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Research paper

Acute phase proteins, interleukin-6, tumor necrosis factor, nitric oxide and oxidative stress markers in horses with cutaneous habronemosis under field condition

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ABSTRACT

Habronemosis is a common parasitic disease of horses worldwide. In order to investigate how haptoglobin (Hp), serum amyloid A (SAA), oxidative stress markers, nitric oxide (NO), interleukin-6 (IL-6) and tumor necrosis factor (TNF- α), varies in cutaneous habronemosis, 30 horses with the clinical disease and 20 clinically healthy horses were included in the current study. The serum levels of Hp, SAA, and proinflammatory cytokines (IL-6 and TNF-α), NO, malondialdehyde (MDA), super oxide dismutase (SOD), glutathione (GSH), and total antioxidant capacity (TAC) were determined in horses before and after two weeks of treatment. The serum levels of Hp, SAA, IL-6, TNF-α and MDA were significantly elevated in infected horses as compared to the controls. Alternately, the serum levels of SOD, GSH, TAC and NO, were recorded low in infected horses as compared to the controls. All tested markers resumed the same levels after treatment as in control group. The Hp, SAA, IL-6, TNF-α, and MDA exhibited a high degree of clinical accuracy of the cases diagnosis. The area under the curve (AUC) for acute phase proteins (SAA, Hp), IL-6, TNF-α, and MDA was 0.87, 0.94, 0.96, 0.96 and 1.0, respectively. These findings showed that Hp, SAA, IL-6, TNF-α, and MDA may be supportive in the diagnosis of cutaneous habronemosis in horses and, simultaneously, they can also be used to monitor the progress of the treatment.

1. Introduction

Habronemosis is a parasitic disease mainly reported in equine species worldwide. Moreover, it has also been reported in canines (Sandersoann and Niyo, 1990). The invasion of stomach worms, *viz.*, *Habronema megastoma* (*Draschia megastoma*), *H. muscae* and *H. majus* is the major cause of the disease. The equine stomach is the major site for the adult parasites (therefore called stomach worm). *H. megastoma* can produce large granulomatous masses that contain adult worms and necrotic materials in the horse stomach. The maggots of intermediate hosts (stable fly, face fly and housefly) ingest the larvae of these pathogenic nematodes present in the horse faeces. Thereafter, the larvae undergo molting within the intermediate host and yield the L3 larvae. The infective larvae spread to horses during feeding of flies on eyes, mouth, vulva, nostrils, teat discharge or any other body secretion (Littlewood, 1999; Pugh et al., 2014).

Habronemosis is highly prevalent in subtropical and tropical regions. The most common sites of cutaneous habronemosis in horses are limbs, prepuce, external genitalia and ventral abdomen. Clinically, it might be introduced as single or multiple injuries, represented by ulceration, exudation, discharge, rich granulation tissue, and pruritus (Pugh et al., 2014).

In animals, acute phase proteins (APPs) are very important in the diagnosis and prognosis of several bacterial, parasitic and viral diseases. Moreover, APPs are used to assess the innate immune system response to inflammation and infection. (Eckersall and Bell, 2010)

Serum amyloid A (SAA) and haptoglobin (Hp) are considered as the most important APPs in horses for assessing immune response against infectious diseases. Interleukin-6 (IL-6) (pro-inflammatory cytokine) is a key inflammatory mediator and regulator of most APPs (Eckersall,

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2010, El-Deeb and El-Bahr, 2014; El-Deeb et al., 2017). However, there are only few reports regarding APPs' response to parasitic infestations in horses (Nielsen et al., 2013; Andersen et al., 2014) those with *Habronema* infection have not been reported yet.

Recently, the role of the free radicals (reactive oxygen and nitrogen species) in the pathogenesis of parasitic infestation has been studied by several authors (Lykkesfeldt and Svendsen, 2007; Celi, 2011; El-Deeb and El-Moslemany, 2015). Moreover, cellular mechanisms involved in the killing of microorganisms have been the major focus of many studies. Previous studies demonstrated that a variety of inflammatory cells are activated and trigger several oxidant-generating enzymes to kill the parasites (extracellular or intracellular) (Murray et al., 1992; Gantt et al., 1992).

There is a need for the assessment of APPs, proinflammatory cytokines, nitric oxide and oxidative stress biomarkers for cutaneous habronemosis in horses. Therefore, the present study is aimed to determine the serum values of SAA, Hp, certain proinflammatory cytokines (IL-6 and TNF-α), nitric oxide (NO) and oxidative stress markers for monitoring progress of treatment of cutaneous habronemosis in horses.

2. Materials and methods

2.1. Animals

Fifty horses from two different farms in the eastern region of Saudi Arabia (from January 2015 to November 2016) were involved in this study. Based on the clinical and histopathological examination, the horses were divided into two groups. The first group of clinically healthy horses (n = 20) was used as control group. The second group (n = 30) included diseased horses with a clinical and histopathological confirmation of cutaneous habronemosis.

2.1.1. Treatment of infested horses

The diseased horses were treated with injectable ivermectin (Ivomec®, Merial LTD, Duluth, GA 30096, USA) administered in the neck muscles, at the dose level of 0.2 mg/kg body weight (Di Pietro et al., 1982), topical corticosteroids, and vitamin AD3E.

2.2. Sampling methods

2.2.1. Blood samples

Blood samples were collected from both groups (*via* jugular vein puncture) into plain vacutainers. Samples were collected twice in the diseased group; the first was before treatment and the second was 14 days after treatment. The serum samples from all horses were collected and stored at −20 °C until analysis.

2.2.2. Skin biopsy for histopathological examination

Skin biopsies obtained from the affected horses were immediately fixed in 10% neutral buffered formalin. The histopathological examination was carried out according the method described by Suvarna et al. (2013).

2.2.3. Biochemical analysis of serum biomarkers

The estimation of serum Hp levels and analysis of SAA in both groups was carried out using ELISA kits (Phase SAA kit, Tridelta Ltd., Ireland) according to the manufacturer's instructions.

The analysis of serum levels of malondialdehyde (MDA), total antioxidant capacity (TAC), reduced glutathione (GSH), superoxide dismutase (SOD) and NO, were performed by using ELISA Kits (Cayman, USA). Moreover, the serum TNF-α and IL-6 levels were estimated using equine ELISA kits (Genorise Scientific, USA). The intra- and inter-assay coefficients of variability for serum TNF-α were 6% and 8%, respectively, while those for IL-6 were 6% and 9%, respectively.

2.3. Statistical analysis of examined biomarkers

Variations in serum biomarkers in the horses before and after treatment were compared using Wilcoxon Mann-Whitney test analysis at P value < 0·05 (since the serum biomarkers were not normally distributed in diseased horses). Selection of cutoff points that optimize sensitivity (Se) and specificity (Sp) for each of Hp, SAA, IL-6, TNF-α, and MDA were determined using receiver operating characteristics (ROC) analyses. The ROC curves were plotted as Se *versus* 1–Sp (false-positive rate) for all possible cut-off points for Hp, SAA, IL-6, TNF-α, and MDA. The area under the ROC curve (AUC) affirms the overall accuracy of the examined biomarker. The differences in the AUC for the Hp, SAA, IL-6, TNF-α, and MDA were assessed using a non-parametric method that explains the correlation resulting from using the same sample for both the tests (DeLong et al., 1988). The complete analyses of data were carried out using Stata version 13 (Stata Corp, College Station TX, USA).

3. Results

3.1. Clinical examination

Habronemosis was characterized by noticeable skin papules, excessive granulation tissue (proud flesh), pruritus, and non-healed wounds. The lesions (with small yellow calcified granules) were solitary in all infected horses. Ulcerations, exudation, intermittent bleeding, and itching were the major clinical symptoms in the diseased horses. The face (below the eye), prepuce, neck, ventral abdomen, and limbs were the most predilection sites for cutaneous habronemosis lesions (Fig. 1A–F).

3.2. Histopathological examination

The dermis was diffusely expanded with a large number of eosinophils as well as capillaries lined with hypertrophied endothelium surround by loose connective tissue containing hypertrophied fibroblasts and granulation tissue (Fig. 2A). Sporadically, few eosinophilic granulomas were also seen within the dermis with eosinophilic cellular and karyorrhectic debris, and degenerated eosinophils in the center that were bound with viable eosinophils, few lymphocytes, histiocytes, plasma cells, and neutrophils (Fig. 2B). Multiple cavities were detected within the sections of dermis containing parasitic larvae. These larvae had a cuticle, polymyarian/coelomyarian musculature (Fig. 2C). The overlying epidermis exhibited focal areas of erosion, ulceration, hyperkeratosis, and acanthosis in adjacent areas (Fig. 2D).

3.3. Biochemical examination

The data presented in Table 1 showed a marked rise in the serum levels of Hp, SAA, IL-6, and TNF-α in infested horses as compared to the control group. Moreover, the serum levels of MDA (lipid peroxidation) were significantly higher in the infected horses than the healthy ones. The serum levels of antioxidant biomarkers (SOD, GSH, and TAC) and NO were much lower in the infested horses than the controls.

The data presented in Table 2 revealed a marked decrease in the serum levels of the acute phase proteins (Hp, SAA) and proinflammatory mediators (IL-6, TNF-α) in the diseased horses after treatment. The serum levels of NO were greatly elevated in the diseased horses and resumed to the same levels after treatment as in the control group (data not shown).

The post-treatment MDA serum levels in the infested horses reduced considerably and resumed to the same levels after treatment as in the control group. Furthermore, the serum levels of antioxidant markers such as SOD, GSH, and TAC were greatly elevated in the diseased horses toward the levels of the control group after two weeks of treatment (data not shown).