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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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) vary 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.

Keywords: Habronema, Haptoglobin, Malondialdehyde, Antioxidant, Cytokines

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.
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 infection has been studied by several authors (Lyykesfeldt 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 surrounded 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).
The comparative account of threshold and test characteristics of Hp, SAA, IL-6, TNF-α, and MDA for supporting the diagnosis of cutaneous habronemosis are shown in Table 3. The area under the ROC curve for Hp, SAA, IL-6, TNF-α, and MDA was 0.87, 0.94, 0.96, 0.96 and 1.0, respectively.

4. Discussion

Habronemosis is a parasitic disease that occurs mostly in equine species worldwide. To the best of our knowledge, this is the first report toward exploring the potential of APPs, proinflammatory cytokines, NO and oxidative stress biomarkers to help the diagnosis of the cutaneous habronemosis in horses and to monitor the response to treatment. In the present study, the serum levels of Hp, SAA, IL-6, and TNF-α were considerably increased in the infested horses than the controls. In healthy animals and those with subclinical infection, the APPs may be undetectable or may be detected in very low quantities (Meling et al., 2012).

In cutaneous habronemosis, the lesions may induce the release of proinflammatory mediators (IL-6 and TNF-α) and subsequently the synthesis of APPs in the liver of infested animals (Radostitis et al., 2007). The key role of APPs is to prevent tissue damage by eliminating harmful molecules and pathogens in the infested horses.

The noticeably elevated serum levels of SAA (the main APP produced by the liver) in the infested horses could be ascribed to stimulating immune cells to the sites of inflammation caused by Habronema (Husby et al., 1994). On the other hand, the prominent increase in the serum Hp (secreted by liver, skin, and other tissues) in the infested horses could be attributed to its role in appropriating the iron within hemoglobin and preventing iron utilization by the pathogen (Petersen et al., 2004). The elevated levels of inflammatory mediators (IL-6 and TNF-α) in infested horses could be due to extensive inflammation of the lesions in the infected horses (Van Miert, 1995; Moreau and Chauvin, 2010). Immune response against parasitic invasion is characterized by skewed Th2 like response (Moreau and Chauvin, 2010). The inflammatory cytokine IL-6 secreted from innate immune cells contributes to the development of protective Th2 cells (Finkelman et al., 1991). It is also considered as a major inducer of APP response (Dilda et al., 2012). In the current study, IL-6 was increased considerably (four-fold) in the infested horses when compared with controls. A similar observation has been recently reported in Alpine ibex with sarcoptic mange (Ráez-Bravo et al., 2015).

Infected horses showed a significant decrease in the levels of NO when compared with control group. Low serum NO levels in infected horses is an indication a typical Th2 response caused by the nematode infection. NO levels increase toward the control levels with treatment indicating that the nematode infection suppresses the microbicidal action of classically activated macrophages. Similar findings were previously reported by Rodríguez-Sosa et al. (2002) in mice infected with Taenia crassiceps.

Interestingly, cutaneous habronemosis induced a state of oxidative stress in the infested horses as illustrated in our study by the high serum levels of MDA (oxidative stress biomarker) and the low serum levels of SOD, GSH, and TAC, (antioxidant markers) in all infected horses. Infested horses respond to this stress by excessive consumption of antioxidants such as SOD and GSH. Therefore, antioxidant supplementation may be a helpful approach in the treatment of such cases. The higher levels of MDA (lipid peroxidation biomarker) and reduced levels of SOD and TAC, (antioxidant biomarker) in a parasitic invasion have also been reported by many authors (Lykkesfeldt and Svendsen, 2007; El-Deeb and El-Moslemany, 2015). A parasitic influx can induce the generation of reactive oxygen and nitrogen species and thereby prompting a state of oxidative stress (Lykkesfeldt and Svendsen, 2007). A parasite-induced inflammatory response results in an inflow of large numbers of circulating eosinophils, which tend to accumulate in the wounded tissues, possibly through the production of ROS (Celi, 2011).

Fig. 1. Horses with different clinical symptoms of cutaneous habronemosis; A, noticeable Habronema lesion and excessive granulation tissue in the submandibular area. B, Habronema lesion and excessive granulation tissue on the neck of 12-years-old horse. C, Habronema lesion on the face (below the eye) in 10-years-old horse. D, Habronema lesion on the prepuce of 11-year-old, Horse. E, Habronema lesion on the prepuce of 9-year-old Horse. F, Habronema lesion on the face (beside the eye) in 11-years-old horse.
The area under the ROC curve for the tested Hp, SAA, IL-6, TNF-α, and MDA showed a high degree of accuracy that support the clinical diagnosis of cutaneous habronemosis in horses. The application of APPs and oxidative stress biomarkers in the prediction of recovery in animals has been documented by many authors (Costantini et al., 2012; Sorci and Faivre, 2009; Dimitrijević et al., 2012; El-Deeb and Iacob, 2012; El-Deeb and El-Moslemany, 2015; El-Bahr and El-Deeb, 2016). Interestingly, it has also been reported that the changes in the status of oxidative stress are correlated with the intensity of parasitic invasion (Dimitrijević et al., 2012). The current findings endorsed by previous reports (Costantini et al., 2012; Sorci and Faivre, 2009; Dimitrijević et al., 2012, El-Deeb and El-Moslemany, 2015) recommend that Hp, SAA, IL-6, TNF-α, and MDA may be used as potential biomarkers that support the diagnosis of the severity of cutaneous habronemosis and simultaneously they can also be used to monitor the progress of treatment.

5. Conclusion

Cutaneous habronemosis in horses induce immune response manifested by higher levels of Hp, SAA, IL-6, TNF-α and lower levels of NO. Moreover, it induced oxidative stress as demonstrated by increased

Table 1
The levels of oxidative stress markers, proinflammatory cytokines and acute phase proteins, in control and horses with cutaneous habronemosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control horses (N = 20)</th>
<th>Infected horses (N = 30)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>25%</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>1.02</td>
<td>1.03</td>
<td>0.96</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>2.95</td>
<td>2.82</td>
<td>2.66</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>112.4</td>
<td>112.4</td>
<td>111.2</td>
</tr>
<tr>
<td>TAC (µmol/L)</td>
<td>0.69</td>
<td>0.65</td>
<td>0.58</td>
</tr>
<tr>
<td>NO (µmol/L)</td>
<td>4.03</td>
<td>3.96</td>
<td>3.72</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>95.14</td>
<td>90.66</td>
<td>85.33</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.43</td>
<td>1.45</td>
<td>1.36</td>
</tr>
<tr>
<td>Hp (g/L)</td>
<td>0.74</td>
<td>0.73</td>
<td>0.66</td>
</tr>
<tr>
<td>SAA (mg/L)</td>
<td>10.75</td>
<td>10.45</td>
<td>9.83</td>
</tr>
</tbody>
</table>

MDA, malondialdehyde; GSH, glutathione; SOD, super oxide dismutase; TAC, total antioxidant capacity; NO, nitric oxide; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; Hp, haptoglobin; SAA, serum amyloid A.

* P-value resulting from non-parametric Wilcoxon Mann–Whitney test.
Table 2

The levels of malondialdehyde (MDA) and acute phase proteins in horses with cutaneous habronemosis before and after treatment with ivermectin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-treatment horses (N = 30)</th>
<th>Post-treatment horses (N = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>8.97</td>
<td>9.1</td>
</tr>
<tr>
<td>Hp (g/L)</td>
<td>1.61</td>
<td>1.3</td>
</tr>
<tr>
<td>SAA (mg/L)</td>
<td>16.05</td>
<td>16.36</td>
</tr>
</tbody>
</table>

MDA, malondialdehyde; Hp, haptoglobin; SAA, serum amyloid A. *P-value resulting from non-parametric Wilcoxon Mann–Whitney test.

Table 3

The threshold and test characteristics of selected acute phase proteins, proinflammatory cytokines, and oxidative stress markers for supporting the diagnosis of cutaneous habronemosis in horses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>%Correctly classified</th>
<th>AUC (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/L)</td>
<td>5.33</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1(1–1)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>262.33</td>
<td>93.3</td>
<td>100</td>
<td>96.08</td>
<td>0.96(0.922–1.015)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.85</td>
<td>96.7</td>
<td>100</td>
<td>98.04</td>
<td>0.96(0.905–1.030)</td>
</tr>
<tr>
<td>Hp (g/L)</td>
<td>0.85</td>
<td>86.67</td>
<td>80.95</td>
<td>84.31</td>
<td>0.87(0.769–0.971)</td>
</tr>
<tr>
<td>SAA (mg/L)</td>
<td>11.66</td>
<td>90</td>
<td>85.71</td>
<td>88.24</td>
<td>0.94(0.889–1.006)</td>
</tr>
</tbody>
</table>

MDA, malondialdehyde; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; Hp, haptoglobin; SAA, serum amyloid A; AUC, Area Under the Curve. *95% CI, 95% confidence interval.

MDA, while reduced antioxidant levels. Therefore, Hp, SAA, IL-6, TNF-α, MDA, SOD, GSH, TAC, and NO may be supportive to facilitate the diagnosis of cutaneous habronemosis in horses. Furthermore, oxidative stress biomarkers, proinflammatory cytokines, and acute phase proteins (SAA and Hp) could also be used to monitor the response of horses to the treatment in a real-time manner. The inclusion of antioxidants in the therapeutic measures could improve the response to the treatment.

Ethics statement

"All the animal procedures were performed according to the guidelines of the Animal Ethics Committee of College of Veterinary Medicine, King Faisal University, Saudi Arabia (committee protocol number: 42/1943)".

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Declaration of interest

The authors declare that they have no competing interests.

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