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RESEARCH ARTICLE

The Evaluation of Important Biomarkers in Healthy Cattle [1]

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Summary

In this study the aim is to determine the blood serum levels of biological markers as procalcitonin, neopterin, TNF-α, MDA, PGE2, IL-8, IFN-γ which are considered as highly beneficial on diagnosing the infections in the veterinary medicine and evaluating the prognosis in the healthy cattle at different ages and in different gender. The materials of this study are 48 (25 female and 23 male) cattle and calf bred (neonatal <1 month, young 12-24 month and mature >24 month) in operations in Sivas region and which are determined to be healthy via the biochemical and hematological findings. Serum procalcitonin level was found lower in neonatal group than the young and adult group (P<0.05). It was realized that neopterin level is reasonably higher in neonatal group than both young and adult group (P<0.05). MDA level in the young and adult group was measured as higher than of the neonatal group (P<0.05). There was found between the adult group and neonatal group a statistically reasonable difference for the serum PGE2 levels (P<0.05). In IL-8 level, there was found a statistically important difference only between young group and neonatal group (P<0.05). There was found no statistic difference among the levels of procalcitonin, neopterin, TNF-α, IFN-γ, MDA, PGE2, IL-8 between the sexes. As a result, it is concluded that determining the levels of markers used in defining the prognosis of the infection in healthy cattle at different ages would be a base data for further studies.

Keywords: *Procalcitonin, Cattle, TNF-α, MDA, Neopterin, PGE2, IFN-γ, IL-8*

Sağlıklı Sığırlarda Önemli Biyomarkerların Değerlendirilmesi

Özet

Bu çalışmada veteriner hekimlikte enfeksiyonların teşhisinde ve prognozunun değerlendirilmesinde önemli faydalar sağlayacağı düşünülen prokalsitonin, neopterin, TNF α, IFN-γ, MDA, PGE2, IL-8 gibi biyolojik markerlerin farklı yaş gruplarında ve cinsiyetlerdeki sağlıklı sığırlardaki kan serumu seviyelerinin belirlenmesi amaçlanmıştır. Çalışmanın materyalini Sivas yöresinde bulunan işletmelerde yetiştirilen biyokimyasal ve hematolojik bulgularıyla sağlıklı olduğu belirlenen neonatal (<1 ay), genç (12-24 ay) ve ergin (>24 ay) 48 adet (25 dişi 23 erkek) sığır ve buzağı oluşturmuştur. Serum prokalsitonin seviyesi, neonatal grupta genç ve ergin gruba göre düşük bulundu (P<0.05). Neopterin seviyesi neonatal grupta hem genç hem de ergin gruba göre anlamlı düzeyde yüksek olduğu görüldü (P<0.05). Genç ve ergin gruptaki MDA seviyesi neonatal gruba göre daha yüksek ölçüldü (P<0.05). Ergin grup ile neonatal grup arasında serum PGE2 seviyeleri açısından istatistiki olarak anlamlı fark belirlendi (P<0.05). IL-8 seviyesinde ise sadece genç grup ile neonatal grup arasındaki fark istatiksel olarak önemli bulundu (P<0.05). Cinsiyetler arasında neopterin, prokalsitonin, TNF-α, IFN-γ, MDA, PGE2, IL-8 seviyelerinde istatistikî olarak fark belirlenemedi (P>0.05). Sonuç olarak farklı yaş aralığındaki sağlıklı sığırlarda enfeksiyonun prognozu belirlenmesinde kullanılan markerlerin seviyeleri belirlenerek sonraki yapılacak farklı çalışmalara temel veri niteliği taşıyacağı düşünülmüştür.

Anahtar sözcükler: *Prokalsitonin, Sığır, TNF-α, MDA, Neopterin, PGE2, IFN-γ, IL-8*

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INTRODUCTION

Despite to all the advantages as too many diagnose methods' defining the inflammatory reactions entering into applications and the increase in the diversity of medicines used against the infections practically, it is still deemed that there have been difficulties in observing the prognosis of infections in domestic animal and there still occurs deaths resulted from sepsis, multiple organ failure and shock developed in parallel with the infections. It is known that a considerable amount of deaths occur because of septic shock both in economically important livestock and both in the pets [1].

Many parameters are used for diagnosis of illnesses caused by infection. C-reactive protein (CRP) as an acute phase reactants and cytokines such as neopterin, interleukins (IL-8, IL-6), tumor necrosis factor (TNF) are major ones used for diagnosis. Procalcitonin, can be used both for of infections despite being sensitive to bacterial inflammations and protein based during the illness it is a long half-life important marker. Among these markers for diagnostic and prognostic the illnesses, procalcitonin stands out for reasons such as its specificity to bacterial infections and unlike cytokines being stabil at room temperature during analyze at plasma concentrations [2].

TNF-α and interferon gamma (IFN-γ) are one of those cytokines activating macrophage. When they are activated, they could phagocytized pathogens since they have turned into the cells which are capable of producing reactive oxygen products and lysosomal enzymes. It is also known that after IFN-γ's stimulating IL-2 receptors in macrophage, IL-2 released from T lymphocyte is increasing the microbicidal effects of macrophages [3-5].

TNF-α stimulates acute inflammation in order to initiate the extrinsic coagulation mechanism and activate the clotting mechanism through stimulating the tissue factor formation in endothelium cells [6]. Moreover, it increases the platelet activating factor (PAF) release by stimulating the endothelium cells and also increases the synthesis of phase proteins by effecting the hepatocyte and it eases chemotaxis and plays an effective role in stimulating the neutrophils for formation of superoxide radicals in phagocytose. There has been various studies conducted on both people and animals demonstrating that there are a positive correlation between TNF-α level and mortality rates and that TNF-α could be used in observing the inflammation [3,7,8].

The release of TNF together with IL-1 both stimulates hypothalamus cells so prostaglandinE_2 (PGE_2) synthesis increases [5] and PGE_2 which are the product of arachidonic acid metabolism are released from mast cells, capillary endothelial cells and macrophages [9]. If the amounts of prostaglandins are low in tissues, then it stimulates the inflammation, however; if high, they behave as if anti-inflammatory. Prostaglandins have some effects such as pain and fever [6].

TNF-α stimulates the formation and release of other cytokines (IL-1, IL-6, IL-8) such cytotoxic factors like free oxygen radicals and nitric oxide (NO) [5].

Reactive oxygen species and free radicals produced by lipid peroxidation in tissue damage has been implicated in the pathogenesis of many diseases. Unsaturated phospholipids and cholesterol appearing in the structure of membranes and free radicals can easily react and so result in lipid peroxidation. During that process a range of reactions occur and as a result of them MDA, an important biological marker used in marking the membrane damage occurs. Through the measuring of the amount of malondialdehyde an indirect data on the level of lipid peroxidation could be gained [10].

IL-8 is produced by inflammatory cells. For leucocytes, potential chemotaxis is an effective cytokine [11]. Cytokines are important proinflammatory mediators during early phase in the sepsis released from macrophages and endothelial cells and monocytes with the stimulation of infections. In numerous studies, in human neonatal and adult sepsis, TNF-α, IL-6 and IL-8 levels are found high [12,13]. IL-8 plays an important role in the organ dysfunction developed in sepsis and lung damage. In studies conducted on determining the diagnostic value of IL-8, Gram has stated that in negative bacteremia the positive predictive value is 73% and the negative predictive value is 94% [8]. In the study done by Martin et al.[12], it was assigned that in newborns serum IL-6, IL-8 and TNF-α levels have increased.

Neopterin is low molecular weight 2-amino-4-hydroxy-(1'2'3'trihydroxypropyl)-pteridin. Neopterin cell is accepted as a marker of cell-mediated immunity [2]. It is produced by active monocyte/macrophage. IFN-γ is a potential neopterin producer and it demonstrates the increase in neopterin concentrations and the existence of IFN-γ in body fluids [2,14].

Procalcitonin is precursor of calcitonin hormone which is produced in thyroid C cells and which is responsible for the calcium homeostasis. Apart from the neuroendocrine in the thyroid gland, procalcitonin which could be released from lung and bowel, as well, in cases of sepsis, is found low in healthy persons. According to studies, procalcitonin in bacterial diseases is reported to increase rapidly in a short time after TNF- α, IL-6 and IL-8. It is known by various researchers that in septicemias, procalcitonin could increase as one hundred times more than normal serum levels [2,15-18].

It is stated that for humans; procalcitonin to be under 0.1 ng/ml in healthy persons, >0.5 ng/m1 in viral infections and 1.5 ng/ml as highest, moreover, in serious bacterial infections this rate might increase to five more times at least, that it could go beyond 10ng/ml and that this rate could even exceed 1.000 ng/ml [2,8].

Biological markers used mostly in determining the type of the ongoing inflammation and observing the respond to the treatment in human medicine. However, in veterinary medicine there known very little parameter used routinely and could be useful in determining the inflammation or observing the respond to the treatment.

In this study the aim is to determine the blood serum levels of the biological markers such as; procalcitonin, neopterin, TNF-α, IFN-γ, MDA, PGE_2, IL-8 in healthy cattle at different ages and of different genders in order to enlighten the studies to be done in the field of veterinary medicine related to the biological markers known for their important benefits for diagnosing the infections and evaluating the prognosis in human medicine.

MATERIAL and METHODS

The materials of this study are heparin containing blood and blood serums taken from 48 (25 female and 23 male) cattle and calf which were separated into three groups as neonatal (<1 month), young (12-24 month) and adult (>24 month) determined as healthy in systematic clinical examinations, whose serum biochemical values and hematological parameters were found among the normal bounds and which were raised in the operations in Sivas region.

In the blood serums taken, glucose, total bilirubin, direct bilirubin, total protein, albumin, globulin and AST levels were determined via auto analyzer device (Mindray BS 200, PRC).

Hematological examinations were given through the heparin containing blood samples taken through the methods acclaimed by literature [19].

Among the blood samples, IFN-γ, IL-8 and procalcitonin levels were determined through the sandwich enzyme immunoassay method, TNF-α, MDA, neopterin and PGE_2 levels were determined through competitive inhibition enzyme immunoassay method and through commercial kits (Cusabio, PRC) and in accordance with the kit procedures and by using the ELISA device (Thermo Multiskan).

In analyzing the facts reached, Student-T Test and ANOVA tests were used. While Levene was evaluating the homogeneity of variance according to the test results, intergroup comparisons were made by using Duncan and Tamhane tests [20] and through the SPSS 14.00 packet program (SPSS Inc, Chicago).

This study was carried out with permission dated 03.04.2013 No. 373 with local Ethics Committee for Animal Experiments of the University of Cumhuriyet.

RESULTS

Serum biochemistry values reached in the study are shown in *Table 1* and the results of clinical examination and hematological values are shown in *Table 2*.

In the statistical analysis of serum biochemistry values determined in the studies, there could not be found any difference among age and gender groups (P>0.05).

The comparison on the age base, of the levels of procalcitonin, neopterin, MDA, TNF-α, IL-8, PGE_2, IFN-γ determined in the serums is shown in *Table 3*. The comparison of determined values to the gender is given in *Table 4*.

The level of serum procalcitonin was found lower in neonatal group than the young and mature groups (P<0.05). The comparison of procalcitonin levels according to age group is shown in *Fig. 1*. Neopterin level was regarded as reasonably high comparing to both young and mature group (P<0.05). The comparison of neopterin levels according to age group is shown in *Fig. 2*. MDA level in young and mature group was measured higher than the

Table 1. *Serum biochemistry evaluations determined in the study*
Tablo 1. *Çalışma gruplarında belirlenen serum biyokimya değerleri*

Parameters	Age Groups						P Value
	Neonatal		Young		Mature		
	n	Mean±SE	n	Mean±SE	N	Mean±SE	
Glucose (mg/dL)	15	61.40±2.85	17	63.88±1.83	16	62.56±1.89	0.729
Creatine (mg/dL)	15	1.13±0.06	17	1.11±0.05	16	1.27±0.07	0.182
T. Bilirubin (mg/dL)	15	0.14±0.03	17	0.12±0.02	16	0.22±0.05	0.234
D. Bilirubin (mg/dL)	15	0.07±0.02	17	0.10±0.02	16	0.14±0.05	0.377
Total Protein (g/dL)	15	7.08±0.11	17	7.01±0.11	16	7.05±0.11	0.898
Albumin (g/dL)	15	3.32±0.12	17	3.42±0.07	16	3.23±0.10	0.394
Globulin (g/dL)	15	3.76±0.14	17	3.59±0.15	16	3.75±0.13	0.611
AST (IU/L)	15	82.73±3.51	17	80.47±3.13	16	83.75±3.27	0.766

Tablo 2. *The clinical examination findings and hematological values determined in the study*
Tablo 2. *Çalışmada belirlenen klinik muayene bulguları ve hematolojik değerler*

Measurements	Neonatal	Young	Mature	Normal Values
Body temperature °C	38.78±0.05	38.73±0.06	38.65±0.02	36.7-39.1
Respiratory rate	49.00±2.15	42.13±1.11	37.75±1.28	26-50
Heart rate	95.00±1.92	74.00±2.07	72.25±1.62	48-84
Packed cell volume %	32.13±0.90	30.75±0.75	30.00±0.93	24-46
Leukocyte (10^3/µl)	5.43±0.27	5.25±0.23	5.13±0.23	4-12
Erythrocyte (10^6/µl)	6.15±0.19	6.34±0.26	6.24±0.28	5-10

Table 3. *The comparison of procalcitonin, neopterin, TNF-α, MDA, PGE_2 IL- 8 and IFN-γ levels according to the age groups*
Tablo 3. *Çalışmada belirlenen prokalsitonin, neopterin, TNF-α, MDA, PGE_2, IL- 8 ve IFN-γ seviyelerinin yaş gruplarına göre karşılaştırılması*

Parameters	Age Groups						
	Neonatal		Young		Mature		P*
	n	Mean±SE	n	Mean±SE	n	Mean±SE	
Procalcitonin (pg/ml)	15	43.257±0.780^{b}	15	52.929±3.71^{a}	15	53.349±3.166^{a}	0.021
Neopterin (ng/ml)	15	4.181±0.266^{a}	17	3.204±0.495^{b}	16	2.493±0.101^{b}	0.004
TNF-α (ng/ml)	13	0.667±0.033	17	0.861±0.123	14	0.707±0.050	0.259
MDA (ng/ml)	13	267.430±20.956^{b}	16	454.379±64.052^{a}	14	473.088±45.813^{a}	0.037
PGE2 (pg/ml)	13	113.343±4.607^{b}	15	138.080±12.968^{ab}	15	150.120±11.887^{a}	0.029
IL-8 (pg/ml)	15	199.187±8.433^{b}	17	293.294±47.031^{a}	16	238.929±9.406^{ab}	0.011
INF-γ (pg/ml)	13	-	15	-	13	-	-

*^{a,b,c} In the same row with different letters are statistically significant differences between the values (P<0.05); -: specified value was not significant for healthy cattle; * Biggest P value of differences*

Table 4. *The comparison of procalcitonin, neopterin, MDA, TNF-α, IL- 8, PGE_2 and IFN-γ levels according to the gender*
Tablo 4. *Çalışmada belirlenen belirlenen prokalsitonin, neopterin, MDA, TNF-α, IL- 8, PGE_2 ve IFN-γ seviyelerinin cinsiyete göre karşılaştırılması*

Parameters	Sex Groups						
	Male		Female		P Value	Total	
	n	Mean±SE	n	Mean±SE		N	Mean±SE
Procalcitonin(pg/ml)	23	50.066±2.800	22	49.614±2.147	0.89	45	49.845±1.755
Neopterin (ng/ml)	24	3.460±0.373	24	3.084±0.224	0.39	48	3.272±0.217
MDA (ng/ml)	22	402.061±53.994	21	405.931±32.292	0.95	43	403.951±31.439
TNF-α (ng/ml)	24	0.758±0.086	20	0.752±0.051	0.95	44	0.755±0.052
IL-8 (pg/ml)	24	217.435±6.735	24	274.093±34.208	0.11	48	245.764±17.734
PGE_2 (pg/ml)	24	132.422±7.755	19	137.806±11.402	0.69	43	134.801±6.571
INF-γ (pg/ml)	21	-	20	-		41	-

neonatal group's. There determined a reasonable difference between the mature group and neonatal group on the basis of PGE_2 levels (P<0.05). In IL-8 level, only the difference between young group and neonatal group was found statistically important (P<0.05). The comparison of PGE_2, MDA and IL-8 levels according to age group are shown in *Fig. 3*. The difference of TNF-α levels between groups was not statistically meaningful (P>0.05). IFN- γ levels were not taken in to account owing to the fact that they were under the sensitivity of the ELISA kit used.

There could not be determined a statistic difference among the levels of procalcitonin, neopterin, MDA, TNF-α, IL-8, PGE_2, IFN-γ between the sexes (P>0.05).

DISCUSSION

It has been reported for many times by various researchers that markers such as IFN-γ, TNF-α, MDA, IL-8, PGE_2, procalcitonin and neopterin which are revealing the

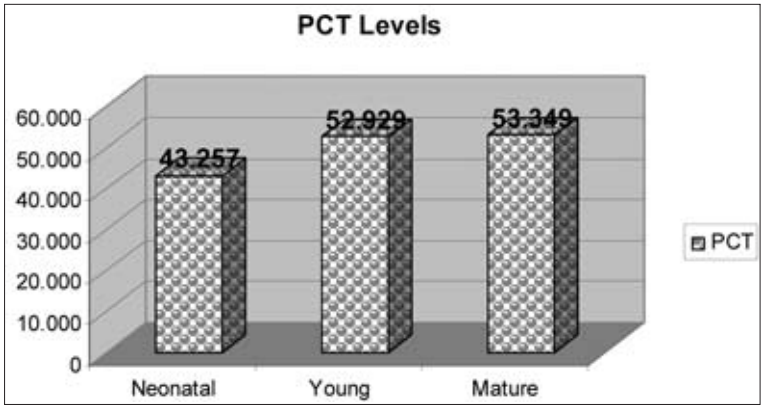
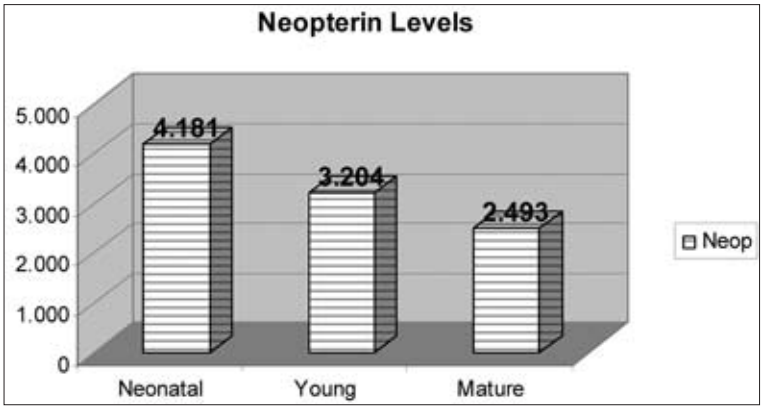


Fig 1. Procalcitonin levels according to age groups

Şekil 1. Yaş gruplarına göre prokalsitonin düzeyleri

Fig 2. Neopterin levels according to age groups

Şekil 2. Yaş gruplarına göre neopterin düzeyleri



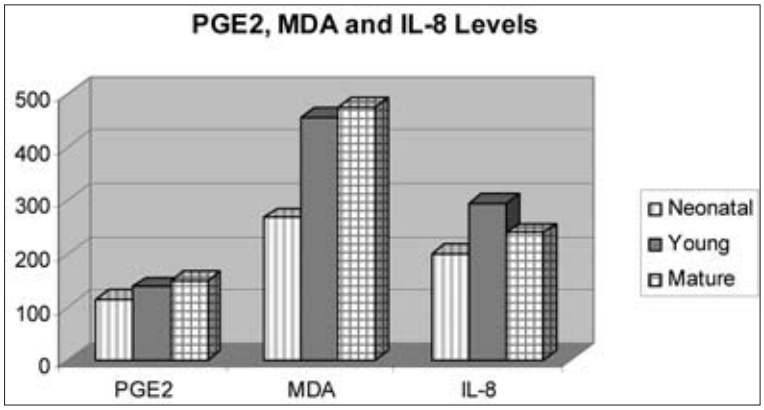


Fig 3. PGE_2, MDA and IL-8 levels according to age groups

Şekil 3. Yaş gruplarına göre PGE_2, MDA ve IL-8 düzeyleri

results of growing immune during the infections could be used in observing the prognosis in infections [3,8,9,14,21,22].

The reported clinical examination results of the healthy cattle and hematologic value ranges [19,23,24] are in parallel with the values determined in the cattle which were the materials of the study as well, and shown in *Table 2*.

It has been stated by numerous researchers that in healthy cattle glucose is 45-75 mg/dl, creatine is 1-2 mg/dl, total bilirubin is 0.01-0.47 mg/dl, direct bilirubin is 0.04-0.44 mg/dl, total protein is 6.7-7.5 g/dl, albumin 3-3.6 g/dl, AST 43-127 IU/L, A/G (albumin/globulin) and its rate is on the range of 0.8 and 0.9 [23,24]. Biochemical values of the serums which are the materials of this study are determined as the in the value ranges accepted for healthy cattle.

IFN-γ, is synthesized by lymphocyte and is one of cytokines that activating macrophage [4]. Hisaeda et al.[3], who claim that IFN-γ increases in cows with mastitis reported that IFN-γ levels in the serums are of the amounts in that could not be reported in the cows of the control group of the study they were doing. This fact coincides with those healthy cows' serum INF-γ levels are not found reasonable.

In the studies conducted by Hisaeda et al.[3] and in the study searching the utility of serum levels in revealing the prognosis of natural coliform mastitis those are stated that; serum TNF-α levels are high in cattle with mastitis however, even though there confirmed no statistical difference of the TNF-α levels between the cattle responding to the treatment and the ones euthanized, TNF-α levels of milk serums have increased statistically reasonably. Moreover,

in the same study they determined that TNF-α level in mature healthy cattle as a control group is lower than 10 ng/ml [3]. In this study, TNF-α levels of neonatal, young and mature are determined respectively as 0.66, 0.86 and 0.70 ng/ml and the average of all groups are calculated as 0.75 ng/ml. These values are in accordance with the values of healthy ones in Hisaeda et al.'s study [3]. In this study, the measured TNF-α levels difference between healthy male and female cattle are not found statistically reasonable (P>0.05).

It is proved by too many studies that procalcitonin could be used in human medicine newborn units as an inflammation mark in pneumonia, septicemia, meningitis, fungal and parasitic infection and that inflammation could be used safely in determining the prognosis. It is demonstrated with various studies that the determination of serum procalcitonin amount caused by bacterial inflammations compared to other cytokines is more specific and sensitive [2,15-18,25-27].

In literature search, it has been found out that that studies on veterinary medicine are limited and that the studies on animals are mostly conducted on experimental animals in order to provide data with the human medicine. In this study the statistical lowness of the serum procalcitonin level in neonatal period relatively to the young and mature animals displays that the age has an effect upon the procalcitonin level (P<0.05) and in statistical analysis of serum procalcitonin measured after grouping the blood serums as male and female it displays that the gender has not got an effect upon procalcitonin serums (P>0.05). In the light of these indications, procalcitonin levels can be detected in the blood of healthy cattle has shown that this parameter can be used in latter studies in the field of veterinary medicine.

INF-γ and other cytokines are effective stimulants in the formation of neopterin by monocytes. In the study done by Stang et al.[14] determined that they were searching the neopterin levels of cattle, horse, lama, dog and cat and it is also determined that the serum neopterin level in both sexes in cattle was 2.85±0.65 nmol L^{-1}. However the serum neopterin levels' not differing between the sex groups are in accordance with the facts of Stang et al.[14], in this study neopterin level is found statistically reasonably higher in neonatal group than young and mature group, apart from Stang et al.[14] (P<0.05). The acceptance as a marker of neopterin immune activation can be associated with its being produced by active monocytes at neonatal period.

Baker et al.[9] have reported that in the study they have arranged to determine PGE_2 levels and histamine and PGD_2 levels in cows with ostertagyiosis, in the healthy cattle assigned as the control group PGE_2 levels are between 178±74 - 266±135 $pgml^{-1}$. In the studies of Fraccaro et al.[28], PGE_2 levels were first measured by immunoenzymatic method for 5 min, 360 min, 720 min and 24 h. While the levels at control were between 500-1.000 pg/ml until min 720, it is indicated that the levels were close to 1.500 pg/ml levels at 24 h measurement. In the study there found no difference according to the sex and though there found reasonable differences according to the age groups, calculated values are lower than of reported in these studies. Higher levels of PGE_2 seen at adults compared to the young and neonatal group was interpreted as antiinflammatory response rised accordingly to the age.

The existence and level of oxidative stress could be set forth by determining the amount of malondialdehyde formed as end product in the process known as lipid peroxidation. The increasing amount of MDA in the serum is an important marker of oxidative stress [10]. In a study researching the markers of oxidative stress and immune system in cattle with anaplasmosis, malondialdehyde level is found as 15.23±2.33 umol/L in 15 healthy cattle whose ages are ranging from 1 to 3 [29]. In cattle infected with *Brucella abortus* as a control group in the study received a mean serum MDA levels in 10 healthy cattle was 1.74±0.25 nmol/mL have been reported [30]. In this study the average MDA level of newborn, young and mature cattle are determined respectively as 264.4, 454.3, 473 ng/ml. It is determined that sex difference hasn't got an effect upon MDA level and that MDA level is reasonably and statistically higher in young and mature cattle than neonatal group (P<0.05).

Due to the fact that IL-8 stimulates neutrophil chemotaxis, its measurement is important at some liquids and serum during the course of certain diseases. The fact that the level of IL-8's normal serum levels show increase in 24 h are reported by many researchers [12,13]. The study comparing viral and bacterial pneumonia IL-8 in cattle have supported the fact that in bronchoalveolar lavage fluid compared to viral IL-8 levels are higher especially in bacterial infections [31]. Serum IL-8 levels are determined in neonatal, young and mature groups respectively as 199.187 pg/ml, 293.294 pg/ml and 238.929 pg/ml. It is also determined that statistically all groups are different from each other and that IL-8 level is higher in young group than other groups (P<0.05). Even though TNF-α levels between groups were not statistically meaningful, when related with IL-8 levels it is seen that these two parameters seem parallel at neonatal, young and adult levels.

As a conclusion, in this study the scope is to determine the blood levels of biological markers such as procalcitonin, neopterin, TNF-α, IL-8, MDA, PGE_2, IFN-γ in healthy cattle at different age and of different sex groups and to put forward the effects of age and sex differences upon serum levels in order to provide base data for further studies in the field of veterinary medicine on biological markers used routinely in diagnosing infectious diseases in human medicine and evaluating prognosis. The results

achieved show that, in the field of veterinary medicine in determining inflammation and prognoses, procalcitonin, neopterin, TNF-α, IL-8, MDA, PGE_2 serum levels can be indicated by ELISA method.

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