

RESEARCH ARTICLE

Evaluation of Acute Phase Response in Blood and Milk Samples of Healthy Holstein Cattle in the Postpartum Period

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Abstract

Normally, birth triggers an acute phase response (APR). In particular, interleukins and proinflammatory cytokines released from activated leukocytes at the site of tissue damage stimulate APR. In the liver, these cytokines also stimulate acute phase proteins (APPs). APPs are one of the options used in monitoring the health status of animals. This study was evaluated in 12 healthy Holstein cattle, 3-6 years old, who calved at least once. Blood and milk samples were collected from the animals' immediately after calving (0th h) and on the 7th, 14th, and 21st days postpartum. Haptoglobin (Hp), serum amyloid A (SAA), ceruloplasmin (Cp), milk amyloid A (MAA), albumin, total protein (TP) and globulin levels were determined in blood serum and milk serum samples. In the findings; when the measurements of blood and milk Hp, Cp and SAA and TP values were compared; there was a statistically significant difference (P<0.05) between 0th h and 21st day measurements. In the correlation findings, a relationship was found between APP's in blood and milk. In conclusion, this study revealed that APR develops after calving and in the postpartum 21-day period, and the developing APR can clearly be seen in blood and milk. In addition, it was shown that the APR can be traced from milk in dairy cattle, in the present study.

Keywords: Acute phase response, Acute phase protein, Ceruloplasmin, Dairy cattle, Haptoglobin, Milk amyloid A, Postpartum period, Serum amyloid A

Postpartum Dönemde Sağlıklı Holstein Sığırların Kan ve Süt Örneklerinde Akut Faz Yanıtın Değerlendirilmesi

Öz

Normalde doğum bir akut faz yanıtını (AFY) tetikler. Özellikle doku hasarı bölgesinde aktive edilmiş lökositlerden salınan interlökinler ve proinflatuar sitokinler AFY'yi uyarır. Karaciğerde bu sitokinler ayrıca akut faz proteinlerini (AFP) de uyarır. AFP'ler, hayvanların sağlık durumunun izlenmesinde kullanılan seçeneklerden biridir. Bu çalışma en az bir kez doğum yapmış, 3-6 yaşlı, 12 adet sağlıklı holstein ırkı sığırdan değerlendirildi. Hayvanlardan doğumdan hemen sonra (0. saatte) ve doğum sonrası 7, 14, ve 21. günlerde kan ve süt örnekleri alındı. Elde edilen kan ve süt serum örneklerinde haptoglobin (Hp), serum amiloid A (SAA) seroplazmin (Cp), süt amiloid A (MAA), albümin, total protein (TP) ve globulin seviyeleri belirlendi. Bulgularda; Kan ve süt Hp, Cp ve SAA ve TP değerlerinin ölçümleri karşılaştırıldığında; 0. saat ile 21. gündeki ölçümler arasında istatistiksel olarak anlamlı fark (P<0.05) vardı. Korelasyon bulgularında ise kan ve sütteki AFP'leri arasında ilişki bulunmuştur. Sonuç olarak bu çalışma ile doğum sonrasında ve postpartum 21 günlük periyotta AFY'in geliştiği ve gelişen AFY'in kanda ve sütte açık bir şekilde görüldüğü ortaya konmuştur. Ayrıca bu çalışma sütçü sığırların sütlerinden de AFY'in takip edilebileceğini göstermiştir.

Anahtar sözcükler: Akut faz yanıtı, Akut faz proteini, Doğum sonrası dönem, Haptoglobin, Seroplazmin, Serum amiloid A, Süt amiloid A, Süt sığırı

INTRODUCTION

The transition period in dairy cattle is known as the period from 3 weeks before calving to 3 weeks after calving. The

first 3 weeks of this period before calving is the prepartum period, and the first 3 weeks after calving is the postpartum period ^[1,2]. There are many factors that predispose dairy cattle to infections and metabolic diseases in the transition

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period. Among these factors; decreased feed consumption, negative energy balance due to high energy loss, lipolysis, weight loss in early lactation, hypocalcaemia following calving and suppression of the immune system that begins 1-2 weeks before calving and continues for 2-3 weeks after calving are among the important metabolic and immune causes. Bacterial contamination of the uterus, which continues for 2-3 weeks after delivery, is among the causes of microbial origin [2-4].

Acute phase response (APR) is a reaction that develops with the disruption of homeostasis in an organism. In particular, interleukins and proinflammatory cytokines released from activated leukocytes at the site of tissue damage stimulate APR. In the liver, these cytokines also stimulate the production of glycoproteins known as acute phase protein (APP) [5,6]. APP differs significantly between species. In cattle, haptoglobin (Hp) and serum amyloid A (SAA) are considered important acute phase proteins. Ceruloplasmin (Cp) is also one of the acute phase proteins considered to be of medium or low importance in cattle [7]. In ruminants, APPs are used to detect inflammation early and definitively [6-9]. In cattle, APPs are widely used in monitoring therapeutic efficacy in diseases [7] and as predictors of retained placenta or metritis [9] and as markers of mastitis [9,10].

Since APPs are one of the options used in monitoring the health status of animals, interest in this field has increased recently [10,11]. For this reason; in this study, it was aimed to evaluate APR in blood and milk samples of healthy Holstein cattle in the postpartum period.

MATERIAL AND METHODS

Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (MAKUHAD-YEK) (Approval no: 20/05/2021-89/774).

Study Design

In this study, 12 healthy Holstein cattle, 3-6 years old, who calved at least once, were evaluated. Blood (from vena jugularis) and milk samples were taken from the animals immediately after calving (0th h) and on the 7th, 14th, and 21st days of postpartum. Blood samples were centrifuged at 3000 rpm for 15 min and serum was obtained. Due to the accumulation of milk fat in the milk samples, the samples were placed in the centrifuge upside down, centrifuged at 3000 rpm for 20 min and stored at -25°C until analysis. In the obtained blood and milk serum samples; Hp, SAA, milk amyloid A (MAA) (Tridelta Development Ltd, Ireland) and Cp were determined by the colorimetric method based on the p-phenylenediamine oxidase activity described by Colombo and Richterich [12]. Albumin and total protein (TP) were determined with a commercial test

kit (Biolabo, France) (Epoch, Biotek, USA). The globulin value was determined by subtraction of the albumin from the total protein according to Doumas et al. [13].

Statistical Analysis

The findings were evaluated using the IBM SPSS 22.0 for Windows package program. Shapiro-Wilk test was used to determine whether the data were normally distributed. As a result of the Shapiro-Wilk test, it was seen that all data were normally distributed. Due to the normal distribution of the data, repeated measurement comparisons within the group were made using one-way repeated measure ANOVA test and Benferoni corrected multiple comparison tests were used. Pearson Correlation analysis was used to determine the correlation between variables.

RESULTS

The results and statistical evaluations of the Hp, Cp and SAA values in blood serum and milk serum performed at the 0th h and on the 7th, 14th and 21st days are given in [Table 1](#). When the measurements of blood and milk Hp, Cp and SAA (MAA in milk) values at 0th h and 7th, 14th and 21st days were compared; a statistically significant differences (P<0.05) were determined between the measurements of both blood Hp and SAA and milk Hp and MAA values at the 0th h and on the 14th and 21st days. On the other hand, statistically significant differences (P<0.05) were observed between the measurements of both blood and milk Cp values only at the 0th h and the 21st day. In addition, a statistically significant differences (P<0.05) were detected between the 7th day and the 14th and 21st days of the milk Hp values. Furthermore, statistically significant differences (P<0.05) were found between the measurements of milk Cp and MAA values on the 7th, 14th and 21st days. It was also revealed that there was a statistically significant difference (P<0.05) between the measurements of milk TP values at the 0th h and the 21st day. The correlation analysis findings of blood and milk Hp, Cp, SAA, MAA, albumin, TP and globulin values are given in [Table 2](#). Hp, Cp, SAA, MAA, albumin, TP, globulin levels in blood and milk at the 0th h and on the 7th, 14th and 21st days are given as [Fig. 1-A,B,C,D,E,F](#).

DISCUSSION

In high milk yielding cows, the stress of birth and the onset of milk production after calving cause a large metabolic load and stress on the animals and adversely affect the metabolism [4]. Normally, birth triggers an acute phase response. Jawor et al. [14] reported that APR increased after calving. In parallel with this information, in our study, it is seen that APPs in blood and milk were higher and statistically significant (P<0.05) after calving (0th h) compared to 7th, 14th and 21st days ([Table 1](#)). These findings demonstrated once again that the acute phase response clearly developed after calving.

Table 1. Evaluation of acute phase response in blood and milk immediately after calving (0th h) and on days 7, 14 and 21

Parameters	Measurement Time			
	After Calving (0 th Day) X±Sd	7 th Day X±Sd	14 th Day X±Sd	21 th Day X±Sd
Hp (Blood) (mg/L or µg/mL)	167.83±59.27 ^a	117.08±44.59 ^{ab}	73.33±28.35 ^b	79.91±30.68 ^b
Hp (Milk) (mg/L or µg/mL)	16.4±0.261 ^a	12.36±2.71 ^b	6.45±2.40 ^c	5.68±2.12 ^c
SAA (mg/L)	47.13±11.53 ^a	36.77±7.09 ^{ab}	33.43±4.39 ^b	28.66±5.87 ^b
MAA (mg/L)	13.03±4.27 ^a	9.73±2.61 ^a	5.67±1.13 ^b	3.70±0.89 ^c
Cp (Blood) (mg/dL)	19.16±2.81 ^a	16.94±3.26 ^{ab}	17.14±4.94 ^{ab}	15.42±2.66 ^b
Cp (Milk) (mg/dL)	6.30±1.93 ^{abc}	6.11±2.49 ^b	3.46±1.87 ^{cd}	2.75±0.89 ^d
Albumin (Blood) (g/dL)	3.56±0.23 ^a	3.63±0.49 ^a	3.57±0.34 ^a	3.73±0.45 ^a
Albumin (Milk) (g/dL)	2.12±0.37 ^a	2.40±0.73 ^a	2.47±0.44 ^a	2.63±0.47 ^a
TP (Blood) (g/dL)	6.94±0.39 ^a	7.07±0.34 ^a	7.21±0.55 ^a	7.18±0.62 ^a
TP (Milk) (g/dL)	3.04±0.46 ^a	3.34±0.69 ^{ab}	3.41±0.44 ^{ab}	3.59±0.23 ^b
Globulin (Blood) (g/dL)	3.37±0.52 ^a	3.44±0.48 ^a	3.64±0.68 ^a	3.45±0.77 ^a
Globulin (Milk) (g/dL)	0.92±0.39 ^a	0.93±0.36 ^a	0.94±0.40 ^a	0.9±0.47 ^a

^{a,b,c,d} Values within a columns with different superscripts differ significantly at $P < 0.05$

Hp: Haptoglobin; SAA: Serum amyloid A; Cp: Ceruloplasmin; MAA: Milk amyloid A; TP: Total protein.

Table 2. Correlation findings between Hp, Cp, SAA, MAA, albumin, TP and globulin values in blood and milk

Variables		Hp (Blood)	SAA (Blood)	Cp (Blood)	Albumin (Blood)	TP (Blood)	Globulin (Blood)	Hp (Milk)	MAA (Milk)	Cp (Milk)	Albumin (Milk)	TP (Milk)	Globulin (Milk)
Hp (Blood)	Pearson Correlation	1	.468**	.266	-.071	-.168	-.088	.615**	.702**	.328*	-.332*	-.144	.250
	Sig. (2-tailed)		.001	.068	.632	.253	.551	.000	.000	.023	.021	.328	.086
SAA (Blood)	Pearson Correlation	.468**	1	.355*	-.238	-.124	.052	.558**	.707**	.281	-.284	-.186	.135
	Sig. (2-tailed)	.001		.013	.104	.401	.725	.000	.000	.053	.050	.207	.361
Cp (Blood)	Pearson Correlation	.266	.355*	1	.105	-.404**	-.385**	.266	.314*	.192	-.433**	-.175	.344*
	Sig. (2-tailed)	.068	.013		.476	.004	.007	.068	.030	.191	.002	.235	.017
Albumin (Blood)	Pearson Correlation	-.071	-.238	.105	1	.023	-.614**	-.023	-.059	-.187	.137	.127	-.017
	Sig. (2-tailed)	.632	.104	.476		.879	.000	.875	.690	.204	.353	.389	.908
TP (Blood)	Pearson Correlation	-.168	-.124	-.404**	.023	1	.776**	-.237	-.224	-.009	.204	.096	-.145
	Sig. (2-tailed)	.253	.401	.004	.879		.000	.105	.126	.953	.164	.517	.326
Globulin (Blood)	Pearson Correlation	-.088	.052	-.385**	-.614**	.776**	1	-.172	-.139	.111	.075	-.005	-.104
	Sig. (2-tailed)	.551	.725	.007	.000	.000		.242	.345	.453	.614	.976	.484
Hp (Milk)	Pearson Correlation	.615**	.558**	.266	-.023	-.237	-.172	1	.772**	.598**	-.239	-.319*	-.095
	Sig. (2-tailed)	.000	.000	.068	.875	.105	.242		.000	.000	.101	.027	.521
MAA (Milk)	Pearson Correlation	.702**	.707**	.314*	-.059	-.224	-.139	.772**	1	.462**	-.228	-.097	.174
	Sig. (2-tailed)	.000	.000	.030	.690	.126	.345	.000		.001	.120	.511	.236
Cp (Milk)	Pearson Correlation	.328*	.281	.192	-.187	-.009	.111	.598**	.462**	1	-.216	-.316*	-.121
	Sig. (2-tailed)	.023	.053	.191	.204	.953	.453	.000	.001		.141	.029	.412
Albumin (Milk)	Pearson Correlation	-.332*	-.284	-.433**	.137	.204	.075	-.239	-.228	-.216	1	.702**	-.412**
	Sig. (2-tailed)	.021	.050	.002	.353	.164	.614	.101	.120	.141		.000	.004
TP (Milk)	Pearson Correlation	-.144	-.186	-.175	.127	.096	-.005	-.319*	-.097	-.316*	.702**	1	.359*
	Sig. (2-tailed)	.328	.207	.235	.389	.517	.976	.027	.511	.029	.000		.012
Globulin (Milk)	Pearson Correlation	.250	.135	.344*	-.017	-.145	-.104	-.095	.174	-.121	-.412**	.359*	1
	Sig. (2-tailed)	.086	.361	.017	.908	.326	.484	.521	.236	.412	.004	.012	

* Statistically significant correlation is found; ** Statistically significant correlation is found

Hp: Haptoglobin; SAA: Serum amyloid A; Cp: Ceruloplasmin; MAA: Milk amyloid A; TP: Total protein.

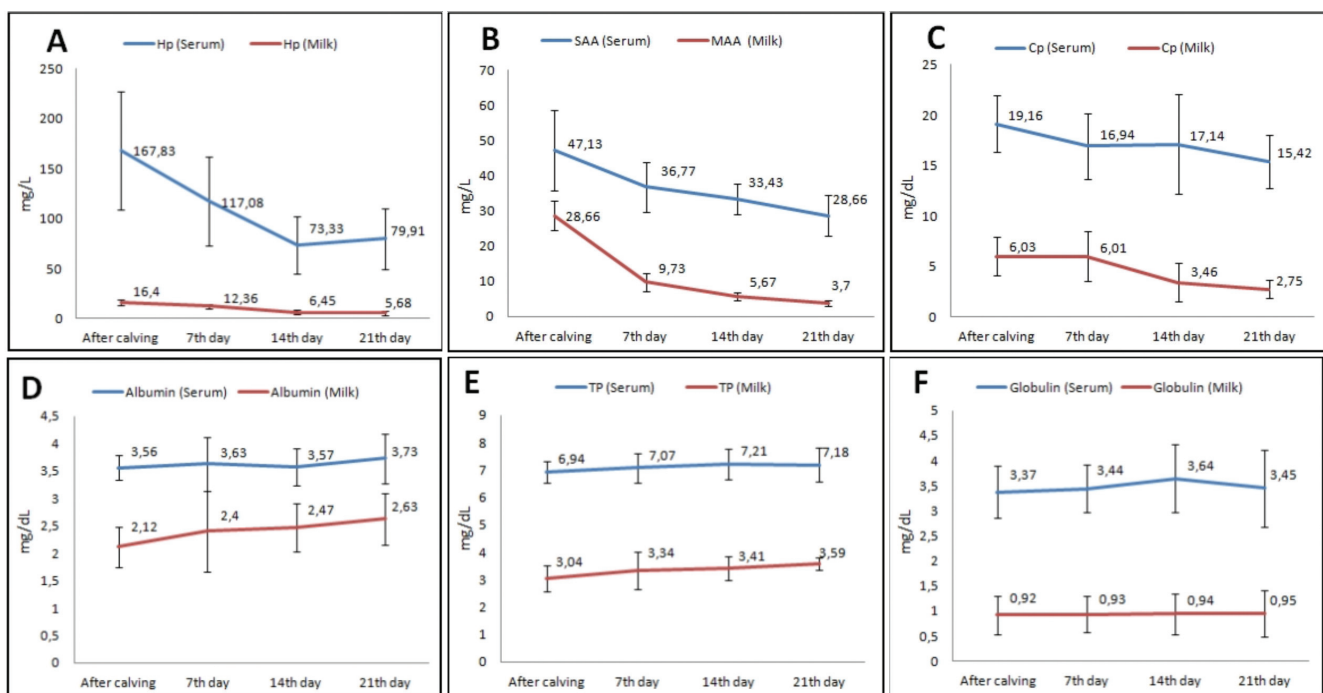


Fig 1. Hp (A), Cp (B), SAA and MAA (C), albumin (D), TP (E), globulin (F) levels in blood and milk at the 0th h and on the 7th, 14th and 21st days. Hp: Haptoglobin; SAA: Serum amyloid A; Cp: Ceruloplasmin; MAA: Milk amyloid A; TP: Total protein

Bertoni et al.^[11] and Humblet et al.^[10] found that serum Hp levels were high in dairy cows, especially in the first 7-day period after calving. Trevisi et al.^[15] determined that serum Hp levels increased in dairy cows within the first 10 days after calving, and decreased after the 10th day. Nightingale et al.^[16] recorded that serum Hp levels increased in 240 dairy cows between 2 and 8 days postpartum. Uchida et al.^[17] and Chan et al.^[18] showed that serum Hp concentration increased after calving in healthy cows and the highest Hp concentration occurred 2-3 days after calving. In addition, Bossaert et al.^[19] found that there was an increase in serum Hp levels especially in the first 3 days after calving and gradually decreased after the first week. In our current study, it was observed that the Hp value in milk and blood was at the highest level after calving, and the Hp value continued to decrease on the 7th and 14th days. A slight increase in serum Hp was observed on day 21. On the other hand, the decrease in milk Hp value continued on the 21st day. It is extremely interesting that while the Hp value in the blood increases on the 21st day, the decrease in milk continues (Table 1, Fig. 1-A). How this develops should be investigated in future studies.

Another important positive APP in cattle is SAA^[20] Uchida et al.^[17] reported that the increased SAA concentration after calving reached its highest level in 2-3 days. But, Chan et al.^[18] determined that SAA levels in healthy cows decreased to normal levels within the first week after calving. On the other hand, in our study, it was observed that SAA concentration was at the highest level after calving, and decreased dramatically on the 7th, 14th and 21st days (Table 1, Fig. 1-B).

Cp is one of the parameters used in the evaluation of animal health and welfare^[21]. Studies in cattle have revealed that Cp can be used for diagnostic purposes in various diseases and conditions^[22]. Trevisi et al.^[15] reported that the Cp value increased after calving and in the first week after calving in high milk yielding cows with low and high LFI index. Furthermore, Bossaert et al.^[19] determined that the level of Cp increased after calving and remained high for 42 days after calving. In supporting this, Hussein et al.^[23] found that there was a significant increase in serum Cp levels in the first week after calving in cows. In our study, it was observed that the serum Cp value increased after calving and decreased to the normal limits within a week. It was also observed that the milk Cp value increased after calving, and decreased dramatically on the 7th, 14th and 21st days (Table 1, Fig. 1-C).

Bayyit and Merhan^[24] determined serum Hp concentration as 0.176 ± 0.007 g/L, Cp concentration as 15.68 ± 0.83 mg/dL, and serum albumin concentration as 3.15 ± 0.08 g/dL in cows with normal calving. In parallel with this information, in our study, Hp concentration was determined as 0.167 ± 0.059 g/L, Cp concentration as 19.16 ± 2.81 mg/dL, and serum albumin concentration as 3.56 ± 0.23 g/dL after calving in dairy cows (Table 1).

Chan et al.^[18] reported serum Hp and SAA concentrations as 630 ± 302 µg/mL and 66 ± 15 µg/mL on days 0-3, 380 ± 250 µg/mL and 48 ± 20 µg/mL on days 4-7, 310 ± 197 µg/mL and 42 ± 18 µg/mL on day 14, and 86 ± 73 µg/mL and 37 ± 19 µg/mL on day 21 respectively in healthy dairy cattle. In our study; serum Hp and SAA concentrations were determined

as 167.83 ± 59.27 $\mu\text{g/mL}$ and 47.13 ± 11.53 $\mu\text{g/mL}$ after calving, 117.08 ± 44.59 $\mu\text{g/mL}$ and 36.77 ± 7.09 $\mu\text{g/mL}$ on day 7, 73.33 ± 28.35 $\mu\text{g/mL}$ and 33.43 ± 4.39 $\mu\text{g/mL}$ on day 14, 79.91 ± 30.68 $\mu\text{g/mL}$ and 28.66 ± 5.87 $\mu\text{g/mL}$ on day 21 respectively (Table 1).

The synthesis of plasma proteins is primarily made in the liver. Especially in some diseases, analysis of TP concentrations and percentage of protein fractions is important [7,24]. Albumin, one of the plasma proteins, is the most osmotically active serum protein and is involved in the transport of many substances. Globulins are a heterogeneous group of proteins. They include antibodies and other inflammatory molecules, haemostatic and fibrinolytic proteins, lipid transporters, vitamins, and hormones. Albumin and globulin concentrations shift during physiological or pathological conditions [25]. Total serum globulin concentrations are component of the organism's defence system. Therefore, it has the property of an indicator of humoral immune status or response. It has been reported that there is decrease in serum globulin concentration in cattle in the peripartum period [26]. After calving, plasma volume increases and albumin synthesis decreases. Therefore, albumin remains at a low level for 2 weeks after calving, and the clinical use of albumin as APP decreases during lactation [27]. Negative APPs are important for albumin in cattle [7]. The amount of APPs produced by each species during its inflammatory response is unique. However, it is speculated that the serum albumin level decreases between 10-30% in all mammalian species [28,29]. In parallel with this information, in our study; although there was no statistical difference, it was observed that the albumin value in blood and milk was at the lowest level at the 0th h and increased on the 7th, 14th and 21st days. It was observed that serum globulin and TP values were low at 0th h, increased on 7th and 14th days, and decreased on 21st day. It was observed that milk albumin, globulin and TP values were low at the 0th h and continued to increase on the 7th, 14th and 21st days. In the measurements taken on the 21st day; while the serum TP value decreased, the milk TP value continued to increase. In addition, there was a statistically significant difference ($P < 0.05$) between 0th h and 21st day in the measurements of milk TP values. It is extremely interesting that the TP value decreases in blood serum and increases in milk, and it should be further investigated (Table 1, Fig. 1-D,E,F).

Hp, SAA, Cp and c-reactive protein (CRP) are among the APPs identified in milk. These APPs have been reported to have the potential to be biomarkers in cases of mastitis [30,31]. The main isoforms of SAA are SAA1, SAA2 and SAA3. SAA1 and SAA2 are produced in the liver. SAA3 is produced in extra hepatic regions. It is more commonly known as mammary-associated amyloid A (M-SAA3), especially since it is predominantly found in milk [32,33]. Cp, which is mainly synthesized from hepatocytes, is also synthesized in the mammary gland and increases in case of infection and

tissue damage [34]. In normal milk, the Hp value is 0.32 $\mu\text{g/mL}$ [35], MAA 3.58 mg/L [36] and Cp levels 0.5 mg/dL [30]. In parallel with this information, in our study, milk Hp, milk Cp and MAA concentrations were determined as 16.40 $\mu\text{g/mL}$, 13.03 $\mu\text{g/mL}$, and 6.30 mg/dL at 0th h, 12.36 $\mu\text{g/mL}$, 9.73 $\mu\text{g/mL}$, and 6.11 mg/dL on 7th day, 6.45 $\mu\text{g/mL}$, 5.67 $\mu\text{g/mL}$, and 3.46 mg/dL on 14th day, 5.68 $\mu\text{g/mL}$, 3.70 $\mu\text{g/mL}$, and 2.75 mg/dL on 21st day respectively. In addition, there was a statistically significant difference ($P < 0.05$) between 0th h and 21st days in the measurements of milk Hp, milk Cp and MAA values (Table 1, Fig. 1-A,B,C). These findings reveal that the acute phase response after calving clearly occurs in milk as well. While SAA is produced in the liver, MAA is produced in non-hepatic regions (mammary tissue) [32,33]. The increase in MAA may be related to the mammary specificity of MAA and postpartum mammary oedema and inflammation. An increase in the SAA (synthesized from the liver) may also be associated with calving. Since there is a strong and statistically significant positive correlation between blood SAA and MAA, it should be investigated in more detail to determine the acute phase response developing in the mammary tissue.

Among the APPs, Hp and SAA increase in parallel in serum and milk [37,38]. Dalanezi et al. [39] reported that there is a positive correlation between milk Hp and SAA values in their study. Similarly, in our study, a moderate and statistically significant positive correlation was found between blood Hp value and SAA value ($r = 0.468$; $P = 0.001$). A strong and statistically significant positive correlation was found between milk Hp value and milk MAA value ($r = 0.772$; $P < 0.001$) (Table 2). Increased activity of Cp in blood and milk has been reported in cases of mastitis in cattle [22,34,40]. Bertoni et al. [11] determined a weak, but significant and linear relationship between serum Cp and serum Hp. Parallel to this information, in our study, a weak positive correlation was found between blood Hp value and blood Cp value ($r = 0.266$; $P = 0.068$). A moderate and statistically significant positive correlation was found between milk Hp value and milk Cp value ($r = 0.598$; $P < 0.001$) (Table 2). Gürlür et al. [41] found that there was a significant positive correlation between milk TP and albumin values in subclinical mastitis cases in buffaloes. In our study, a strong and statistically significant positive correlation was found between milk, albumin value and milk TP value ($r = 0.702$; $P < 0.001$) (Table 2). According to these results, it was clearly demonstrated that negative and positive acute phase proteins increase in parallel in serum and milk.

The high amount of MAA in colostrum binds to Gram-negative bacteria with high affinity, as well as initiating or increasing mucin secretion by stimulating neonatal intestinal cells to secrete mucus, which will reduce bacterial colonization and increase resistance to disease in newborn calves [32,38]. Similarly, the antioxidant activity of high Hp in colostrum will be beneficial in countering

bacterial invasion of the intestine^[38]. Since Cp in colostrum is an extracellular antioxidant, it will be necessary for the anti-oxidative defence of the organism^[42,43]. The results obtained in this study once again demonstrate the importance of APPs in a passive transfer of colostrum-induced immunity in newborns calves.

In conclusion, this study revealed that APR develops after calving and in the postpartum 21-day period, and the developing APR can clearly be seen in blood and milk. In addition, it was shown that the APR can be traced from milk in dairy cattle, in the present study.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study available from the corresponding author (K. Varol) on reasonable request.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Design of the study: VK. EEH. MO. BK. Data collection: VK. Data analysis; EEH. MO. BK. Article writing VK. EEH. MO. BK.

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