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Changes in the levels of acute-phase protein and other serum protein fractions in Santa Inês ewes fed with a high-concentrate diet

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ABSTRACT

The aim of this study was to evaluate the effects of subacute ruminal acidosis (SARA) on total serum protein (TP), plasma fibrinogen (Fb), and serum protein fractions in seven hay-adapted Santa Inês ewes fed with a high-concentrate diet for 133 days. Blood was extracted, before feeding, by external jugular venipuncture into tubes with ethylenediaminetetraacetic acid dipotassium anticoagulant, to evaluate the Fb concentration by heat precipitation, and into plain tubes, for TP analysis by the Biuret method and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE obtained the following proteins: immunoglobulin A (IgA) and G (IgG); ceruloplasmin (Cp); transferrin (Tf); albumin; α_1-antitrypsin (α_1AT); haptoglobin (Hp); α_1-acid glycoprotein (α_1AG); and 140, 101, 95, 46, 36, 34, 31, and 23 kDa molecular weight proteins (MWP). Associations among variables and time were evaluated using regression analysis. Fb concentrations did not change ($P > 0.05$); IgA, α_1AT, and Hp levels increased, while Cp decreased throughout the period. Mean Tf levels increased, reaching the highest value on the 72nd day. The other protein levels oscillated during the study. A positive correlation, measured using Pearson's coefficient, was found between TP and IgG levels ($r = 0.77$; $P < 0.0001$). IgA, Hp, Tf, and α1AT showed greater potential for use in the diagnosis of SARA. The occurrence of proteins with unknown functions, but that have been highly influenced by the high-grain diet, increases the possibility of identifying inflammatory markers for SARA.

1. Introduction

The growing sheep meat industry has resulted in increasing numbers of feedlot animals, which are susceptible to disorders such as ruminal acidosis that cause economic problems owing to treatment costs, reduced herd productivity, disposal of animals, and injury. Ruminal acidosis is often categorized into several types including acute and subacute (also called chronic, latent, or subclinical) types (Kleen et al., 2003; Owens et al., 1998).

Subacute ruminal acidosis (SARA) is more common than its acute manifestation, but more difficult to characterize (Cannizzo et al., 2012; Enemark et al., 2002). SARA is defined as an intermittent decrease of ruminal pH to non-physiological levels after intake of a low-structure, high-energy diet, owing to the inability of a non-adapted ruminal environment to adequately ferment and absorb the resulting short-chain fatty acids (SCFA) (Kleen et al., 2003). However, the rumen microbial population fits diet rich in grains with many amylolytic and lactolytic microorganisms so that the lactic acid does not accumulate (Enemark et al., 2002). The clinical symptoms of SARA appear after a delay from the time of the initial insult (Kleen et al., 2003), manifesting as reduced feed intake and performance; however, animals may not appear to be ill (Owens et al., 1998).

Inflammation due to grain-induced SARA could result from the translocation of lipopolysaccharides (LPS) derived from gram-negative bacteria from the rumen to body circulation (Plaizier et al., 2009; Plaizier et al., 2012). Translocation of LPS may also occur in the large intestine because grain-induced SARA increases its acidity and LPS content; further, the barrier function of the large intestine is more easily compromised than that of the rumen (Dong et al., 2011; Plaizier et al., 2012). LPS interacts with mononuclear, endothelial, and smooth muscle cells; polymorphonuclear granulocytes; and thrombocytes. It also stimulates the production of pro-inflammatory mediators such as

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cytokines (Plaizier et al., 2012). Other immunogenic factors in the digestive tract may contribute to inflammation following ingestion of a high-grain diet (Dong et al., 2011; Plaizier et al., 2009); as SARA can lead to rumenitis, this inflammation could also induce an acute-phase response (APR) (Plaizier et al., 2009; Plaizier et al., 2012).

Acute-phase proteins (APPs) are blood proteins of the innate immune system that change concentration in response to external or internal challenges such as infection, inflammation, surgical trauma, or stress (Eckersall and Bell, 2010; Murata et al., 2004) such as that caused by the transport of ruminants (Giannetto et al., 2011; Piccione et al., 2012). They act as modulators of inflammatory response by interacting with both defensive cells and pathogens (Ceciliani et al., 2012). Because circulating concentrations of APPs reflect disease severity, they may be useful for in vivo disease monitoring (Danscher et al., 2011; Murata et al., 2004), providing an alternative method for assessing disease pathogenesis and monitoring animal health, including diagnosis, prognosis, response to therapy, and welfare status (Ceciliani et al., 2012; Eckersall, 2000; Eckersall and Bell, 2010; Tothova et al., 2014). APPs are non-specific biomarkers (Danscher et al., 2011; Eckersall and Bell, 2010; Tothova et al., 2014) with species-specific responses (Eckersall and Bell, 2010; Murata et al., 2004; Tothova et al., 2014).

APR in ruminants differs significantly from other species, in which haptoglobin (Hp) is a major APP (Eckersall and Bell, 2010). APR in experimental and natural diseases in cattle has been investigated for decades (Ceciliani et al., 2012). Information on APR and the relationships between APP levels and production parameters in sheep is limited (Pfeffer et al., 1993), but Hp and serum amyloid A (SAA) are considered major APPs, α_1-acid glycoprotein (α_1AG) moderate APP, fibrinogen (Fb) and ceruloplasmin (Cp) minor APPs, and albumin negative APP in this species (Gómez-Laguna et al., 2011). Hp, Cp, and Fb levels may be more useful diagnostic markers than the number of circulating neutrophils in sheep (Pfeffer and Rogers, 1989).

Metabolic disorders in animals may cause activation of the immune system, including initiation of inflammatory responses (Tothova et al., 2014). APPs have been used to investigate the pathophysiology of the gastrointestinal tract in cattle and the effects of diet variation on its function (Ceciliani et al., 2012). Despite recent study with some APPs that indicated that SARA did not stimulate the immune response in dairy cows under field conditions (Cannizzo et al., 2012), they also seem to be informative adjuncts to pH measurements in ruminal acidosis when other sources of inflammation can be excluded (Danscher et al., 2011) and important in early detection of SARA (Gozho et al., 2006). Thus, the aim of the present study was to evaluate the effects of long-term high-concentrate diet on APPs and other serum protein fractions in Santa Inês sheep.

2. Materials and methods

Seven ewes were kept in pens under appropriate hygiene conditions and maintained for 60 days on a basal diet of coast cross hay (*Cynodon* spp.), drinking water, and mineral mixture (Tortuga Ovinofós com minerais orgânicos, Tortuga Companhia Zootécnica Agrária, São Paulo, Brazil) ad libitum. The basal values were the means of three weekly assessments: 15 days, 7 days, and immediately before (D0) the dietary change. During induction to SARA, based on their average consumption during the basal diet (2 kg organic matter/animal/day), their diet was replaced with 10% roughage for concentrate feed (76.8% ground corn, 18.2% soybean meal, and 5% mineral core; Guabi Ruminúcleo Ovinos 40 ADE, Mogiana Alimentos S/A, Campinas, Brazil), daily, up to 80% concentrate feed (D0–D7). This percentage was maintained until day 133 (D133). No buffer was included in the rations. After changes to the diet were made, measurements were performed daily for the first 14 days (D1–D14), every other day until the 28th day (D16, D18, D20, D22, D24, D26, D28), and weekly for the remainder of the 5-month experiment (D35, D42, D49, D56, D63, D70, D77, D84, D91, D98, D105, D112, D119, D126, D133). Sampling was performed in the morning, prior to feeding.

In order to evaluate the occurrence of SARA, 10-mL ruminal fluid samples were collected through the rumen cannula using a disposable pipette. Each sample was packed in a sterile, 15-mL conical tube and analyzed immediately for the physical characteristics (viscosity, color, odor), sedimentation and flotation test – SFT (seconds; Arcuri et al., 2006), methylene blue reduction time – MBR (seconds; Dirksen et al., 1993), and pH. During spontaneous urination, induced by vulvar massage (Enemark et al., 2002) or by brief restriction of breathing (Benech et al., 2015), urine samples were collected in sterile, 80-mL lidded containers. Urine and ruminal fluid pH values were immediately measured using a digital pH meter (Digimed DM20, Digicrom Analítica Ltda., São Paulo, Brazil).

From each animal, blood samples were collected by external jugular venipuncture into 10 mL plastic tubes without anticoagulant (BD Vacutainer, BD Diagnostics – Preanalytical Systems, São Paulo, Brazil), and into 4 mL tubes with ethylenediaminetetraacetic acid dipotassium anticoagulant (K_2EDTA 7.2 mg). Blood samples collected into plain tubes were used for serum total protein (TP) and protein electrophoresis analysis. The blood samples collected into tubes with K_2EDTA were used to evaluate the plasma fibrinogen (Fb) concentration. Serum samples were separated quickly by centrifugation at 2500*g* for 10 min and stored at −70 °C for two months before laboratory analysis.

Protein fractionation was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein fraction percentages were measured using a computerized scanning densitometer (Shimadzu CS 9301, Shimadzu Corporation, Tokyo, Japan), with a reference marker solution (Sigma-Marker, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA) with molecular weights from 6.5 to 200 kDa. Serum protein concentrations (g/L) were determined by multiplying the percentage of each fraction by the TP concentration obtained by a semi-automatic spectrophotometer (Labquest, Labtest, Brazil) using the Biuret method with a bovine albumin standard (4 g/dL) provided with the commercial kit. Fb was determined by heat precipitation (Allison, 2015) and measured using a refractometer (Atago T2-NE Clinical, Atago Co. Ltd., Japan). These analyses were performed at the Research Laboratory of the Department of Veterinary Clinic and Surgery, School of Agrarian and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil.

Data analysis was performed using commercial statistical software (SAS 9.1, SAS Institute Inc., Cary, North Carolina, USA) to assess associations among variables and time by linear, quadratic, and cubic regressions (y = variable value, x = day after dietary change). Maximum and/or minimum points were obtained by the derivative of the function. Correlation among protein levels was measured using Pearson's coefficient. Score classifications of the correlation between variables were considered weak ($0.01 \leq r \leq 0.39$), moderate ($0.40 \leq r \leq 0.69$), or strong ($0.70 \leq r \leq 1.00$). Statistical significance was set at $P \leq 0.05$.

This study was approved by the Ethics Commission in Use of Animals of the School of Agrarian and Veterinary Sciences (Protocol no. 008891/12).

3. Results

The ruminal fluid remained liquid, sometimes viscous, and its odor changed from characteristic to predominantly acid from the day 4. It remained olive green until the third day, then the yellowish color frequency increased until it predominates from the day 18. The MBR ($y = 0.00365x^2 - 0.72524x + 66.81923$; $P \leq 0.05$) and SFT ($y = -0.32541x + 75.73218$; $P < 0.0001$) decreased during the observation period. Ruminal pH decreased until day 20, increased until day 95, and decreased thereafter ($y = -0.00000187x^3 + 0.00032076x^2 - 0.01052x + 6.15389$; $P \leq 0.05$). Urine pH markedly decreased throughout the study ($y = -0.00002244x^3 + 0.00558x^2 - 0.33641x + 75.08852$; $P < 0.001$).