused by impaired intracellular communications between growing oocytes and the surrounding cumulus cells, there was no difference in the number of TZPs between the control and heat shock groups. These results suggest that communication between oocytes and surrounding somatic cells could be maintained at the same level as the control group in the heat shock group, while the production of GSH in granulosa cells or oocytes themselves diminished in the heat shock group. In a future study, I should examine the enzymes related to GSH synthesis and consumption in OCGCs and their metabolism of amino acids, which is necessary for GSH synthesis in the IVG culture medium.

OCGCs in the heat shock group showed lower developmental competence concurrent with reduced intracellular GSH levels in the oocytes than those in the control group. In addition, the supplementation of cysteine, which stimulates GSH synthesis, increased the intracellular GSH level, growth, and developmental competence of the oocytes exposed to high temperature during IVG. These findings are consistent with previous reports indicating a relationship between low GSH levels in oocytes before or after IVM and their impaired developmental competence. Heat exposure during IVM [54] or the addition of a GSH synthesis inhibitor to the pre-IVM culture medium [55] decreased the intracellular GSH levels in oocytes and their developmental competence. This reduced developmental competence of oocytes subjected to intracellular GSH depletion is probably attributable to the decrease in antioxidant capacity during subsequent embryonic development. The results suggest that heat exposure to OCGCs in the growth phase impairs the growth and the developmental competence by depletion of GSH in the oocyte

cytoplasm.

The study suggests that OCGCs derived from early antral follicles are susceptible to high temperature in the physiological range. It takes about one month for an early antral follicle (0.5-1 mm in diameter) to develop into a large dominant follicle [47]; therefore, impaired oocyte developmental competence caused by heat exposure to early antral follicles during summer could be associated with low fertility in the subsequent cooler autumn. Some treatments or feeding management to improve the antioxidative capacity during the summer could ameliorate the negative effects of heat stress on the early antral follicles, thereby improving oocyte quality and fertility in the subsequent autumn. The culture system developed in the present study could replace *in vivo* trials to look for possible antioxidants to improve low oocyte quality and fertility caused by persistent effects of summer heat stress.

In conclusion, heat exposure during the IVG culture of OCGCs derived from early antral follicles impaired the growth of oocytes and the percentage of oocytes developing to the blastocyst stage. The study in this chapter suggested that the intracellular GSH depletion in oocytes (decrease in the antioxidative capacity) is one cause of the low oocyte developmental competence caused by summer heat stress, which can lead to impaired fertility in the subsequent autumn. However, it is necessary to keep in mind that I focused on the effects of high temperature within the physiological range on the cultured oocytes at growing phase in the present study. Not only high body temperature, but also alterations in gonadotropin secretion and metabolic system would be involved in low developmental competence of GV oocytes caused by seasonal heat stress in *in vivo*. Further studies are necessary to clarify more detailed mechanisms by which summer heat stress reduces oocyte competence during the oocyte growth.

Tables and figures

Table II-1. Effects of heat exposure during *in vitro* growth (IVG) culture of oocyte-cumulus-granulosa complexes (OCGCs) on their oocyte developmental competence.

Group	No. of oocytes (replicates)	Cleavage (%)	Blastocyst (%) *	Cell no. in blastocysts (n)
Control	47 (4)	55.3	27.7[a]	104.5 ± 9.3 (13)
Heat shock	48 (4)	45.8	0.0[b]	-

* Blastocyst rates were calculated based on the number of inseminated oocytes.

Cell number in the blastocysts is presented as means ± SEM.

[a, b] Different superscripts indicate significant differences between the groups ($P < 0.05$).

Table II-2. Effect of cysteine supplementation on the developmental competence of oocytes exposed to high temperature during *in vitro* growth (IVG) culture

Treatment	No. of oocytes (replicates)	Cleavage (%)	Blastocyst (%)*	Cell no. in blastocysts (n)
Cysteine (-)	49 (4)	57.1	6.1^{b}	$74.3 \pm 6.8\ (3)^{B}$
Cysteine (+)	43 (4)	67.4	27.9^{a}	$122.1 \pm 10.8\ (12)^{A}$

* Blastocyst rates were calculated based on the number of inseminated oocytes.

Cell numbers in the blastocysts are presented as means ± SEM.

$^{a, b}$ Different superscripts indicate significant differences between the groups ($P < 0.05$).

$^{A, B}$ Different superscripts indicate slight differences between the groups ($P = 0.06$).

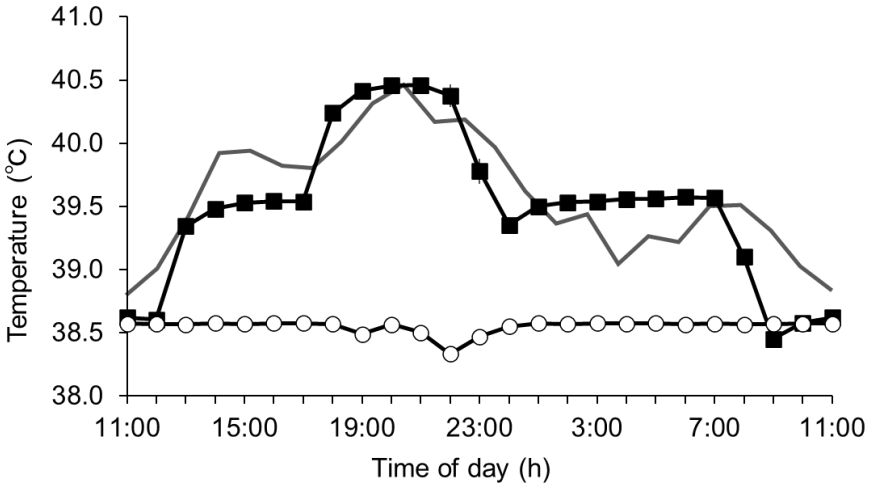

Figure II-1. Temperature conditions during *in vitro* growth (IVG) culture in the control and heat shock groups

Daily changes in the rectal temperature of lactating dairy cows under heat stress (solid line), and culture temperatures for the heat shock group (■) and control group (○) monitored by a data logger placed in the incubator (averages of temperature measured every hour for 12 days). The horizontal axis indicates the time of temperature change in the incubator. Oocyte-cumulus-granulosa complexes (OCGCs) in the heat shock group were cultured at a range of temperatures (38.5°C for 5 h, 39.5°C for 5 h, 40.5°C for 5 h, and 39.5°C for 9 h) similar to those experienced by heat-stressed cows [108]. OCGCs in the control group were cultured at a constant temperature of 38.5°C for 24 h, mimicking the body temperature of cows without heat stress [65].

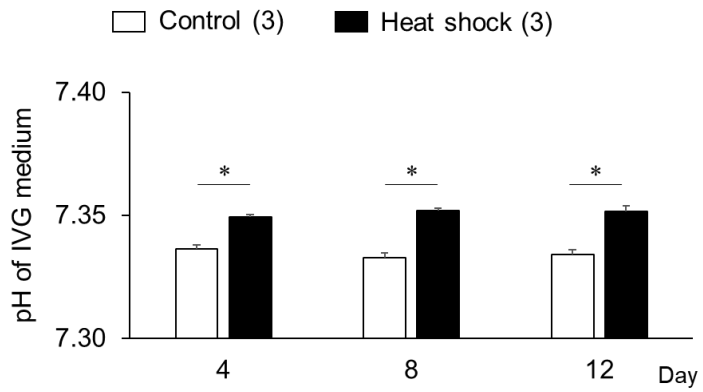

Figure II-2. Effect of elevated culture temperature on the pH value of *in vitro* growth (IVG) medium without oocyte-cumulus-granulosa complexes (OCGCs).

Numbers in parentheses indicate the number of IVG medium used for pH measurement. The experiment was repeated thrice. Error bars indicate SEM. * An asterisk indicates a significant difference between the control and heat shock groups on the same day ($P < 0.05$).

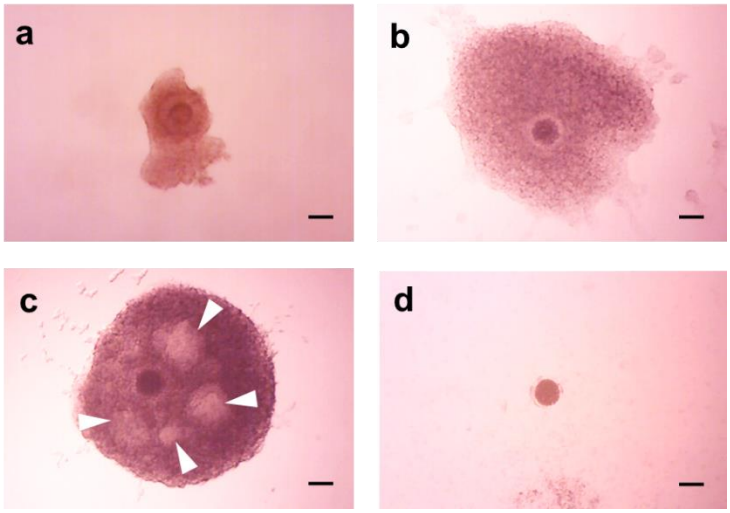

Figure II-3. Morphology of oocyte-cumulus-granulosa complexes (OCGCs) before and after 12 days of *in vitro* growth (IVG) culture.

(**a**) Isolated OCGC before IVG culture. (**b**) Surviving OCGC without antrum formation in the granulosa cell layer after 12 days of IVG culture. (**c**) Surviving OCGC with antrum formation (white arrowhead) in the granulosa cell layer. (**d**) Degenerated OCGCs after 12 days of IVG culture. Scale bar = 100 µm.

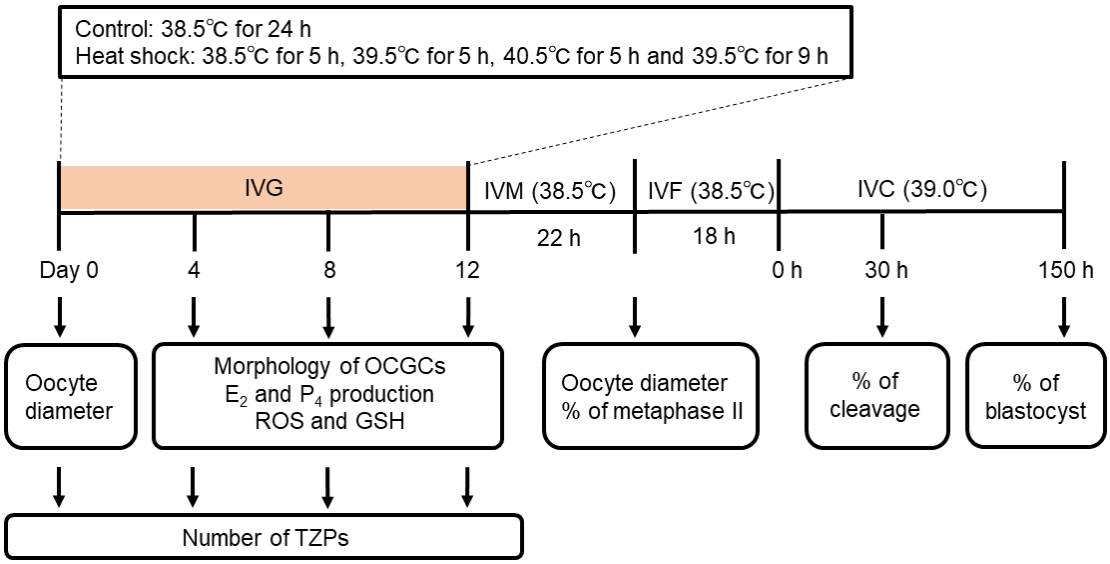

Figure II-4. Schematic illustration of the experimental design

OCGCs derived from early antral follicles (0.5-1 mm in diameter) were cultured for 0, 4, 8, or 12 days in an IVG culture. The oocyte diameter was evaluated on day 0 of the IVG culture. The morphology of OCGCs (viability of OCGCs and antrum formation in granulosa cell layers) was evaluated every four days during the IVG culture (days 4, 8, and 12). After 12 days of IVG, some surviving OCGCs were subjected to *in vitro* maturation (IVM). After IVM, the diameter and nuclear status of some oocytes were evaluated. Some oocytes after IVM were subjected to *in vitro* fertilization (IVF) and an *in vitro* culture (IVC) to evaluate developmental competence. OCGCs cultured until IVM (evaluation of growth and nuclear status) or IVC (evaluation of developmental competence) were derived from different culture sessions. The concentrations of estradiol-17β (E_2) and progesterone (P_4) in the IVG media, and the intracellular reactive oxygen species (ROS) and reduced glutathione (GSH) levels in the oocytes were evaluated every four days during IVG culture (days 4, 8, and 12). The number of transzonal projections (TZPs) was evaluated on day 0 and every four days during IVG culture (days 4, 8, and 12).

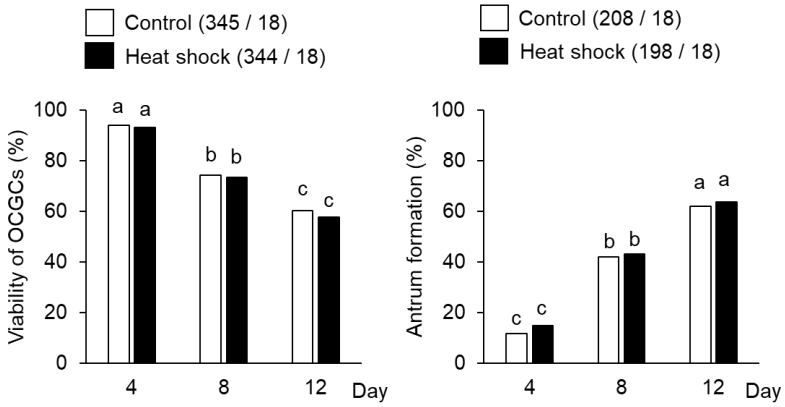

Figure II-5. Effects of heat shock during *in vitro* growth (IVG) culture on the viability, antrum formation of oocyte-cumulus-granulosa complexes (OCGCs).

Numbers in parentheses indicate the number of OCGCs and replicates.

The viability of OCGCs was calculated based on 689 OCGCs that were cultured until the end of IVG culture (12 days) (345 from the control group and 344 from the heat shock group). The percentage of antrum formation in the granulosa cell layer was calculated based on 406 OCGCs surviving on day 12 (208 from the control group and 198 from the heat shock group). [a-c] Different letters indicate a significant difference between the duration of culture in the same group ($P < 0.05$).

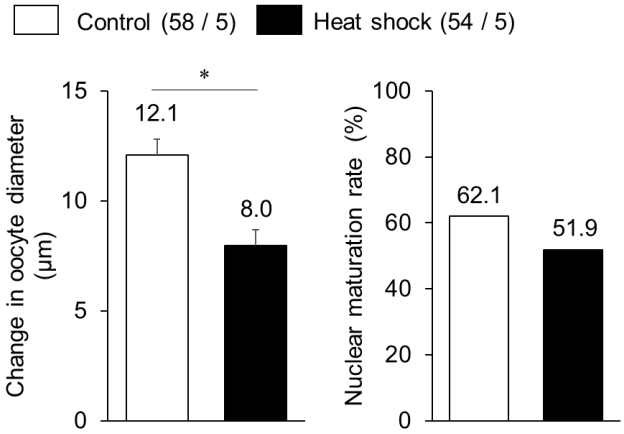

Figure II-6. Effects of heat shock during *in vitro* growth (IVG) culture on the growth and nuclear status of oocytes derived from early antral follicles.

Values on the bar graphs indicate the changes in oocytes diameters before and after culture, and the percentage of nuclear maturation, respectively. Numbers in parentheses indicate the number of oocytes and replicates. * An asterisk indicates a significant difference between the control and heat shock groups ($P < 0.05$). Error bars indicate SEM.

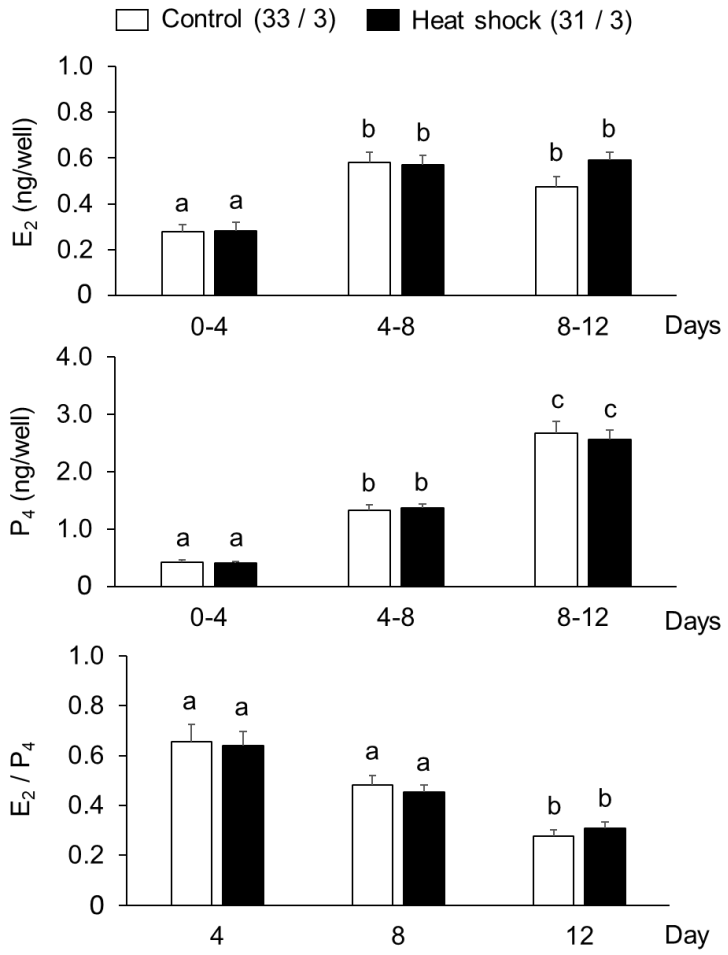

Figure II-7. Effects of heat shock during *in vitro* growth (IVG) culture on the production of estradiol-17β (E_2) and progesterone (P_4) by oocyte-cumulus-granulosa complexes (OCGCs), and the E_2/P_4 ratio in culture media.

Numbers in parentheses indicate the number of OCGCs and replicates. The culture media for the hormone assays were derived from some OCGCs used to evaluate the nuclear status after *in vitro* maturation (three replicates). $^{a\text{-}c}$ Different letters indicate significant differences between culture periods in the same group ($P < 0.05$). Error bars indicate SEM.

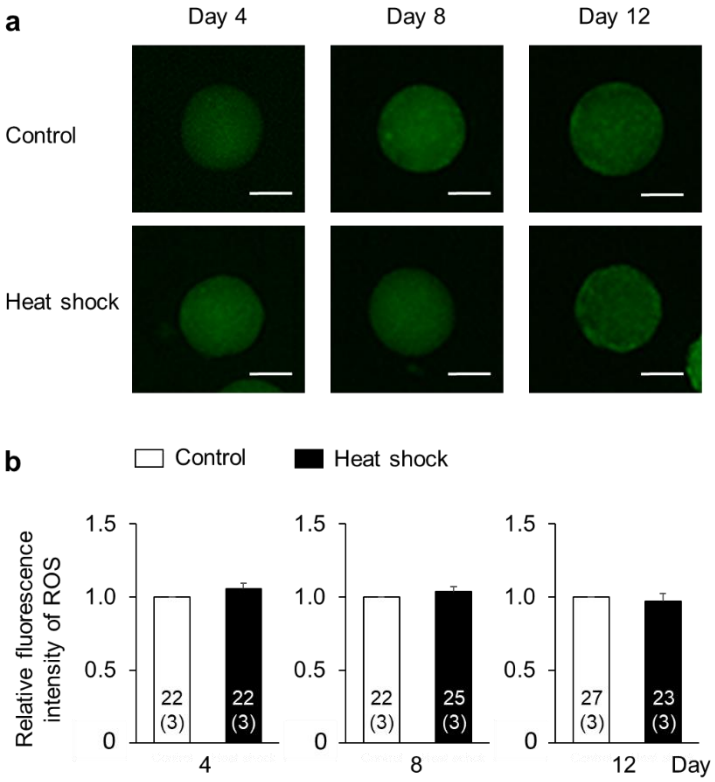

Figure II-8. Effects of heat shock during *in vitro* growth (IVG) culture on the intracellular reactive oxygen species (ROS) levels in oocytes.

(**a**) Representative fluorescent photomicrographs of IVG oocytes detected with 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA). The intracellular ROS levels in oocytes were evaluated every four days during IVG culture (days 4, 8, and 12) in the control group (upper panels) and the heat shock group (lower panels). Scale bar = 50 μm.

(**b**) The relative fluorescent intensity for ROS levels from the control and heat shock groups every four days during IVG culture (days 4, 8, and 12). The fluorescent intensity of ROS was measured using a total of 141 oocytes (three replicates each). Numbers in the bar graph indicate the number of oocytes, while the number of replicates is shown in parentheses. Fluorescence intensity of the heat shock group was normalized to that of the control group on the same culture day. Error bars indicate SEM.

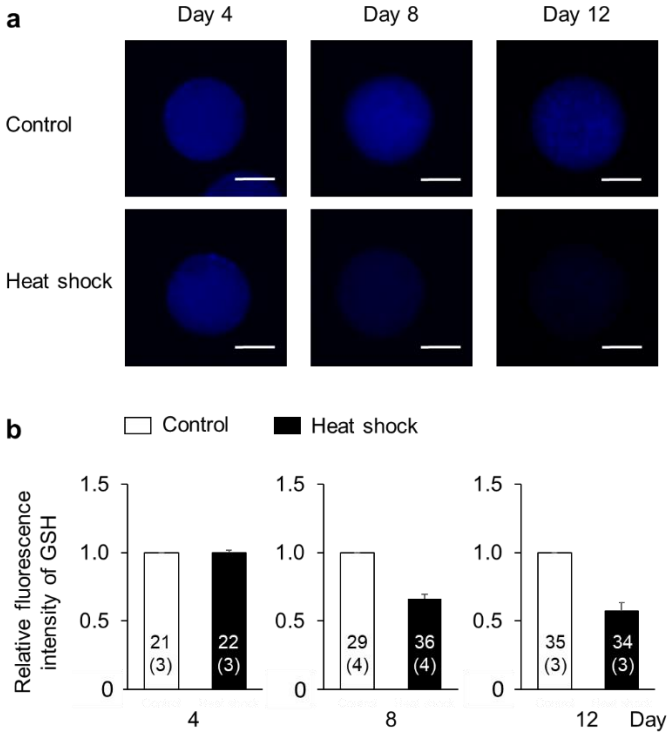

Figure II-9. Effects of heat shock during *in vitro* growth (IVG) culture on the intracellular reduced glutathione (GSH) levels in oocytes.

(**a**) Representative fluorescent photomicrographs of IVG oocytes detected with CellTracker Blue. The intracellular GSH levels in the oocytes were evaluated every four days during IVG culture (days 4, 8, and 12) in the control group (upper panels) and the heat shock group (lower panels). Scale bar = 50 μm.

(**b**) The relative fluorescent intensity for GSH levels from the control and heat shock groups every four days during IVG culture (days 4, 8, and 12). The fluorescent intensity of GSH was measured using a total of 177 oocytes (three-four replicates each). Numbers in the bar graph indicate the number of oocytes, while the number of replicates is shown in parentheses. Fluorescence intensity of the heat shock group was normalized to that of the control group on the same culture day. Error bars indicate SEM.

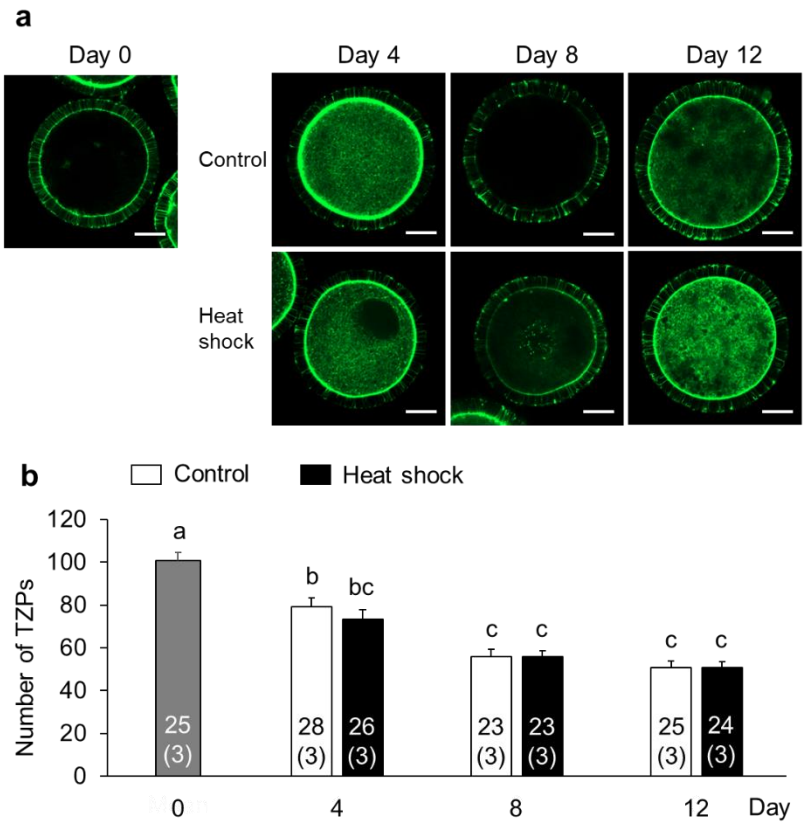

Figure II-10. Effects of heat shock during *in vitro* growth (IVG) culture on the number of transzonal projections (TZPs) between oocytes and cumulus cells.

(**a**) Fluorescence staining of TZPs between oocytes and surrounding cumulus cells detected with fluorescein isothiocyanate-labeled Phalloidin. The number of TZPs was evaluated on days 0 (upper left panel), and every four days during IVG culture (days 4, 8, and 12) in the control group (upper panels) and heat shock group (lower panels). Scale bar = 25 μm.

(**b**) The number of TZPs in IVG oocytes from the control and heat shock groups at day 0, and every four days during IVG culture (days 4, 8, and 12). The number of TZPs was evaluated using a total of 159 oocytes (three replicates each). Numbers in the bar graph indicate the number of oocytes, while the number of replicates is shown in parentheses. Error bars indicate SEM. $^{a\text{-}c}$ Different letters indicate significant differences ($P < 0.05$).

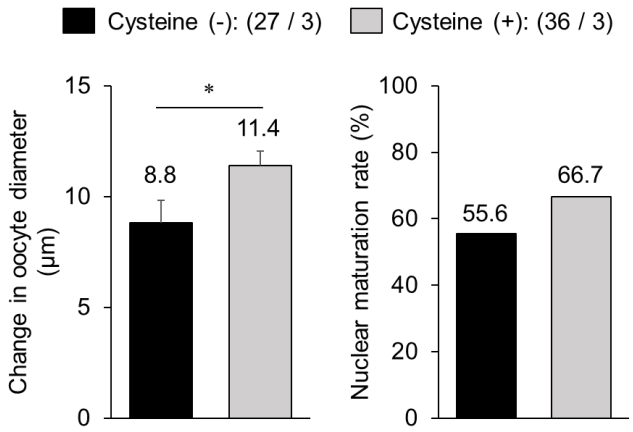

Figure II-11. Effect of cysteine supplementation on the growth and nuclear status of oocytes exposed to high temperature during *in vitro* growth (IVG) culture.

Values on the bar graphs indicate the changes in oocytes diameters before and after culture, and the percentage of nuclear maturation, respectively. Numbers in parentheses indicate the number of oocytes and replicates. * An asterisk indicates a significant difference between the control and heat shock groups on the same day ($P < 0.05$). Error bars indicate SEM.

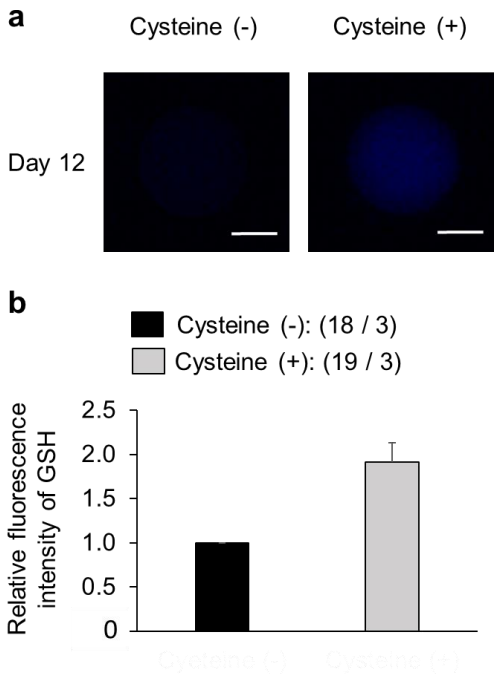

Figure II-12. Effect of cysteine supplementation on the GSH levels of oocytes exposed to high temperature during *in vitro* growth (IVG) culture.

(**a**) Representative fluorescent photomicrographs of IVG oocytes detected with CellTracker Blue. The intracellular GSH levels were evaluated on day 12 of IVG culture in the cysteine untreated group (left panels) and cysteine treated group (right panels). Scale bar = 50 μm.

(**b**) The relative fluorescent intensity for GSH levels from the cysteine untreated and treated group after day 12 of IVG culture. The fluorescent intensity of GSH was measured using a total of 37 oocytes (three replicates each). Numbers in parentheses indicate the number of oocytes and replicates. The fluorescence intensity of oocytes in the cysteine treated group was normalized to those in the cysteine untreated group. Error bars indicate SEM.

Summary

In dairy cows, low fertility caused by summer heat stress continues into the cooler autumn season. This can be caused by the damage to oocytes enclosed in small growing follicles during summer. Here, I subjected oocyte–cumulus–granulosa complexes (OCGCs) derived from early antral follicles (0.5–1 mm) to *in vitro* growth (IVG) culture under two different temperature settings (the control group; 38.5℃ for 24 h and heat shock group; 38.5℃ for 5 h, 39.5℃ for 5 h, 40.5℃ for 5 h, 39.5℃ for 9 h) for 12days, and evaluated effects of heat exposure on growth and developmental competence of oocytes, factors affecting the developmental competence of oocytes (steroidogenesis of granulosa cells, oxidative stress in oocytes, and cell-to-cell communication between oocytes and somatic cells). Changes in oocyte diameters before and after culture were smaller in the heat shock group than in the control group ($P < 0.05$). Although nuclear maturation and cleavage rates were similar between the groups, blastocyst rates were lower in the heat shock group (0.0%) than in the control group (27.7%) ($P < 0.05$). Steroidogenesis of granulosa cells, reactive oxygen species levels in oocytes, and number of transzonal projections did not differ between the groups, while reduced glutathione (GSH) levels in oocytes were lower in the heat shock group. Further, I examined the effects of cysteine, which stimulates GSH synthesis, on the growth, developmental competence, and GSH levels of oocytes exposed to high temperature during IVG. The cysteine supplementation increased the GSH levels and improved the growth and blastocyst rate of oocytes exposed to high temperature during IVG culture (27.9%) ($P < 0.05$). These results suggest that heat exposure impairs the growth and developmental competence of oocytes in early antral follicles through GSH depletion, which can induce low fertility during summer and the following autumn.

Summary and conclusions

In dairy cows, summer heat stress reduces conception rate of artificial insemination (AI) through multifactorial causes. Previous studies have shown that oocytes during maturation or fertilization and early embryos before 8 cells were highly susceptible to high temperature, and actions of heat stress during these stages increased the early embryonic loss. On the other hands, morula or blastocyst stage embryos have resistance to high temperature and transferring these embryos into uterus (embryo transfer: ET) is known to be an effective method to improve fertility during summer. However, heat stress possibly impairs the uterine function, and also reduces the efficacy of ET during summer. Moreover, low conception rate of AI is not only confined to summer season, but also continues into the cooler autumn season. This is considered to be caused by the damage to oocytes in the small growing follicles during summer. Therefore, in the present study, I have investigated the effects of heat stress on the uterine endometrial function (Chapter I), and growth and developmental competence of oocytes in the small follicles (Chapter II) in dairy cows.

Endometrial epidermal growth factor (EGF) concentrations peak on Days 2-4 and 13-14 during the estrous cycle in fertile cows. The loss of these peaks has been linked to an increased incidence of embryonic loss and, thus, reduced fertility in repeat breeder and high-yielding cows. Alterations in the endometrial EGF profile may be induced by changes in ovarian steroid hormone levels in both types of cows. Similar changes may occur with heat stress, which is associated with reductions in ovarian steroid production and increases in embryonic loss. In chapter I, I examined the incidence of alterations in the endometrial EGF profile and the conception rate after ET in Holstein lactating cows to confirm whether the EGF alteration are related to reductions in fertility under heat stress. The endometrial EGF profiles of 365 lactating Holstein cows in the Hokkaido (cold zone) and Kyushu (temperate zone) regions in Japan were examined between June and September (heat stress period, 90 cows in Hokkaido and 121 cows in Kyushu) and between October and January (control period, 86 cows in Hokkaido and 68 cows in Kyushu). EGF profiles were examined using uterine endometrial tissues obtained by biopsy on Day 3 (estrus = Day 0). The proportion of cows with an altered EGF profile in both regions was higher in the heat stress period than in the control period (41.2 vs. 16.2%, $P < 0.05$). This proportion in the heat stress period increased by 2- and 3-fold in the Hokkaido and Kyushu regions, respectively. The proportion of cows with an altered EGF profile was slightly higher in Kyushu than in Hokkaido throughout the study period (34.9 vs. 26.1%, $P = 0.07$). The effects of rectal temperature on Days 0 and 3 on the endometrial EGF profile were also examined in lactating cows ($n = 79$) between June and September in Kyushu. Some of these cows ($n = 67$) were subjected to ET on Day 7 to evaluate fertility. Regardless of rectal temperature on Day 3, the proportion of cows with an altered EGF profile was higher (64.1 vs. 30.0%, $p < 0.05$) and the

conception rate after ET was lower (26.7 vs. 51.4%, $p < 0.05$) in cows with a high rectal temperature (≥ 39.5°C) on Day 0. The present results indicate that the alterations induced in the endometrial EGF profile by an elevated body temperature due to heat stress on Day 0 contributed to reductions in fertility in dairy cows during the heat stress period.

As mentioned above, low fertility in dairy cows caused by summer heat stress continues into the subsequent cooler autumn seasons. For this reason, it is considered that oocytes enclosed in the small growing follicles are damaged during summer and their developmental competence are impaired. Especially, the early antral follicle stage is the critical for oocytes to acquire the developmental competence, however, there are no reports focusing on the effect of heat stress on the growth of early antral follicles. In chapter II, I subjected oocyte–cumulus–granulosa complexes (OCGCs) derived from early antral follicles (0.5–1 mm) to *in vitro* growth (IVG) culture under two different temperature settings (the control group; 38.5℃ for 24 h and heat shock group; 38.5℃ for 5 h, 39.5℃ for 5 h, 40.5℃ for 5 h, 39.5℃ for 9 h) for 12 days, and evaluated effects of heat exposure on growth and developmental competence of oocytes, factors affecting the developmental competence of oocytes (steroidogenesis of granulosa cells, oxidative stress in oocytes, and cell-to-cell communication between oocytes and cumulus cells). Changes in oocyte diameters before and after the culture were smaller in the heat shock group than in the control group ($P < 0.05$). Although nuclear maturation and cleavage rates were similar between the groups, blastocyst rates were lower in the heat shock group (0.0%) than in the control group (27.7%) ($P < 0.05$). Steroidogenesis of granulosa cells, reactive oxygen species levels in oocytes and number of transzonal projections did not differ between the groups, while reduced glutathione (GSH) levels in oocytes were lowered in the heat shock group. Further, I examined the effects of cysteine, which stimulates GSH synthesis, on the growth, developmental competence, and GSH levels of oocytes exposed to high temperature during IVG. The cysteine supplementation increased the GSH levels and improved the growth and blastocyst rate of oocytes exposed to high temperature during IVG culture (27.9% vs. 6.1%) ($P < 0.05$). These results suggest that heat exposure impairs the growth and developmental competence of oocytes in early antral follicles through GSH depletion, which can cause low fertility during summer and the following autumn.

In the present study, I have clarified that seasonal heat stress impaired the uterine endometrial function and reduced the conception rate after ET, which is recognized as the most effective method for improving summer fertility. In addition, I have developed the experimental model mimicking the follicular growth under high temperature and revealed that GSH depletion in oocytes is one of the mechanisms by which heat exposure reduces the growth and developmental competence of oocytes in the small follicles. Although further study is necessary in the future, reproductive performance of dairy cows during the summer and subsequent autumn can be improved by management or treatment targeting the uterine endometrial function and

growth of small follicles.

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Summary in Japanese

夏季の暑熱ストレスによる乳牛の受胎率低下の原因は多岐にわたる。これまでの研究では、卵子の成熟および受精の過程、8 細胞期以前の初期胚などが高温に感受性であり、これらの時期の卵子および初期胚への暑熱負荷によって早期胚死滅が起こることが主な原因とされてきた。一方、桑実胚期以降の胚は高温に対して抵抗性であり、これらの胚を子宮内へ移植する胚移植は、夏場の受胎率向上に有効であることが明らかにされている。しかし、暑熱ストレスは子宮の機能異常を引き起こすことで、胚移植による受胎率も低下させる可能性が考えられる。また、人工授精による受胎率の低下は、涼しくなる秋季においても継続する。これは夏季の暑熱ストレスによって発育途中の小さな卵胞内の卵子がダメージを受けているためであると考えられる。したがって、本研究では、第 1 章において暑熱ストレスによる乳牛の子宮内膜機能への影響について、第 2 章においては小卵胞内の卵子の発育および発生能への影響について評価した。

牛では子宮内膜における上皮成長因子 (Epidermal growth factor: EGF) 濃度は受胎性および子宮内膜機能の指標とされ、その発情周期中の変化が消失すると、早期胚死滅が増加して受胎性が低下する。子宮内膜 EGF 濃度異常の発生には、リピートブリーダー牛や高泌乳牛において共通してみられる、血中の卵巣ホルモン濃度の変化が関わっていることが示されている。また、この血中卵巣ホルモン濃度の異常は、暑熱ストレスを受けている牛においても同様にみられる変化である。そこで第 1 章では、子宮内膜の EGF 濃度異常が暑熱ストレスによる受胎率の低下に関与しているか調べるために、ホルスタイン種泌乳牛において、暑熱環境下における EGF 濃度異常の発生頻度と胚移植後の受胎率の関係について調べた。北海道および九州において飼養されているホルスタイン種泌乳牛 365 頭を用い、6-9 月 (暑熱期、北海道: 90 頭、九州: 121 頭) および 10-1 月 (対照期、北海道: 86 頭、九州: 68 頭) において試験を行った。発情後 3 日目に子宮内膜組織を採取し、組織中の EGF 濃度を測定した。その結果、北海道および九州いずれの地域においても、EGF 濃度異常を示す牛の割合は、10-1 月に比べて 6-9 月の方が高かった ($P < 0.05$)。10-1 月と比較した 6-9 月の EGF 濃度異常を示す牛の割合は、北海道および九州において、それぞれ 2 倍および 3 倍に増加した。試験期間を通じた (6-1 月) EGF 濃度異常を示す牛の割合は、北海道 (26.1%) と比較して九州 (34.9%) において高い傾向にあった ($P = 0.07$)。次に、6-9 月において九州地方のホルスタイン種泌乳牛 79 頭を用い、発情日および発情後 3 日目の直腸温度による EGF 濃度異常の発生率への影響を調べた。試験牛の一部 (67 頭) には、受胎性を評価するために、発情後 7 日目に胚移植を行った。発情後 3 日目の直腸温度にかかわらず、発情日の直腸温度が高い (≥39.5℃) 牛において、EGF 濃度異常の発生頻度は高く (64.1 vs. 30.0%、$P < 0.05$)、胚移植による受胎率は低かった (26.7 vs. 51.4%、$P < 0.05$)。これらの結果から、発情日の暑熱ストレスによって引き起こされる子宮内膜 EGF 発現の異常が、暑熱期における乳牛の受胎率低下の一因となることが示された。

夏季の暑熱ストレスによる乳牛の受胎率低下が、涼しくなる秋季にも持続することは、夏季の暑熱ストレスによる小卵胞内の卵子へのダメージにより、卵子の発生能が低下することが原因であると推測されている。小卵胞の中でも、初期胞状卵胞 (直径 0.3-1 mm) の時期は、卵子が発生能を獲得する上で重要な時期であるが、暑熱ストレスによる初期胞状卵胞の発育および卵胞内卵子の品質への影響を調べた報告はない。そこで第 2 章では、初期胞状卵胞 (直径 0.5-1 mm) に由来する卵子-卵丘-顆粒層細胞複合体 (oocyte-cumulus-granulosa complexes: OCGCs) の体外発育培養系 (in vitro growth: IVG) を用いて、暑熱期の乳牛の日内体温変化を模した条件を設定しその影響を調べた。OCGCs を牛の正常な体温に近い 38.5℃で培養する対照群と、暑熱環境下の乳牛の体温変化を模した温度条件 (38.5℃: 5 h, 39.5℃: 5 h, 40.5℃: 5 h, 39.5℃: 9 h) で培養する暑熱群に分け、12 日間の IVG に供し、暑熱負荷が卵子の発育、発生能、および卵子発生能と関連がある指標 (顆粒層細胞のステロイドホルモン産生能、卵子の酸化ストレス状態、および卵子と卵丘細胞との細胞間結合) に及ぼす影響を調べた。培養前後の卵子直径の増加は、対照群と比べて暑熱群の方が小さかった ($P < 0.05$)。卵子の核成熟率、受精後の卵割率、顆粒層細胞のステロイドホルモン産生能、卵子中の活性酸素種量および卵子と卵丘細胞との細胞間結合の程度には、群間で差がみられなかった。一方、胚盤胞発生率は対照群 (27.7%) と比べて暑熱群 (0.0%) で低く、卵子中の還元型グルタチオン (reduced glutathione: GSH) 量も暑熱群において低かった ($P < 0.05$)。また、GSH の合成を促進するシステインの培地への添加が、IVG において暑熱負荷を受けている卵子の発育、発生能および GSH 量に及ぼす効果を調べたところ、システインの添加によって卵子中の GSH 量が増加し、卵子の発育と胚盤胞への発生率 (27.9 vs. 6.1%) も改善された ($P < 0.05$)。これらの結果から、夏季の暑熱ストレスが卵子中の GSH 量を減少させることによって、初期胞状卵胞中の卵子の発育および発生能を低下させ、冷涼な秋季においても受胎率を低下させる可能性が考えられた。

本研究において、夏季の暑熱ストレスが乳牛において子宮内膜機能の異常を引き起こし、夏場の受胎率改善に最も有効とされる胚移植による受胎率も低下させることを示した。さらに、暑熱ストレスは卵子中の GSH の枯渇を介して、小卵胞中における卵子の発育および発生能を低減させることを示した。夏季と秋季における乳牛の繁殖成績は、子宮内膜機能と小卵胞の発育に着目した対策や治療法によってさらに改善できる可能性がある。