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Original article

FIELD STUDY ON PLASMA HAPTOGLOBIN CONCENTRATIONS AND TOTAL MILK SOMATIC CELL COUNTS IN COWS WITH UNTREATED AND TREATED MASTITIS

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Summary

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The aim of the present study was to determine plasma haptoglobin concentrations in cows in field conditions. Experiments were carried out a total of 106 cows, divided in 5 groups. The 1st group comprised 25 cows with low milk somatic cell counts (SCC), without microbiological findings used as controls; the IInd group –23 cows with untreated subclinical mastitis with high milk SCC and without microbiological findings; the IIIrd group: 13 cows with untreated subclinical mastitis with high milk SCC and with microbiological findings. The IVth group consisted of 17 cows with subclinical mastitis, high milk SCC, microbiological findings, treated with tetracycline HCL 200 mg, neomycin 250 mg, bacitracin 2000 IU, prednisolone 10 mg (Mastijet fort®, MSD Animal Health), and the Vth group – 28 cows with clinical mastitis treated with Mastijet fort®, MSD Animal Health. Our results showed that the mean plasma haptoglobin concentrations in the I (control) group was 0.054±0.003 g/L. In the IInd group (0.288±0.04 g/L) Hp was 5.2 times higher that the mean values of controls ($P<0.001$). Mean Hp concentration of group III was also statistically significantly different ($P<0.001$) than that of the control group. The value of Hp in group IV (0.215±0.04 g/L) was significantly lower ($P<0.05$) that mean values of IInd group but substantially higher ($P<0.001$) vs controls. The concentration of haptoglobin in cows of the Vth group was statistically significantly lower ($P<0.001$) compared both with control and IInd groups. In this study, total somatic cell count showed statistically significant differences ($P<0.001$) between the control and experimental groups.

Key words: acute phase response, cows, haptoglobin, mastitis, total somatic cell count

INTRODUCTION

Mastitis is one of the most widely spread disease affecting lactating cows with considerable economic importance. That is why the accurate diagnosis is crucial be-

cause it optimises the effect of treatment and minimises the recovery time, thereby reducing production losses and improving the animals' welfare (Nielsen *et al.*, 2004). The diagnosis of mastitis is based predominantly on clinical examination, measurements of somatic cell counts and the circulation of pathogens in milk, but the demand for objective and rapidly assessable markers of udder health has increased with the introduction of robotic milking systems (Nielsen *et al.*, 2004). In mammals, local tissue damage or inflammation leads to systemic changes known as acute phase response (APR). Among the varied physiological alterations, producing this response, a change in the circulating levels of a number of liver derived proteins, collectively known as the acute phase proteins (APP) occurs (Koj, 1974).

The determination of serum Hp levels is a very useful diagnostic tool in clinical medicine especially in cattle: in cows at parturition (Uchida *et al.,* 1993); in cows with fatty liver (Nakagawa *et al*., 1997); in plasma of bull calves after surgical castration (Fisher *et al*., 1997); clinical mastitis and extrammary inflammatory conditions (Nielsen *et al.*, 2004).

Godson *et al.* (1996) stated that Hp, in particular, which is absent from normal bovine serum, is detected at various amounts in cattle suffering from numerous bacterial, but nor viral diseases, like bovine respiratory disease.

The aim of the study was to investigate plasma haptoglobin concentrations in several groups cows: I group – cows with low milk somatic cell counts without microbiological findings, which are clinically healthy, II group – cows with untreated subclinical mastitis with high milk somatic cell counts without microbiological findings, III group – with untreated subclinical mastitis, high milk somatic cell counts and microbiological findings, IV group – cows with treated subclinical mastitis, high somatic cell counts and ith microbiological findings, and V group – with treated clinical mastitis, high somatic cell counts and microbiological findings.

MATERIALS AND METHODS

Animals and experimental design

The experimental procedure was approved by the Ethic Committee at the Faculty of Veterinary Medicine. Experiments were carried out totally with 106 cows, divided in 5 groups. The Ist group comprised 25 cows with low milk somatic cell counts (SCC), without microbiological findings used as controls; the IInd group – 23 cows with untreated subclinical mastitis with high milk SCC and without microbiological findings; the IIIrd group: 13 cows with untreated subclinical mastitis with high milk SCC and with microbiological findings. The IVth group consisted of 17 cows with subclinical mastitis, high milk SCC, microbiological findings, treated with tetracycline HCL 200 mg, neomycin 250 mg, bacitracin 2000 IU, prednisolone 10 mg (Mastijet fort®, MSD Animal Health), and the Vth group – 28 cows with clinical mastitis treated with Mastijet fort®, MSD Animal Health. Total somatic cell counts were counted only in I, II and III groups, because in IV and V groups the number was huge.

At the Land O'Lakes cattle farm, Brown Swiss breed (imported and local crosses) dairy cows were housed in free stalls, fed concentrate, corn silage, meadow hay and had a constant access to water. The milking scheme was 2×4 in compliance to hygienic requirements – previous cleaning, drying, milking and post

milking disinfection. The daily milk yield of lactating cows was 17–22 L.

Clinical examination

The clinical examination of the mammary gland included inspection and palpation of the teats and the udder, test milking to determine the patency of milk canals, as well as macroscopic examination of the milk secretion. The acute mastitis was characterised by a painful, warm, reddened and swollen udder.

In August 2012, a survey on the prevalence of subclinical mastitis was conducted via the rapid mastitis test and collection of sterile milk samples for bacteriological examination from udder quarters with ++ and +++ results from the CMT (increased somatic cell counts).

Somatic cell counts in individual milk samples were determined by direct counting as per IDF Standard 148 A:1995 (Milk: Enumeration of Somatic Cells). The isolation and differentiation of mastitis pathogens was done according to the accredited method of the National Mastitis Council (NMC, 1999|0, described in the Laboratory Handbook on Bovine Mastitis) (Hogan *et al*., 1999).

Biochemical analysis

Ten mL of blood were collected from each cow from *v. jugularis*. The blood was taken into sterile heparinised tubes and centrifuged immediately (1500 rpm for 10 min, 4 °C) to obtain plasma. Plasmas were decanted and stored at –20 ^{o}C until assayed. Hp concentration (g/L) was measured using a commercial kit (Tridelta), in which haptoglobin from samples was incubated with haemoglobin (Hb) to form an Hp-Hb complex. The pH was then reduced by addition of chromogene, destroying the peroxidase activity of the unbound Hb. The generated hydrogen peroxide developed a deep blue colour which was measured at a wavelength 630 nm by a biochemical analyser at the Faculty of Veterinary Medicine in Milano. The preservation of the peroxidase activity of the Hb is directly proportional to the amount of Hp present in the sample. Samples with unknown Hp concentration were quantitated vs standards with known concentrations of Hp .

Statistical analysis

The statistical analysis of the data was performed using one way analysis of variance (ANOVA). The significance of differences was evaluated by LSD test. All data were expressed as mean ± standard error (SE) and the differences were considered as significant when P value was less than 0.05.

RESULTS

As shown on Fig. 1, the mean plasma Hp concentrations of group I (control) comprising cows with with low milk somatic cell counts without microbiological findings was 0.054±0.003 g/L, and varied between 0.020 and 0.86 g/L. In the II group (cows with untreated subclinical mastitis, high milk somatic cell counts without microbiological findings) the mean Hp concentration was 0.288±0.04 g/L and varied between 0.093 and 0.589 g/L. The value of group II was 5.2 times higher than that of the control group ($P<0.001$).

The mean haptoglobin concentration in the IIIrd group from cows with untreated subclinical mastitis, high milk somatic cell counts and microbiological findings was 0.302±0.02 g/L which was also statistically significantly different ($P<0.001$) and several times higher than control mean. The minimum Hp value in this

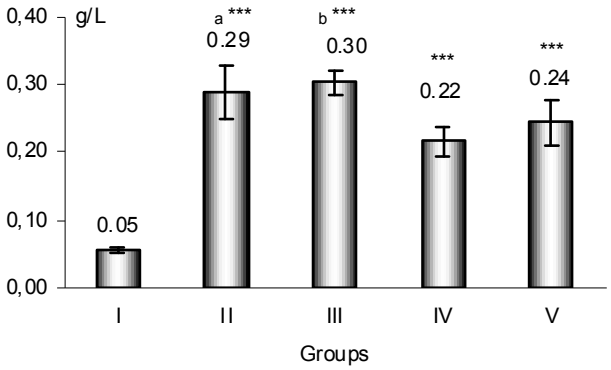

Fig. 1. Mean values (g/L) of haptoglobin concentration in cows: I group – cows with with low milk somatic cell counts without microbiological findings (control); II group – cows with untreated subclinical mastitis with high milk somatic cell counts without microbiological findings; III group – cows with untreated subclinical mastitis, high somatic cell counts and microbiological findings; IV group – cows with treated subclinical mastitis, high somatic cell count and microbiological findings; V group – cows with treated clinical mastitis with high somatic cell counts and microbiological findings. *** statistically significant difference at P<0.001 vs group I (control); [a] statistically significant difference at P<0.05 between groups II (untreated subclinical) and IV (treated subclinical); [b] statistically significant difference at P<0.01 between groups II (untreated subclinical) and V (treated clinical).

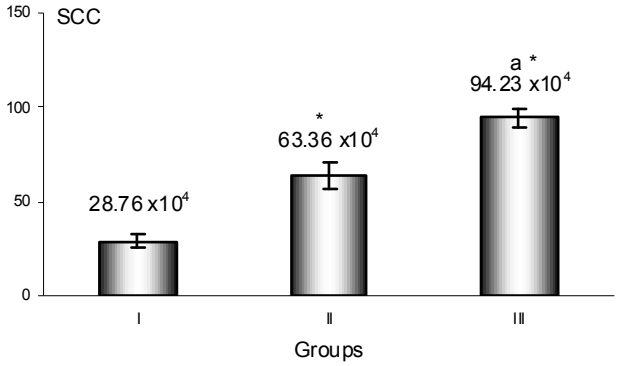

Fig. 2. Total somatic cell counts (TSCC; $\times 10^4$) in cows: I group – cows with with low milk somatic cell counts without microbiological findings (control); II group – cows with untreated subclinical mastitis with high milk somatic cell counts without microbiological findings; III group – cows with untreated subclinical mastitis, high somatic cell counts and microbiological findings; * statistically significant difference at P<0.001 vs group I (control); [a] statistically significant difference at P<0.01 between groups II and III.

group was 0.178 g/L and the maximum value was 0.433 g/L.

The average value of Hp from cows with treated subclinical mastitis (IV group) was 0.215±0.04 g/L, significantly lower (P<0.05) than plasma Hp of the group with untreated subclinical mastitis, high somatic cell counts and without microbiological findings (II group). It was

also substantially higher ($P<0.001$) compared with the level of Hp in controls.

The average concentration of haptoglobin in cows with treated clinical mastitis (V^{th} group) was 0.243±0.03 g/L. It was significantly different to values of cows from the control group ($P<0.001$) as well as to group II ($P<0.01$). Maximum Hp concentrations were recorded in the II^{nd} group – 0.589 g/L (30 times higher) versus the lowest concentration, measured in the control group – 0.02 g/L.

In the control clinically healthy group, the mean of Hp was 0.054±0.003 g/L. The mean result was significantly lower than all other experimental groups.

The results for total somatic cell counts (TSCC) in this study (Fig. 2) showed statistically significant differences between experimental groups and controls. The mean value of SCC in group I (control) was 28.76±3.34 $\times 10^4$/mL. In group II (cows with untreated subclinical mastitis, high somatic cell count without microbiological findings) and group III (cows with untreated subclinical mastitis with high somatic cell count and microbiological findings) TSCC were 63.36± 6.91 $\times 10^4$/mL and 94.23±4.53 $\times 10^4$/mL respectively. The average values of these two groups differed at $P<0.01$, and were also statistically significantly different from control TSSC ($P<0.001$).

DISCUSSION

Haptoglobin is one of the acute phase proteins that are non-specific markers of inflammation, whose serum concentration increase in response to several infectious and inflammatory conditions. Hp and serum amyloid A belong to major APP in cows, acid glycoprotein and α_1 antiprotease are moderate APP and fibrinogen and α_2-macroglobulin – minor APP in this species (Olfert & Godson, 2000). Moreover, APP are more sensitive to the inflammatory process than blood analysis, which is now recommended as a diagnostic marker for the assessment of inflammation. They also are stable and can be measured in frozen samples (Horadagoda *et al.,* 1999). In their study, Skinner & Roberts (1994) found that haptoglobin is a better indicator of bacterial infection than haematological examination. Other authors (Solter *et al*., 1991) also claim that ceruloplasmin and haptoglobin are more sensitive for the detection of inflammation in the dog compared to haematological study. Especially serum haptoglobin is very useful in cows because in this animal species it normally has very low values in healthy animals (McNair *et al*., 1995) or is not detected in healthy cattle but greatly increase during acute inflammation (Bremner, 1964). For this reason, Hp has been used as a marker for infections, diseases and trauma in beef and dairy herds (Horadagoda *et al.,* 1999). The reason for this is not clear but perhaps bovine haptoglobin is only needed for removal of haemoglobin whereas in other species haptoglobin plays a more constant homeostatic role to capture Fe^{2+}. According to Makimura & Suzuki (1982), the values obtained for Hp in normal cows range between 0 and 5.1 mg haemoglobin.100 mL^{-1}. The same authors reported between 13 and 127 mg haemoglobin.100 mL^{-1} for cows with a variety of pathological conditions. We assume that a Hp value below 0.1 g/L was normal and the mean value for the control group was 0.054±0.003. The mean values for cows with untreated subclinical mastitis with high somatic cell counts without microbiological findings (II^{nd} group) and those with microbiological findings (III group) were very close (0.288±0.04 g/L and 0.302±0.02 g/L respectively), there-

fore no differentiation between the two conditions is possible by measurement of haptoglobin. In these two groups, the onset of the disease is usually unknown. There was a significant difference in haptoglobin concentration ($P<0.05$) between cows with untreated (II group) and treated subclinical mastitis (0.22±0.04 g/L – IV group). Conner *et al.* (1986) found out that Hp level in healthy cows was 0 mg Hb per 100 mL blood while 70 mg Hb/100 mL blood was the level detectable in samples with mastitis. Therefore, the authors suggested that Hp is one of the most useful bovine acute phase proteins. Hp appears in disease conditions and according to Skinner *et al*., (1991) Hp is a valuable marker for detecting bovine bacterial infections. The magnitude of Hp increase after infection can be up to 1000-fold and is associated with the severity of the disorder. The results for haptoglobin concentration are in agreement with the earlier data of Salonen *et al.* (1996) demonstrating that Hp is a major bovine APP exhibiting a high relative increase during acute phase reaction in dairy cows with experimentally induced *E. coli* mastitis. Measurement of haptoglobin as a major acute phase protein in cows is therefore recommended to detect inflammation processes in general. The significant difference between haptoglobin concentration in subclinical untreated and subclinical treated mastitis was a better parameter to distinguish between these two conditions than somatic cells counts.

Studies on acute phase reactions in field conditions are more complicated, because the onset of initial infection is often unknown, and it is difficult to determine the phase of infection (Paulina & Tadeusz, 2011). In this study, it was shown that plasma haptoglobin could be helpful to distinguish between treated subclinical and clinical mastitis and untreated subclinical mastitis with high milk somatic cell counts and microbiological findings. Also, it could be useful to differentiate healthy cows from cows with inflammatory conditions.

It is interesting to comment the data concerning the earlier decrease of Hp in the treated groups – IV (subclinical) and V (clinical) mastitis compared to TSCC in the same groups, in which the number of somatic cells was still huge. According to Andersson *et al.* (2015) in naturally occurring chronic sub-clinical mastitis, a large variation in APP expression in milk, and a discrepancy between the levels of APP and adenosine triphosphate, an indirect measure of the milk somatic cell counts, was observed. Hirvonen (1999) observed highly increased Hp level after experimental *E. coli* mastitis within 36 h and peak values 1–3 days after challenge. Hp levels were normalised within one week.

According to Eckersall & Conner (1988), bovine Hp differs substantially from human Hp and was firstly recognised by Polonovski & Jayle (1940) who gave the name of Hp to a component of the α_2-globulins in plasma which had the ability to bind firmly to haemoglobin (Hb). Bremner (1964) reported that plasma samples from healthy calves contained very little if any Hp, but inflammation caused by the injection of oil of turpentine caused a remarkable increase in plasma Hp with at least a five-fold increase, which was demonstrated on the third day after injecting turpentine. Our results showed that Hp concentration in cows with milk with high somatic cell counts and microbiological findings reached 30.2 mg per 100 mL, which was 6 times higher than in the plasma of healthy cows. The absence of Hp from the plasma of healthy

cattle is in striking contrast to humans when Hp is a normal serum protein in other species. In man, the normal value of Hp is 1 mg/mL whereas the low level of Hp in cows is usual (Conner *et al.,* 1986). Alsemgeest *et al*. (1994) determined Hp concentration as a haemoglobin binding capacity per 100 mL plasma and described that in healthy cattle Hp was not detectable, in cows with acute inflammation the mean Hp value was 21.6±14.5; in cows with subacute inflammation: 52.0±5.32 and in cows with chronic problems – 100.3±11.9. This study agrees with our results which indicated that values obtained for Hp concentration in cows with untreated subclinical mastitis with high milk somatic cell counts was higher than that in treated clinical mastitis.

Bovine haptoglobin has two subunits with molecular weights of 20–23 (α-subunit) and 35–37 kDa (β-subunit) (Morimatsu *et al.,* 1992). In the circulation, it is highly polymerised having a molecular weight of approximately 1000–2000 kDa (Godson *et al.*, 1996). Bovine Hp could exists also as polymers associated with albumin (Eckersall & Conner, 1996). Spooner & Miller (1971) reported that haemoglobin-reactive protein was detected only in 0.6% of clinically healthy cattle. The diagnostic potential of measurement of acute phase proteins in cattle serum is well documented (Conner *et al.*, 1986; Eckersall & Conner, 1988; Alsemgeest *et al.,* 1994). These authors reported increased serum amyloid A and haptoglobin concentrations o in the serum and milk of cows with acute mastitis. Raised haptoglobin levels have been reported in diseases where acute inflammation, particularly of bacterial origin, is involved. High haptoglobin was reported in cattle with mastitis (Conner *et al.*, 1986; Spooner & Miller, 1971), liver abscesses, *C. pyogene*s infected abscesses, in calves exposed to the *Pasteurella haemolytica* (Spooner & Miller, 1971), pyometra, traumatic reticulitis, traumatic pericarditis and abomasal displacement (Makimura & Suzuki, 1982; Godson *et al.,* 1996). Changes in the levels of some acute phase proteins including haptoglobin in cows with mastitis were studied by Turk *et al*. (2012). The increased APP were attributed by the authors to an increase of pro-inflammatory cytokines and oxidative stress during mastitis.

One disadvantage of the haptoglobin analysis is that once bound to haemoglobin, it is rapidly removed from the circulation so that any associated haemolytic disease will give false low results (Conner *et al*, 1986).

According to Eckersall & Conner, (1988) the acute phase proteins have been extensively studied in humans and in laboratory animals. Previous studies (Skinner *et al*., 1991; Alsemgeest *et al*., 1994) have observed increases in the concentration of haptoglobin in serum in a variety of inflammation conditions. Nielsen *et al.* (2004) have studied APP in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions and found significant differences between healthy animals and those affected with mastitis. They found that the acute phase proteins in milk increased significantly with increasing somatic cell counts, suggesting that they may be indicators of the severity of an infection.

The results of our study showed that the measurement of haptoglobin as a major acute phase protein in cows is a good indicator for the incidence of subclinical mastitis, because the values in groups II and III were 5– 6 times higher than those in the control group. The significant dif-

ference in haptoglobin concentration in cows with untreated subclinical and treated subclinical mastitis was a better parameter to distinguish between these two conditions than somatic cells counts.

In our study, we found that blood haptoglobin concentration was highly dependent on the results of treatment, because it decreased earlier than did somatic cell counts. While the number of somatic cells was still huge, the concentration of haptoglobin started to decrease. The problem is in the lack of available rapid test like Californian mastitis test for TSCC, allowing for field determination of major APPs, for example haptoglobin, in cows. The determination of TSCC is a much less expensive alternative to haptoglobin assay and it is used for detection of an increased milk somatic cell counts. The development and optimisation of rapid field tests for analysis of major APP in all species within a short time after collection of blood or milk samples will be rather helpful for field diagnostics.

CONCLUSION

Trustworthy information about the body condition could be obtained by appropriate combination of reliable markers, such as the acute phase proteins (APP), total somatic cell counts (TSCC) and bacteriological examination.

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