The use of inflammatory markers as a prognostic aid for traumatic reticuloperitonitis in water buffalo (*Bubalus bubalis*)

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ABSTRACT: The present study was conducted to evaluate the prognostic significance of selected inflammatory markers for prediction of clinical outcomes of traumatic reticuloperitonitis (TRP) in water buffalo (*Bubalus bubalis*). Acute local TRP was initially diagnosed in 32 buffalo by clinical examination and confirmed by ultrasonography (USG), laparo-rumenotomy and/or necropsy findings in non-surviving cases. Ten clinically healthy buffalo were randomly selected and served as controls. Blood was drawn from all examined buffalo to measure the respective levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-10, interferon gamma (INF)-γ, serum amyloid A (SAA), haptoglobin (Hp), fibrinogen (Fb), C-reactive protein (CRP) and serum sialic acid (SSA). Clinically, the heart rates, but neither respiratory rate nor rectal temperature, were significantly higher in non-survivors compared with survivors (*P* < 0.05). In addition, the non-surviving buffalo were more likely to have anorexia and weakness compared with survivors. However, rumen stasis, recurrent ruminal tympany, lacrimation, lordosis, bruxism, and decreased milk production were commonly observed in all diseased animals. Biochemically, TNF-α, IL-1β, IL-6, IL-10, SAA, Hp, Fb, CRP, and SSA levels were significantly higher in diseased buffalo compared with controls, and were higher in non-survivors than survivors (*P* < 0.05). The data herein indicate an ongoing cascade of systemic inflammatory responses in buffalo with TRP with concomitant compensatory anti-inflammatory reactions and the overall degree of cytokine network disruption may be an important prognostic indicator. Medical strategies to modulate inflammation must take into account the complex of cytokine biology in buffalo with TRP.

Keywords: cytokine profile; prognosis; traumatic reticulo-peritonitis; water buffalo

Traumatic reticuloperitonitis (TRP) is one of the most frequently occurring digestive diseases of bovines, which has drawn the attention of animal health professionals over the past years (Mousavi et al. 2007). It is caused by a combination of a lapse in good management and the non-selective eating habits of large ruminants. The buffalo is considered to be at higher risk of TRP than cattle (Misk et al. 2001). It has been suggested that TRP should be considered in any bovine animal with indigestion (Radostits et al. 2007); however, the variability of the clinical settings represents a significant challenge. The overall clinical symptoms of TRP are anorexia, recurrent tympany, fever, tachypnoea, and lordosis with abducted elbows (Saleh et al. 2008; Abdelaal et al. 2009). However, the observed signs are dependent upon the site of reticular perforation and lesions caused by the foreign body, as visceral and muscular contractions promote migration within the body. Due to the complexity of TRP and possible coexistence with other syndromes, diagnosis is often difficult (Jafarzadeh et al. 2004). In practice, an initial diagnosis of TRP is mainly based upon clinical signs and laboratory findings, while it is confirmed by radiography and USG (Saleh et al. 2008; Abdelaal et al. 2009; Aref and Abdel-Hakiem 2013).

Systemic inflammation is a normal response to altered homeostasis and has an important role in several pathophysiological processes, such as infection or trauma. It is characterised by the endocrine release of different cytokines normally confined to paracrine regulation of a local inflammatory response (Koj 1997). These cytokines stimulate the release of acute phase proteins (APPs) from the
liver (Yoshioka et al. 2002). The initiation of the acute phase response starts when the animal is subjected to different internal or external stimuli (Johnson 1997). APPs have been widely used as a diagnostic tool for several human diseases, and recently in different animal species. In bovine species, the most commonly studied APPs include Hp, SAA, CRP, Fb, ceruloplasmin, alpha 1-antitrypsin and alpha 1-acid glycoprotein (Morimatsu et al. 1989; Jafarzadeh et al. 2004; Orro et al. 2011; El-Deeb and Jacob 2012). Although APPs constitute an element of the non-specific innate immune response which aims to restore homeostasis, their serum levels could reflect the degree of tissue damage in the diseased animal (Murata et al. 2004). Until recently, many clinicolaboratory advancements have been conducted for the diagnosis of TRP in water buffalo (Saleh et al. 2008; Abdelaal et al. 2009; Abouelnasr et al. 2012; Aref and Abdel-Hakiem 2013; Abouelnasr et al. 2013; El-Ashker et al. 2009; Abouelnasr et al. 2012; Aref and Abdel-Hakiem 2013). However, the prognostic significance of immunological markers in buffalo with TRP has not been previously addressed. Therefore, this study aimed at evaluating the prognostic significance of selected inflammatory markers to predict clinical outcome in buffalo with TRP.

MATERIAL AND METHODS

Animal population and clinical examination. The study was conducted on thirty-two water buffalo cows (Bubalus bubalis). Twenty-four were within six weeks post partum and eight animals were in their final gestation period; their ages ranged from five to seven years. Ten clinically healthy peri-parturient buffalo cows, at three to five years of age, were randomly selected and served as controls. The clinical examination of all animals was carried out at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Egypt during 2012 and 2013. All examined buffalo were subjected to thorough clinical examination, in which all cardinal signs were recorded. Pain tests were also performed, including abdominal palpation caudal to the xiphoid, pinching of the withers, by placing a rod underneath the abdomen to elicit a grunt, and by using a foreign body detector that was applied over the ventral aspect of the abdomen to detect ferromagnetic foreign bodies. The diseased buffalo exhibited clinical signs of disturbed appetite, recurrent ruminal tympany, decreased defecation and milk yield, and mild pyrexia or euthermia. Acute local TRP was initially diagnosed on the basis of clinical examination, and confirmed by USG, laparo-rumenotomy, and/or necropsy of non-surviving cases. Cases of chronic local TRP or diffuse peritonitis were not diagnosed during this investigation. The selection criteria were applied to the examined buffalo, excluding those with concomitant ailments and thereby removing potential confounding variables (n = 6). Once the diagnosis of acute local TRP had been made, medical and surgical management were subsequently offered to the owners. Conservative medical management included the immobilisation of the diseased animal, oral administration of a magnet, and intramuscular injection of broad spectrum antibacterial agents (penicillin and streptomycin twice daily at a dose of 22 000 IU/kg b.w. and 25 mg/kg b.w., respectively), concurrently with i.v. injection of flunixin meglumine at a dose of 2.2mg/kg b.w. once daily. Rehydrating i.v. fluids were also administered on an individual basis. In cases where an improvement in clinical signs was not observed within three days of the prescribed treatment, a laparo-rumenotomy was performed. Follow-up information was obtained via clinical examination, owner communication, and referring veterinarians with respect to the health status of animals following intervention. According to the clinical outcome, the diseased buffalo were allocated into two groups, survivors (n = 12), and non-survivors (n = 20).

Blood sampling and measurements. Blood was collected from all investigated buffalo by jugular vein puncture. The collected samples were added to heparinised tubes and tubes without anticoagulant to yield plasma and serum, respectively. Blood plasma and serum samples were separated by centrifugation at 3000 g for 10 min and kept frozen at –20 °C until required. Plasma samples were used to determine SAA, Hp, Fb and CRP by using commercially available bovine kits, according to Orro et al. (2011) (Kamiya Biomedical Company, USA) and an automated microplate reader (Bio TEC, ELX800G, USA) according to the manufacturer’s instructions. Bovine cytokine kits were used for estimation of serum TNF-α, IL-6 (Kamiya Biomedical Company, USA), IL-1β (Thermo Scientific Pierce Protein Biology Products-Rockford, Illinois), IL-10 (Genorise Scientific Inc., Philadelphia, USA), and IF-γ (BioSource Europe S.A.Rue de l’Industrie, Belgium). The cross-reactivity between the aforementioned buffalo and bovine cytokines has previously been documented by Mingala et al. (2007).
and Suzuki et al. (2011). Cytokine levels were determined according to the manufacturers’ instructions; the plates were read at 450 nm and a correction wavelength of 550 nm was measured on a computerised automated microplate reader (Bio TEC, ELX800G, USA). Values were expressed in picograms per millilitre (pg/ml). Samples were run in duplicate for all of the examined cytokines and APPs. SSA was measured in the serum samples according to the method described by Shamberger (1984) using Ehrlich’s reagent (made by adding 0.7 g of p-dimethylaminobenzaldehyde (Sigma Aldrich) to 150 ml of concentrated HCl and 100 ml of distilled water). The absorbance of samples was measured at 525 nm using a Jenway 7305 UV-VIS spectrophotometer (Jenway Scientific Equipments, UK). Data were expressed as mmol/l.

**USG examination.** USG was performed on all examined animals in standing position using 3.5 and 5.0 MHz transducers (Chison Medical imaging Co. Ltd., Waxi, China 214142) according to Braun (2009) and Abouelnasr et al. (2013). The reticulum was examined by placing the transducer on both sides of the sternum and thorax up to the level of the elbow. The lung area was examined with the transducer held parallel to the 3rd to 11th intercostal space. For echocardiography, the 3rd, 4th and 5th intercostals were examined on both sides of the thorax after moving the forelimbs cranially to help improve the contact between the probe and the intercostal space. Within the cardiac region, the heart, major blood vessels, valves, and the mediastinal region were scanned.

**Laparo-rumenotomy.** A left flank laparo-rumenotomy was performed in three cases in standing position under linear infiltrating anaesthesia using Lidocaine 2% according to Ducharme and Fubini (2004).

**Gross post mortem examination (PM).** Upon the approval of the Faculty Ethical Committee, four buffalo were subjected to complete PM examination and the findings were recorded.

**Statistical analysis.** Data were statistically analysed using the SPSS statistical software program (SPSS, version 15, USA). Significant differences \( P < 0.05 \) among groups were compared using one-way ANOVA and the Duncan test. Means and standard deviation for each variable were calculated.

### RESULTS

An overview of the clinical and laboratory findings in the diseased buffalo as well as controls is provided in Table 1 and in Figures 1–3. Clinically, the heart rates, but neither respiratory rate nor rectal

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control ((n = 10))</th>
<th>Survivors ((n = 12))</th>
<th>Non survivors ((n = 20))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>63.0 ± 8.07\textsuperscript{a}</td>
<td>87.66 ± 6.18\textsuperscript{b}</td>
<td>88.80 ± 6.79\textsuperscript{b}</td>
</tr>
<tr>
<td>Respiratory rate (breath/min)</td>
<td>12.66 ± 1.96\textsuperscript{a}</td>
<td>13.00 ± 2.28\textsuperscript{a}</td>
<td>14.33 ± 1.63\textsuperscript{a}</td>
</tr>
<tr>
<td>Rectal temperature (^{\circ})C</td>
<td>37.85 ± 0.17\textsuperscript{a}</td>
<td>38.80 ± 1.13\textsuperscript{a}</td>
<td>38.90 ± 0.67\textsuperscript{a}</td>
</tr>
<tr>
<td>Weakness and poor performance</td>
<td>absent ((n = 10))</td>
<td>absent ((n = 7))</td>
<td>present ((n = 9))</td>
</tr>
<tr>
<td></td>
<td>present ((n = 5))</td>
<td>present ((n = 5))</td>
<td>present ((n = 11))</td>
</tr>
<tr>
<td>Abdominal distention</td>
<td>absent ((n = 10))</td>
<td>absent ((n = 11))</td>
<td>absent ((n = 18))</td>
</tr>
<tr>
<td></td>
<td>present ((n = 1))</td>
<td>present ((n = 1))</td>
<td>present ((n = 2))</td>
</tr>
<tr>
<td>Appetite</td>
<td>normal ((n = 10))</td>
<td>inappetence ((n = 4))</td>
<td>inappetence ((n = 4))</td>
</tr>
<tr>
<td></td>
<td>anorexia ((n = 8))</td>
<td>anorexia ((n = 8))</td>
<td>anorexia ((n = 16))</td>
</tr>
<tr>
<td>Ruminal motility</td>
<td>normal ((n = 10))</td>
<td>hypomotile ((n = 4)), hypomotile ((n = 5)),</td>
<td>ruminal stasis ((n = 15))</td>
</tr>
<tr>
<td></td>
<td>ruminal stasis ((n = 8))</td>
<td>ruminal stasis ((n = 8))</td>
<td></td>
</tr>
<tr>
<td>Recurrent tympany</td>
<td>absent ((n = 10))</td>
<td>absent ((n = 2))</td>
<td>absent ((n = 1))</td>
</tr>
<tr>
<td></td>
<td>present ((n = 10))</td>
<td>present ((n = 10))</td>
<td>present ((n = 19))</td>
</tr>
<tr>
<td>Lacrimation</td>
<td>absent ((n = 10))</td>
<td>absent ((n = 4))</td>
<td>absent ((n = 6))</td>
</tr>
<tr>
<td></td>
<td>present ((n = 8))</td>
<td>present ((n = 8))</td>
<td>present ((n = 14))</td>
</tr>
<tr>
<td>Lordosis/ bruxism</td>
<td>absent ((n = 10))</td>
<td>absent ((n = 4))</td>
<td>absent ((n = 8))</td>
</tr>
<tr>
<td></td>
<td>present ((n = 8))</td>
<td>present ((n = 8))</td>
<td>present ((n = 12))</td>
</tr>
<tr>
<td>Changes in milk yield</td>
<td>absent ((n = 10))</td>
<td>sharp declined ((n = 12))</td>
<td>sharp declined ((n = 12))</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b}variables with different superscripts in the same row are significantly different at \( P < 0.05 \); *the remaining eight patients were dry cows in late gestation*
temperature, were significantly higher in non-survivors compared to survivors (Table 1). In addition, the non-surviving buffalo were more likely to develop anorexia and weakness compared with survivors. However, rumen stasis, recurrent ruminal tympany, lacrimation, lordosis, bruxism and decreased milk production were commonly observed in both survivors and non-survivors. Biochemically, TNF-α, IL-1β, IL-6, IL-10, SAA, Hp, Fb, CRP, and SSA levels were significantly higher in non-survivors compared with survivors as well as controls; on the other hand, serum IF-γ showed no difference between diseased buffalo and controls (Figure 2 and 3).

**USG findings**

In clinically healthy buffalo, the reticulum appeared as a semi-circular shape with a smooth contour. However, in buffalo with TRP, the USG examinations showed fibrin deposits interspersed with fluid pockets between the reticulum, the dorsal ruminal sac and the diaphragm (Figure 1). Reticular abscesses were also observed as echogenic capsules with a hypoechogenic centre. None of the foreign bodies could be visualised. The amplitude and frequency of reticular contraction were decreased. With extensive adhesions, the frequency of contractions was decreased to one or zero contractions every two minutes.

**Exploratory laparo-rumenotomy findings**

Variable numbers and sizes of metallic foreign bodies (nails and wires) were removed from the ventral sac of the rumen and reticulum. Rumen contents were not fully macerated (impacted rumen). The animals were postoperatively treated with parenteral antibiotics, anti-histaminics, and anti-inflammatory drugs for five days. Complete recovery was achieved by 12 days postoperatively.

**Necropsy findings**

PM examination of slaughtered buffalo revealed the existence of foreign bodies (including pieces of wire and nails) that had perforated the reticular wall, were embedded in the diaphragm and formed peri-reticular abscess.

**DISCUSSION**

While significant progress has been made over the past years in the application of APPs as biomarkers in several animal diseases, the prognostic significance of inflammatory markers in buffalo...
with TRP has not yet been fully addressed. Our hypothesis was that inflammatory markers can help predict clinical outcome in buffalo with TRP. TRP in buffalo has become increasingly common and is particularly challenging to manage as its clinical presentation is often vague. In this study, a tentative diagnosis of TRP in diseased buffalo was initially based on clinical signs and laboratory findings, and later confirmed by USG evaluation, exploratory laparo-rumenotomy and/or necropsy in non-surviving cases. The classical clinical findings of TRP in diseased buffalo were consistent with previous reports (Saleh et al. 2008; Abdelaal et al. 2009; El-Ashker et al. 2013). Relative to cattle, indicators of abdominal pain including stiff gait, lordosis, and painful urination/defecation (Radostits et al. 2007) were less common in diseased buffalo, whereas lacrimation was very prominent. Thus, early diagnosis of TRP is more difficult in buffalo compared to cattle. These findings are consistent with previous reports (Saleh et al. 2008; Abdelaal et al. 2009). Although clinical examination and USG were essential for diagnosis of TRP, they contribute little to discriminating non-survivors from survivors. Nonetheless, USG provides clear information with respect to the location and extent of the insult(s). Our findings in this regard were in part consistent with other reports (Abdelaal et al. 2009; Braun 2009; Abouelnasr et al. 2012).

Measurements of APPs in buffalo and cattle have been performed using standard commercial kits (El-Deeb and Iacob, 2012), and (Orro et al. 2011), which are similar to our methodology. On the other hand, the cross reactivity of some inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) between buffalo and cattle has been previously described by Mingala et al. (2007). These authors reported the cloning, sequencing and phylogenetic analy-
which the anti-inflammatory cytokines, including the IL-1α, IL-1β, IL-6 and TNF-α genes from swamp buffalo and two bubaline breeds, and the Bulgarian Murrah buffalo. The multiple sequence comparison showed a high homology between the bubaline breeds, which ranged from 99.3% to 100.0% similarity, whereas the comparison with cattle ranged from 98.6% to 99.0%. In addition, Suzuki et al. (2011), documented a cross reactivity between IL-2, IFN, IL-4 and IL-10 between African buffalo and cattle, and reported that the nucleotide sequence homology of IL-2, IFN-c and IL-4 was more than 98%, which resulted in identical polypeptides. Also, the IL-10 gene of African buffalo and cattle exhibited 95% homology in nucleotide sequence, corresponding to thirteen amino acid residue substitutions. In this study, buffalo with TRP had higher values of all measured acute phase cytokines as well as inflammatory markers than controls, with higher levels in non-survivors compared with survivors. This intensive inflammatory reaction is followed by a compensatory anti-inflammatory response, during which the anti-inflammatory cytokines, including IL-10 are liberated into the bloodstream. The inhibitory effect of IL-10 on IL-1β, IL-6, TNF-α, and IF-γ supports a role for this cytokine in not only the regulation of T-cell responses, but also in acute inflammatory responses (Fiorentino et al. 1991). It is now recognised that pro-inflammatory cytokines are required for the initiation of an effective inflammatory response to infection and/or tissue injury. These pro-inflammatory cytokines are able to induce each other in a signalling cascade, thereby resulting in synergistic potentiating of pathological effects. Our findings were in part similar to those reported by Horadagoda et al. (2002) in buffalo following intravenous inoculation of Pasteurella multocida serotype B:2 endotoxin. In such report, serum concentrations of TNF-α rose within one hour post inoculation (p.i) to reach peak values at 1–2 h p.i. and then declined rapidly to baseline levels 3–5 h p.i.; however, hp showed a delayed but prolonged increase in its values from 12 h p.i. and reached a plateau at 60 h p.i. It was reported that high concentrations of pro-inflammatory cytokines correlate with the prognosis of sepsis and the development of multiple organ dysfunction syndromes (Simmons et al. 2004). Likewise in animal models, the infusion of high concentrations of pro-inflammatory cytokines can lead directly to the development of multi-organ system failure (Kusawa et al. 1988). Several, but not all, studies have suggested that levels of circulating pro-inflammatory cytokines can be used as a measure of the severity of illness and/or as a prognostic marker for patients with sepsis and multiple organ system failure (Makimura and Suzuki 1982; Rauchhaus et al. 2000; Orro et al. 2011; El-Ashker et al. 2013; El-Sebaei et al. 2013). It was also proposed that the quantification of blood APP levels can serve as both a diagnostic and prognostic tool when appropriate sampling schedules are performed (Murata, et al. 2004; Cray et al. 2009; Orro et al. 2011). Similarly, Jafarzadeh et al. (2004) demonstrated that serial interpretation of total plasma protein and plasma Fb with suggested cut-off points is useful for differentiating TRP from other gastrointestinal problems in cattle. Several studies have previously demonstrated the significance of Fb, Hp, and CRP as useful biochemical parameters for evaluating the incidence and severity of inflammatory reactions in bovine species with inflammatory conditions (McSherry et al. 1970; Makimura and Suzuki 1982; Makimura and Usui 1990; Lee et al. 2003; Jafarzadeh et al. 2004, Orro et al. 2011). CRP was also found to be both a useful marker for evaluating the health status of a herd, and a parameter to assess individual stress levels. CRP may be useful in early surveillance of intra-herd TRP (Lee et al. 2003), and may also fulfill an important function in defense against infection and control of the inflammatory response (Mold et al. 2002). It is suggested that CRP can be used as a sensitive biomarker of TRP in water buffalo.

The results of our study suggest that the dysregulation of pro-inflammatory and anti-inflammatory cytokine networks may occur in parallel in buffalo with TRP, and imply that the overall degree of cytokine network disruption may be an important prognostic indicator. Therapeutic strategies to modulate inflammation must take into account the complex cytokine biology in buffalo patients with TRP.

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