—Original Article—

Comparison of the global gene expression profiles in the bovine endometrium between summer and autumn

Ryosuke SAKUMOTO[1], Ken-Go HAYASHI[1], Shiori SAITO[1], Hiroko KANAHARA[1], Keiichiro KIZAKI[2] and Kosuke IGA[3]

[1]*Animal Physiology Research Unit, National Institute of Agrobiological Sciences, Ibaraki 305-0901, Japan*
[2]*Laboratory of Veterinary Physiology, Iwate University, Iwate 020-8550, Japan*
[3]*NARO Tohoku Agricultural Research Center, Iwate 020-0198, Japan*

Abstract. Heat stress compromises fertility during summer in dairy and beef cows by causing nutritional, physiological and reproductive damages. To examine the difference in endometrial conditions in cows between summer and autumn, gene expression profiles were compared using a 15 K bovine oligo DNA microarray. The trial was conducted in the summer (early in September) and autumn (mid-November) seasons of two consecutive years (2013–2014) in Morioka, Japan. Endometrial samples were collected from the cows using a biopsy technique. The expressions of 268 genes were significantly higher in the endometrium collected in summer than those collected in autumn, whereas the expressions of 369 genes were lower ($P < 0.05$ or lower). Messenger RNA expressions of *glycoprotein 2 (GP2)*, *neurotensin (NTS)*, *E-cadherin (CDH1)* and *heat shock 105kDa/110kDa protein 1 (HSPH1)* were validated by quantitative real-time PCR. Transcripts of *GP2* and *NTS* were more abundant in the endometrium from summer than in the endometrium from autumn ($P < 0.05$). In contrast, the mRNA expressions of *CDH1* were lower ($P < 0.05$) and those of *HSPH1* tended to be low ($P = 0.09$) in the endometrium from summer. Immunohistochemical staining showed that GP2, NTS and HSPH1 were expressed in the endometrial epithelial or glandular epithelial cells. The serum concentrations of NTS collected from the cows in summer were higher than those collected from cows in autumn ($P < 0.05$). Collectively, the different gene expression profiles may contribute to functional differences in the endometrium between summer and autumn, and the increases in GP2 and NTS may have a relationship with the endometrial deficiency that causes infertility of cows in summer.
Key words: Cow, Endometrium, Heat stress, Infertility, Microarray analysis

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Conception rate to first service declined from 60% to 30–40% over the past 25 years in the world [1]. There are clear seasonal patterns in conception rate, especially in dairy cows [2–4] and lower conception rates are consistently observed in summer months compared with winter months [5, 6]. Both appetite and dry matter intake are reduced by heat stress [7], thus prolonging the period of postpartum negative energy balance and increasing the calving–conception interval [8, 9]. Heat stress reduces summer fertility both in dairy and beef cows by causing poor estrous expression due to reduced estradiol secretion from the dominant follicle developed in a low luteinizing hormone (LH) environment [10–12]. Moreover, heat stress inhibits oocyte growth by altering progesterone, LH and follicle-stimulating hormone secretion during the estrous cycle [7]. Abnormal profiles of progesterone as well as prolactin (PRL) may induce infertility in buffaloes during a hot summer [13]. Heat stress has also been associated with impaired embryo development and increased embryo mortality in cattle [14–16]. Understanding the physiological differences in endometrial function between summer and autumn might help to determine a way to improve reproductive efficiencies and reduce the number of defective fetuses.

Therefore, in the present study, we looked for differences in global gene expression patterns in the bovine endometrium obtained by biopsy in summer and autumn seasons using a custom-made 15 K bovine oligo DNA microarray. Since the expressions of some genes including *glycoprotein 2 (GP2)*, *neurotensin (NTS)*, *E-cadherin (CDH1)* and *heat shock 105kDa/110kDa protein 1 (HSPH1)* changed drastically between summer and autumn, gene and protein expressions of these substances in the endometrium or serum were also investigated.

Materials and Methods

Animals

All procedures for animal experiments were carried out in accordance with guidelines approved by the Animal Ethics Committee of the NARO Tohoku Agricultural Research Center (Morioka, Japan) for the use of animals.

Sampling procedures were carried out in the summer (early in September) and autumn (mid-November) seasons of two consecutive years (2013–2014) in Morioka, Japan. These periods were selected because empirically derived conception rate are low in the summer

Table 1. Temperature (C) and humidity (%) for 0700–1700 h during 45 days before the day of sampling in summer and autumn

		Temperature (C)			Humidity (%)		
		Mean	Max	Min	Mean	Max	Min
Summer	2013	25.6	34.6	15.4	71.1	99.9	30.3
	2014	25.7	34.8	16.4	74.0	99.9	37.2
Autumn	2013	11.5	27.4	−1.7	73.0	99.9	33.2
	2014	13.0	24.4	1.1	68.4	99.9	25.9

and are high in the autumn. Daily high, low and mean ambient temperatures and humidity were recorded every 1 h from 0700 to 1700 h in the pasture area of NARO Tohoku Agricultural Research Center using a temperature and humidity SD data logger (AD-5696, A&D, Tokyo, Japan). The data for ambient temperature and humidity (mean, maximum and minimum) during 45 days before the sampling day are shown in Table 1.

Three nonpregnant Japanese Black cows (2.5 to 4 years of age with normal estrous cycles) were used in each year (individual ID #206, #207 and #321 in 2013 and #217, #230 and #326 in 2014). The cows used in 2013 and 2014 weighed 399 ± 8.0 kg and 447 ± 22.4 kg, respectively. The cows were housed in identical chambers in the same shelter under the same husbandry conditions from 1700 to 0700 h and had access to native grass pasture and hay from 0700 to 1700 h. Cows were housed in groups under the same nutritional conditions and received water from metal tanks *ad libitum* in the pasture area. Endometrial tissues and blood samples were obtained from three cows (#206, #207, #321) in both the summer and autumn seasons of 2013. The same operations were conducted using another three cows (#217, #230, #326) in 2014. A total of six cows were used in the experiments.

For synchronization of estrus, a controlled internal drug release device (CIDR; EAZI-BREED, Surge Miyawaki, Tokyo, Japan) was inserted for 4–6 days at day 10–14 of the estrous cycle, and 0.5 mg cloprostenol, a prostaglandin (PG) $F_{2\alpha}$ analogue (Estrumate; Nagase Medicals, Itami, Hyogo, Japan), was injected i.m. on the same day as the CIDR treatment. Estrus was checked visually by heat mount detector (QuickStick; Kamar Products, Zionsville, IN, USA). Cows expressing estrous behavior within 39 h after removal of the CIDR were included in the experiment. On day 2 of the estrous cycle, ovulation was confirmed by ultrasonography with a 9 MHz linear array transducer (Apron EUB-7000HV, Hitachi Medical, Tokyo, Japan).

Collection of bovine endometrial tissues

Endometrial biopsies were obtained with a biopsy instrument (Yamauchi Methods, FB141, Fujihira, Tokyo, Japan) using a previously described method with slight modifications [17, 18]. Samples were obtained from the cows at day 15 of the estrous cycle. The day of estrus was designated as day 0. Briefly, after cleaning the perineum and external genitalia, the biopsy instrument in a protective sheath was introduced into the vagina. The instrument was guided into the cervix by manipulation per rectum. Then the instrument alone was introduced into the uterus after rupturing the sheath at the external cervical orifice and guided into the previously gravid horn approximately 3 cm past the uterine bifurcation. To ensure

reproducible tissue procurement, the tip of the biopsy instrument inside the uterus was identified using the nonoperating hand per rectum. Then the biopsy jaws were opened, and, with the help of the hand in the rectum, the medial uterine wall was gently pressed into the instrument jaws. Endometrial tissue was clipped off by closing the jaws and withdrawing the instrument. All biopsies were performed by the same operator. The tissue was then placed on sterilized dishes. For molecular analysis, endometrial tissues were cut into small pieces (< 0.5 cm^3), cryopreserved in liquid nitrogen and then stored at −80 C for later processing. For endocrinological analysis, blood samples were collected from the jugular vein at just before biopsy and centrifuged at $3,300 \times g$ at 4 C. Plasma samples were stored at −30 C until use. For histologic assessment, bovine endometrial tissues were obtained from Japanese Black cows at the institute ranch within 10–30 min of exsanguination, and tissue samples were collected from cows on day 15 of the estrous cycle (n = 3).

Microarray analysis

A custom-made 15 K bovine oligo DNA microarray (GPL9284) was used for the microarray analysis, which was performed as described previously [19]. After verifying the quality of the RNA with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and an Experion RNA StdSens Analysis Kit (700-7104JA, Bio-Rad Laboratories, Hercules, CA, USA), we performed one-color microarray analysis. RNA integrity was confirmed; all samples had an A260/280 ratio greater than 1.8 and an RNA integrity number greater than 8.5. The oligo microarray produced by Agilent Technologies (Palo Alto, CA, USA) was used in this study. Sixty-mer nucleotide probes for the customized microarray were synthesized on a glass slide. cDNA synthesis, Cy3-labeled cRNA preparation, hybridization and washing and scanning of the array slides were performed according to the Agilent one-color microarray-based gene expression analysis protocol. Briefly, 400 ng of total RNA from each sample were reverse transcribed into cDNA using a Quick Amp Labeling Kit (Agilent Technologies) with an oligo dT-based primer, and then Cy3-labelled cRNA was prepared by *in vitro* transcription. Labeled cRNA was purified with an RNeasy Mini Kit, and the concentration and Cy3 dye incorporation (pmol Cy3/µg cRNA) were measured with a spectrophotometer. Labeled cRNA (600 ng) was fragmented and hybridized using a Gene Expression Hybridization Kit (Agilent Technologies), according to the manufacturer's instructions. The arrays were washed using a Gene Expression Wash Pack Kit and scanned using an Agilent Microarray Scanner. Feature Extraction ver. 9.5 was used for image analysis and data extraction. The microarray data from each sample were imported into GeneSpring 12 (Agilent Technologies) for further data characterization. The GEO accession numbers are as follows: Platform, GPL9284; samples, GSM1613709 to GSM1613720; series, GSE66057.

Real-time PCR

Total RNA isolation and subsequent reverse transcription and real-time PCR steps were carried out as previously described [20]. The primers encoding the bovine sequences were chosen using an online software package (http://primer3.ut.ee/) and synthesized as listed in Table 2. The primer length (20 bp) and GC contents

Table 2. Primers used in real-time PCR

Gene		Sequence (5'–3')	GenBank accession no.	bp
Glycoprotein 2 (GP2)	For	AGTGAGGATGCCAGAGGACT	NM_001075950	149
	Rev	TCTTCCAGAGGCAGCAGTTG		
Neurotensin (NTS)	For	TCCTGCTGCCTTAGATGGTT	NM_173945	105
	Rev	TCCTGAATCAACTCCCAGTGT		
E-cadherin (CDH1)	For	GATTGCAAGTTCCCGCCATC	NM_001002763	145
	Rev	ACATTGTCCCGGGTGTCATC		
Heat shock protein 1 (HSPH1)	For	TGCAATCCTTTCCCCAGCAT	NM_001075302	141
	Rev	GGAGCAGCGTGGTTTCTACT		
RN18S1	For	AAACGGCTACCACATCCAAG	DQ222453	141
	Rev	TCGCGGAAGGATTTAAAGTG		

of each primer (50 to 60%) were selected to avoid primer dimer formation. Gene expression was measured by real-time PCR using an Mx3000P Real-time PCR analyzing system (Agilent Technologies) and QuantiFast SYBR Green PCR Kit (204054, Qiagen) starting with 200 ng of reverse-transcribed total RNA. The expression of *18S ribosomal RNA* (*RN18S1*) was used as an internal control. The PCR was performed under the following conditions: (first step) 95 C for 5 min; 40 cycles of 95 C for 15 sec, 60 C for 30 sec and (second step) 95 C for 60 sec; and then 60 C for 30 sec. The reaction was then held at 25 C. Each PCR was followed by obtaining melting curves to ensure single-product amplification. As standard curves, serial dilutions of appropriate cDNA were used for gene quantification. The obtained data were normalized on the basis of *RN18S1* mRNA content. Use of the Mx3000P Real-time PCR analyzing system at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient r > 0.95). To exclude any contaminating genomic DNA, all experiments included controls lacking the reverse transcription enzyme. As a negative control, water was used instead of RNA for the PCR to exclude any contamination from buffers and tubes.

Immunohistochemistry

Immunohistochemistry for glycoprotein 2 (GP2), neurotensin (NTS), E-cadherin (CDH1) and heat shock 105kDa/110kDa protein 1 (HSPH1) in the bovine endometrium was performed using an automated Ventana HX System Discovery with a Dab Map Kit (Roche Diagnostics, Mannheim, Germany) as described previously in detail by our laboratory [21]. The endometrial samples for immunohistochemistry were fixed with 10% neutral formalin for 24 h. Five-micrometer-thick sections from paraffin embedded tissue were incubated at room temperature with rabbit polyclonal anti-human GP2 antibody (LS-B6834, LifeSpan BioSciences, Seattle, WA, USA), rabbit polyclonal anti-bovine NTS antibody (bs-6631R, Bioss, Woburn, MA, USA), rabbit polyclonal anti-bovine CDH1 antibody (bs-1519R, Bioss) or rabbit polyclonal anti-hamster HSPH1 antibody (LS-B7471, LifeSpan BioSciences) diluted 1:25 or 1:50 each in Discovery Ab diluents (Roche) for 12 h. The signals were detected using anti-rabbit IgG-Biotin conjugate (Sigma-Aldrich, St Louis, MO, USA) diluted 1:100 for 1 h and then counterstained with hematoxylin. Negative controls were performed using normal rabbit IgG (NBP2-24891, Novus Biologicals, Littleton, CO, USA) diluted at concentrations equivalent to the primary antibodies. The sections were observed with a Leica DMRE HC microscope (Leica Microsystems, Tokyo, Japan) and a Nikon Digital Sight DS-Fi1-L2 (Nikon, Tokyo, Japan).

ELISA

The NTS and CDH1 concentrations in the plasma were determined using commercial bovine ELISA kits (NTS, CSB-EL016136BO, CUSABIO Biotech Wuhan, PR China; CDH1, CSB-EL005034BO, CUSABIO). The ranges of the standard curves were 25–1600 pg/ml for NTS and 7.8–500 ng/ml for CDH1, respectively. The intra-assay coefficients of variation were 8.8% for NTS, and 10.6% for CDH1. The plasma samples were directly used for the ELISA.

Statistical analyses

Microarray data were analyzed statistically with the Student's *t*-test and summarized using GeneSpring 12 (Agilent Technologies). All extracted microarray data were analyzed with one-way ANOVA followed by the Turkey-Kramer multiple comparison test using the JMP 7 software (SAS Institute, Cary, NC, USA). The correlations between the flow cytometric collection and gradient collection were determined by the Pearson product–moment correlation coefficient using the JMP software, and the correlation coefficients were calculated as r-values. Experimental data for real-time PCR and ELISA are presented as the mean ± SEM. The statistical significance of differences in the abundance of mRNA expressions and in protein concentrations was assessed by a Wilcoxon-Mann-Whitney U-test with KaleidaGraph 4.1 (Synergy Software, Reading, PA, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Gene expression profiles in the bovine endometrial tissues

The expressions of many genes in the endometrium obtained in summer statistically differed by a factor of > 1.0-fold (P < 0.05) from their expressions in the endometrium obtained in autumn. These included 268 and 369 genes with higher and lower expressions in the endometrium in summer than autumn, respectively. Some of the genes were differentially expressed between the endometria of summer

Table 3. Fold change in mRNA levels of selected genes in the endometrium obtained from cows in summer and autumn

Gene	Change (fold)	P-values
(Summer > autumn): total 268 genes		
Glycoprotein 2 (GP2)	16.48	0.035
Neurotensin (NTS)	10.45	0.037
Insulin-like growth factor binding protein 1 (IGFBP1)	2.55	0.049
Chemokine C-X-C motif ligand 3 (CXCL3)	2.45	0.019
ISG15	1.88	0.007
Placenta-specific 8 (PLAC8)	1.76	0.020
BAX	1.61	0.004
MX1	1.54	0.025
Cluster of differentiation 83 (CD83)	1.54	0.025
TCRA	1.52	0.025
Endothelin converting enzyme 1 (ECE1)	1.34	0.025
ESAM	1.30	0.036
IL10RB	1.24	0.010
Tissue inhibitor of metalloproteinase 2 (TIMP2)	1.24	0.006
Caspase 4 (CASP4)	1.20	0.042
BOLA-DMB	1.20	0.040
(Summer < autumn): total 369 genes		
E-cadherin (CDH1)	1.80	0.037
Heat shock 105kDa/110 kDa protein 1 (HSPH1)	1.41	0.009
Insulin-like growth factor 2 (IGF2)	1.36	0.044
Prostaglandin D2 synthase (PTGDS)	1.29	0.014
Oviductal glycoprotein 1 (OVGP1)	1.27	0.030
Calmodulin (CALM)	1.19	0.010
Minichromosome maintenance complex component 5 (MCM5)	1.19	0.013
Heat shock 70kDa protein 8 (HSPA8)	1.18	0.010
Progesterone receptor membrane component 2 (PGRMC2)	1.16	0.016
Minichromosome maintenance complex component 7 (MCM7)	1.11	0.003

and autumn (Table 3). The *glycoprotein 2 (GP2)* and *neurotensin (NTS)* were the genes with the highest expression, and *E-cadherin (CDH1)* and *heat shock 105kDa/110 kDa protein 1 (HSPH1)* were those with the lowest expression in the endometrium in summer compared with autumn.

Expressions of GP2, NTS, CDH1 and HSPH1 in the endometrial tissues

Messenger RNA expressions of *GP2*, *NTS*, *CDH1* and *HSPH1* were checked by real-time PCR (Fig. 1). Transcripts of *GP2* and *NTS* appeared to be more abundant in the endometrium obtained in summer than that obtained in autumn (Fig. 1A and 1B; P < 0.05). In contrast, the expressions of *CDH1* were lower (Fig. 1C; P < 0.05) and those of *HSPH1* tended to be low (Fig. 1D; P = 0.09) in summer compared with autumn.

Immunohistochemical staining showed that GP2, NTS and HSPH1 were clearly expressed in either the endometrial epithelial or glandular epithelial cells (Fig. 2), whereas CDH1-positive immunostaining was not observed in the endometrium used in this study. NTS concentrations were significantly higher in the plasma obtained from the cows in summer than those in the plasma obtained from the cows in autumn

(Fig. 3A; P < 0.05). In contrast, the CDH1 concentrations in the plasma did not change significantly between summer and autumn.

Discussion

The preceding results are the first to demonstrate the number of genes whose expressions changed in the endometrium during the transition between summer and autumn. In addition, some of the genes, including *GP2* and *NTS*, showed drastically upregulated expressions in the endometrium in summer. These observations suggest that the different expressions of these specific substances were related to infertility of the cows during summer.

It has been demonstrated that heat stress during the hot months continues to suppress antral follicles on days 40–50 [22, 23]. Heat stress reduces gonadotropin-releasing hormone (GnRH) and LH secretion [24, 25] and causes low blood estradiol concentrations by reducing the steroidogenic capacity of theca and granulosa cells in the dominant follicles [26, 27]. In contrast, the effects of heat stress on plasma progesterone concentrations in dairy cows differ depending on the study, with some showing that they were increased [28, 29], decreased [7] or unchanged [27, 30]. These endocrine
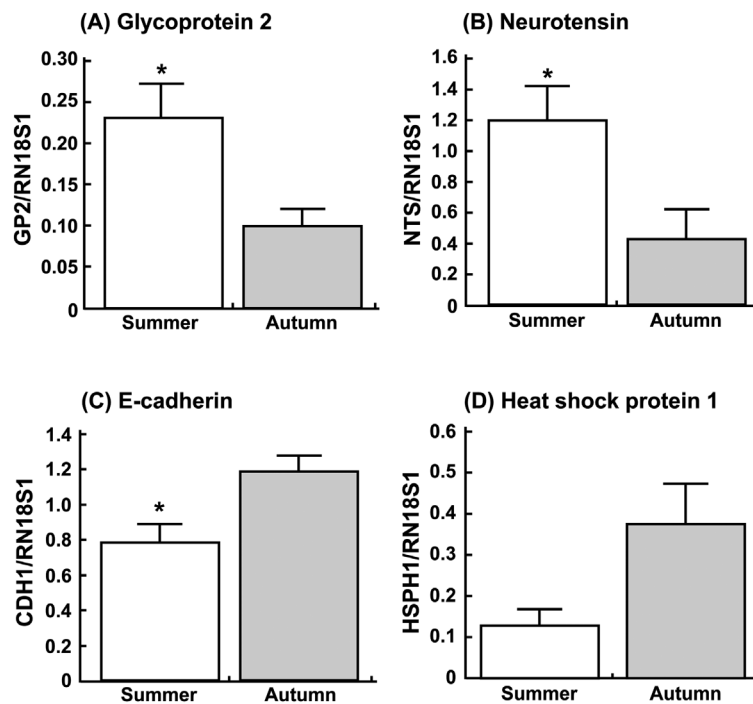
Fig. 1. Changes in relative amounts of (A) *glycoprotein 2*, (B) *neurotensin*, (C) *E-cadherin* and (D) *heat shock protein 1* mRNA expressions in the endometrium obtained from the cows in summer and autumn. Data are the means ± SEM for six cows per season and are expressed as relative ratios of the mRNAs to *18S ribosomal RNA* (*RN18S1*). Asterisks show significant differences between summer and autumn (* $P < 0.05$).

changes may cause, at least, reduced follicular activity, altered ovulatory mechanisms and decreased oocyte and embryo quality. In addition, heat stress increases $PGF_{2\alpha}$ production in the endometrium, which might result in early regression of the corpus luteum or death of the embryos [31, 32]. For example, elevation of the tissue incubation temperature from 39 to 43 C induced a large reduction in conceptus protein synthesis and stimulated release of $PGF_{2\alpha}$ by the endometrium. These *in vitro* results suggest that exposure of pregnant cows to high environmental temperature and humidity may disrupt the balance between the conceptus and endometrial biochemical factors responsible for maintenance of pregnancy [31]. Thus, one might speculate that heat stress during summer affects endometrial conditions as well as follicular development in nonpregnant and pregnant cows as demonstrated in the present study.

The microarray analysis demonstrated that the gene expressions of *GP2*, *NTS*, *CDH1* and *HSPH1* in the endometrial tissues on summer were significantly different from their expressions in autumn (Table 3). GP2, which is known as zymogen granule membrane protein, is the major membrane protein expressed in the pancreatic zymogen granules. GP2 is cleaved from the membrane and secreted into the pancreatic duct along with the other digestive enzymes [33]. In the present study, both GP2 mRNA and protein were expressed in the bovine endometrial epithelial cells. The relevance of GP2 to reproduction in mammals is not yet known, and thus the physiological meanings of GP2 in reproductive function remain to be studied. NTS is a 13 amino acid neuropeptide that is closely associated with numerous biological activities in the central nervous system and periphery [34]. Beside the opioid-independent analgesic system involved in the pathophysiology of various mental disorders, NTS affects food intake, glucose homeostasis, body temperature, behavior and cortisol secretion [34, 35]. NTS is also known as an anti-apoptotic factor in a variety of cells [36]. In rats, intraventricular injection of NTS resulted in decreasing plasma LH and PRL concentrations [37], and thus NTS is thought to have critical effects on the LH/PRL surge in regulation of the ovulation process. Hence, the higher levels of NTS expressions in the endometrial epithelial and glandular epithelial cells and high concentrations of NTS in plasma during the hot season in this study may account for the suppressed follicular development and subsequently compromised ovulation in the cows.

Heat shock proteins (HSPs) were first identified in *Drosophila* salivary glands after exposure to elevated temperature [38]. HSPs are classified into different families according to their molecular weights, e.g., 27, 60, 70, 90 and 105/110 kDa [39, 40]. The HSPs have been studied for their roles in protecting cells from elevated temperatures in the process of acclimation to stress or immune responses [41]. Although HSP60 and 70 are known as important factors that protect cells from heat stress in a variety of reproductive organs including the oocyte and placenta [42], there is very little information on HSPH1 (105/110kDa) in the reproductive system. This study is the first to demonstrate that both the gene and protein of HSPH1 were expressed in the bovine endometrium. On the other hand, CDH1 is expressed within the cytoplasm of trophoblast binuclear cells in the bovine placentome [43]. *CDH1* mRNA in day 22 conceptuses was less abundant than in day 17 and day 20 conceptuses [44]. Although
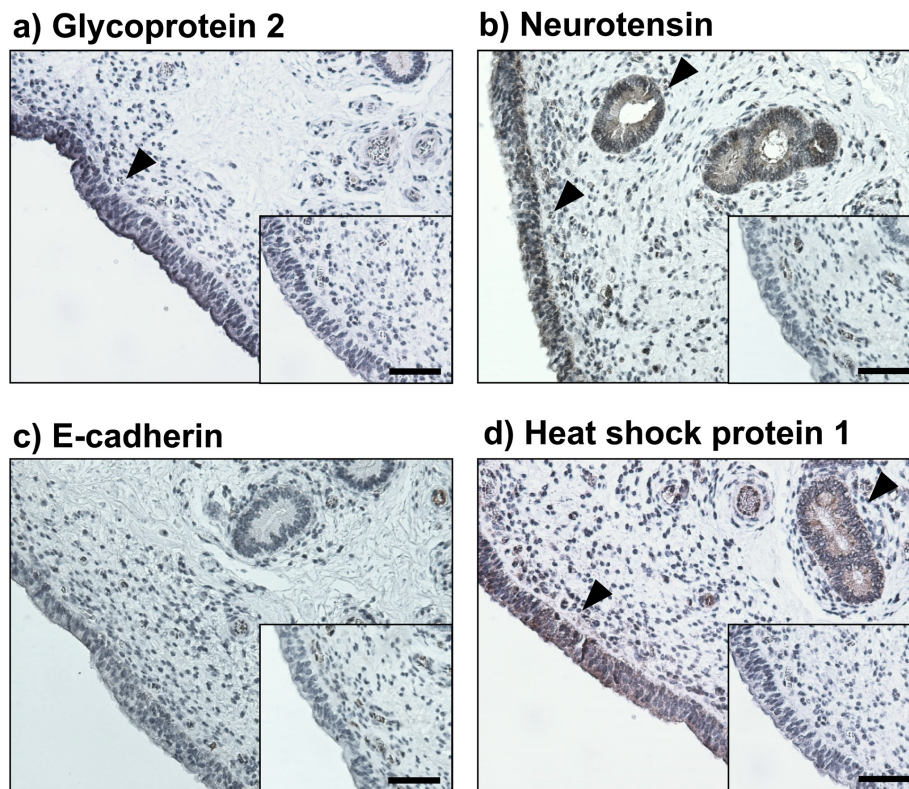
a) Glycoprotein 2

b) Neurotensin

c) E-cadherin

d) Heat shock protein 1

Fig. 2. Localization of a) glycoprotein 2, b) neurotensin, c) E-cadherin and d) heat shock protein 1 in the bovine endometrium evaluated by immunohistochemistry. Intensive immunoreactivities were observed in epithelial or glandular epithelial cells (black arrowheads). No positive immunoreactivity was observed in the negative control (inserted panels). Scale bar = 50 μm.
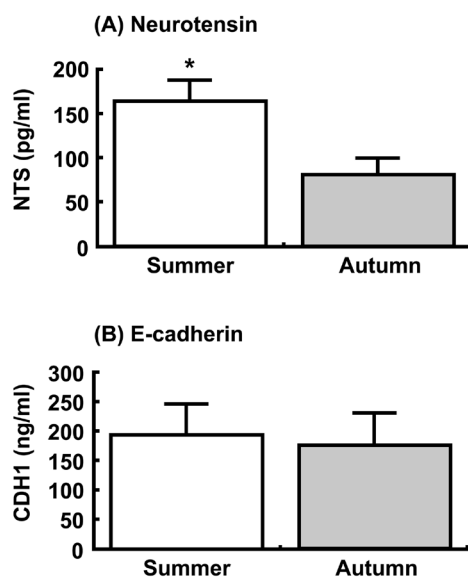

Fig. 3. Concentrations of (A) neurotensin and (B) E-cadherin in the plasma obtained from the cows in summer and autumn evaluated by ELISA. Data are the means ± SEM for six cows per season. Asterisks show significant differences between summer and autumn (* P < 0.05).

CDH1 mRNA was expressed in the bovine endometrium in this study, positive staining of CDH1 could not be observed by using our immunohistological analysis. Further studies are needed to determine how HSPH1 and CDH1 affect the functional and morphological properties of the endometrium during summer in cows.

Collectively, the changes in gene and protein expressions that we observed may contribute to functional differences in the bovine endometrium between summer and autumn. The substances with altered expression levels in summer may play a pivotal role(s) in regulating uterine function or fetus survival in cows. In addition, since plasma NTS concentrations were higher in the cows under warm conditions rather than cool conditions in this study, NTS as well as GP2 may become useful tools for diagnosis of infertility during summer in cows.

Acknowledgments

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