

Serum acute phase proteins in cows with SARA (Subacute Ruminant Acidosis) suspect

[*Proteínas séricas de fase aguda em vacas com suspeita de SARA (Subacute Ruminant Acidosis)*]

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ABSTRACT

The aim of this study was to evaluate the variations of Acute Phase Proteins (APPs) and other blood constituents during the onset of the sub-acute ruminal acidosis (SARA) pathological status. A total of 108 cows from 12 dairy herds were randomly selected and divided into three Groups of 36 animals each. All animals were subjected to a rumenocentesis. Group A was composed by subjects with a rumen pH > 5.8, Group B was composed by subjects with a rumen pH $\leq 5.5 \leq 5.8$ and Group C was composed by subjects with a rumen pH < 5.5. Blood samples were collected by jugular venipuncture and Haptoglobin (Hp), Serum Amyloid A (SAA), Total Proteins, Albumin and White Blood Cells (WBC) were determined. One-way ANOVA showed a statistical significance on Rumen pH, Hp, SAA. SARA seems not stimulate the APPs production from liver.

Keywords: acute phase protein, dairy cow, haptoglobin, serum amyloid A, SARA

RESUMO

O objetivo deste estudo foi avaliar as variações de Proteínas de Fase Aguda (APPs) e outros constituintes sanguíneos durante o início do status patológico de sub-acute ruminal acidosis (SARA). O total de 108 vacas de 12 rebanhos leiteiros foram aleatoriamente selecionados e divididos em três grupos com 36 animais cada. Todos os animais foram submetidos a ruminocentesis. O Grupo A foi composto por sujeitos com rumen pH > 5.8, o Grupo B foi composto por sujeitos com rumen pH $\leq 5.5 \leq 5.8$ e o Grupo C foi composto por sujeitos com rumen pH < 5.5. Amostras sanguíneas foram coletas por punção venosa jugular e Haptoglobina (Hp), Serum Amyloid A (SAA), Proteínas totais, Albumina e células brancas do sangue (WBC) foram determinadas. ANOVA em via única mostrou significância estatística em Rumen pH, Hp, SAA. SARA não parece estimular a produção de APPs pelo fígado.

Palavras-chave: proteína de fase aguda, gado leiteiro, haptoglobina, serum amyloid A, SARA

INTRODUCTION

In recent years an increasing interest was addressed to the animal welfare of dairy cows. The sub-acute ruminal acidosis (SARA) is one of the most frequent and insidious pathology in dairy cattle. This is due to an unbalance between

the production of short chain fatty acids (SCFA) in the rumen and their absorption and neutralization (Enemark *et al.*, 2004). This unbalance is directly dependent on an increase of the easily digestible carbohydrates in the feed ration, with an increased production of butyric acid, despite the starch that instead leads to a production of propionic acid as final result of the

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decomposition of cellulose (Garrett *et al.*, 1999; Enemark *et al.*, 2004). The diagnosis of SARA in a group or in a dairy herd is possible by measuring the rumen pH in a subsample of cows (Enemark *et al.*, 2004). Clinical signs are usually absent or, when present, tend to be ambiguous (Enemark *et al.*, 2004). The symptoms of SARA are largely variable, but often the first pathological event and the most common is a reduction of appetite and rumination (Ghoso *et al.*, 2005). Infection, inflammation, trauma and tumours can induce an acute phase reaction, in which the production of various acute phase proteins (APPs) by the liver is increased and these proteins are secreted into the blood (Kujala *et al.*, 2010). An elevation of Haptoglobin (Hp) and Serum Amyloid A (SAA) was previously suggested as a useful parameter for controlling SARA (Enemark *et al.*, 2008; Mohebbi *et al.*, 2010). It has been suggested that low rumen pH could result in death and lysis of gram-negative bacteria that are in the rumen and hence increase free endotoxins in the rumen (Nagaraja *et al.*, 1978; Andersen *et al.*, 1994). The acidic rumen environment, changes in osmotic pressure, and ruminal LPS may render the rumen epithelium susceptible to injury (Brent, 1976; Enemark *et al.*, 2002; Kleen *et al.*, 2003), resulting in the translocation of rumen endotoxin into the bloodstream (Kleen *et al.*, 2003). Ruminal endotoxin was implicated in the etiology of multiple metabolic disorders (Andersen, 2003; Ametaj *et al.*, 2005). The presence of LPS in the bloodstream results in the production of multiple proinflammatory cytokines, reactive oxygen and nitrogen intermediates, and bioactive lipids, which affect the host's metabolic response to inflammation (Baumann and Gauldie, 1994). Hp is a system of globulins circulating bound to haemoglobin which in response to various stimuli releases haptoglobin intravascularly from erythrocytes (Maes *et al.*, 1995). SAA, instead, is an apolipoprotein which has an important role during inflammation which is still not well understood and is one of the most reactive during the acute phase response both in humans and in animals (Badolato *et al.*, 1994; Murata *et al.*, 2004; Baghshani *et al.*, 2010; Deguchi *et al.*, 2010). Some of these functions include detoxification of endotoxins, inhibition of T lymphocyte adhesion and a down regulation of the inflammatory process (Murata *et al.*, 2004). In cows the APPs are elevated in stress conditions such as delivery, parturition and particular

diseases as in the fatty liver syndrome (Murata *et al.* 2004; Huzzey *et al.*, 2009). The significance of the individual differences in the APP response remains to be elucidated. In cattle, SAA is generally perceived as an indicator of acute inflammation, whereas haptoglobin is more slowly reacting and thus reflects the presence of chronic inflammatory conditions (Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1999).

In the past, few researchers studied the relationship between SARA and the serum acute phase proteins (Khafipour *et al.*, 2009; Ghoso *et al.*, 2005). Based on these considerations, the aim of this study was to evaluate the variations of APPs, and some blood parameters in relation to the onset of this pathological status.

MATERIALS AND METHODS

12 Italian intensive dairy herds were selected from different areas throughout northern Italy, some of which were considered potentially at high risk of SARA. All herds had a high average milk production (about 10000kg per year); the dairy cows were housed in free stalls and, in the early part of their lactation, used a total mixed ration (TMR) and adopted "steaming up" in the final part of the dry period as standard farming practice. 108 cows were randomly selected from all farms and were divided into three Groups of 36 animals each, based on ruminal pH. All animals were Holstein breed in the first 60 days of lactation, and were healthy at clinical examination. Table 1 shows the chemical composition analysis of the diet administered during the study. The Body Condition Score (BCS) average values were 3.03 ± 0.07 , in a 1 to 5 scale, according with the procedure of Edmonson (Edmonson *et al.*, 1989). All cows which participated in the study were subjected to a rumenocentesis, using a 13 G 105-mm needle (Intranule PP, Vygon, France), the most commonly used technique which provides accurate results (Garrett *et al.*, 1999; Duffield *et al.*, 2004; Morgante *et al.*, 2007). The time of sampling was between 4 and 6 hours post TMR distribution as recommended by Morgante *et al.* (2007). An area in the left flank of 20x20cm, 20cm caudal to the last costae, and on the level of the top of the stifle joint was prepared with an aseptic technique by disinfection with ethanol and iodine. The farmer was instructed to restrain the dairy cows by means of a tail grip and the

needle was introduced into the rumen by a veterinary surgeon. The rumen pH was immediately determined by means of a portable pHmeter (Piccolo, Hanna Instruments). All animals were classified into three different Groups according to the acidosis risk, depending on the rumen pH. Group A was composed by subjects with a rumen pH>5.8, Group B was composed by subjects with a rumen pH ≤5.5≤5.8 and Group C was composed by subjects with a rumen pH<5.5.

Table 1. Mean values of the chemical composition of the diet administered to 108 cows from 20 dairy herds

Parameters	Mean±SD
Crude protein	15.21±1.06
Ethereal extract	4.52±0.43
Ash	7.48±0.85
NDF	35.30±1.83
NSC	37.46±2.47
Digestible dry matter (%ss)	65.48±1.85
ADF	20.58±1.66
Starch	22.87±1.97
Calcium	1.00±0.05
Phosphorus	0.45±0.01
Magnesium	0.33±0.02
Sodium	0.58±0.05
Potassium	1.45±0.10
Chlorine	0.32±0.09
Anions/Cations (meq)	41.78±2.09
NDF/NSC	0.94±0.10
NSC/NDF	1.06±0.11
NDF/Proteins	2.33±0.22
Starch/Proteins	1.50±0.14

Blood samples were collected through jugular venipuncture before rumenocentesis (to avoid stress effect on blood parameters) under aseptic conditions and placed in tubes containing 1.2 mg anhydrous salt of ethylenediamine tetraacetic acid (K₃EDTA) *per* ml of blood. A white blood

cells (WBC) count was performed on collected blood samples through an automatic analyser for haematology (Abbott Cell Dyn 3500 - Abbott Diagnostic Division, CA; software 6.1)). The hematochemical profile was determined on plasma obtained from blood collected in tubes with lithium-heparin and the total proteins and albumin were measured through the Boehringer Mannheim/Hitachi 911 automated chemistry analyser (Roche, Basel, Switzerland) The Hp determination was performed on serum, at the Reactivlab ltd (Glasgow University), through an automated biochemical assay validated by Eckersall *et al* (1999) which measures the increase in absorbance. The principle of this test is based on the peroxidase activity of haemoglobin, inhibited at low pH. The Haptoglobin binds free haemoglobin and preserves, even at low pH, the peroxidase activity, which is therefore directly proportional to the level of haptoglobin in the sample.

The concentration of SAA in bovine serum was quantified by using a commercial ELISA obtained from Tridelta Development Ltd. (Dublin, Ireland), according to the manufacturer's instructions.

Total proteins and Albumin were determined from an aliquot of blood stored into vacutainer tubes without anticoagulant, through an automated analyser (Boehringer Mannheim/HITACHI 911, Roche, Basel, Svizzera).

One-way Analysis of Variance (ANOVA) was applied to compare all Groups. Bonferroni's test was applied for post hoc comparison. A *P* value <0.05 was considered statistically significant. Correlation and linear regression between Rumen pH and other parameters were performed. All data was analysed using Statistica 7 software (Statsoft Inc.).

RESULTS

Table 2 shows mean values (±SD) of all studied parameters with the statistical significances, expressed in their conventional units of measurement.

Table 2. Mean values(\pm SD) of the parameters analyzed, expressed in their units of measurements, with the statistical significance, from 108 dairy cows, divided into three groups by the rumen pH range: Group A (rumen pH >5.8), Group B (rumen pH $\leq 5.5 \leq 5.8$) and Group C (rumen pH <5.5)

Experimental Conditions				
Parameters	Group A	Group B	Group C	Reference Values
Rumen pH	6.20 \pm 0.39	5.77 \pm 0.35	5.57 \pm 0.31*	6.8-5.8
Haptoglobin(g/L)	0.24 \pm 0.04	0.11 \pm 0.08*	0.12 \pm 0.07*	0.08 \pm 0.03
Serum Amyloid A(μ g/ml)	46.09 \pm 15.72	107.13 \pm 35.51*	67.50 \pm 15.22*	4.50 \pm 0.18
Total Proteins(g/dL)	78.51 \pm 7.40	82.29 \pm 6.93	80.29 \pm 8.99	67.4-74.6
Albumin(%)	34.63 \pm 3.12	36.00 \pm 3.73	36.71 \pm 3.96	30.30-35.50
White Blood Cells (10^3 /ml)	7.33 \pm 1.39	7.34 \pm 1.78	6.77 \pm 1.08	4-12

Statistical significances:

* Vs Group A(P $<$ 0.001)

• Vs Group B(P $<$ 0.001)

One way ANOVA showed a statistical significance on Rumen pH (P $<$ 0.001; $F_{(2;107)}=13.84$), Hp (P $<$ 0.001; $F_{(2;107)}=43.33$), SAA (P $<$ 0.001; $F_{(2;107)}=59.54$). ANOVA showed no statistical significances on total proteins. All the results are graphically presented in Figure 1. No statistical significances emerged from the analysis of the correlation and the linear regression.

DISCUSSIONS

In contrast to monogastric mammals, about 50% of healthy cattle show an undetectable Hp level in plasma, and in the remaining healthy animals the concentrations reach 0.1g/L (Jawor *et al.*, 2010). In our study the Hp levels were higher in Group A, while we observed lower and similar values in the other groups. During an inflammatory event, the Hp rises rapidly and decreases in the following days, until returning to normal levels in about 10 days (Jawor *et al.*, 2010). High levels of Hp can be detected not only in ill subjects, but also in stress conditions (Murata *et al.*, 2004; Carroll *et al.*, 2009). In fact, stress has a role in activating latent infections resulting in acute phase response (Chan *et al.*, 2004) and we know that acute phase proteins could also be released in a stressful event: it is widely accepted that, in humans and experimental animals, physical and psychological stress elevates plasma IL-6 and APPs levels (Deak *et al.*, 1997; Nukina *et al.*, 2001). There is also evidence in cattle that physical stress can induce Acute Phase

Response(APR) (Murata and Miyamoto, 1993; Alsemgeest *et al.*, 1995; Lomborg *et al.*, 2008). The activation of the hypothalamic-pituitary-adrenal (HPA) axis by stress signals may be a trigger of systemic or local (intra-pituitary) cytokine production, thereby augmenting hepatic APP synthesis and release into the bloodstream (Murata *et al.*, 2004). In ruminants, the basal Hp values oscillate between 0.08 and 0.30 g/L (Chan *et al.*, 2004; Nowroozsi-Asl *et al.*, 2008; Baghshani *et al.*, 2010). The hp values we found in this trial were within the physiological range for Hp, and did not reach the serum levels found in severe diseases, just as in the retained placenta or a post partum metritis, where 1.13g/L of Hp are expected (Chan *et al.*, 2004). Both the groups at risk of SARA (B and C) showed lower values than the basal and this suggests that this metabolic condition does not affect the Hp production from liver. This situation is very different from what is described in literature, probably because in an experimental condition, grain-induced SARA determines a defined inflammatory status more comparable to an acute/inflammatory situation, while in field conditions this does not happen. Moreover, some authors showed a similar result demonstrating that SARA is a metabolic pathology that does not stimulate the immune response (Khafipour *et al.*, 2009). Regarding the higher levels of Hp in group A, we could probably assist to a physiological-induced increase since this group had the lower days in milk average and haptoglobin has been cited as calving-induced protein.

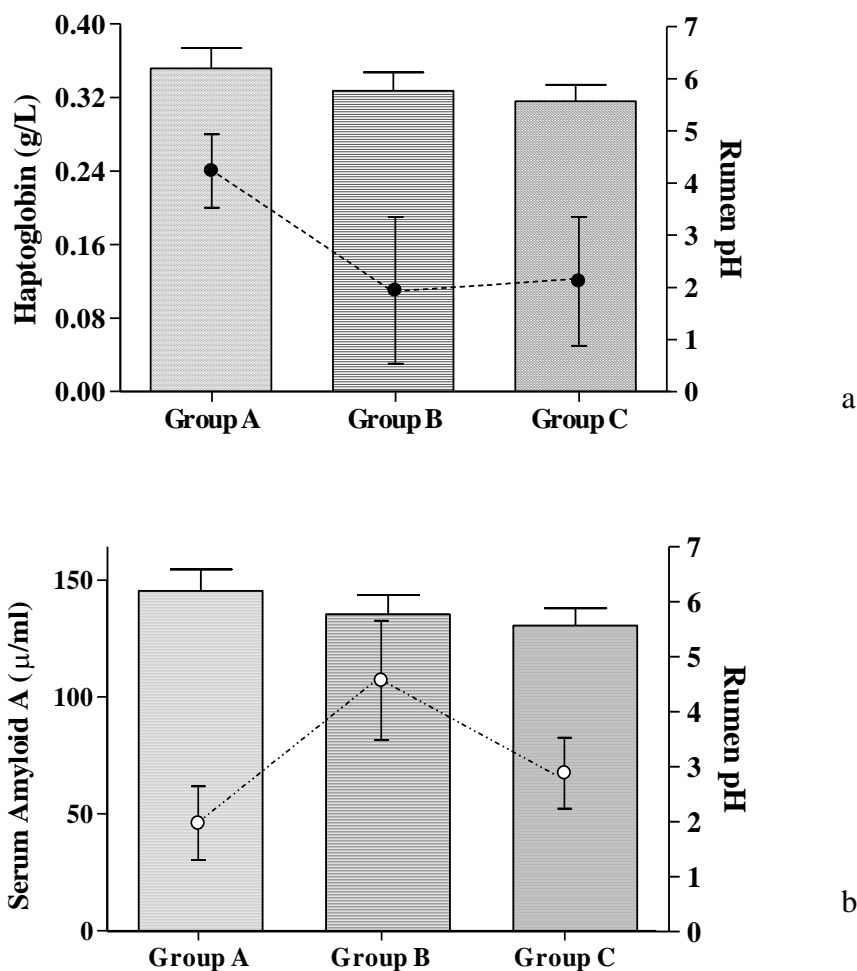


Figure 1. Patterns of the Rumen pH, some Acute Phase Proteins (Haptoglobin(a) and Serum Amyloid A(b)) in relation to the sub-acute ruminal acidosis (SARA), in 108 dairy cows, divided into three groups based on the rumen pH values: Group A (pH>5.8), Group B, (pH ≤5.5≤5.8) and Group C (pH<5.5).

SAA values found were higher than the mean values reported by other authors and had an opposite trend in relation to the values of Hp (Nazifi *et al.*, 2009). Elevated SAA serum levels can be found following inflammation and also under conditions unrelated to inflammation, such as physical stress or parturition (Baghshani *et al.*, 2010; Murata *et al.*, 2004). Group B, composed by medium risk acidosis cows with a rumen pH mean value of 5.75, showed the most elevated SAA level, while Group C, composed by high risk cows, had a SAA level lower than Group B. Positive acute phase response was reportedly associated to a change in metabolism (Gruys *et al.*, 2005). Abrupt SARA induction in heifers and

SARA induced by gradual adaptation from a forage based diet to a concentrate diet was associated to an increase of SAA in many trials, but some authors indicated that while grain-induced SARA has reportedly increased APP concentration in blood, SARA induced by reducing fiber particle size did not (Mulligan and Doherty, 2008) and this second situation is probably more similar to the field situation in North Italy. The cows used in our research were animals subjected to an intensive metabolic activity, including calf production. We hypothesized that, in any case, our results are not related to SARA, since the higher serum level does not correspond to the lower rumen pH

value, and elevated SAA serum levels are found in cows at parturition and in those with mastitis (Alsemgeest *et al.*, 1994; Murata *et al.*, 2004, Safi *et al.*, 2009). Moreover, since Hp decreases in group B and C and albumin (that is a negative APP) lightly increased at the same time, we could exclude an inflammatory status or an acute phase response.

CONCLUSIONS

The situation we found in field conditions is very different from what many authors described inducing SARA with barley grain and from studies in which LPS were infused directly regardless of sequelae before they are supposed to translocate in portal vein. It is still unclear by which mechanism LPS, when it passes a ruminal barrier, can pass portal circulation and hepatic barrier and stimulate an APR, since LPS can be detossified by the liver before reaching general circulation (Andersen *et al.*, 2003) but many receptors for cytokines are present in kupfer cells (Bode e Heinrich, 2001) and then the first proinflammatory cytokines could be released before the detossification starts.

Our results seem to indicate that SARA does not stimulate the immune response, even if the subjects in our study were in lactation and few cows were in the SARA threshold. We must consider also that clinical signs attributed to SARA become manifest after a certain delay to the initial insult: since we don't know if cows that we diagnosed with SARA are having problems afterwards and if normal cows have had subclinical problems before, it is difficult to relate APP variations to other clinical signs or more difficult to subclinical signs. We can then suggest that SARA should be considered a group-disease, since individual variations can disturb the diagnosis and it is necessary to exclude individual effect using a statistically significant number of animals.

Further investigation should be done to assess the influence of lower rumen pH values on the immune response, and it would be interesting to investigate more on causes and effects of SARA to understand if this fermentative disturb could be the consequence of stressful situations. If the main cause to develop SARA was linked to welfare, we could easily connect SARA to structures and management that could determine

lameness or other problems and explain many related pathologies that probably are not caused by SARA, but are the cause.

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