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Original Article

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

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Abstract. The endometrial epidermal growth factor (EGF) profile is an indicator of uterine function and fertility in cattle. The present study 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 and Kyushu 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 between June and September than between October and January (41.2 *vs*. 16.2%, P < 0.05). 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 present results 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.

Key words: Dairy cow, Embryo transfer, Endometrial epidermal growth factor, Heat stress

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Heat stress is one of the major contributing factors to low fertility in dairy cows. It is defined as an environment that increases body temperature to above the set-point temperature [1]. Ambient temperature (AT), humidity, wind, and solar radiation are some of the factors contributing to heat stress [2]. A negative correlation has been reported between an elevated body temperature and fertility in lactating dairy cows. The conception rate of artificial insemination (AI) begins to decline when uterine temperature at insemination increases by approximately 0.5°C above the normal range (38.3–38.6°C) [3]. The temperature-humidity index (THI), which is calculated from AT and relative humidity (RH), has been widely used as an indicator of heat stress in dairy cows. The typical stress threshold of THI is 72 [4]. THI higher than 72 may be reached in tropical and subtropical zones, and recently in temperate and some cold zones [5]. 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, impaired corpus luteum (CL) function, the suppressed expression of estrus, and embryonic loss [6, 7]. The detrimental effects of heat stress on oocytes and early embryos are considered to be the main cause of increased embryonic loss [8]. 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 [9–12]. Accordingly, studies using *in vitro* embryo production systems demonstrated that the developmental competence of oocytes obtained from cows exposed to heat stress [13–15] or a high temperature (41.0°C) during *in vitro* maturation cultures [16, 17] was reduced. 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) [18, 19].

Early embryos after Day 3 were found to be less sensitive to heat stress [10, 18, 19]. Consequently, heat stress decreases the pregnancy rate of AI more than that of embryo transfer (ET) [20, 21] and, thus, ET has been used to compensate for low fertility during the hot season [22]. The effects of heat stress on pregnancy after ET currently remain unclear. A previous study reported a decreased pregnancy rate after ET between the hot and cool seasons [23], while other studies found no or only slight differences in pregnancy rates [24–27]. Nevertheless, changes in the production and circulating levels of ovarian steroid hormones [4], and the synthesis and secretion of proteins [28] and prostaglandins [29–31] in the endometrium by heat stress may increase the incidence of embryonic loss even after ET due to an improper endocrine environment [7] or uterine dysfunction [32, 33].

In cattle, the epidermal growth factor (EGF) profile in the uterine endometrium has been identified as an indicator of endometrial function and fertility [34, 35]. Endometrial EGF concentrations exhibit a cyclic change with two peaks on Days 2–4 and 13–14

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during the estrous cycle [34, 36]. The loss of these peaks reduces fertility with an increase in embryonic loss [37] in repeat breeder (RB) and high-yielding dairy cows [35, 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].

Alterations in the endometrial EGF profile have been linked to changes in circulating estradiol (E_2) and progesterone (P_4) concentrations in RB and high-yielding cows [39]. 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 [43]. This may cause a slower increase and lower peaks in E_2 and P_4 concentrations in the circulation [44]. Although RB cows may not necessarily be high producers, they show similar alterations in ovarian steroid hormone profiles to those in high-yielding cows [35]. Since the expression of EGF in the endometrium is primarily regulated by E_2 and P_4 [45, 46], changes in circulating E_2 and P_4 concentrations may be amplified in the endometrium as an altered EGF profile [35]. Seasonal heat stress was also found to suppress the production and circulating concentrations of E_2 and P_4 in dairy cows [4]; therefore, reduced fertility during the heat stress period may be attributed, at least in part, to uterine dysfunction caused by alterations in the endometrial EGF profile.

The present study examined the relationship between decreased fertility during the heat stress period and uterine dysfunction caused by an altered endometrial EGF profile in dairy cows. We initially investigated the effects of seasons and regions on the EGF profile on Day 3. We then examined the effects of an elevated body temperature on Day 0 (estrus) and Day 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 (≥ 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 [34]. All tissue samples were obtained from the uterine horns on the contralateral side to 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, 47] 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 CVs 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 [36, 38].

Measurement of rectal temperature

Rectal temperature was measured using a clinical thermometer once a day between 1300 and 1700 h 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 [48]. The primary antibodies used for the E_2 and P_4 assays were anti-estradiol-17β-6-carboxymethiloxime (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; Jacson Immuno Research Laboratories, West Grove, PA, USA) was used as the secondary antibody. The inter- and intra-assay coefficients of variations 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 [49].

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

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. 1). Mean AT and THI between June and September and between October and January in each of the four areas are summarized in Table 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 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: 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 Day3. 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 1 and Fig. 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: 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.

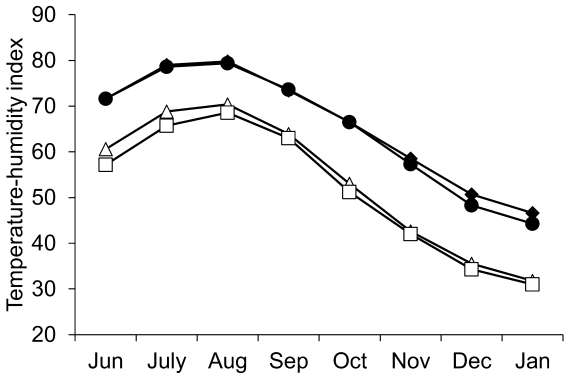

Fig. 1. Mean monthly temperature humidity index (THI) of the study period (2015–2017) in two regions (Hokkaido: Sapporo △ and Tomakomai □; Kyushu: Fukuoka ◆ and Kumamoto ●).

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 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

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 [36, 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

Table 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 ± SDs.

< 0.05) (Table 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

Rectal temperature between Days 0 and 3 was similar in both rectal temperature groups (Table 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 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 profie. 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 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

Table 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 based on previous findings [36, 38].

Table 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 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 (11)	6.46 ± 2.23 (16)
< 39.5ºC	< 39.5ºC	24	29.2	2.86 ± 1.30 (7)	8.02 ± 1.15 (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 [36, 38].

Table 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 [36, 38].

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 [35]; 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 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 [35]. 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 [45, 46]. Heat stress suppresses ovarian steroid hormone production by inhibiting the systemic endocrine system and ovarian cell activity [4]. Heat stress was previously shown to reduce the number of luteinizing hormone (LH) pulses in lactating dairy cows [50]. 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) [51]. Therefore, plasma concentrations of E_2 at the time of luteolysis [52] and estrus [53] decrease under heat stress conditions. Furthermore, heat stress was found to suppress the LH surge during the natural estrous cycle in Guernsey heifers [54] and its release in response to gonadotropin-releasing hormone administration in dairy cows [55]. 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 [56, 57]. However, the redistribution of blood flow from visceral organs, including the ovary and uterus, to the periphery occurs for thermoregulation during heat stress [32]. An elevated uterine blood flow in response to treatment with E_2 in ovariectomized cows decreased under the heat stressed condition [58]. 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 [32] 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 [32, 59]. The exposure of the cultured bovine endometrium to a high temperature increased the synthesis of heat shock protein (HSP) 70 and HSP90 [28]. Since HSPs are part of the complex of proteins that associate with P_4 and estrogen receptors [60–62], 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 ER-dependent transcription [62].

The rectal temperature showed a relatively wide range from 38.2°C to 40.8°C 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 [63]. Further, genetic variation for tolerance of heat stress in dairy cows [64] could be a potential cause since the specific single nucleotide polymorphisms of genes for tolerance to heat stress has been identified [65, 66].

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 in 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 $PGE_2/PGF_{2\alpha}$ [67] in the cultured endometrium and PGE_2 functions as a luteotropic agent [68]. 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 [69, 70].

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) [71]. 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%) [20, 21, 23–26, 72–75], 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 [76].

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 [32]. 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.

Conflict of interests: The authors declare that there is no conflict of interests.

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