Assessment of Inflammatory Cytokine Concentrations During Diagnosis and After Treatment of Postpartum Dairy Cows with Clinical and Subclinical Endometritis

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SUMMARY

The objectives of the present study were (1) to compare serum inflammatory cytokine [Tumor necrosis factor-alpha (TNF-α), Interleukin-1beta (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), and Interleukin-10 (IL-10)] levels between healthy dairy cows, cows with clinical endometritis (CE) and subclinical endometritis (SCE) during early postpartum (pp), (2) to observe the treatment and time-related alterations of these cytokines. A total of 127 Holstein-Friesian dairy cows were evaluated in the study, and each animal was subjected to three consecutive examinations [between 21-27 (E1), 28-34 (E2), and 35-41 (E3) days in milk (DIM)] with 7-day intervals. General physical controls and blood collection were performed at E1, E2, and E3. Reproductive controls were performed only at E1 and E3. Cows were divided into three categories as endometritis-negative, CE, and SCE based on their vaginal discharge scores (VDS) and percentage of endometrial polymorphonuclear cells (PMNs) at the end of the E1. Immediately following diagnosis, a single dose of intrauterine 500 mg cephapirin benzathine was administered to cows with CE and SCE. The VDS and PMNs of the cows were re-evaluated and the treatment responses were determined at E3. Endometritis-negative, CE, and SCE cows were divided into six sub-categories according to the last examination findings and treatment responses at the end of the E3. The CE and SCE cows were divided into two sub-categories as cured and uncured. Also, endometritis-negative cows were divided into two sub-categories as healthy cows and the cows that later developed a uterine infection. The cows that later developed uterine infection were not evaluated in the study. Finally, five subgroups to observe the treatment and time-related alterations, and three main groups to evaluate the diagnostic potential of the cytokines were established. All cytokine levels were measured by ELISA. Diagnostically, TNF-α ($P < 0.05$), IL-1β ($P < 0.001$) and IL-8 ($P < 0.05$) levels were significantly higher in cows with CE than in healthy cows. Nevertheless, IL-6 and IL-10 levels were similar between healthy, CE, and SCE cows ($P > 0.05$). Similarly, no differences were observed in all cytokine levels between healthy and SCE cows ($P > 0.05$). In repeated measurements concerning subgroups, no apparent relationship was observed between inflammatory cytokine levels and treatment response. Also, all cytokine levels showed a linear course regardless of treatment response and uterine health condition within subgroups between 21-41 DIM ($P > 0.05$). As a result, high early pp serum IL-1β, TNF-α, and IL-8 levels may reflect a persistent clinical uterine infection. However, no inflammatory changes have emerged to the extent that could affect the systemic inflammatory cytokine levels of SCE cows.

KEY WORDS

Endometritis, cytokine, dairy cow, diagnosis, treatment.

INTRODUCTION

Bacterial contamination of the uterus occurs in 80-90% of dairy cows after parturition. This activates innate immune response which is required for the elimination of uterine inflammation and infection.[1] Nevertheless, the improper balance between intrauterine antimicrobial self-defense mechanisms and infection mostly leads to main postpartum (pp) reproductive diseases, including endometritis.[2]

Endometritis is an inflammation of the endometrium not associated with systemic signs and occurring at least 21 days after calving. Clinical endometritis (CE) is characterized by the presence of purulent uterine discharge detectable in the vagina 21 days or more after parturition, or mucopurulent discharge detectable in the vagina after 26 days pp.[3,4] Subclinical endometritis (SCE) is an inflammation of the endometrium in the absence of CE and the rate of polymorphonuclear cells (PMNs) in the total number of endometrial cells is indicative for SCE. It is defined as the presence of > 18% PMNs at 21-33, or > 10% PMNs at 34-47 days in milk (DIM) collected in uterine cytology samples of cows in the absence of purulent or mucopurulent vaginal discharge.[4-6] Endometritis is one of the most prevalent infections in high-producing dairy cows and causes serious economic losses.[3,5,7] The early diagnosis and treatment are essential for the reproductive performance of affected cows. Cephapirin appeared to be a suitable antibacterial agent for intrauterine treatment.[6,8]

Endometrial bacterial infection induces an inflammatory response by secretion of inflammatory cytokines. The inflam-

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matory mediators including pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and chemokines (e.g., IL-8) stimulate the diapedesis and chemoattraction of neutrophils and monocytes, and promote phagocytosis to remove microbial agents and damaged host cells.[9-11] In the later stages of the inflammation, anti-inflammatory cytokines such as IL-10 are released for tissue repair, timely resolution of inflammation, and minimizing the harmful effects of chronic inflammation.[11] Recently, the role of the inflammatory cytokines in endometritis, and the relationships between these mediators and endometritis have gained importance. Thus, local or systemic inflammatory cytokine levels and mRNA expressions of cows have been examined for different purposes.[10,12-27] Although inflammatory cytokines mediate uterine infections by functioning locally and/or systemically, it remains unclear whether they are a diagnostic tool for endometritis, especially for SCE, and variations can be seen between studies. Also, no study has been conducted to evaluate the treatment-related changes in inflammatory cytokines in cows with endometritis. Therefore, the aims of the present study were (1) to evaluate the serum TNF-α, IL-1β, IL-6, IL-8, and IL-10 levels between healthy and endometritic cows at the 4th week of pp, which is the earliest period when the endometritis is defined, (2) to monitor the treatment and time-related alterations of these cytokines in endometritic cows together with healthy cows. The results of the present study may provide useful information about threshold levels, diagnostic efficacy, and treatment/time-dependent changes in the serum inflammatory cytokines of cows.

MATERIALS AND METHODS

Animals

The animals were selected from one commercial dairy farm located in Aydin/Turkey. The study was conducted on 130 Holstein-Friesian dairy cows (1-6 lactations) between January 2017-January 2018. The average 7-day milk yields of the animals were between 24.8-52.9 L.

Management of Animals

The cows were fed twice daily with a balanced TMR adjusted to actual milk yield and gestation period, and had access to the water ad libitum. Cows were housed in free-stall barns and milked three times per day at 8-h intervals. The animal data were recorded from the computerized herd management system (Alpro, DeLaval, Sweden).

Inclusion and exclusion criteria

At the moment of enrollment, as described below, general physical controls were performed and only cows with no clinical infection and no history of abortion, stillbirth, C-section, fetotomy, parturient paresis, or acute septic metritis were included. Cows were not included if they received antibiotic and hormonal treatment or had clinical mastitis, pneumonia, abomasal displacement, clinical hypocalcemia, and ketosis within 14 days before the first examinations. Also, cows diagnosed with the same problems throughout the study were excluded.

Case definition and experimental design

Case definition: All of the cows included in the study were subjected to three consecutive examinations [between 21-27 (E1), 28-34 (E2), and 35-41 (E3) DIM] seven days apart. In all of these three examinations, general physical controls and blood collection were performed as explained below. Reproductive controls were performed only at E1 and E3, as explained below. Uterine infections were determined based on the vaginal discharge score (VDS) and endometrial PMNs of the cows. The character of vaginal discharge was assessed according to the combination of two endometritis scoring systems[4,28], and classified as follows; (VDS0: Clear or translucent mucus, VDS1: Discharge containing flecks of white or off-white mucus, VDS2: Discharge containing less than 50% white or off-white mucopurulent material, VDS3: Discharge containing equal to or more than 50%, usually white or yellow and sometimes bloody mucopurulent material). Clinical endometritis was defined as the presence of VDS2 or VDS3 at E1 and E3. The endometrial PMNs of the cows were evaluated using the modified cytobrush technique.[7] Subclinical endometritis was defined as the presence of > 18% PMN at E1 or > 10% PMN at E3 in the absence of CE. Endometritis-negative cows were defined as the presence of ≤ 18% PMN at E1 in the absence of CE. Cows having ≤ 18% PMN at E1, and ≤ 10% PMN at E3 in the absence of CE were defined as healthy. The cows that later developed uterine infection were defined as endometritis-negative at E1 but developed CE/SCE at E3.

Experimental Design: Cows were divided into three categories as endometritis-negative (VDS0/1 and ≤ 18% PMN) (n = 65), CE (VDS2/3) (n = 34), and SCE (VDS0/1 and > 18% PMN) (n = 31) based on their VDS and endometrial PMNs at the end of the E1. Immediately following the diagnosis, a single dose of intrauterine 500 mg cephapirin benzathine was administered to cows with CE and SCE. The cows were examined two more times at E2 and E3. Due to the exclusion criteria, three cows (2 endometritis-negative, 1 CE) were excluded from the study during E2. The treatment responses were evaluated at E3, 14 days later from the diagnosis. Endometritis-negative, CE, and SCE cows were divided into six sub-categories according to the last examination findings and treatment responses at the end of the E3. Cows with CE were divided into two sub-categories as cured CE (VDS0/1, and ≤ 10% PMN) (n = 20) and uncured CE (VDS2/3, or VDS0/1 and > 10% PMN) (n = 13). Similarly, cows with SCE were divided into two sub-categories as cured SCE (VDS0/1 and ≤ 10% PMN) (n = 22) and uncured SCE (VDS2/3, or VDS0/1 and > 10% PMN) (n = 9). Endometritis-negative cows were also divided into two sub-categories as healthy cows (VDS0/1 and ≤ 10% PMN) (n = 52) and the cows that later developed a uterine infection (VDS2/3, or VDS0/1 and > 10% PMN) (n = 11). Although the cows that later developed uterine infection were endometritis-negative at E1 and examined throughout the study, their data were not evaluated. Because CE/SCE were detected at E3 in these cows, and it was thought that cytokine levels might be misleading At the end of the study, to observe the treatment and time-related alterations of cytokines, subgroups were formed from previously sub-categorized animals. It was aimed to establish subgroups with a parallel number of animals from each sub-category, and to complete the sampling number of two kits provided for each cytokine. For this purpose, five subgroups [Healthy subGroup (HsG) (n = 13), cured-Clinical Endometritis Group (cCG) (n = 11), uncured-Clinical Endometritis Group (uCG) (n = 11), cured-Subclinical Endometritis Group (cSG) (n = 11), and uncured-Subclinical En-

dometritis Group (uSG) (n = 9)] were randomly established from previously sub-categorized animals [healthy (n = 52), cured CE (n = 20), uncured CE (n = 13), cured SCE (n = 22), and uncured SCE cows (n = 9)] respectively.

Finally, to evaluate the diagnostic potential of cytokines, three main groups [Clinical Endometritis Group (CG) (n = 22), Subclinical Endometritis Group (SG) (n = 20), and Healthy Control Group (HG) (n = 22)] were established. Clinical Endometritis Group was formed by the combination of cCG and uCG. Subclinical Endometritis Group was formed by the combination of cSG and uSG. To establish the groups with a parallel number of animals, Healthy Control Group was formed by randomly adding new cows to HsG from previously subcategorized healthy cows (n = 52).

General physical controls

All examination findings were recorded on the individual follow-up forms throughout the study. Data including age, calving date, parity, DIM, 7-day average milk yield, calving status, and clinical history of cows in the current lactation were recorded. Rectal temperatures were measured and body condition scores (BCS) of the cows were assessed by using 5 point scale.[29]

Reproductive controls

Following general physical controls, cows were subjected to vaginal, rectal, ultrasonographical, and endometrial cytological examinations, respectively.

Vaginal examination: The VDS of the cows were determined with a Metricheck device (Metricheck, Simcro Tech, Hamilton, New Zealand) as defined by McDougall et al.[30] The vaginal discharge accumulated on the device was assessed visually with regards to volume, color, and proportion of pus.

Rectal and ultrasonographic examination: Rectal examination was performed before the ultrasonography (USG). Transrectal USG was performed with a portative ultrasound having a 6.5 MHz linear transducer (KX5100V, Kaixin Elec. Inc. Co. Ltd., Xuzhou, Jiangsu, China). Uterine lumen was screened for fluid accumulation and echogenicity changes. Uterine horn asymmetry was evaluated by transversal scanning for determining previous pregnant/non-pregnant horns. Presence/absence of ovarian structures [corpus luteums, follicles (> 5 mm fluid-filled structures), cysts (> 25 mm fluid-filled structures)] were also visualized.

Endometrial cytology: Endometrial cytology was used to determine the proportion of endometrial PMNs of cows, not detected as CE. The samples were taken and evaluated using modified cytobrush technique.[7] The samples were taken from previously gravid uterine horn which was larger and/or had echogenicity changes in the lumen, and content accumulation was detected at USG. When there was no difference between uterine horns, sampling was performed from any horn or corpus uteri. Two slides were prepared from each sample to the clean glass microscope slides and allowed to dry. Smears were fixed in absolute methanol for 10 minutes, stained with Wright-Giemsa for 45 minutes, and evaluated under a bright light microscope (B203, Soif Optical Instruments, China) using 400x magnification. A total of 300 cells (endometrial cells and PMNs) were counted by a single examiner to determine the percentage of PMNs.

Blood collection

Blood samples were taken from each cow during E1, E2, and E3 with 7-day intervals. The samples were collected from the external jugular vein to the clot activator tubes (Vacuette, Grainer Bio-One, Kramsmünster, Austria), immediately placed on ice, and transferred to the laboratory. The samples were kept at +4 °C for two hours in the laboratory for clotting and then, centrifuged (M 4808 PR, Elektromag, Istanbul, Turkey) at 3000x g at 4°C for 10 minutes. The separated serums were transferred to the 1.5 ml microcentrifuge tubes and stored at -20°C until the cytokine measurements.

Intrauterine treatments

Following diagnosis, CE and SCE cows were treated with a single dose of intrauterine 500 mg cephapirin benzathine (Metricure, Intervet, Turkey) on the same day.

Measurements of cytokines

Serum TNF-α, IL-1β, IL-6, IL-8, and IL-10 levels were measured with bovine ELISA kits (Sunred Biotechnology Company®, Shanghai, China) in a commercial laboratory (Farmasina Medical and Chemical Products Industry, Istanbul, Turkey) according to the manufacturer's guidelines. The serum samples were transferred to the laboratory in dry ice.

The assay ranges of Bovine TNF-α, IL-1β, IL-6, IL-8, and IL-10 kits were between 15-4000 ng/L, 1.5-400 pg/ml, 30-6000 ng/L, 2-600 ng/L, and 2-600 pg/ml, respectively. The sensitivities of Bovine TNF-α, IL-1β, IL-6, IL-8 and IL-10 kits were 14.155 ng/L, 1.053 pg/ml, 28.725 ng/L, 1.911 ng/L, and 1.887 pg/ml respectively. The measured TNF-α, IL-6, and IL-8 concentrations were converted from ng/ml to pg/ml, and all the cytokine levels were presented as pg/ml.

Statistical Analysis

Power analysis was performed to determine the sample size based on the data obtained in the study. The effect size, alpha, and power values were taken as 0.40, 0.05, and 0.80, respectively. The minimum number of samples required was determined as 55 cows.

Statistical analysis of the study was performed using SPSS 22.0 packet program (SPSS, IBM SPSS Statistics, Chicago, IL, USA). All the data were presented as Mean ± SEM, and $P < 0.05$ was considered statistically significant. Distribution analyzes were performed with the Shapiro-Wilk test. Homogeneity of the variances was evaluated with the Levene's test. Parametric tests were applied to the data showing normal distribution, and non-parametric tests were applied to the data that did not show normal distribution after logarithmic transformation.

One-Way ANOVA was used to evaluate data including age, milk yield, DIM, parity, body temperatures, presence of ovarian structures, and serum IL-1β, IL-6, IL-8, and IL-10 levels between groups, and posthoc Tukey test was applied, when the difference was significant. Kruskal-Wallis test was used to evaluate the BCS and serum TNF-α levels between groups, and Mann Whitney U test was applied, when the difference was significant.

One-Way ANOVA was used to evaluate data including age, DIM, milk yield, parity, body temperatures, presence of ovarian structures, and cyclic activity rates between subgroups, and posthoc Tukey test was applied, when the difference was significant. Kruskal-Wallis test was used to evaluate the BCS between subgroups, and the Friedmann test was used to evaluate the time-related BCS changes of each subgroup. Two-Way ANOVA was used to evaluate repeated measurements of all serum cytokine

levels and body temperatures of subgroups. Posthoc evaluations were performed according to GLM procedures when differences were significant between subgroups or time.

RESULTS

A total of 161 calvings occurred; however, 31 of these cows were not included due to non compliance with the inclusion criteria. Thus, 130 cows were examined throughout the study. Three cows (2 endometritis-negative, 1 CE) were excluded from the study during the E2 clinical examination; thus, the final dataset included 127 cows. The BCS of the cows ranged from 2.50 to 4.00, and involved cows were between 24.5-108.3 months of age.

Data including the mean age, 7-day average milk yield, parity, and DIM were similar between both groups ($P > 0.05$) and subgroups ($P > 0.05$). During the diagnosis, the mean BCS, body temperature, and proportions of ovarian structures did not significantly differ between groups ($P > 0.05$). The mean body temperatures and BCS of cows were found to be similar on the same examination times between subgroups ($P > 0.05$). Also, the mean body temperatures were similar between different time intervals within subgroups ($P > 0.05$). However, the mean BCS of all subgroups significantly decreased between different time intervals from E1 to E2 and E3 ($P < 0.01$, $P < 0.05$). The distribution of ovarian structures was similar between subgroups both at E1 and E3 ($P > 0.05$). The cycling cows were assessed via ovarian ultrasound, made at 14-day intervals. Cows with corpus luteum, detected at least in one examination and one ovarium, were evaluated as cycling. Accordingly, no significant difference was observed in cyclic activity rates between subgroups ($P > 0.05$).

The mean diagnostic serum cytokine levels of groups were presented in Figure 1. Interleukin-8 and TNF-α levels were found to be higher in cows with CE than in healthy cows ($P < 0.05$). Also, IL-1β levels were significantly higher for CE than both healthy and SCE cows ($P < 0.001$). However, none of the cytokines showed a significant difference between healthy and SCE cows ($P > 0.05$). Also, IL-6 and IL-10 levels were similar between all groups ($P > 0.05$).

The mean serum cytokine levels of subgroups regarding the treatment and time-related alterations were presented in Table 1. None of the cytokines showed time-related changes within subgroups ($P > 0.05$). At E1, TNF-α and IL-6 levels were similar between subgroups ($P > 0.05$). Interleukin-10 levels were higher for uCG than healthy controls ($P < 0.05$). However, IL-1β and IL-8 levels of cured CE cows were higher than healthy controls ($P < 0.05$). At E2, IL-1β and IL-6 levels were similar between subgroups ($P > 0.05$). The TNF-α levels were higher in uCG compared with HsG and uSG ($P < 0.05$). However, IL-8 and IL-10 levels were higher for cCG than healthy controls ($P < 0.05$). At E3, TNF-α, IL-6, IL-8, and IL-10 levels were similar between subgroups ($P > 0.05$). Only IL-1β levels were found to be higher in cCG than in healthy controls ($P < 0.05$).

DISCUSSION

An increase in pro-inflammatory cytokines during the physiological uterine involution period, which returns to normal within 2-3 weeks pp, is considered a reflection of healthy in-

Table 1 - Treatment and time-related serum inflammatory cytokine (TNF-α, IL-1β, IL-6, IL-8, and IL-10) profiles between and within subgroups at 21-27 (E1), 28-34 (E2), 35-41 (E3) days in milk.

	Subgroups	E1	E2	E3	P values
TNF-α (pg/ml)	HsG	355.53±41.93	337.52±32.77^{ac}	354.68±48.23	Group: P < 0.05
	cCG	400.77±23.67	454.30±36.80^{ab}	405.29±24.52	Time: P = 0.428
	uCG	483.90±25.78	491.57±31.44^{b}	442.15±28.63	Group x Time: P = 0.556
	cSG	376.75±39.71	373.25±36.28^{ab}	375.73±23.48	
	uSG	445.35±64.88	330.47±29.06^{a}	349.71±34.16	
IL-1β (pg/ml)	HsG	32.32±3.47^{a}	33.27±2.87	32.52±3.71^{a}	Group: P < 0.05
	cCG	51.55±4.81^{b}	48.03±5.99	49.20±5.13^{b}	Time: P = 0.938
	uCG	47.28±4.09^{ab}	42.78±4.83	46.08±3.51^{ab}	Group x Time: P = 0.302
	cSG	37.47±2.79^{ab}	36.57±2.54	37.64±2.94^{ab}	
	uSG	36.90±3.90^{ab}	46.29±7.45	38.75±3.28^{ab}	
IL-6 (pg/ml)	HsG	447.32±48.04	432.05±51.14	444.94±48.28	Group: P = 0.096
	cCG	559.87±39.65	707.28±84.31	633.03±67.48	Time: P = 0.496
	uCG	554.33±67.94	549.43±72.60	569.75±57.33	Group x Time: P = 0.934
	cSG	557.06±45.55	562.04±29.08	584.93±45.49	
	uSG	530.69±120.19	483.92±87.90	458.47±55.78	
IL-8 (pg/ml)	HsG	44.64±4.24^{a}	45.76±3.40^{a}	48.10±6.39	Group: P < 0.05
	cCG	69.37±6.43^{b}	72.68±11.69^{b}	71.57±8.36	Time: P = 0.850
	uCG	67.43±5.78^{ab}	70.98±6.30^{ab}	68.24±5.42	Group x Time: P = 0.956
	cSG	59.61±4.33^{ab}	56.17±4.25^{ab}	54.82±4.89	
	uSG	59.18±10.72^{ab}	57.97±7.46^{ab}	53.45±9.44	
IL-10 (pg/ml)	HsG	54.55±4.03^{a}	55.54±5.23^{a}	57.09±5.82	Group: P < 0.05
	cCG	79.94±11.49^{ab}	84.25±11.31^{b}	80.93±9.75	Time: P = 0.908
	uCG	82.21±6.10^{b}	74.33±6.74^{ab}	77.47±7.13	Group x Time: P = 0.257
	cSG	62.57±5.18^{ab}	55.22±3.71^{ab}	59.60±3.91	
	uSG	65.99±13.35^{ab}	76.96±12.01^{ab}	65.03±9.86	

HsG: Healthy subGroup (n = 13); cCG: cured-Clinical Endometritis Group (n = 11); uCG: uncured-Clinical Endometritis Group (n = 11); cSG: cured-Subclinical Endometritis Group (n = 11); uSG: uncured-Subclinical Endometritis Group (n = 9). Values are shown as mean ± SEM. $^{a, b, c}$: Represents significance at the same time points between subgroups ($P < 0.05$).

flammatory reactions.[25] However, higher or excessive expressions of pro-inflammatory cytokines in the same period were frequently associated with the infection.[12,13] It was also reported that decreased expressions of these cytokines could impair the bacterial cleaning and cause endometritis by reducing the activation/chemotaxis of immune cells.[10] Regarding these data, it was thought in the present study that early pp serum pro-inflammatory cytokine levels could be measured higher or lower in cows with endometritis compared to healthy animals.

Tumor necrosis factor-alpha and IL-1β stimulate the expression of chemokines.[31,32] Postpartum endometrial TNF-α mRNA expressions were reported to be increased in cows with CE and SCE.[14,23] Loyi et al.[20] found that endometrial TNF-α mRNA expression of endometritic buffaloes increased independently from the stage of the oestrus cycle, and correlated with the severity of inflammation. In addition to gene expression studies, a study found that cows with CE and SCE had higher serum TNF-α levels compared to healthy cows measured at the 5th week of pp.[19] Brodzki et al.[22] investigated early pp TNF-α levels of healthy and SCE cows in both serum and uterine flush samples, and only serum TNF-α levels were found to be higher in cows with SCE. However, serum TNF-α levels were found to be similar between healthy, CE and SCE cows throughout the first 8 weeks of pp in another study.[21] In the present study, high diagnostic serum TNF-α levels of CE cows compared to healthy cows were associated with the severity of infection. Besides, similar TNF-α levels between healthy and SCE cows should be verified by future research.

Interleukin-1 stimulates the production of IL-8, responsible for the chemoattraction of neutrophils/monocytes for clearing pathogens.[31,32] Several studies showed that bovine endometrial IL-1β mRNA expressions increased in endometritis,[14,15] and correlated with the endometrial PMN rate.[15,26] Serum IL-1β levels of CE[18,19] and SCE[27] cows were also found to be significantly increased compared to healthy cows at the first month of pp. Also, Adnane et al.[25] found that cervicovaginal mucus samples of CE cows had higher IL-1β levels than healthy cows. However, according to the results of another study, serum IL-1β levels of both CE and SCE cows were found to be similar with healthy cows.[21] Interleukin-1β results of the current study were in accordance with the majority of literature and the reflection of endometritis to the serum IL-1β levels could occur only in clinical cases. Thus, IL-1β may be a useful marker in the diagnosis of CE.

Interleukin-6, produced during the early stages of inflammation, has important tasks including stimulation of acute-phase response and regulation of immune cell functions.[9] In a study, which covers the same pp interval as our study, endometrial IL-6 mRNA expressions were found to be similar between CE, SCE, and healthy cows.[14] However, Johnson et al.[23] stated that endometrial IL-6 expression increased in cows with endometritis. Contrasting results are also found in enzyme studies. Interleukin-6 levels measured from cervical mucus[25] and uterine flush[21] samples were reported to be high in cows with CE. Kasimanickam et al.[19] stated that cows with CE and SCE had higher serum IL-6 levels compared to healthy cows at the 5th week of pp. While Kim et al.[21] reported similar serum IL-6 levels between healthy cows and endometritic cows at the first 8 weeks of pp. Also, serum and uterine flush IL-6 levels of healthy and SCE buffaloes were found to be similar during early pp.[27] Interleukin-6 is also produced by adipose tissue and released into the systemic circulation,[33] and endometrial expression of

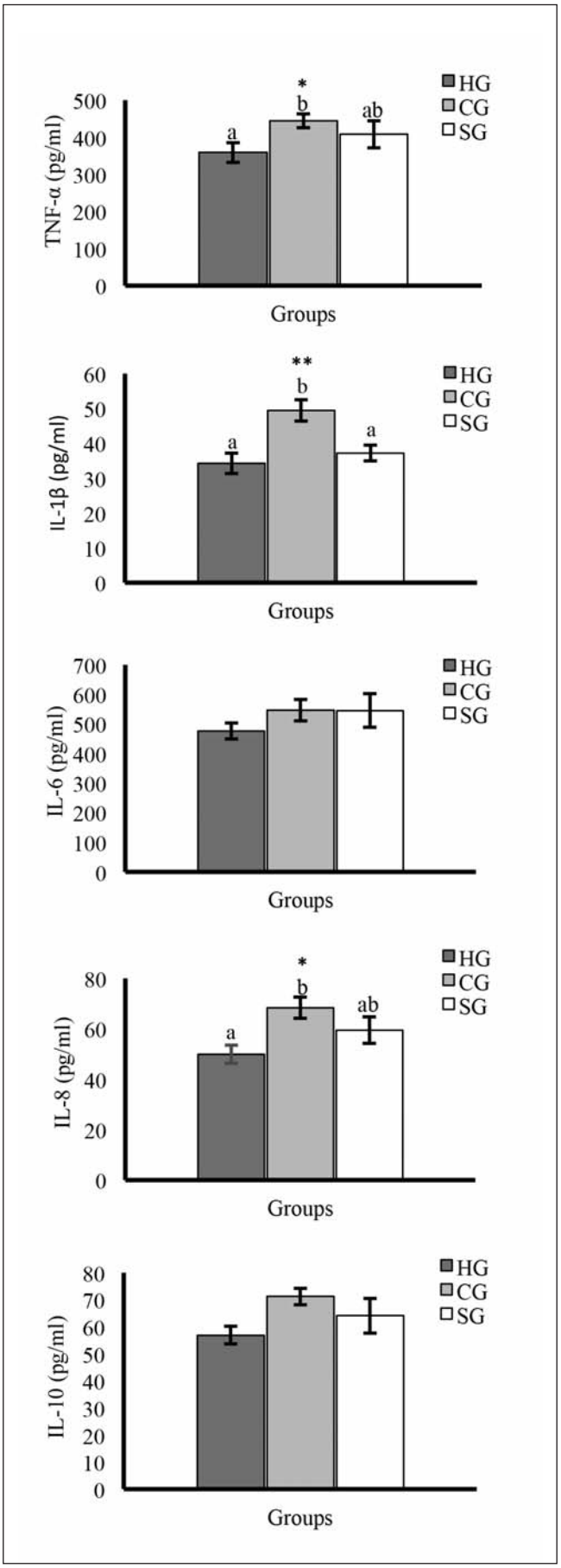

Figure 1 - Diagnostic serum cytokine (TNF- , IL-1 , IL-6, IL-8, and IL-10) profiles (pg/ml) of groups. HG: Healthy Control Group (n = 22); CG: Clinical Endometritis Group (n = 22); SG: Subclinical Endometritis Group (n = 20). Values are shown as mean ± SEM. **a, b:** Represents significance at the same time points between groups (**P < 0.001; *P < 0.05).

IL-6 may be affected by alteration of body condition.[34] However, the mean BCS of our groups were similar and the IL-6 levels were evaluated independently from this effect. Diagnostic IL-6 levels of the current study were similar between healthy and endometritic animals. Considering the present and previous studies, the effect of endometritis on local or systemic IL-6 levels may be variable and serum IL-6 measurements may not provide sufficient information about endometritis.

Interleukin-8 induces further immune cell migration to the site of infection or tissue damage.[35] This was proven experimentally by human recombinant and anti-IL-8 administrations in cows and mares.[36] Endometrial epithelial IL-8 mRNA expressions of cows were found to be increased in CE[14] and SCE[17,24,26] This increase was correlated with uterine bacterial density[24] and endometrial PMN rate.[37] In enzyme studies, IL-8 levels of local samples were reported to be high in cows with CE[21,25] and SCE.[21,27] Galvao et al.[10] observed similar plasma IL-8 levels between healthy and SCE cows throughout the first seven weeks of pp. However, to our knowledge, there is no study investigating the systemic IL-8 levels of CE cows. Our study showed that the CE cows had higher serum IL-8 levels than healthy cows. Similar IL-8 levels between healthy and SCE cows were also consistent with Galvao et al.[10]

The pro-inflammatory response is mediated by anti-inflammatory factors to prevent excessive stimulation of the immune system.[13] Interleukin-10 inhibits the cytokine production of monocytes/macrophages and neutrophils.[38] Postpartum blood monocyte or endometrial biopsy IL-10 mRNA expressions were reported to be similar between healthy and SCE cows.[10,16] Herath et al.[13] also reported similar endometrial IL-10 mRNA expressions between healthy fertile cows and infertile cows with a persistent uterine infection. In a study, IL-10 levels measured from serum and uterine flush samples were found to be increased in SCE during early pp. It was reported that this may adversely affect the uterine self-defense mechanism, resulting in endometritis.[22] However, Kim et al.[21] found that IL-10 levels of uterine flush samples were similar between healthy, CE, and SCE cows at the first month of pp. Diagnostic serum IL-10 levels of our study were found to be similar between study groups at 21-27 DIM. This was interpreted as the local chronic infections of the endometrium might not generate an apparent systemic anti-inflammatory response during early pp.

Inflammatory cytokines have been investigated for prognostic purposes in various diseases including sepsis and systemic inflammatory response syndrome.[39,40] According to Bannerman[41], cytokines might be effective in determining the severity and outcome of the disease. The cows with the highest plasma pro-inflammatory cytokine levels in the last month of pregnancy were associated with a worse early pp inflammatory and metabolic state, and measurement of these cytokines in late pregnancy was suggested as prognostic markers.[42] Besides the diagnostic evaluation, we aimed to monitor treatment and time-related alterations of cytokines with the repeated measurements in the subgroups. Considering the pre and post-treatment measurements; some cytokines were higher in cured subgroups and some cytokines were higher in uncured subgroups compared to healthy animals. Also, we obtained similar cytokine levels between cured and uncured CE and SCE animals during all examination times. It is thought that the early pp inflammatory cytokine measurements may not provide sufficient data for monitoring the treatment response of endometritis and similarly planned future studies including a higher number of animals are needed.

It was reported that pp endometrial inflammatory cytokine expressions show a time-dependent manner.[15] In a study, endometrial inflammatory cytokine expressions were found to be decreased progressively during the normal involution but continued to elevate in cows with endometritis.[26] The present study hypothesized that the cytokine levels of healthy and successfully treated cows might decrease in time, but may continue to be elevated in uncured cows. However, none of the cytokines showed significant time-related changes within subgroups during the repeated measurements between 21-41 DIM, and seem not to be affected by the uterine health status or treatment. In a former study, no relation was found between sampling time and serum cytokine (TNF-α, IL-1β, and IL-6) levels of healthy, CE, and SCE cows throughout the first two months of pp.[21] A similar situation was observed in plasma IL-8 levels of cows in the first 3 weeks of pp.[16] The findings of these studies are consistent with our research.

Body condition score is used to assess body fat, and the change in BCS indirectly reflects the fat metabolism and metabolic parameters of dairy cattle.[43] In a study, Kasimanickam et al.[19] found significantly higher serum TNF-α, IL-1β, and IL-6 levels in cows with low BCS compared to the high ones. They speculated that an increase in inflammatory cytokines might be mediated by BCS loss. However, the mean BCS of the cows was similar between both groups and subgroups in the present study. Thus, it can be said that the possible effect of BCS on cytokines was eliminated, and only endometritis-related changes were evaluated.

Local cytokine expressions/levels of the uterus might vary depending on whether the research is gene- or enzyme-based. Also, while the first step of production and secretion of proteins is mRNA synthesis, ultimate circulatory protein levels could be affected by several interprocess.[21,44] Collection of washing samples requires perfusion of solution into the uterus, which results in the emergence of unknown dilution factors and the inability to collect all of the given solutions. This might be associated with underestimated cytokine levels.[25] While local sampling from the uterus may require technical equipment and expertise, blood samples can be obtained quickly and easily under farm conditions. Thus, evaluation of inflammatory cytokines in serum can provide an advantage. Also, samples of the present study were obtained from a uniform population having similar conditions (feeding, caring, environment, age, milk yield, parity, DIM) and clinical status (body temperatures, presence of ovarian structures, cycling rates). These can be suggested as an important factor, increasing the reliability of results. However, it should not be ignored that different concurrent subclinical infections may change the systemic levels of these cytokines.

Brodzki et al.[22] states that when there is no or a low level of the pathogen in the uterus, local immune mechanisms are not activated. The lowest cytokine levels measured in healthy cows throughout the present study, and the numerical increase in diagnostic cytokine levels of SCE cows support this idea. Indeed, this increase became more evident, and statistical differences occurred in TNF-α, IL-1β, and IL-8 levels of CE cows. The expression of IL-8 is stimulated by TNF-α and IL-1β.[31,32] The significant increase in TNF-α, IL-1β, and IL-8 levels of CE cows confirms this information. Higher cytokine levels of CE cows reveal that these animals have a more severe systemic inflammatory response. It is thought that IL-1β levels may be used

in the detection of CE in the early pp, supported by other studies in the future. Although TNF-α, IL-6, IL-8, and IL-10 provide important data regarding immunity, similar levels of these cytokines between healthy and SCE or SCE and CE cows make it difficult to use for diagnostic purposes in both types of endometritis.

CONCLUSION

In conclusion, serum inflammatory cytokine measurements showed that there were significant increases in TNF-α, IL-8, and especially IL-1β levels in CE at the 4th week of pp, and this may indicate a persistent clinical uterine infection. In the same period, there were no significant alterations occurred in the serum cytokine levels between healthy and SCE cows. In repeated measurements, the differences in serum cytokine levels between subgroups could not be adequately associated with the endometritis treatment response. Time-related changes of cytokines within subgroups were similar and showed parallel course regardless of infection or treatment response at the 4th, 5th, and 6th weeks of pp. The present research gives important information about the systemic reflection of pp uterine immune response of healthy and endometritic cows and may serve as a reference for future research, especially in the treatment of endometritis. Similarly planned studies need to be performed in more cases, in the same or different pp intervals, and in different body fluids.

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