

**EVALUATION OF PLASMA AND MILK HAPTOGLOBIN CONCENTRATIONS IN THE DIAGNOSIS AND TREATMENT FOLLOW-UP OF SUBCLINICAL MASTITIS IN DAIRY COWS**

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*The purpose of this study was to examine whether plasma and milk haptoglobin (Hp) concentrations could be an alternative method for the diagnosis and treatment follow-up of subclinical mastitis (SM) in dairy cows. In the study, 14 cows with subclinical mastitis (SM) in more than one udder quarter and 5 healthy control cows were used. Cows in the non-treated group (GNT; n=6) did not receive any treatment while cows in the treated group (GT; n=8) were treated with intramammary cefquinom sulphate on the second week. Healthy cows were evaluated as Control group (GC). Plasma Hp concentrations did not differ within groups and between groups ( $p>0.05$ ). When compared milk Hp concentrations within groups, there was a slight increase in GT on the third week ( $p<0.05$ ) and the concentrations in GNT and GC did not reveal any difference ( $p>0.05$ ). A relationship between CMT scores, SCC values, plasma Hp and milk Hp concentrations was not established. It was concluded that plasma Hp and milk Hp are not useful parameters to diagnose and monitor the treatment efficacy of subclinical mastitis in dairy cows.*

*Key words: cow, haptoglobin, subclinical mastitis*

## INTRODUCTION

Mastitis is the most important disease in the dairy industry that causes economical losses due to decreased milk production and increased health care costs (Melchior *et al.*, 2006). In addition, mastitis alters milk composition thus leads to technological problems especially in cheese-making (Le Roux *et al.*, 2003). Furthermore, the subsequent milk yield is reduced after severe cases of clinical mastitis occurring during lactation (Bhutto *et al.*, 2010). Clinical mastitis is easily defined with symptoms of the teat and udder. In contrast, subclinical mastitis requires laboratory analysis and is characterized by an increase in somatic cell count (SCC). SCC of milk samples is determined to monitor the level of subclinical mastitis in herds or individual cows. For that purpose, California Mastitis Test (CMT) is available as an indirect method and microscopic somatic cell count as a direct method (Dohoo and Meek, 1982). CMT is accepted as a

quick and simple test that indicates the severity of intramammary infection and both CMT and SCC scores have good sensitivity to predict mastitis (Bhutto *et al.*, 2010). Composite SCC data is generally found in many farms and is valid to estimate infection dynamics in a herd (Cook *et al.*, 2002) and SCC of 200.000 cells/ml is regarded as a proof of bacterial infection (Schepers *et al.*, 1997). Though SCC is routinely monitored with automated tools, or the more practical CMT is performed weekly, other biomarkers of cow's health are needed.

Infection, inflammation, trauma or stress stimulate acute phase response characterized by an increase in acute phase proteins (APP) (Murata *et al.*, 2004). Recently the use of APP to assess the response to infection or trauma is becoming more common. Among proteins that can be used in the diagnosis and treatment follow-up (Eckersall and Bell, 2010) haptoglobin (Hp) is the major APP in cattle as its concentration increases up to 1.000 fold in inflammatory conditions (Eckersall *et al.*, 2006). Increased serum or plasma bovine Hp concentrations are associated with inflammatory diseases such as foot and mouth disease (Höfner *et al.*, 1994), pneumonia (Hall *et al.*, 1992), severe metritis (Smith *et al.*, 1998) and also delayed uterine involution (Sheldon *et al.*, 2001). Besides, invasive surgery may lead to increased Hp concentrations (Morimatsu *et al.*, 1992). Bovine Hp concentration is determined to be higher in moderate to severe cases of clinical mastitis than mild cases (Wens *et al.*, 2010). Serum Hp is also known to be useful in distinguishing between acute and chronic inflammation (Petersen *et al.*, 2004; Horagoda *et al.*, 1999) and it is an effective APP in cattle in the diagnosis of mastitis (Eckersall and Bell, 2010). Also milk Hp was detected to be synthesized in the mammary tissue (Eckersall *et al.*, 2006) and evaluated as a mastitis marker in many studies (Akerstedt *et al.*, 2007; 2009, Grönlund *et al.*, 2005). The aim of the present study was to research the accuracy of plasma Hp and milk Hp concentrations for the diagnosis and treatment efficacy in spontaneous subclinical mastitis of dairy cows.

## MATERIAL and METHODS

### *Animals and Study Design*

The study included 14 cows with subclinical mastitis (SM) and 5 healthy control cows, 2-5 years of age from the same dairy Holstein-Friesian herd in Bafra, Samsun province (Turkey). The cows were milked twice a day, at 8 am and 6 pm by milking machines. Mean total daily milk yield was 25.5 kg. Clinical diagnosis of SM was done by CMT. Selected cows were divided into three groups: nontreated SM group (GNT, n=6), treated SM group (GT, n=8) and healthy control group without abnormalities in the udder or in the milk (GC, n=5). GNT and GT cows had subclinical mastitis in two or more quarters with CMT positive results for two consecutive weekly examinations and all cows including GC had complete physical examination indicating that they were clinically free of disease, estrus signs, pregnancy or were inseminated recently. Blood and milk samples of all cows were collected once a week during three weeks after performing CMT at the same week day. GT cows were treated with intramammary 75 mg cefquinom sulphate (Cobactan LC<sup>®</sup>, Intervet) for three days after taking samples at the

second week. Forty ml milk samples from udder quarters were collected in GNT, GT and GC i.e. 20 mL for SCC and 20 mL for determination of Hp. After disinfection of the teat 4 streams of milk were removed from the affected quarter before sample collection. Blood samples were collected from the jugular vein of each cow into EDTA tubes. Blood and milk samples collected every week were transferred to the laboratory in a thermos flask with ice cubes within an hour for sampling. In the laboratory quarter milk samples were gently mixed and composite milk samples were prepared by pooling 200  $\mu$ L milk from each quarter. The Ethics Committee of University of Ondokuz Mayıs approved the study protocol.

#### *California Mastitis Test*

CMT was carried out according to the method described by Schalm and Noorlander (1957). Milk samples from each quarter were taken into clean test paddles that have four separate wells. Equal volume of milk was mixed with the test solution containing bromocresol purple and anionic detergents. By rotating the plate gently, alterations such as colour changes or gel formation were evaluated. CMT scores were given as 0 for no reaction, 1 for a weak positive, 2 for a distinct positive and 3 for a strong positive reaction.

#### *Definition of Somatic Cell Count*

Microscopic somatic cell count was performed according to the method described by Kilicoglu *et al.* (1989) in order to confirm CMT results. For that purpose 10 mL of composite milk sample was centrifuged at 1550 g, the cream was removed and tubes were turned over and left for twenty minutes. Sediments accumulated at the bottom of tubes were taken and samples were prepared by putting one drop of saline solution. Slides were dried at room temperature and stained with 0.2 % toluidin blue for 3-5 minutes, washed with water and dried. Immersion oil was put on the slides, somatic cells were counted on 20 areas and the mean cell numbers in the microscope area were evaluated as: 0-1 cells: 100.000 - 200.000 cell/mL, 1-5 cells: <300.000cells/mL, 6-20 cells: >300.000 cells/mL, >20 cells: >million cells/mL.

#### *Seperation of Milk Sera*

One mL of 0.3 % chymosin was added to 20 mL milk samples, then the samples were incubated for 20 minutes in a water bath at 37°C to obtain clot. Clots were left for 80 minutes for the serum to drain easily and to provide optimum separation. After that the milk serum was filtered into glass tubes. Supernatants in glass tubes were centrifuged at 1550 g for 5 minutes and the separated cream was eliminated. Clear milk serum was taken into plastic microcentrifuge tubes and kept at -80°C until analysis.

#### *Seperation of Blood Plasma*

In order to separate plasma, blood samples were centrifuged at 1550 g for 10 minutes at 4°C. Plasma were taken into plastic microcentrifuge tubes and kept at -80°C until analysis.

#### *Assays of Plasma and Milk Hp Concentrations*

The concentrations of plasma Hp and milk Hp were determined using ELISA kits (Bovine Haptoglobin ELISA Test Kit, Life Diagnostics, West Chester, PA). Samples were analysed according to the procedure suggested by the manufacturer. Optic density of plates was detected on a microplate reader at 450 nm (Digital and Analog Systems, RS 232, Roma, ITALY). Hp concentrations were calculated by a standard calibration curve obtained from haptoglobin standards.

#### *Statistical Analysis*

Data were analysed with one-way ANOVA and Duncan's multiple range test were used to evaluate for differences of means with 0.05 alpha. Also, Pearson correlations were calculated for variables (CMT, plasma and milk Hp). SAS statistical package program (2009) were used for data analysis.

### RESULTS

CMT scores of the nontreated SM group (GNT) and treated SM group (GT) at the first and second week were determined to be higher than the scores at the third week ( $p < 0.01$ ;  $p < 0.05$ , respectively). CMT results for three weeks were negative in GC and there was no difference when compared CMT values of GC ( $p > 0.05$ ) during three weeks. CMT scores of subclinical mastitis groups in the first week (GNT=1.62 and GT=1.50) were found to be significantly higher than the control group (GC=0.0) ( $p < 0.01$ ). This difference decreased in the second week, but it was still statistically significant ( $p < 0.05$ ). CMT values of all groups in the third week did not show any statistical difference ( $p > 0.05$ ) (Table 1).

Table 1. California Mastitis Test scores

Group	CMT		
	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week
GNT (n=6)	1.62±1.13 <sup>a,**</sup>	1.45±1.14 <sup>A,**</sup>	0.75±1.18 <sup>a,#</sup>
GT (n=8)	1.50±1.19 <sup>a,*</sup>	1.25±1.04 <sup>A,*</sup>	0.50±0.80 <sup>a,#</sup>
GC (n=5)	0±0 <sup>b,*</sup>	0.28±0.61 <sup>B,*</sup>	0.14±0.36 <sup>a,*</sup>

GNT: nontreated SM group; GT: treated SM group; GC: control group

Scores shown with different superscripts are statistically different at the same column (a,b:  $p < 0.01$  and A,B:  $p < 0.05$ ).

Scores shown with different asterisks are statistically different at the same line (\*\*,#:  $p < 0.01$  and \*,#:  $p < 0.05$ ).

In the first week, SCC values of GNT and GT revealed mastitis (300.000-1.000.000 cell/mL) while SCC of GC indicated that there was no infection (100.000-200.000 cell/mL). Though SCC of GNT and GT in the second week decreased ( $> 300.000$  cell/mL) again they were higher than GC (100.000-

200.000cell/mL). In the third week, SCC of all groups was between 100.000-200.000 cells/mL.

When compared milk Hp concentrations within groups, the concentrations in GNT and GT did not reveal any difference ( $p>0.05$ ); but there was a slight increase in GT in the third week despite intramammary antibiotic treatment ( $p<0.05$ ). Comparison among the groups showed that there was no difference between milk Hp concentrations in the first and third week ( $p>0.05$ ) and the concentrations of GNT and GT at the second week were higher than GC ( $p<0.05$ ) (Table 2). Plasma Hp concentrations did not differ within groups and among the groups ( $p>0.05$ ) (Table 3). In addition there was no relationship between CMT values of plasma Hp and milk Hp concentrations.

Table 2. Milk haptoglobin concentrations

Group	Milk Hp (mg/mL)		
	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week
GNT (n=6)	0.025±0.025 <sup>a,*</sup>	0.023±0.020 <sup>a,*</sup>	0.028±0.015 <sup>a,*</sup>
GT (n=8)	0.022±0.016 <sup>a,*</sup>	0.023±0.020 <sup>a,*</sup>	0.028±0.020 <sup>b,*</sup>
GC (n=5)	0.016±0.016 <sup>a,*</sup>	0.011±0.013 <sup>a,#</sup>	0.018±0.018 <sup>a,*</sup>

GNT: nontreated SM group; GT: treated SM group; GC: control group  
 The results shown with different superscripts are statistically different at the same column (<sup>a,b</sup>:  $p<0.05$ )  
 The results shown with different asteriksces are statistically different at the same line (\*, #:  $p<0.05$ )

Table 3. Plasma haptoglobin concentrations

Group	Plasma Hp (mg/mL)		
	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week
GNT (n=6)	0.038±0.010 <sup>a,*</sup>	0.037±0.002 <sup>a,*</sup>	0.050±0.023 <sup>a,*</sup>
GT (n=8)	0.047±0.034 <sup>a,*</sup>	0.053±0.013 <sup>a,*</sup>	0.039±0.005 <sup>a,*</sup>
GC (n=5)	0.033±0.011 <sup>a,*</sup>	0.032±0.023 <sup>a,#</sup>	0.031±0.007 <sup>a,*</sup>

GNT: nontreated SM group; GT: treated SM group; GC: control group  
 The results shown with the same superscripts are not statistically different at the same column (<sup>a</sup>:  $p>0.05$ )  
 The results shown with the same asteriksces are not statistically different at the same line (\*:  $p>0.05$ )

## DISCUSSION

In the present study CMT values at the first and second week in GNT and GT were higher than in the third week. Similarly CMT values in the third week in GT was lower than in the second week. This significant difference between pretreatment period and posttreatment day 4 made us think that CMT as a classical mastitis test is effective both for diagnosis and treatment follow-up. GNT also revealed a decrease in both CMT scores and SCC values in the third week indicating a naturally healing process. SCC of GNT and GT in the first week were

found to be higher than GC. SCC of GNT and GT at the second week during which was decreased, but still indicating inflammation. In the third week, SCC of all groups was between 100.000-200.000 cells/mL. The fact that SCC of GNT and GT in the first and second week (which was the pretreatment period of the study) was higher than in the third week, in other words posttreatment day 4 was normal and showed that mastitis cases were on the way of recovery not only in the treated, but also in the nontreated group. Therefore, a positive correlation between SCC results and CMT scores was found in GNT and GT. A decrease in CMT score corresponded with a decrease in SCC.

It is known that serum or plasma Hp concentrations increase after experimentally induced or naturally occurring inflammation and trauma (Petersen *et al.*, 2004) and APPs play an important role in defence related activities such as fighting with infection agents and repair of tissue damage (Murata *et al.*, 2004). Mastitis was determined to induce an increase in plasma concentration of Hp (Eckersall *et al.*, 2001). As major APP in ruminants, circulating Hp concentration is negligible in normal animals, but can increase up to 100 fold on immune stimulation (Murata *et al.*, 2004). Tourlomoussis *et al.* (2004) determined that mean plasma Hp concentration in cows without pathological conditions was  $0.02 \pm 0.03$  mg/mL while the mean Hp concentration in cows with pathological conditions was  $0.27 \pm 0.40$  mg/mL. Similarly, Conner *et al.* (1986) stated that Hp concentrations in healthy cattle is often undetectable. Eckersall and Bell (2010) stated that serum Hp concentration of healthy cattle is  $<20$  mg/L, but can increase to  $>2$  g/L in acute infections. Eckersall *et al.* (2006) reported that serum Hp concentration in cows rose to a level of  $0.54 \pm 0.12$  mg/mL in experimentally induced subclinical mastitis while its concentration was below 0.01 mg/mL in control cows. Khoshvaghti *et al.* (2009) reported that serum Hp was  $0.570 \pm 0.063$  mg/mL in cows with subclinical mastitis while it was  $0.108 \pm 0.017$  mg/mL in healthy cows. In our study, plasma Hp concentrations were above 0.02 mg/mL during the three weeks study period not only in GNT and GT, but also in GC and no statistical difference was determined both within groups and among groups. Though there was no statistical significance, plasma Hp concentration of GNT in the third week was 1.6 times higher than the concentration in the first week. In spite of this plasma Hp concentration of GT in the third week was 1.2 times less than in the first week.

Haptoglobin increases in bovine milk during intramammary infections though its sources are not fully understood. Milk Hp was detected to originate from the circulation (Pedersen *et al.*, 2003). Lai *et al.* (2009) found that increased milk Hp was related to neutrophils associated with biosynthesis and release of Hp. Besides it was determined to be localized within the epithelial cells of the mammary glands with mastitis (Lai *et al.*, 2009). Hiss *et al.* (2004) also suggested that Hp could be locally synthesized within the mammary gland. Several researchers have demonstrated that bovine milk Hp elevates in mastitis (Eckersall *et al.* 2006; Akerstedt *et al.*, 2009; Grönlund *et al.*, 2005). Eckersall *et al.* (2006) determined that Hp concentrations in milk samples rose within 12 hours of *Staphylococcus aureus* infusion and reached its peak level 3 days after the infusion. Milk Hp concentrations were detected to be higher in the infected quarter

than in milk from healthy quarters (Eckersall *et al.*, 2001). Akerstedt *et al.* (2007) reported detectable levels of Hp in bulk tank milk samples. In another study conducted by Grönlund *et al.* (2005) a substantial variation in milk Hp concentrations was determined in udder quarters with chronic subclinical mastitis while its concentration below the detection limit was accepted as an indicator for healthy udder quarters. In the present study, milk Hp concentrations in groups with subclinical mastitis (GNT and GT) were slightly higher than in the control group (GC) in the second week before the intramammary treatment started. In contrast to our expectations milk Hp concentrations of GT was slightly higher in the third week despite the application of intramammary antibiotic therapy indicating an activation of the acute phase response during the treatment period in the mammary gland. Thus, it might be necessary to study Hp concentrations a few weeks after therapy.

Akerstedt *et al.* (2007) reported a significant relationship between Hp and SCC at quarter and composite milk levels in cows with subclinical mastitis. They found Hp concentrations to be high in quarter and composite milk samples with high SCC and similar results were detected by Safi *et al.* (2009). In our study, there was no relationship among plasma Hp, milk Hp, SCC values and CMT scores though SCC results and CMT scores decreased in the third week. Based on these findings we concluded that detailed studies should be carried out including cows with subclinical mastitis and a longer observation period after the treatment in order to create a clear relationship between the studied parameters and to understand whether evaluating plasma and milk Hp concentrations would be useful to monitor the treatment efficacy in subclinical mastitis.

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#### ODREĐIVANJE KONCENTRACIJE HAPTOGLOBINA U PLAZMI I MLEKU PRI DIJAGNOZI I PRAĆENJU TRETMANA SUBKLINIČKIH MASTITISA MLEČNIH KRAVA

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#### SADRŽAJ

Cilj ovog istraživanja je bio da se ispita da li haptoglobin (Hp) u plazmi i mleku može da bude alternativna metoda za dijagnozu i praćenje tretmana kod subkliničkog mastitisa (SM) mlečnih krava. U studiji je korišćeno 14 mlečnih krava sa subkliničkim mastitisom (SM) koji je zahvatao dve i više četvrtina vimena i 5 zdravih (kontrolnih) krava (GC). Šest obolelih krava nije tretirano lekovima (GNT), dok su krave iz tretirane grupe (GT; n=8) bile tretirane intramamarnom aplikacijom cefquin sulfata u drugoj nedelji od pojave bolesti. Koncentracije Hp u plazmi se nisu razlikovale između grupa, kao ni u okviru jedne grupe ( $p > 0.05$ ). Kada je upoređena koncentracija Hp u mleku pojavio se blagi porast u GT grupi u trećoj nedelji ( $p < 0.05$ ) ali između koncentracija registrovanih u GNT i GC nije bilo značajnih razlika ( $p > 0.05$ ). Nije utvrđena korelacija između CMT rezultata, SCC vrednosti i koncentracije Hp u krvnoj plazmi i mleku. Zaključeno je da određivanje koncentracije Hp u mleku i plazmi nije koristan parametar za dijagnozu i praćenje efikasnosti tretmana kod subkliničkog mastitisa mlečnih krava.

