Acute Phase Biomarkers for Inflammatory Response in Dairy Cows with Traumatic Reticuloperitonitis

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ABSTRACT

The traumatic reticuloperitonitis (TRP) model was used to investigate the acute phase biomarkers (APBs) in the inflammatory response of dairy cows. Fourteen Swiss Brown cows were diagnosed with TRP based on the clinical findings, ferroscopy and ultrasonography as well as positive responses to pain tests. Four of the cows were necropsied and TRP was confirmed. Additionally, 10 healthy cows were used as the control group. Blood samples were obtained from cows during the clinical stage of TRP. Mean serum haptoglobin (Hp) (1.19±0.37 vs. 0.03±0.01 mg/mL) (P<0.05) and plasma fibrinogen (Fb) (205.1±18.1 vs. 101.1±17.6 ng/mL) (P<0.001) concentrations of TRP group were found higher compared to control group. There was an insignificant increase in mean serum amyloid A (SAA) (165±63 vs. 67.9±34 μg/mL) and α-1 acid glycoprotein (α-1 AGP) (1069±220 vs. 663±121 μg/mL) levels (P>0.05). Mean total white blood cell (WBC) count of TRP group (10.8±1.4 vs. 6.9±0.6 x103 /μL) was significantly higher compared to control group (P<0.001). Moreover, neutrophil counts of the TRP group showed a tendency to increase compared to the control group, however no statistical difference was detected (P>0.05). While positive correlation was detected between WBC count and Hp concentration of TRP group (r=0.636; P=0.01); there was no correlation between WBC count and Fb concentration (r=0.395; P>0.05). There was no statistical difference (P>0.05) between groups for routine biochemical parameters. In conclusion, significant increases in Fb and Hp values were found to be related to the inflammatory response of dairy cows with TRP. The tendency of increase in the SAA and α-1 AGP were evaluated as nonspecific for the response. In addition, high Hp values were consistent with the correlation of high WBC counts due to the inflammatory response.

Keywords: Acute Phase Biomarkers; Dairy Cow; Inflammation; Leukogram; Traumatic Reticuloperitonitis.

INTRODUCTION

Traumatic reticuloperitonitis (TRP) is a common condition in adult cattle caused by the ingestion and migration of foreign bodies in the reticulum. Perforation of the wall of the reticulum allows leakage of ingesta and bacteria, which contaminate the peritoneal cavity, resulting in local or diffuse peritonitis (1, 2). In particular pica occurring as a result of malnutrition is a risk factor for TRP (3). The clinical signs of cattle with TRP are variable depending on the severity, duration, and involvement of other organs. Fever, increased heart and respiratory rates, anorexia, dehydration, decreased milk production, weight loss, ruminal stasis, tympani, abdominal tension, abdominal pain and grunting are the most common clinical signs observed in cattle with TRP (1, 2). It is difficult to make an early diagnosis as clinical findings of TRP are non-specific and clinical signs only appear after a protracted period (1, 4). Rumenotomy often fails because TRP diagnosis is made at a late stage by which time the prognosis is poor (5).
It is stated in previous studies that many hematological and biochemical changes may be observed in animals with TRP (2, 6, 7). It has been further reported that acute phase proteins (APPs) are far more sensitive than the leukogram and routine biochemical changes for determining inflammatory responses in cattle (7, 8). It has been proposed that the use of laboratory analyses such as APPs are required for early diagnosis and determination of the stage, complications and prognosis of the disease (5).

Acute phase response (APR) occurs during infection, inflammation, tissue injury and trauma, and is the leading systemic reaction seen during disease. One of the main features of APR is hepatic production of APPs. The aim of the generation of these proteins is to isolate and destroy the infectious agents, prevent ongoing tissue damage and restore homeostasis. The secretion of APPs is regulated by proinflammatory cytokines. Blood concentration of APPs generally increase within 8 hours of stimulation and reaches maximum level in 24-48 hours and gradually decreases to their normal levels in 4-7 days relative to the inflammatory response (9). However, blood concentration of APPs continues to stay at high levels in chronic cases where stimulation continues to occur. APPs may be used for separation of bacterial and viral infections, differential diagnosis of clinical, subclinical, acute and chronic diseases, determination of prognosis of sick animals and tracking patients during treatment (9, 10). APPs for determination of cattle diseases are listed as fibrinogen (Fb), haptoglobulin (Hp), serum amyloid A (SAA) and α-1 acid glycoprotein (α1-AGP) (9-11).

The number of studies on APPs is limited for APR in cattle with TRP (5, 12-14), and there is only one study in which all APPs were evaluated (5). In the present study, investigation of the acute phase biomarkers (APBs) in the inflammatory response in Swiss Brown cows with TRP was studied. The inflammatory response was further evaluated along with clinical findings, leukogram, and routine biochemical parameters and ultrasonographic examinations.

MATERIAL AND METHODS

Animals

Fourteen Swiss Brown cows with TRP referred to the clinic were included in the study as the experimental group. Ten clinically healthy Swiss Brown cows were obtained from the dairy farm of the Faculty of Veterinary Medicine and served as a control group. All cows were adults of 3 to 8 years of age.

The protocol was approved by the university ethics committee. The approval number was 2010/58.

Diagnosis of TRP and collection of samples

Diagnosis of TRP was made according to clinical findings, ferroscopy, (Hauptner Ferroscope, Art-Nr 39500; H. Hauptner & Richard Herberholz GmbH & Co. KG, Solingen, Germany) glutaraldehyde coagulation test and responses to pain test. The clinical signs for the definition of animals with TRP were reduction or lack of rumen contraction, tympani, constipation, grunting and abdominal pain. Physical examination of cows with TRP consisted of rectal temperature (RT), heart rate (HR) and respiratory rate (RR) and measurement of the frequency of rumen contractions (RC). Sick animals were brought to the clinic generally 3-5 days after the emergence of clinical signs. Blood samples were taken from the external jugular veins into vacuum tubes with no anticoagulant (Vacutainer, BD-Plymouth, UK) for serum analyses. Serum samples were separated by centrifugation at 3000 g for 10 minutes at room temperature and stored at -20°C until analyses. Blood samples were also collected into tubes with EDTA (3.6 mg K2E, Vacutainer, BD-Plymouth, UK) for the determination of hematological parameters.

Glutaraldehyde coagulation test

Glutaraldehyde coagulation test (GCT) was used to evaluate the indicative concentrations of fibrinogen and immunoglobulin semiquantitatively. GCT test solution was prepared using GCT 5.6 ml 1.4% glutaraldehyde (Merck, USA), 200 mg sodium EDTA and 94.4 ml 0.9% NaCl. This test solution was mixed with equal volume of blood (i.e. 4 ml to 4 ml). Immediately after peripheral blood sample was obtained from jugular vein of each cattle into sterile syringes, GCT test solution was mixed to determine the clotting time. Clotting times of 0-5 min, 5-10 min, and 10-15 min were accepted as indications of severe, moderate and mild positive results of the test, respectively. The clotting time was evaluated as negative after 15 minutes (25).

APBs of determination

The concentration of Hp was assessed using a commercial colorimetric kit (Tridelta Development Plc, Wicklow, Ireland) in microplates, based on Hp–haemoglobin binding
and preservation of the peroxidase activity of the bound hæmoglobinat at a low pH. SAA was analyzed by the method of sandwich enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kit (Tridelta Development Plc, Wicklow, Ireland) in microplates. The optical densities were read on automatic microplate reader Opsys MR (Dynex Technologies, USA) at 630 nm for Hp, and at 450 nm using 630 nm as reference for SAA. The concentration of α-1 AGP was detected by bovine radial immunodiffusion assay kits (Tridelta Development Plc, Wicklow, Ireland). Fb was measured by using a solid-phase sandwich ELISA (Life Science Inc. Uscn, Wuhan, China).

**Hematological analyses**

Venous blood samples were collected in tubes with EDTA (3.6 mg K$_3$E, BD Vacutainer, BD-Plymouth, UK., 2ml). A cell counter (Abacus Junior Vet5, Hungary) was used to establish basophil (BAS), eosinophil (EOS), lymphocyte (LYM), monocyte (MON), neutrophil (NEU) and total leucocyte counts (WBC).

**Serum biochemistry**

Serum enzyme activities [alkaline phosphatase (ALP), aspartate aminotransferase (AST), gama glutamyl transferase (GGT), lactate dehydrogenase (LDH)], total bilirubin (TBIL), albumin (ALB), glucose (GLU) and total protein (TP) concentrations were determined with commercial test kits by a biochemistry autoanalyzer (Cobas 6000/Roche Diagnostics, Germany). The concentration of total globulin (GLOB) was calculated by subtracting the ALB concentration from the TP concentration (16, 17).

**Statistical analysis**

Data were expressed as mean ± standard error of the mean (SEM). The level of statistical significance was set at P<0.05. Statistical comparisons of values between the two groups were analyzed using ANOVA (SPSS, Version 11.5 Microsoft, Chicago, IL, USA). Correlations between parameters were calculated with the Pearson Correlation test.

**RESULTS**

**Clinical signs**

Mean values of clinical signs of cows with TRP and control group were shown in Table 1. Body temperature and respiration rate were unaffected however the heart rate of cows with TRP significantly increased compared to the control group (82±4.7/min vs. 60±2.8/min) (P<0.001) and the numbers of rumen contraction significantly decreased (3.6±0.6/5min vs. 8.6±0.3/5min) (P<0.001). Additional findings of the cows with TRP were anorexia, grunting, constipation, tympani, ruminal stasis, impaction, abdominal pain and tension. Ferroscopy detected metallic foreign bodies with different response to the device (as 10-30 $\mu$A) around the reticulum and the cranio-ventral region of the rumen in left side of the cows with TRP.

**APBs concentrations and GCT results**

Mean values of APBs levels of cows with TRP and control group were presented in Table 2. It was determined that Fb (205.1±18.1 ng/mL vs. 101.1±17.6 ng/mL) (P<0.001) and Hp (1.19±0.37 mg/mL vs. 0.03±0.01 mg/mL) concentrations significantly increased in TRP group compared to control group (P<0.05). Although there was an increase in SAA and α-1 AGP P levels, there was no statistical difference between two groups (P>0.05). Additionally, GCT was determined as severe positive within 1 to 5 min clotting time of blood samples in cows with TRP. The blood samples of healthy cows responded to the test without clotting for up to 15 minutes.

**Table 1:** Mean values and standard error of the mean of clinical signs in dairy cows with TRP and control group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control ($n=10$)</th>
<th>TRP ($n=14$)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT (°C)</td>
<td>38.4 ± 0.1</td>
<td>38.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>HR (beat/min)</td>
<td>60 ± 2.8</td>
<td>82.3 ± 4.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RR (breaths/min)</td>
<td>24 ± 1.6</td>
<td>26.6 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>RC (cycle/5 min)</td>
<td>8.6 ± 0.3</td>
<td>3.6 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS: not significant.
RT: rectal temperature; HR: heart rate; RR: respiration rate; RC: rumen contractions.

**Table 2:** Mean values and standard error of the mean of APBs in dairy cows with TRP and control group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control ($n=10$)</th>
<th>TRP ($n=14$)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fb (ng/mL)</td>
<td>101.1±17.6</td>
<td>205.1±18.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hp (mg/mL)</td>
<td>0.03±0.01</td>
<td>1.19±0.37</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SAA (µg/mL)</td>
<td>67.9±34</td>
<td>165±63</td>
<td>NS</td>
</tr>
<tr>
<td>α-1 AGP (µg/mL)</td>
<td>663±121</td>
<td>1069±220</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: not significant.
Fb: fibrinogen; Hp: haptoglobin; SAA: serum amyloid A; α-1 AGP: alfa-1 acid glycoprotein
Leukogram values

Mean values of leukogram findings of cows with TRP and control group were presented in Table 3. It was found that total WBC counts of TRP group significantly increased compared to control group (10.8±1.4x10^3/μL vs. 6.9±0.6x10^3/μL) (P<0.05). Moreover, neutrophil counts of TRP group showed a tendency to increase compared to the control group however without statistical significant differences detected (P>0.05). There was positive correlation between total WBC count and Hp concentrations in TRP group (r=0.636, P=0.01) but there was no correlation between total WBC count and Fb concentrations (r= 0.395, P>0.05).

Biochemical parameters

Mean values of biochemical parameters of cows with TRP and control group are shown in Table 4. Although there were tendencies to increase in means of ALP, AST, GGT, LDH activities and TBIL levels in TRP group, there were no statistically significant differences detected (P>0.05). There was no statistically significant difference between two groups regarding the means of ALB, GLOB, GLU and TP concentrations (P>0.05).

Necropsy Findings

In post-mortem examination of the cows, different size of metallic foreign bodies was found in reticulum and the cranio-ventral region of the rumen. Prominent gross findings were diffuse thickening of the reticular and ruminal wall around the perforation caused by the foreign body with local peritonitis, inflammation and fibrotic adhesions.

DISCUSSION

Traumatic reticuloperitonitis progresses in cattle as reticulitis, acute or chronic, local and diffuse peritonitis. Besides, contamination to surrounding organs, inflammation of the reticulum and complications may also result as peritonitis, pericarditis, myocarditis, endocarditis, pneumonia, pleuritis, hepatitis and septicemia (1, 2). The development of inflammation in the reticulum from the acute process to the chronic process makes the treatment and healing course complex. For this reason it was decided to evaluate acute phase biomarkers as a measure of the inflammatory response in cows with traumatic reticuloperitonitis.

The increase in heart rate may be associated with systemic effects of acute inflammation. Similarly, the reason for significant decrease of numbers of rumen contraction of TRP group compared to control group may be a result of negative effect of inflammation on the reticulum and the rumino-reticular movements as stated by Radostits et al. (1) and Constable (2). There was no statistical difference between mean values of rectal temperature in the groups indicating body temperature is not significantly affected by local inflammation of reticulum at the stage of clinical findings.

The most frequent APP evaluated in cattle is fibrinogen (Fb). Plasma Fb level in cattle increases in 2 days after inflammation (7, 15, 16). In cattle, Fb has been used for many years to evaluate inflammatory and traumatic diseases and is characterized by a marked increase in the synthesis of the protein in response to infection (12). Hirvonen and Pyörälä, have stated that Fb is useful for distinguishing TRP from...
other gastrointestinal diseases and pre-determination of the healing process of abdominal disorders (12). Gokce et al. (17) stated that TRP is indicative of hyperfibrinogenemia. Therefore, Fb concentration is known to be useful for the diagnosis of TRP (5, 13).

Fb level of cows in the TRP group was significantly higher than in the control group (205.1±18.1 vs. 101.1±17.6 μg/mL, p<0.001). These obtained values were determined by solid-phase sandwich ELISA method. The increase in the Fb level was associated with the severity of inflammation process. Furthermore, there was no correlation between Fb concentration and WBC count in TRP group. This suggests that Fb concentration is preferable as a marker as compared to the leukogram when evaluating traumatic inflammatory processes in cattle (7, 8).

GCT is a non-specific test for the indication of any diffuse inflammatory acute diseases including TRP. It is utmost important to note here that GCT be evaluated by using clinical signs of cows. The cows diagnosed as TRP showed clinical signs such as reduction or lack of rumen contraction, tympani, constipation, grunting and abdominal pain. Severe positive GCT and increased levels of Hp and Fb were detected in these cows with the clinical signs of TRP revealing acute diffuse inflammation.

Hp levels are increased during acute infection but decreased with treatment or chronicity (9, 10). However, the level remains high in chronic cases if stimulation continues (5, 10). Plasma Hp levels in healthy cattle have been reported to be less than 0.35 g/L; it increases in 24-48 hours following inflammation and remains high for two weeks (8). It has also been reported that traumatic stimulation, surgery, starvation and stress increase Hp level in cattle with TRP (5, 10, 12, 18). In several studies on Hp levels in cattle with TRP, Hp levels increased to the range of 0.86-2.16 g/L. According to these studies, Hp levels may be useful for TRP diagnosis (5, 13, 14).

In accordance with the reports above, it has been observed in this study that Hp level of cows with TRP is significantly increased as compared to the control group. It has been proposed that prognosis is considered good when Hp plasma levels are about 0.1-1 g/L and prognosis is poor and treatment is required when this level is over 1 g/L (19). Similar to previous reports, the mean level of Hp was over 1 g/L in cows with TRP in this study. Furthermore, a positive correlation has been detected between Hp level and WBC count. Therefore it is considered important to evaluate Hp concentration and WBC counts simultaneously to determine the severity of inflammation in animals with TRP.

SAA is an APP which plays roles in detoxification of endotoxins, proliferation of endothelial cells and activation of immune response (20). The level is less than 8.8 mg/L in healthy cattle and increases in the first 10 hours following bacterial infection (8). It was determined that SAA increased in 31 of 31 cattle with acute inflammation and in 27 of 50 cattle with chronic inflammation (8). Mean SAA level were 74.3 mg/L in cattle with acute inflammation and mean SAA level was 11.7 mg/L in cattle with chronic inflammation. These results suggest that SAA is a more determinative marker as compared to total leukocytes and neutrophil counts for distinguishing acute and chronic inflammations of cattle (8). It has been stated in several studies on SAA levels of cattle with TRP that SAA concentrations ranges within 85.02-312.40 μg/mL and that it can be useful for TRP diagnosis (5, 13, 14). The increase of SAA in the cattle in this study with TRP was not been found statistically significant contrary to previous studies. The reason for this may be due to the large intergroup variations in the TRP group for SAA levels (67.9±34 vs. 165±63 μg/mL). The wide variability of this parameter may be a limiting factor affecting its diagnostic value.

α-1 AGP inhibits lymphocyte blastogenesis and suppresses systemic immune responses and it is mostly used to indicate a chronic inflammatory state since it causes a slight increase with a slow response (18, 21). Serum α-1 AGP level is about 200-450 mg/L in healthy cattle, and increases in 24-72 hours following inflammatory conditions (7). Horadagoda et al. (8) stated that α-1 AGP was found to be increased in 25 of 28 cattle with acute inflammation (89%) and in 36 of 50 cattle with chronic inflammation (72%). The mean α-1 AGP value was 1101.4 mg/L in acute inflammation and 815.2 mg/L in chronic inflammation, and α-1 AGP was considered to be more of a determinative marker as compared to WBC count, especially in chronic inflammation.

Bozukluhan and Gokce (5) stated that mean α-1 AGP level was 972.5 μg/mL in cattle with TRP and 378 μg/mL in healthy cattle. Therefore it may be useful for TRP diagnosis. There was a tendency for an increase in the concentration of α-1 AGP as a result of TRP in the present study. This increase was not found to be statistically significant probably due to the high intergroup variations (663±121 vs. 1069±220 μg/mL).
The changes in the hemogram have been found to be significant for determining the inflammation of cattle with TRP (7, 15). The total and differential leukocyte counts provide useful diagnostic and prognostic data (1). Leukocytosis with left shift neutrophilia is a common hematological finding in cattle with TRP (17). At the initiation of severe inflammation, WBC count may be normal or lower than the normal value due to lymphopenia based on the migration of neutrophils to the inflammatory area and due to stress (7). In the study presented, WBC count of the TRP group significantly increased compared to control group and the increase was observed associated with neutrophilia. Although a tendency to increase was detected in TRP group compared to control group related with neutrophil count, a statistical difference was not found. It appeared that neutrophil left shift may have occurred in the acute period of TRP resulting from an increased band neutrophil count relative to segmented neutrophils (1, 2). Total leukocyte, neutrophil and lymphocyte counts can revert to normal in chronic periods. Therefore, it has been stated that leukocyte changes cannot be useful indicators for the diagnosis of chronic inflammation in cattle (7, 22).

Morphological changes of the leukocytes such as band neutrophils were not determined in the animals used in this study therefore limiting the interpretation of leukocyte counts. On the other hand, the tendency of an increase in neutrophil count obtained in TRP group (4.7±1.1 vs. 2.0±0.3 10^3 /μL, P=0.06) suggests a regenerative response of neutrophils, which may be related to an increase in band neutrophils. It should be noted that the differential leukocyte counts should be used to evaluate the stage of the inflammatory response.

Changes in total protein, globulin and albumin levels were expected in response to inflammation during the clinical form of TRP. In previous studies, total protein levels have been determined as normal (6, 23), low (24) or high (17, 25, 26) under these circumstances. Total protein levels were not changed in the TRP group in this study. Reticular abscess associated with TRP have been found to result in hyperglobulinemia (6). Ok and Aslan (25) stated that total globulin levels decreased during the disease as a result of protein migration into the inflammatory area, and consequently, blood protein concentration decreased temporarily until new protein synthesis begun. In this present study, mean globulin levels in TRP group were not found to be statistically different than those of the control group. The decrease in albumin may be linked to the synthesis of APPs, digestive failure, starvation and/or malnutrition (6, 26). In this study, mean albumin levels in TRP group were not different from control group. This result may reflect that hepatic albumin synthesis was not affected by APR synthesis.

No statistically differences in serum liver enzyme activities (ALP, AST, GGT and LDH), total bilirubin and glucose levels were found in TRP group as compared to the control group. These findings indicate that hepatocyte integrity of the liver was not impaired in the animals with TRP.

The results of this study evaluated the clinical findings, APPs, leukogram and biochemical parameters in Swiss Brown cows. Ocal et al. (3) have reported concomitant changes with hematologic parameters in the same breed of cows. Additionally, previous investigations have determined comparable serum and hematologic findings in Swiss Brown cows along with Holstein, Water Buffalo, Ayrshire and Friesian breed cows (6, 12, 27, 28). Further research is required with different strains of cattle, males versus females and other parameters, which may affect the results.

In conclusion, the cows with TRP responded with several of the APBs. APBs and in particular Hp, increase in the cows showing clinical signs of TRP. The tendency for increases in the SAA and α-1 AGP were found to be nonspecific. Significant increases in Fb and Hp values were related with the inflammatory response of cattle with TRP. In addition, high Hp values were consistent with the correlation of high WBC count as confirmed by the inflammation.

ACKNOWLEDGEMENTS

The authors would like to thank to Ataturk University for supporting this research financially (Ataturk University Scientific Research Office, Project Number: 2010/160).

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