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Research Article

Evaluation of Serum C-Reactive Protein and Natural Antibodies in Cows with Endometritis [1]

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Abstract

The aim of this study is to assess serum natural antibody (NAb) bindings keyhole limpet hemocyanin (KLH) titer and C-reactive protein (CRP) levels, two of the most important elements of the natural defense system in the uterus. The study was conducted on 77 Brown Swiss cows (3-7 years of age) with body condition scores (BCS) of 2.6±0.1. The diagnosis of endometritis in cows on day 30-32 postpartum was made by rectal examination, vaginoscopy, ultrasonography and cytobrush technique. Blood samples were collected immediately after the examinations to measure serum NAb titer and CRP concentrations. NAb binding KLH titers were determined using the indirect ELISA method and the CRP level by using the bovine CRP ELISA kit. Mean NAb titers were 5.36±0.16, 7.43±0.15, 8.55±0.19 and 9.64±0.27 respectively in healthy cows (Control, n = 29), cows with mild endometritis (ME, n = 21), cows with moderate endometritis (E, n = 17) and cows with severe endometritis (SE, n = 10). Mean serum CRP concentrations were 48.88±3.92 µg/mL, 82.86±4.28 µg/mL, 98.86±8.2 µg/mL and 122.34±12.72 µg/mL respectively in groups control, ME, E and SE. NAb binding KLH titer and CRP levels increased significantly in cows with endometritis (P<0.001). BCS did not change serum NAb titer and CRP level (P>0.05). Parity did not cause a significant difference in serum NAb titer and CRP levels (P>0.05), but serum NAb level were higher in multiparous cows with ME (P<0.05). In conclusion, it is thought that serum NAb titer and CRP level can be used effectively as an indicator to determine uterus infections.

Keywords: *Cow, Endometritis, NAb, CRP*

Endometritisli İneklerde Serum C-Reaktif Protein ve Doğal Antikor Düzeylerinin Değerlendirilmesi

Özet

Sunulan çalışmada uterusun doğal savunma sisteminin en önemli bileşenlerinden olan keyhole limpet hemosiyanine (KLH) serum doğal antikor (NAb) titresi ve C-reaktif protein (CRP) düzeyinin belirlenmesi amaçlanmıştır. Çalışma 3-7 yaşlı, ortalama vücut kondisyon skoru (BCS) 2.6±0.1 olan 77 İsviçre Esmeri inekte yapıldı. Postpartum 30-32. günlerde bulunan ineklerde endometritis teşhisi; rektal, vaginoskopik, ultrasonografik muayene yöntemleri ve cytobrush tekniğiyle yapıldı. Muayenelerden hemen sonra KLH bağlı NAb titresi ve CRP konsantrasyonlarının belirlenmesi için kan örnekleri alındı. Serum NAb titresi indirekt ELISA yöntemiyle, CRP düzeyi ise bovine CRP ELISA kiti kullanılarak belirlendi. Serum NAb titresi sağlıklı (Kontrol, n = 29), hafif endometritis (ME, n = 21), orta derecede endometritis (E, n = 17) ve şiddetli endometritisli (SE, n=10) ineklerde sırasıyla ortalama 5.36±0.16, 7.43±0.15, 8.55±0.19 ve 9.64±0.27 olduğu belirlendi. Serum CRP konsantrasyonlarının ise kontrol, ME, E ve SE'de sırasıyla ortalama 48.88±3.92 µg/mL, 82.86±4.28 µg/mL, 98.86±8.2 µg/mL ve 122.34±12.72 µg/mL düzeyinde olduğu tespit edildi. Endometritisli ineklerde serum NAb titresi ve CRP düzeyinin önemli oranda arttığı belirlendi (P<0.001). BCS'nin serum NAb ve CRP düzeylerini değiştirmediği saptandı (P>0.05). Doğum sayısının serum NAb titresi ve CRP düzeyinde önemli bir değişikliğe neden olmadığı (P>0.05), sadece ME grubundaki multipar ineklerde serum NAb titresini artırdığı belirlendi (P<0.05). Sonuç olarak ineklerde uterus enfeksiyonlarının belirlenmesinde serum NAb titresi ve CRP düzeyinin indikatör olarak etkin bir şekilde kullanılabileceği düşünülmektedir.

Anahtar sözcükler: *İnek, Endometritis, NAb, CRP*

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INTRODUCTION

Uterus infections result economic losses by causing decreases in reproductive performance and milk production, extending the time intervals between parturition-first insemination and between parturition-conception [1-5]. The incidence of clinical endometritis reportedly ranges between 9-31% (mean 17%) depending on the diagnostic method used, the way the herd being studied is managed and the season in which the study is being conducted [2,6-8]. *Trueperella pyogenes, Fusobacterium necrophorum, Escherichia coli* [9,10] and *Campylobacter fetus* the most important micro-organisms causing endometritis [11]. The regional and systemic defense system of the uterus attempt to destroy [12] the microorganisms that enter the uterus due to the fact physical protective barriers are removed at parturition [13,14]. Neutrophils and lymphocytes play a fundamental role in the uterine immune system [13,15]. Pathogens opsonized with the humoral defense system are increasingly phago-cytosed by neutrophils [15]. Natural antibodies (NAb) are the most significant elements of the humoral defense system from birth [16]. They are present in body serum even in non-vaccinated organisms [17]. They are produced by B1 cells without needing any antigenic stimulation [18]. They constitute the first line of defense against pathogens and activate the complement system [19]. Acute phase proteins also play an important role in the system that fights infection. One of the most important acute phase proteins is C-reactive protein (CRP) [20,21]. CRP is a pentameric protein capable of binding to various pathogenic bacteria [20]. It regulates the immune system in the early periods of infection. It also plays a significant role in the destruction of infectious agents, protecting the tissue against greater damage, and tissue repair and regeneration [22]. Furthermore, it mediates phagocytosis by binding to specific receptors on phagocytic cells, stimulates the production of anti-inflammatory cytokines and contributes to the acquired defense system by activating the complements [23]. In reviewing the literature, we encountered a limited number of studies measuring serum CRP level and NAb titer in cows diagnosed with different severities of endometritis and reporting the differences observed in CRP level and NAb titer according to the severity of endometritis. The aim of this study were to determine the serum NAb titer as a humoral marker and serum CRP level as an acute phase protein in cows with endometritis.

MATERIAL and METHODS

Animals

The present study was conducted on 77 Brown Swiss cows ages 3-7 (between their 1st and 5th parity) at the Kafkas University Veterinary School Research and Application Farm fed on meadow grass, silage (live weight x 1.5/100) and dairy cattle feed (20% crude protein, 2700 energy) with mean body condition score (BCS) of 2.6±0.1.

The Classifications and Determination Endometritis

Cows with a history of caesarean operation, retentio secundinarum, vaginal tear and severe systemic-metabolic disease were excluded. Postpartum examinations were carried out between postpartum days 30-32. Initially the tail, vulva and perineum was examined for the presence of discharge. Then a vaginoscopy was performed, and the character of the discharge on the vaginal wall or coming from the cervix uteri was recorded. Rectal palpation findings (location of the uterus, symmetry of the cornua, etc.) and ultrasonographic (USG, 7.5 MHz, Titan®, Sonosite, USA) images of the uterus (diameter of the cornua, the presence of fluid in the uterine lumen, uterine wall thickness) were recorded. The diameter of the cervix uteri was measured using USG. Endometrial samples were obtained using the cytobrush technique from cows that showed no evidence of discharge, had a normal cervix uteri diameter and presented no symptoms of endometritis in the USG. The cytobrush technique was performed using a brush approximately 3 cm long on a stainless steel rod, 65 cm in length and 4 mm in diameter. Plastic sheaths were used to prevent contamination of the cytobrush in the vagina. Endometrial cytology samples were obtained by rotating cytobrushes a few times in the uterine endometrium. The samples obtained were smeared on slides and dried. They were fixed in methanol (Sigma Aldrich®, Turkey) for fifteen minutes and stained with Giemsa solution (Merck®, Turkey) for 30 min. Then the neutrophil to leukocyte ratio was calculated using microscopy (Olympus CX23®, Olympus corp, Japan) (by counting a minimum of 100 cells at 400x magnification). Cows having a neutrophil to leukocyte ratio >18% [24] were classified as subclinical endometritis. Cows that were determined to be healthy were put in the control group. Endometritis was classified and evaluated as reported by LeBlanc et al.[2] Cows' body condition scores were assessed immediately after the examinations as reported by Edmonson et al.[25] (1=thin; 5=fat; in increments of 0.25). Cows classified according to the degree of endo-metritis were later separated into subgroups based on BCS and number of parities; their serum NAb titer and CRP level were compared. Blood samples were collected from the vena coccygea of all animals into vacuum gel serum tubes (8.5 mL). The blood samples were centrifuged at 1200 g for 10 min. The serums were then transferred into eppendorf tubes and stored at -20°C until the measurements were made.

Analysis of NAb Binding KLH Titer and CRP Level

C-reactive protein was estimated using the bovine CRP ELISA kit purchased from Mybiosource® (USA). Since KLH is a metalloprotein found in the hemolymph, cows are sensitive to it [26]. Therefore, it is reported that unlike the determination of specific serum immunoglobulins of cows, KLH is a good antigen with which to measure NAb [27]. NAb binding KLH titer were determined using an indirect ELISA procedure described by van Knegsel et al.[28]. Plates were coated with 2 µg of KLH/mL (Sigma Aldrich®, USA)

(100 µL/well) dissolved in carbonate buffer (10.6 g/L Na_2CO_3, pH 9.6). After incubating overnight at 4°C and then washed once with tap water and 0.05% Tween-20 (Sigma Aldrich®, USA). After washing, serial dilutions of plasma (1:4) in PBS, 0.05% Tween-20, and than 2.5% rabbit serum (Sigma Aldrich®, USA) were added. Plates were incubated with in PBS with 0.05% Tween-20 for at 1 h at room temperature. Serial dilutions of plasma (1:4), and 2.5% rabbit serum were added, dilutions started at 1:40 for plasma. After being washed with tap water and 0.05% Tween-20, binding of bovine antibodies was detected using 1:20.000 diluted rabbit-anti-bovine IgG conjugated to peroxidase (Sigma Aldrich®, USA) 100 µL/well. After washing, tetramethyl-benzidine (Sigma Aldrich®, USA) 100 µL/well were added and incubated for 10 min at room temperature. The reaction was stopped by adding 1.25 M H_2SO_4 50 µL/well. Extinctions were measured with ELISA Reader (Epoch®, Biotek, USA) at a wavelength of 450 nm.

This study was conducted after obtaining approval from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK - Submission: 18.12.2014/040).

Statistical Analysis

Statistical analyses were performed using the SPSS® (SPSS 20, IL, USA) software program. NAb titer and CRP level observed in each group were tested for normality using the Shapiro-Wilk test. Differences in NAb titer and CRP level between the groups were compared using ANOVA - Tukey HSD tests. The effects of parity and BCS on NAb titer and CRP level were compared using the Independent T-test. The results were analyzed and found to be X±SE. P values <0.05 were considered statistically significant.

RESULTS

Postpartum Examinations Results

In postpartum examinations, mild endometritis (ME) was diagnosed in 21 cows, moderate endometritis (E) in 17 cows, severe clinical endometritis (SE) in 10 cows, healthy (control) in 29 cows. No differences were found between control group (2.61±0.11) and cows with endometritis (ME = 2.63±0.12, E = 2.74±0.12, SE = 2.28±0.13) in terms BCS (P>0.05).

NAb Binding KLH Titers

Serum NAb titer in each group are illustrated in *Fig. 1*. Serum NAb titer were higher in groups with endometritis (ME = 7.43±0.15, E = 8.55±0.19, SE = 9.64±0.27) than in control group (5.36±0.16) and this difference was statistically significant (P<0.001). The more severe the endometritis, the higher the serum NAb titer raise and this difference was considered statistically significant (ME-E = P<0.001; ME-SE = P<0.001; E-SE = P<0.005).

CRP Levels

Serum CRP level in control group and groups with endometritis are presented in *Fig. 2*. Serum CRP level were significantly higher in groups with endometritis (ME = 82.86±4.28 µg/mL, E = 98.86±8.2 µg/mL, SE = 122.34±12.72 µg/mL) than in control group (48.88±3.92 µg/mL, P<0.001). Serum CRP level in the ME group were lower than the SE group (P=0.002). However, no statistically significant difference was observed between the groups ME and E and the groups E and SE in terms of serum CRP level (P>0.05).

Serum NAb Binding KLH Titer and CRP Levels were Compared According to BCS

The number of cows with a body condition score ≤2.5 was control = 14, ME = 10, E = 8, SE = 8, and the number of cows with BCS >2.5 was control = 15, ME = 11, E = 9, SE = 2. When serum NAb titer and CRP level were compared according to BCS, it was found that NAb titer and CRP level were higher in the group with a BCS ≤2.5 (7.23±0.29, 81.94±6.86 µg/mL respectively) than the group with a BCS >2.5 (7.14±0.28, 75.24±4.81 µg/mL); however, this difference was not considered statistically significant (P>0.05). When groups were compared (cows with a BCS ≤2.5 and those with a

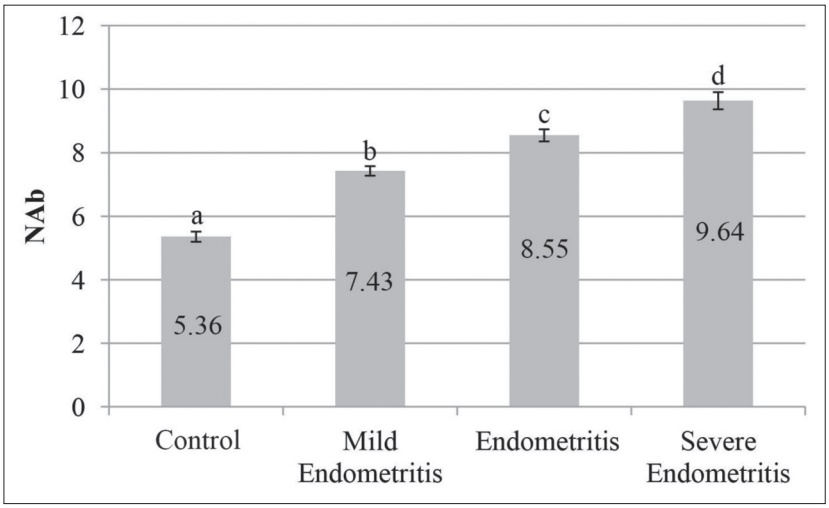

Fig 1. Comparison of NAb titer binding KLH for the groups (a:b:c:<0.001; a:b:d<0.001; c:d=0.005)

Şekil 1. Gruplarda KLH'ye bağlanan NAb titrelerinin karşılaştırılması (a:b:c:<0.001; a:b:d<0.001; c:d=0.005)

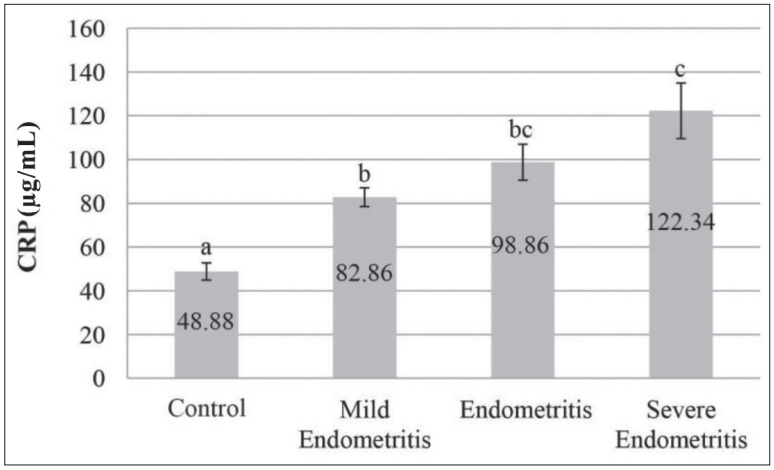

Fig 2. Comparison of serum CRP levels in each group (a:b<0.001; a:bc<0.001; a:c<0.001; b:c=0.002; b:bc>0.05; bc:c>0.05)

Şekil 2. Gruplarda serum CRP seviyelerinin karşılaştırılması (a:b<0.001; a:bc<0.001; a:c<0.001; b:c=0.002; b:bc>0.05; bc:c>0.05)

Table 1. *Determination of NAb titer and CRP level based on body condition scores*
Tablo 1. *Vücut kondisyon skoruna göre NAb titresi ve CRP düzeyinin belirlenmesi*

Groups	BCS		Variables	BCS		P
	≤2.5 (n)	>2.5 (n)		≤2.5	>2.5	
Control	14	15	NAb	5.26±0.20	5.46±0.24	>0.05
			CRP (µg/mL)	41.51±4.09	55.76±6.15	>0.05
Mild Endometritis	10	11	NAb	7.20±0.20	7.64±0.22	>0.05
			CRP (µg/mL)	83.00±6.44	82.74±5.99	>0.05
Endometritis	8	9	NAb	8.33±0.28	8.76±0.24	>0.05
			CRP (µg/mL)	101.33±13.56	96.67±10.49	>0.05
Severe Endometritis	8	2	NAb	9.61±0.27	9.75±1.05	>0.05
			CRP (µg/mL)	131.99±13.66	83.75±13.75	>0.05

n *= cows number,* ***BCS*** *= Body condition score*

Table 2. *Determination of NAb titer and CRP level based on parity*
Tablo 2. *Doğum sayısına göre NAb tiresi ve CRP düzeyinin belirlenmesi*

Groups	Primiparous (n)	Multiparous (n)	Variables	Parity		P
				Primiparous	Multiparous	
Control	16	13	NAb	5.48±0.22	5.22±0.21	>0.05
			CRP (µg/mL)	45.53±4.80	53.00±6.46	>0.05
Mild Endometritis	12	9	NAb	7.17±0.2*	7.78±0.20*	0.043*
			CRP (µg/mL)	77.46±5.09	90.06±6.92	>0.05
Endometritis	9	8	NAb	8.63±0.31	8.46±0.20	>0.05
			CRP (µg/mL)	96.26±9.47	101.79±14.47	>0.05
Severe Endometritis	4	6	NAb	9.60±0.50	8.74±0.52	>0.05
			CRP (µg/mL)	133.3±18	115.03±18.13	>0.05

n *= cows number, * istatistically significant*

BCS >2.5), no statistically significant difference was observed in serum NAb titer and CRP level *(Table 1*; P>0.05)

Serum NAb Binding KLH Titer and CRP Level were Compared According to Parity

The number of primiparous cows in the study was control = 16, ME = 12, E = 9, SE = 4 while the number of multiparous cows was control = 13, ME = 9, E = 8, SE = 6. Serum NAb titer were significantly higher in multiparous cows with ME than in primiparous cows (P<0.05). Although a lower mean serum NAb titer was found in multiparous cows compared to primiparous cows in other groups, no statistically

significant difference was observed (P>0.05). When groups were compared in terms of serum CRP levels, no statistically significant difference was found (P>0.05; *Table 2)*.

DISCUSSION

The purpose of this study was to assess serum CRP level and NAb titer according to the degree of endometritis in cows. As is known, the innate defense system serves as a barrier against infections and fights organisms causing the infection. The most important element of this defense system is NAb [29]. Furthermore, NAb is also related to the acquired defense system. It helps clean out pieces of circulating pathogens in order to prevent them from damaging vital organs [18]. Nutrition, environmental conditions and factors related to the cow may individually change the NAb titer. In order to eliminate such differences in the titer of NAb, cows at similar postpartum days, raised under the same conditions of care and feeding were included in the present study. Natural antibodies (IgM, IgG and IgA) are present in the blood serum and do not require any antigenic stimulation [30]. They prevent bacterial load from binding to the uterine endometrium [31] and opsonizes bacteria for phagocytosis [31,32]. They increase the phagocytic capacity of neutrophils by opsonizing microorganisms [15,33]. The titer of NAb binding to KLH in plasma shows the efficiency of the innate humoral defense system [29]. Resistance to disease is reportedly higher in cows with a high titer of NAb [27,34,35]. Machado et al.[27] reported an association between higher circulating titer of serum NAb around parturition and decreased incidence of clinical endometritis. van Knegsel et al.[28] report in their study that an increase in the titer of NAb in milk may indicate a mammary infection. The NAb titer in plasma has been shown to decrease in the peripartum period [36]. The mean titer of NAb binding to KLH in healthy controls on days 27-195 postpartum was 8.5±0.35 [29]. In the present study, the mean titer of NAb binding to KLH in control group was 5.36±0.16. NAb binding KLH titer were higher in groups with endometritis than in control group, and the more severe the endometritis was the higher the NAb titer rose. These results imply that postpartum uterine infections may increase NAb levels in blood. This increase in NAb titer is thought to arise from NAb production by the natural defense system of the body to facilitate phagocytosis by neutrophils.

C-reactive protein is one of the most important elements of the innate defense system playing a role in pathogen response [23]. CRP is an important acute phase protein in humans [37], dogs [38,39], horses [40] and pigs [41]. Since peripheral CRP level changes minimally in cows during inflammation [42] it is not generally considered an acute phase protein for cows [42,43]. However, some studies have shown that infections increased serum CRP levels in cows [44,45]. A lower level of CRP is reported in cows raised under the best conditions and management methods compared to other cows. It is known that the potential for diseases is higher under poor conditions. In this respect, it is thought that there is a relation between health conditions in dairy farming and CRP levels [45]. The increase in CRP levels are reported to be useful in the diagnosis of mastitis [44]. The mean CRP level was found to be 39.8±47.5 µg/mL in healthy cows [46]. In an another study was found 22.4 ng/mL in CRP level of healthy cows [44]. Li et al.[47] reported in their study that serum CRP levels were significantly higher in cows with clinical endometritis on day 28 postpartum (262.47±8.69 µg/mL) than in healthy cows (1.39±0.04 µg/mL). Consistent with the findings of Li et al.[47], our study found that serum CRP levels were higher in groups with endometritis than in control group (48.88±3.92 µg/mL). CRP levels in the SE group were approximately 3 times higher than in the control group. This difference in CRP levels suggests that serum CRP levels are sensitive to uterine infections and can be a significant indicator in the diagnosis of endometritis.

It is known that the energy balance in animals cannot be measured directly and that BCS is the most important factor showing the amount of food consumed. The negative energy balance (NEBAL) resulting from the reduction in food intake and the start of lactation causes BCS to decline [48,49]. The increase in NEBAL and NEBAL-associated variables in the postpartum period affects innate cellular and humoral activities. The titer of NAb binding to KLH is negatively related to the plasma non-esterified fatty acid (NEFA) concentration increased in NEBAL. This indicates a significant relationship between metabolic health and plasma NAb titer [28]. Similarly, a study conducted by Lee et al.[45] showed a significant relation between serum CRP levels and BCS (serum CRP level is higher in cows with a low BCS than in other cows). However, since cows were classified according to their BCS in the present study (BCS≤2.5 and BCS>2.5), no significant difference could be detected in the serum NAb titer and CRP level. Absence of a difference is thought to be due to the fact that the cows inlcuded in the study had similar BCS (generally between 2.50 and 3.25).

There are studies which report that parity raises NAb binding to KLH [36] but that it has no effect on serum CRP levels [45]. The increase of NAb titer depend on parity in cows corresponds with the that exogenous stimuli enhance the NAb formation [50]. In this study, NAb titer were found to be significantly higher only in multiparous cows in the ME group compared to primiparous cows (P<0.05). Parity had no effect on serum NAb titer in the other groups. Smilarly, parity had no effect on serum CRP levels (P>0.05).

In conclusion, serum NAb titer increased significantly in uterine infections, and the severity of endometritis was significant in this increase. Furthermore, it was determined that CRP, which has been shown to change only minimally in inflammatory disease in cows, increased significantly in uterus infections. It was determined that BCS did not

change serum NAb titer and CRP levels. It is thought that serum NAb titer and CRP level can be effectively used as an indicator for detecting uterus infections in cows. Future studies should confirm these results and shed light on the value of serum NAb titer and CRP levels in evaluating of uterus health.

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