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Association of the severity of colic in horses with oxidative stress biomarkers, acute-phase proteins, and certain trace elements

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Sixty-one horses were enrolled in this study and divided into 3 different groups according to their severity of colic (heart rate, oral mucous membrane color, and abdominal distention): a strangulating colic (SC) group (n=21), non-strangulating colic (NC) group (n=20), and control group (n=20) consisting of randomly selected normal horses without signs of colic. The serum concentrations of haptoglobin, tumor necrosis factor-α (TNFα), nitric oxide (NO), malondialdehyde (MDA), zinc, iron, and copper were evaluated in all horses. The average concentration of TNFα in the SC group was higher than that in the control group (P<0.001). The TNFα concentration was higher in the NC group compared with the control group (P<0.001). Furthermore, the average concentration of TNFα tended to be higher in the SC group compared with the NC group (P=0.052). The average concentration of haptoglobin in the SC group was higher than that in the control group (P<0.001). The average concentration of NO was higher in the SC group compared with the NC group. (P=0.016) The average concentration of MDA was higher in the SC group compared with the control group (P=0.042). Furthermore, the concentration of MDA was higher in the SC group compared with the NC group (P=0.048). TNFα in horses with signs of colic may be a reliable indicator of prognosis and the severity of clinical signs. The haptoglobin concentration may be a useful marker in cases where animals are referred to clinicians a few days after the onset of colic. The concentrations of MDA and NO should be interpreted with caution.

Key words: *acute-phase proteins, clinical signs, colic, oxidative stress*

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One of the most frequent causes of mortality in horses is colic [31]. The aims of clinical examinations in horses with colic symptoms are to determine whether the digestive system is the organ responsible for the discomfort and to specify the type of lesion if this is the case, offer a prognosis and appropriate treatment, and predict how the condition will progress. There are multiple causative agents responsible for the occurrence of this particular clinical

syndrome, including diseases based on the digestive system that classify the cause of colic as obstruction, obstruction with strangulation, infarction, or inflammation [5]. Simple obstruction is a partial blockage of the ingesta pathway in the digestive system by food, enteroliths, parasites, foreign body, or sand, resulting in disruption of ingesta movement downwards. This obstruction makes the horse show mild to moderate clinical signs of colic and often needs medical intervention. Obstruction with strangulation occurs due to intestinal accidents such as torsion and intussusception, which cause severe intolerable pain, and shock due to intestinal barrier damage and bacterial toxins that pass into the bloodstream, and it requires urgent surgical intervention [11]. An accurate diagnosis of the origin of the colic have yield prognostic value. Furthermore, more precise prognostication is made possible by assessing a horse's physiologic status, such as measurement of heart and respiratory rates, mucous membrane color and capillary refill time, arterial blood pressure, hematocrit, and serum total protein concentration. As a consequence, only around 20% of field cases have the source of colic identified [5]. Most of the moralities due to colic are associated with circulatory collapse due to the absorption of bacterial lipopolysaccharide after a series of events such as ischemic gut injury and rapid degeneration of the intestinal barrier [13]. Intestinal ischemia as a consequence of colic may lead to the accumulation of harmful oxidative nitrogen and oxygen products [19]. Assessment of oxidative stress markers may be a useful therapeutic target and a prognostic indicator in several conditions in horses, such as orthopedic-related conditions, Cushing's syndrome, endometritis, and gastrointestinal diseases [21]. The severity of oxidative stress can be assessed using oxidative stress parameters such as nitric oxide (NO) and malondialdehyde (MDA) [9]. NO is involved in a variety of physiological functions, including vasodilation, immune responses, and neurotransmission. Moreover, mononuclear phagocytes release NO following inflammatory conditions. NO has anti-inflammatory properties, but following the production of reactive compounds such as nitrite peroxide, detrimental repercussions may occur [2]. MDA might be an indicator of intestinal ischemia and rebleeding, which is an oxidative stress factor produced as a byproduct of fat cell peroxidation. Therefore, the presence of MDA might be a sign of lipid peroxidation and free radical damage to the phospholipid membranes of cells [15]. Colic is commonly accompanied by inflammation, reduction of tissue perfusion, and various organ failures [3, 7]. Inflammation as a result of colic may induce the acute-phase response [6, 32]. This response is a nonspecific systemic immune response that releases cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNFα). It also increases the production of positive acute-phase proteins, which are involved in restoring hemostasis; and decreases the concentration of negative acute-phase proteins, like albumin and transferrin. Haptoglobin (Hp) is an acute-phase protein (APP) that has been studied recently in horses with colic [24]. It is a reliable biomarker of chronic inflammatory conditions that binds to free hemoglobin to prevent iron loss and rises 12 to 24 hr after an inflammatory event [6]. It has been shown that the removal of the haptoglobin-hemoglobin/myoglobin complex by the liver is associated with a sudden decrease in plasma haptoglobin concentration [27, 28]. Several physiological functions, including the immune system, depend on trace metals like iron, copper, and zinc, deficiencies of which may impair the efficiency of various organs' functions, particularly when diseases occur [4]. The total iron concentration in the blood of horses with systemic inflammation, including intestinal disorders, has been regarded as an acute-phase reactant with prognostic value [1]. Insufficiency of zinc and copper metalloenzymes, which are involved in the body's antioxidant defense mechanisms, might result from an insufficiency of zinc and copper [29]. We hypothesized that the concentrations of inflammatory and oxidative stress biomarkers and those of trace minerals like iron, copper, and zinc are associated with the severity and classification of colic, including non-strangulating colic (NC) and strangulating colic (SC). Therefore, the current study aimed to assess the association of the severity of colic with inflammation and oxidative stress and the correlation of trace mineral concentration with inflammatory and oxidative stress biomarkers to improve our capabilities in diagnosing the clinical state and providing a reliable prognosis for affected animals.

Materials and Methods

Compliance with ethical standards

The protocols of the study were approved by the *Ethics Committee of Shiraz University* (IACUC no: 4687/63). We additionally noted the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the standards for protecting animals used for experimental purposes. Also, all horses were treated in accordance with the regulations in the guidelines of the Iranian Council of Animal Care (1995), and the experiment was approved by the Iranian Ministry of Agriculture (*experiment permission no.* 1828).

Animals and study procedures

This study included 61 horses that were referred to the large animal clinic of School of Veterinary Medicine, Shiraz University and had a mean body weight of 550 ± 25 kg (mean ± SD) and mean age of 7 ± 3 years (mean ± SD). The horses were dewormed and vaccinated in accordance with the standard operating procedures of the facilities they

belonged to. The study was conducted over a 180-day period in the winter and spring of 2017–2018. Categorization of colic in this study was based on clinicals signs, and the referred horses were divided according to the non-strangulation and strangulation forms of colic, for which medical and surgical treatments were considered, respectively. The 61 horses were clinically examined by Three veterinarians and divided into two groups, simple obstructive colic (NC group) and obstructive colic with congestion and vascular injury (strangulation; SC group), based on their histories and clinical symptoms, such as signs of pain, heart rate, temperature, abdominal distention, mucosal discoloration, capillary refill time, gastrointestinal sounds, presence or absence of gastric reflux, and rectal examination. In the SC group, strangulation lesions were confirmed by celiotomy or necropsy [5, 16]. Unfortunately, all the horses in this group died. Sixteen horses did not undergo surgery, and their strangulation lesions were confirmed during necropsy. Five horses were confirmed to have strangulation lesions by celiotomy, but they unfortunately died during or after surgery. Heart rate was categorized into four groups: group 1, 20–50 beats/min; group 2, 50–60 beats/min; group 3, 60–80 beats/min, and group 4, more than 80 beats/min. Abdominal distension was categorized into two groups: group 1, animals with abdominal distension, and group 2, animals without abdominal distension. Mucus membrane color and capillary refill time were categorized into 3 groups: group 1, pink mucus membrane with a capillary refill time of less than 2 sec; group 2, red mucus membrane with a capillary refill time of 2 to 3 sec; and group 3, purple mucus membrane with a capillary refill time of more than 3 sec (Table 1). Twenty healthy horses randomly selected from among horses referred to the hospital for routine check-ups were used as the control group. Blood samples (10 m*l*) were taken from the jugular vein and divided into two vacutainers with and without an anticoagulant (heparin, Becton-Dickenson, Mississauga, ON, Canada). After clot formation in tubes without the anticoagulant, samples were centrifuged at 3,000 rpm to separate the serum, and the serum was frozen at −20°C and stored until analysis. Commercially available kits were used to measure serum nitric oxide by the sandwich ELISA method (QuantiChrom Nitric Oxide Assay Kit, catalog no. D2NO-100, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions (assay range, 3–200 mmol/*l*; sensitivity, 1.52 mmol/*l*). A multispecies malondiadehyde (MDA) assay kit (cat. no. ZB-MDA96A, ZellBio GmbH, Ulm, Germany) was used to measure MDA based on the reaction with thiobarbituric acid in an acidic condition and at high temperatures. The color of the complex was then measured calorimetrically at 535 nm. Serum haptoglobin was measured by a solid-phase sandwich ELISA method with a Horse Haptoglobin ELISA Assay Kit (catalog no. HHG91-K01, Eagle Biosciences, Nashua, NH, USA ; assay range, 3–200 μg/m*l*; sensitivity, 2.46 μg/m*l*). TNF-α was measured with an equine ELISA kit (equine TNFa BioAssay^{TM} ELISA kit, catalog no. 370270, United States Biological, Salem, MA, USA). Atomic absorption spectrophotometry (Shimadzu AA-670, Shimadzu, Kyoto, Japan) was used to measure iron, zinc, and copper concentrations.

Table 1. Descriptive statistics for age, gender, and colic-related clinical findings in the different observation groups

Clinical findings	Type of colic		
	Control (%)	Non-strangulating (%)	Strangulating (%)
Age			
5 years ≥	45	19	16.7
5–10 years	40	57	66.7
10 years ≤	15	19	16.7
Gender			
Male	45	50	33
Female	55	50	67
Abdominal distension			
No	100	18.8	75
Yes	0	81.2	25
Mucosal membrane color and capillary refill time			
Pink and ≤ 2 sec	100	81.8	0
Red and 2–3 sec	0	18.2	73.7
Purple and 3 sec ≤	0	0	26.3
Heart rate[1]			
20–50	100	12.5	5
50–60	0	18.8	15
60–80	0	68.8	25
80 ≤	0	0	55

[1]Heart beats per minute.

Statistical analysis

All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A priori overall sample size calculation and power analysis were performed using the PROC POWER procedure. The calculations considered a power of 80%, alpha of 0.05, standard deviation of 4 units, confidence of 95%, and mean difference of 3 units in haptoglobin concentration between normal and horses diagnosed with colic based on the data reported by Cray *et al*. [6]. Fifty-eight samples were deemed necessary to prevent type I and type II errors in total, and 18 samples were deemed necessary per group. Data were analyzed by ANOVA using the GLIMMIX procedure in SAS followed by calculation of the differences of least square means for multiple comparisons to compare differences among the independent variable. Data are presented with the respective standard error of the mean using the Tukey-Kramer method. The Pearson student residual plot distribution for

each response variable was evaluated for normality and homoscedasticity. Variables with violations of assumptions of normality (NO and TNFα) were subjected to Box-Cox power transformation using the TRANSREG procedure of SAS. To transfom estimated mesures of NO and TNFα, the logarithms of the these variables were used for the analysis and presented after back transformation. To evaluate the association between the type of colic and inflammatory and oxidative biomarkers, a model containing fixed effects of group (control, NC, and SC), age, gender, the interaction of age and group, and the interaction of group and gender was designed. The significance of the fixed effects (age, gender, interaction of age and group, and interaction of age and group) was checked, the explanatory variable with the highest *P*-value was removed from the equation, and the model was reconstructed with the remaining fixed effects until all the fixed effects had a *P*-value >0.05. To determine the degree of relationship between inflammatory and oxidative response variables (TNFα, Hp, NO, and MDA) and the variables of interest (Fe, Zn, and Cu), correlation analysis was performed with Pearson correlations using JMP Pro 15 (JMP Statistical Discovery, SAS Institue Inc., Cary, NC, USA). *P*=0.05 was considered significant, and trends were declared at 0.05 <*P*≤0.10.

Results

Blood parameters

TNFα: The TNFα concentration was (*P*<0.001) significantly different among the observation groups. No association was observed between the TNFα concentration and age or gender. Furthermore, it was not associated with the interaction of age and group or the interaction of sex and group. The TNFα concentration was higher in the SC group (mean=53.21 ± 3.14 mg/*l*, 95% CI=47.26, 59.91) compared with the control group (mean=16.49 ± 0.97 mg/*l*, 95% CI=14.64, 18.57; *P*<0.001). It was also higher in the NC group (mean=42.86 ± 2.92 mg/*l*, 95% CI=37.37, 49.15) compared with the control group (*P*<0.001). Finally, it tended to be higher in the SC group compared with the NC group (*P*=0.052).

We also constructed a model to check the parameters correlated with the TNFα concentration in each group. The TNFα concentration was not correlated with the Zn concentration in the control group (*P*=0.462) or SC group (*P*=0.615), but it tended to show a negative correlation (r= −0.41, 95% CI= −0.72, 0.03) with the Zn concentration in the NC group (*P*=0.066). The TNFα concentration was positively correlated (r=0.44767, 95% CI=0.006, 0.74) with the Fe concentration in the control group (*P*=0.047), but it was not correlated with the Fe concentration in the NC group (*P*=0.255) or SC group (*P*=0.797). The TNFα concentration was not correlated with the Cu concentration in the control group (*P*=0.432). On the other hand, it was positively correlated (r=0.53, 95% CI=0.12, 0.79) with the Cu concentration in the NC group (*P*=0.015). It also tended to show a positive correlation (r=0.39, 95% CI= −0.04, 0.70) with the Cu concentration in the SC group (*P*=0.079) (Fig. 1A).

Haptoglobin: Haptoglobin was associated with the observation groups (*P*<0.001) and age (*P*=0.044), but there was no association between Hp and the interaction of age and group. Moreover, no association was observed between the Hp concentration and the interaction of gender and group. The Hp concentration was higher in the SC group (mean=82.58 ± 6.40 μg/m*l*, 95% CI=69.72, 95.43) compared with the control group (mean=51.37 ± 6.12 μg/m*l*, 95% CI=39.07, 63.68; *P*<0.001). It tended to be higher in the SC group compared with the NC group (mean=58.79 ± 7.22 μg/m*l*, 95% CI=44.29, 73.29; *P*=0.054). However, there was no difference in Hp concentration between the NC group and control group (*P*=0.708). The Hp concentration was associated with age (*P*=0.044), with a one-year increase in age decreasing the Hp concentration by 3.05 ± 1.48 μg/m*l*. We constructed a model to check the parameters correlated with the Hp concentration, taking into consideration the interaction of the age and observation groups. Hp was not correlated with Fe in the control (*P*=0.630), NC (*P*=0.310), and SC groups (*P*=0.992). It was also not correlated with Zn in the control group (*P*=0.227) but tended to be negatively correlated (r= −0.39, 95% CI= −0.71, 0.05) with Zn in the NC group (*P*=0.082); it was not correlated with Zn in the SC group (*P*=0.519). Finally, Hp was not correlated with Cu in the control group (*P*=0.291) or SC group (*P*=0.272), but it was correlated (r=0.49, 95% CI=0.06, 0.76) with Cu in the NC group (*P*=0.027) (Fig. 1B).

Nitric oxide: The NO concentration was different among the observation groups (*P*=0.050). No association was observed between the NO concentration and age, gender, the interaction of group and age, or the interaction of group and gender. The NO concentration was lower in the NC group (mean=21.78 ± 1.98 μmol/*l*, 95% CI=18.13, 26.16) compared with the SC group (mean=29.45 ± 2.32 μmol/*l*, 95% CI=25.13, 34.51; *P*=0.041). However, no difference was observed between the NC group and control group (mean=25.04 ± 1.97 μmol/*l*, 95% CI=21.37, 29.35; *P*=0.484) or between the SC group and control group (*P*=0.323). The concentration of NO was not correlated with the concentration of Zn in the control group (*P*=0.128), NC group (*P*=0.395), or SC group (*P*=0.571). It was also not correlated with the concentration of Fe in the control group (*P*=0.726), NC group (*P*=0.600), or SC group (*P*=0.718). Finally, the concentration of NO was not correlated with the concentration of Cu in the control group (*P*=0.653) or

SC group (P=0.569), but it was positively correlated with the Cu concentration (r=0.58, 95% CI=0.19, 0.81) in the NC group (P<0.001) (Fig. 1C).

Malondialdehyde: The concentration of MDA was significantly different among the observation groups (P=0.021). No association was observed between the MDA concentration and age, gender, the interaction of group and age, or the interaction of group and gender. The concentration of MDA was higher in the SC group (mean=9.17 ± 0.81 nmol/*l*, 95% CI=7.52, 10.81) compared with the control group (mean=6.29 ± 0.81 nmol/*l*, 95% CI=4.65, 7.93; P=0.042). Furthermore, it was higher in the SC group compared with the NC group (mean=6.14 ± 0.94 nmol/*l*, 95% CI=4.24, 8.03; P=0.048). There was no difference in MDA concentration between the NC group (mean=6.15 ± 1.22) and control group (P=0.991) (Fig. 1D). The concentration of MDA was not correlated with Zn in the control group (P=0.382), NC group (P=0.111), or SC group (P=0.551). MDA was not correlated with Fe in the control group (P=0.538) or SC group (P=0.699), but it was negatively correlated (r= −0.44, 95%CI= −0.74, −0.002) with Fe in the NC group (P=0.049). The concentration of MDA was not correlated with Cu in the control group (P=0.348) or SC group (P=0.335), but it was positively correlated (r=0.49, 95% CI=0.06, 0.76) with Cu in the NC group (P=0.027). We did not observe significant differences in mineral concentration in our groups (Fig. 2).

Association of blood parameters and clinical findings: The average concentration of TNFα was significantly different among the different categories of heart rate (P<0.001). The concentration of TNFα was lower in heart rate group 1 (mean=18.29 ± 1.50 mg/*l*, 95% CI=15.50, 21.59) compared with heart rate group 2 (mean=51.25 ± 9.65 mg/*l*, 95% CI=35.06, 74.91; P<0.001), group 3 (mean=44.69 ± 4.71 mg/*l*, 95% CI=36.14, 55.26; P<0.001), or group 4 (mean=50.82 ± 5.82 mg/*l*, 95% CI=40.35, 64.01; P<0.001). Moreover, the average concentration of TNFα was significantly different among the different categories of mucus membrane color/capillary refill time (P<0.001). The concentration of TNFα was lower in mucus membrane color group 1 (mean=21.89 ± 1.86 mg/l, 95% CI=18.45, 25.96) compared with mucus membrane color group 2 (mean=50.44 ± 5.66 mg/*l*, 95% CI=40.24, 63.22; P<0.001) or group 3 (mean=57.47 ± 11.535 mg/*l*, 95% CI=38.37, 86.08; P<0.001). The average concentration of TNFα was significantly different among the two categories of abdominal distension (P<0.001). The concentration of TNFα was lower in non-abdominal distention group 2 (mean=25.34 ± 2.34 mg/*l*, 95% CI=21.04, 30.51) compared with abdominal distension group 1 (mean=50.43 ± 6.69 mg/*l*, 95% CI=38.62, 65.84). The average concentration of Hp was significantly different among the different categories of heart rate (P<0.001). The concentration of Hp was higher in heart rate group 2 (mean=128.87 ± 9.04 μg/m*l*, 95% CI=110.62, 147.12) compared with heart rate group 1 (mean=54.14 ± 3.93 μg/m*l*, 95% CI=46.21, 62.07; P<0.001), group 3 (mean=54.80 ± 5.17 ± μg/m*l*, 95% CI=44.35, 65.24; P<0.001), or group 4 (mean=75.54 ± 5.65 μg/m*l*, 95% CI=64.12, 86.95; P<0.001). Furthermore, the average concentration of Hp was significantly higher in heart rate group 4 compared with heart rate group 1 (P=0.017) and significantly lower in heart rate group 3 compared with heart rate group 4 (P=0.046). The average concentration of Hp was significantly different among the different categories of mucus membrane color (P<0.001). The concentration of Hp was lower in mucus membrane color group 1 (mean=53.62 ± 5.55 μg/m*l*, 95% CI=42.45, 64.80) compared with mucus membrane color group 2 (mean=81.6 ± 7.39 μg/m*l*, 95% CI=66.73, 96.48; P<0.001).

The average concentration of NO was significantly different among the different categories of heart rate (P<0.001). The concentration of NO was higher in heart rate group 2 (mean=72.17 ± 5.03 μmol/*l*, 95% CI=62.012, 82.32) compared with heart rate group 1 (mean=24.67 ± 2.18 μmol/*l*, 95% CI=20.26, 29.08; P<0.001), group 3 (mean=22.30 ± 2.88 μmol/*l*, 95% CI=16.49, 28.11; P<0.001), or group 4 (mean=27.27 ± 3.14 μmol/*l*, 95% CI=20.92, 33.62; P<0.001).

The average concentration of MDA was significantly different among the different categories of heart rate (P<0.001). The concentration of MDA was higher in heart rate group 2 (mean=17.49 ± 1.17 nmol/*l*, 95% CI=15.13, 19.85) compared with heart rate group 1 (mean=6.18 ± 0.51 nmol/*l*, 95% CI=5.16, 7.21; P<0.001), group 3 (mean=5.92 ± 0.67 nmol/*l*, 95% CI=4.57, 7.27; P<0.001), or group 4 (mean=7.62 ± 0.73 nmol/*l*, 95% CI=6.14, 9.09; P<0.001).

Discussion

Colic is a condition associated with hemodynamic changes due to tissue injuries that affect horses frequently in equine practice, and it is a major cause of death in this species. To establish an appropriate prognosis for colic, acute-phase protein, oxidative stress parameters, and other laboratory findings that are observed along with systemic signs should be better investigated. Therefore, the current study aimed to evaluate parameters that may provide a better diagnosis and prognosis in horses with non-strangulating colic and those with strangulating colic. We observed that the TNFα concentration was associated with an increase in the severity of symptoms, with the TNFα concentration being highest in the SC group compared with the control group. Furthermore, the concentration of TNFα was higher in the NC group compared with the control group (Fig. 1A). Others have reported that the TNFα concentration was

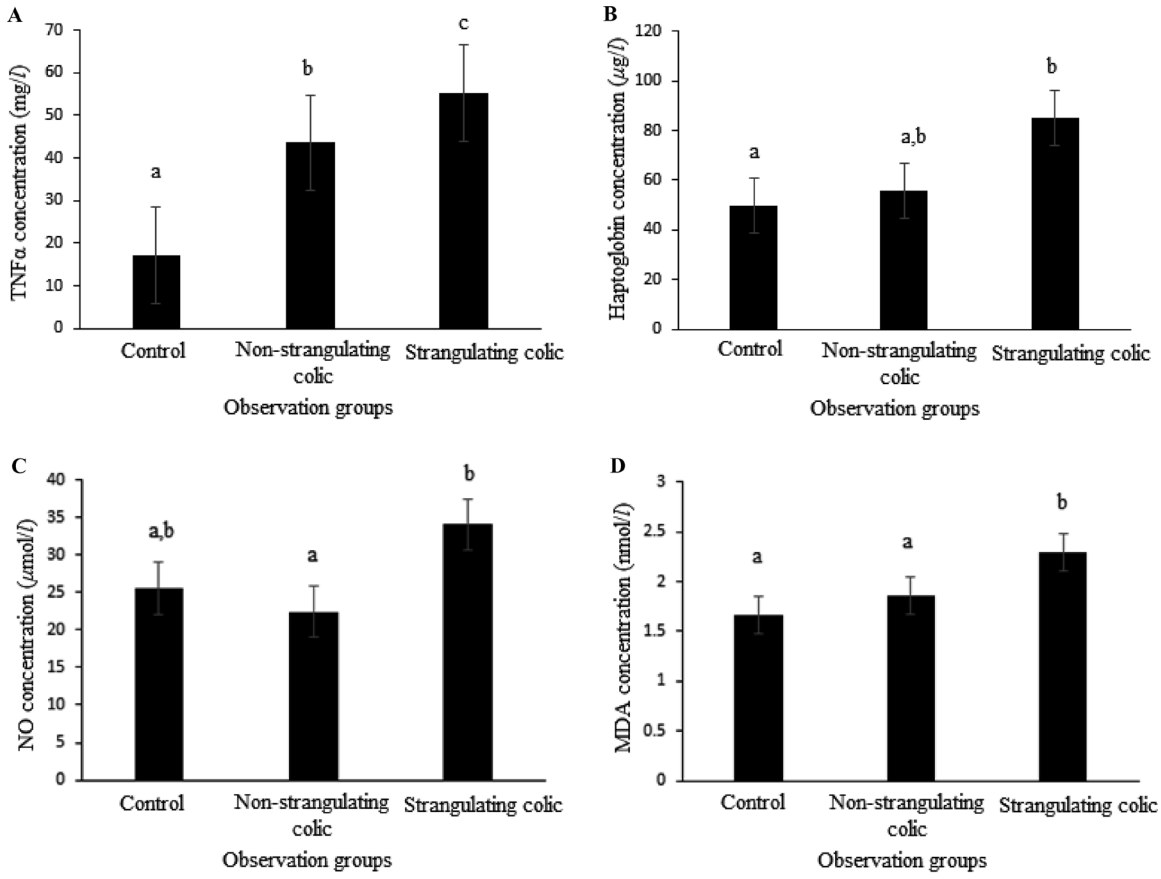

Fig. 1. A: Serum concentrations of tumor necrosis factor-α (TNFα) in the control, non-strangulating colic, and strangulating colic groups. B: Serum concentrations of haptoglobin in the control, non-strangulating colic, and strangulating colic groups. C: Serum concentrations of NO in the control, non-strangulating colic, and strangulating colic groups. D: Serum concentrations of MDA in the control, non-strangulating colic, and strangulating colic groups. [a,b,c]Different letters between groups indicate significant differences ($P \leq 0.05$).

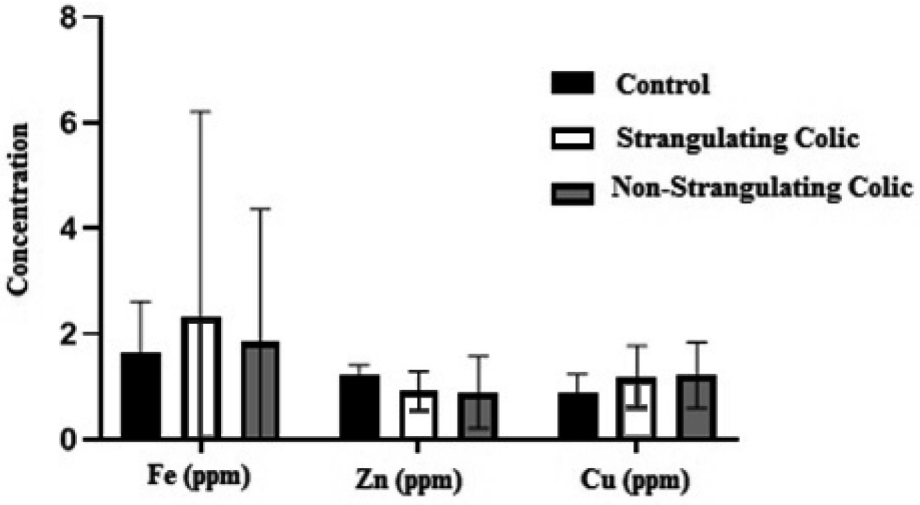

Fig. 2. Serum concentrations of Fe, Zn, and Cu in the control, non-strangulating colic, and strangulating colic groups.

significantly higher in horses with strangulation obstruction colic and horses with acute colitis compared with horses with non-strangulation obstruction and healthy horses [17, 22, 23]. In agreement with the results of this study, it has been shown that there is a direct correlation between disease severity/lethality and the TNFα concentration [18, 22, 23]. Many inflammatory processes, such as infection, stress, trauma, and allergy, may cause an increase in Hp concentration [6, 7, 14]. The Hp concentration was higher in horses in the SC group compared with the control group and tended to be higher in the SC group compared with the NC group (Fig. 1B). On the other hand, several studies have reported Hp as a parameter to monitor and diagnose inflammatory processes in horses even though it has a late response and a relevant variety in healthy horses [24, 25, 28]. Others have found no difference in Hp concentration among horses with clinical surgical colic or healthy horses [28, 30]. Hp has recently been studied in cases of colic, but its role is still unknown [24]. Since all of the horses referred to the large animal hospital had developed colic symptoms at least a day before arrival at the hospital, we decided to evaluate the Hp concentration due to its late response. In agreement with studies that observed a late response of Hp, we suggest that the Hp concentration may be a good indicator of inflammation in cases that are referred for clinical examination more than a day after the onset of clinical symptoms of colic. However, our evaluations of acute-phase proteins in this study should be considered with caution, as our cases were at different stages of the disease and we were unable to determine the amount of time between the onset of colic and hospitalization. In our study, significant correlation was observed between the severity of the critical clinical symptoms, including heart rate, mucous membrane color, and abdominal distention, and elevated levels of TNFα. Furthermore, significant correlation was observed between increased levels of Hp and mucous membrane color severity as well as a moderately elevated heart rate. These associations of TNFα and Hp with clinical findings may explain the use of these acute-phase proteins as a predictor for the severity of colic and vice versa. When practitioners do not have access to laboratory facilities, it may be assumed that some acute-phase proteins, such as TNFα, may be increased based on the severity of clinical signs, such as heart rate, mucous membrane color, and abdominal distention. Furthermore, the above results are consistent with earlier studies [3, 16, 28, 30] that highlighted the significance of clinical findings for the diagnosis and prognosis of colic in horses and focused on the relevance of clinical findings in colic categories. According to the findings of Marañón *et al*. [19], the ischemic intestine releases proinflammatory molecules into the portal and systemic circulation, including hydrogen peroxide, superoxide radicals, cytokines, and arachidonic acid metabolites, causing direct tissue damage and increasing oxidative stress biomarkers. Zuluaga *et al.* demonstrated that 96 hr after inducing gastric mucosal injury in horses, the average plasma concentration of MDA increases [33]. Furthermore, Ibrahim found an increase in superoxide dismutase and MDA activity in horses with flatulent and impaction colic [15]. In this study, the MDA concentration was higher in the SC group compared with the NC and control groups. Furthermore, there was no significant difference in MDA concentration between the NC and control groups, suggesting that the MDA concentration may be an indicator of tissue injury and its severity, as it was elevated in horses that had strangulating obstruction colic (Fig. 1D). However, this did not support the severity of the clinical findings we observed for heart rate, as MDA and NO were markedly elevated in heart rate group 2 but not in heart rate group 4. Future investigations with a larger sample size could clarify the associations of MDA and NO with the severity of clinical symptoms in colic horses. Bacterial infection may cause lipid peroxidation and, as a result, the release of oxygen-free radicals like superoxide radicals that inhibit NO activity. If these radicals are not removed by endogenous antioxidants, such as superoxide dismutase and glutathione, the rate of oxidation will exceed the rate of antioxidation, resulting in oxidative stress [9]. El-Deeb *et al.* claimed that horses with cutaneous habronemosis had lower levels of NO than healthy horses [10]. Moreover, El-Bahr and El-Deeb demonstrated that NO levels decrease in Arabian mares with pyometra [9]. We observed that the NO concentration tended to be lower in the NC group compared with the SC group. We speculate that the rate of oxidation exceeded the rate of antioxidation, causing a reduction in the NO concentration in the NC group (Fig. 1C). Mirza *et al.* reported that NO concentrations increase in horses after small intestinal strangulation obstruction due to an increase in mucosal and submucosal NO synthases in response to ischemia-related stimuli [20]. We speculate that the higher concentration of NO in the SC group compared with the NC group may have been an indicator of the severity of ischemia-related gastrointestinal injuries. We also speculate that the lack of a significant difference in NO among our observation groups was due to the small sample size. Future studies using a larger sample size might be able to highlight the relationship between MDA and the severity of clinical symptoms in colic horses. The mineral requirements of animals rise in response to stress. As a result, a decrease in minerals like copper and zinc may result in insufficiency of copper and/or zinc metalloenzymes, which are involved in the antioxidant defense system [29]. Zinc serves as an indirect antioxidant, so concurrent dietary inadequacy and/or deficiency may contribute to the occurrence of a variety of diseases, complicate clinical features, negatively affect

the immunologic status, increase oxidative stress, and increase the production of inflammatory cytokines [8, 26]. Under oxidative stress conditions, iron mediates the formation of superoxide anion radicals and hydrogen peroxide, which oxidize cellular macromolecules and membrane proteins [12]. Iron mediates the formation of superoxide anion radicals and hydrogen peroxide under oxidative stress conditions, which oxidize cellular macromolecules and membrane proteins. Since this was an observational study and mineral concentrations are highly associated with an animal's diet, the absence of control over the diets of the referred animals with colic symptoms may have prevented the detection of differences in the concentrations of the measured minerals across animals.

In conclusion, we found that horses with strangulating colic had a greater concentration of TNFα. We suggest that the TNFα concentration in horses with colic symptoms may be a helpful predictor for the severity of colic. If clinicians do not have access to laboratory facilities, it can be expected that some acute-phase proteins, such as TNFα, may increase depending on the severity of clinical symptoms such as the heart rate and mucous membrane color. As a result of the late response of Hp, we propose that the Hp concentration might be a helpful biomarker in horses with colic symptoms that are referred for clinical assessment more than a day after the onset of clinical symptoms. In horses with colic signs, the concentrations of MDA and NO should be interpreted with caution when used to categorise the severity of colic. Further investigation using a larger samples size could highlight the associations of MDA and NO with clinical signs of colic. Additional controlled trials could identify the roles of minerals in horses with colic symptoms when dietary components are taken into account.

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References

1. Borges, A.S., Divers, T.J., Stokol, T., and Mohammed, O.H. 2007. Serum iron and plasma fibrinogen concentrations as indicators of systemic inflammatory diseases in horses. *J. Vet. Intern. Med.* **21**: 489–494. [Medline] [CrossRef]
2. Borunova, S.F., Tkachev, N., Iolchiev, B., Artyushina, Z., Abramov, P., Nikitina, M., Silanteva, A., Khusnetdinova, N., and Serezhenkov, V. 2020. Estimation of nitrite-nitric oxide derivative-in horses with intestinal colic by ESR Spectroscopy. *Vet. Sci.* **7**: 191. [Medline] [CrossRef]
3. Cesarini, C., Monreal, L., Armengou, L., Delgado, M.A., Ríos, J., and Jose-Cunilleras, E. 2010. Association of admission plasma D-dimer concentration with diagnosis and outcome in horses with colic. *J. Vet. Intern. Med.* **24**: 1490–1497. [Medline] [CrossRef]
4. Chaturvedi, U.C., Shrivastava, R., and Upreti, R.K. 2004. Viral infections and trace elements: a complex interaction. *Curr. Sci.* **87**: 1536–1554.
5. Constable, P.D., Hinchcliff, K.W., Done, S.H., and Gruenberg, W. 2017. Veterinary Medicine: A Textbook of the Diseases of Cattle. pp. 1115–1128. Horses, sheep, pigs and goats, 11 th ed., Elsevier, St. Louis.
6. Cray, C., and Belgrave, R.L. 2014. Haptoglobin quantitation in serum samples from clinically normal and clinically abnormal horses. *J. Equine Vet. Sci.* **34**: 337–340. [CrossRef]
7. Crisman, M.V., Scarratt, W.K., and Zimmerman, K.L. 2008. Blood proteins and inflammation in the horse. *Vet. Clin. North Am. Equine Pract.* **24**: 285–297, vi. [Medline] [CrossRef]
8. Eide, D.J. 2011. The oxidative stress of zinc deficiency. *Metallomics* **3**: 1124–1129. [Medline] [CrossRef]
9. El-Bahr, S.M., and El-Deeb, W.M. 2016. Acute-phase proteins, oxidative stress biomarkers, proinflammatory cytokines, and cardiac troponin in Arabian mares affected with pyometra. *Theriogenology* **86**: 1132–1136. [Medline] [CrossRef]
10. El-Deeb, W., Iacob, O., Fayezd, M., Elgioushy, M., Shawaf, T. and Ibrahim, A. 2018. Acute phase proteins, interleukin-6, tumor necrosis factor, nitric oxide and oxidative stress markers in horses with cutaneous habronemosis under field condition. *Vet. Parasitol.* **225**: 20–25. [Medline] [CrossRef]
11. Ferraro, G.L. 2008. Colic: an age-old problem. *CEHHorse Report*. **26**: 3–16.
12. Galaris, D., Mantzaris, M., and Amorgianiotis, C. 2008. Oxidative stress and aging: the potential role of iron. *Hormones (Athens)* **7**: 114–122. [Medline] [CrossRef]
13. Grosche, A., Morton, A.J., Graham, A.S., Sanchez, L.C., Blikslager, A.T., Polyak, M.M., and Freeman, D.E. 2011. Ultrastructural changes in the equine colonic mucosa after ischaemia and reperfusion. *Equine Vet. J. Suppl.* **43**: 8–15. [Medline] [CrossRef]
14. Hultén, C., Grönlund, U., Hirvonen, J., Tulamo, R.M., Suominen, M.M., Marhaug, G., and Forsberg, M. 2002. Dynamics in serum of the inflammatory markers serum amyloid A (SAA), haptoglobin, fibrinogen and alpha2-globulins during induced noninfectious arthritis in the horse. *Equine Vet. J.* **34**: 699–704. [Medline] [CrossRef]
15. Ibrahim, H.M.M. 2014. Oxidative stress associated with spasmodic, flatulent, and impaction colic in draft horses. *J. Equine Vet. Sci.* **34**: 1205–1210. [CrossRef]
16. Kaufman, J.M., Nekouei, O., Doyle, A.J., and Biermann,

N.M. 2020. Clinical findings, diagnoses, and outcomes of horses presented for colic to a referral hospital in Atlantic Canada (2000–2015). *Can. Vet. J.* **61**: 281–288. [Medline]

17. Kyaw, W.O., Uhlig, A., Köller, G., Sack, U., and Schusser, G.F. 2008. Free hemoglobin and tumor necrosis factor-alpha in the blood of horses with colic or acute colitis. *Berl. Munch. Tierarztl. Wochenschr.* **121**: 440–445 (in German). [Medline]
18. Lavoie-Lamoureux, A., Beauchamp, G., Quessy, S., Martin, J.G., and Lavoie, J.P. 2012. Systemic inflammation and priming of peripheral blood leukocytes persist during clinical remission in horses with heaves. *Vet. Immunol. Immunopathol.* **146**: 35–45. [Medline] [CrossRef]
19. Marañón, G., Manley, W., Cayado, P., García, C., de la Muela, M.S., and Vara, E. 2009. Alterations in the glutathione metabolism could be implicated in the ischemia-induced small intestinal cell damage in horses. *BMC Vet. Res.* **5**: 10. [Medline] [CrossRef]
20. Mirza, M.H., Oliver, J.L., Seahorn, T.L., Hosgood, G., and Moore, R.M. 1999. Detection and comparison of nitric oxide in clinically normal horses and those with naturally acquired small intestinal strangulation obstruction. *Can. J. Vet. Res.* **63**: 230–240. [Medline]
21. Molinari, L., Basini, G., Ramoni, R., Bussolati, S., Aldigeri, R., Grolli, S., Bertini, S., and Quintavalla, F. 2020. Evaluation of oxidative stress parameters in healthy saddle horses in relation to housing conditions, presence of stereotypies, age, sex and breed. *Processes (Basel)* **8**: 1670. [CrossRef]
22. Morris, D.D., Moore, J.N., and Crowe, N. 1991. Serum tumor necrosis factor activity in horses with colic attributable to gastrointestinal tract disease. *Am. J. Vet. Res.* **52**: 1565–1569. [Medline]
23. Morris, D.D., and Moore, J.N. 1991. Tumor necrosis factor activity in serum from neonatal foals with presumed septicemia. *J. Am. Vet. Med. Assoc.* **199**: 1584–1589. [Medline]
24. Pihl, T.H., Andersen, P.H., Kjelgaard-Hansen, M., Mørck, N.B., and Jacobsen, S. 2013. Serum amyloid A and haptoglobin concentrations in serum and peritoneal fluid of healthy horses and horses with acute abdominal pain. *Vet. Clin. Pathol.* **42**: 177–183. [Medline] [CrossRef]
25. Pollock, P.J., Prendergast, M., Schumacher, J., and Bellenger, C.R. 2005. Effects of surgery on the acute phase response in clinically normal and diseased horses. *Vet. Rec.* **156**: 538–542. [Medline] [CrossRef]
26. Prasad, A.S. 2009. Zinc: role in immunity, oxidative stress and chronic inflammation. *Curr. Opin. Clin. Nutr. Metab. Care* **12**: 646–652. [Medline] [CrossRef]
27. Schaer, D.J., Buehler, P.W., Alayash, A.I., Belcher, J.D., and Vercellotti, G.M. 2013. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. *Blood* **121**: 1276–1284. [Medline] [CrossRef]
28. Souto, P.C., Fonseca, L.A.D., Orozco, A.M.O., Lopez, C.J.R., Ermita, P.A.N., Carvalho Filho, W.P., and Girardi, F.M. 2019. Acute-phase proteins of healthy horses and horses naturally affected by colic syndrome. *J. Equine Vet. Sci.* **80**: 1–4. [Medline] [CrossRef]
29. Wagner, E.L., Potter, G.D., Gibbs, P.G., Eller, E.M., Scott, B.D., Vogelsang, M.M., and Walzem, R.L. 2011. Copper and zinc balance in exercising horses fed 2 forms of mineral supplements. *J. Anim. Sci.* **89**: 722–728. [Medline] [CrossRef]
30. Westerman, T.L., Foster, C.M., Tornquist, S.J., and Poulsen, K.P. 2016. Evaluation of serum amyloid A and haptoglobin concentrations as prognostic indicators for horses with colic. *J. Am. Vet. Med. Assoc.* **248**: 935–940. [Medline] [CrossRef]
31. White, N.A. 2006. Equine colic: I. Introduction (San Antonio, ed.), 52 Annual convention of the American Association of Equine Practitioners−AAEP American Association of Equine Practitioners, Lexington, Internet: International Veterinary Information Service, Ithaca (www.ivis.org).
32. Zak, A., Siwinska, N., Elzinga, S., Barker, V.D., Stefaniak, T., Schanbacher, B.J., Place, N.J., Niedzwiedz, A., and Adams, A.A. 2020. Effects of equine metabolic syndrome on inflammation and acute-phase markers in horses. *Domest. Anim. Endocrinol.* **72**: 106448. [Medline] [CrossRef]
33. Zuluaga, A., Silveira, G., and Martinez, J. 2016. Nitric oxide and malondialdehyde in gastric contents and blood in an equine model of gastric ulcer induced by phenylbutazone. *RCCP* **29**: 43–50.