Original Research

Selected Biochemical Indicators of Equine Rhabdomyolysis in Arabian Horses: Acute Phase Proteins and Trace Elements

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ABSTRACT

Although creatine kinase (CK), aspartate transaminase (AST), cytokines, and oxidative stress parameters were shown to be useful biomarkers for diagnosis of equine rhabdomyolysis, additional biomarkers of the disease may be of interest to indicate prognosis of the disease. Therefore, the present study investigated acute phase proteins and trace elements as additional biomarkers of ER. Sixty male horses (4-6 years old) of 2 equal groups were used. Horses of the first group were clinically healthy and served as a control group, whereas horses of the second group were ER-diseased animals. Harvested sera were used for estimation of the activities of CK, AST, lactate dehydrogenase (LDH), serum amyloid A (SAA), haptoglobin (Hp), ceruloplasmin (Cp), copper, iron, and zinc, whereas plasma samples were used for determination of fibrinogen. The present findings revealed a significant ($P \leq .05$) increase in values of CK, AST, and LDH in diseased horses compared to those in control values. In addition, a significant ($P \leq .05$) increase in SAA (162.6 $\pm$ 5.32 mg/L), Hp (3.6 $\pm$ 0.54 g/L), Cp (39.32 $\pm$ 2.31 mg/L), and copper (28.36 $\pm$ 1.23 μmol/L) along with a significant ($P \leq .05$) reduction in levels of iron (9.32 $\pm$ 0.23 μmol/L) and zinc (8.65 $\pm$ 1.02 μmol/L) was recorded in diseased horses compare to that in controls (11.3 $\pm$ 2.2 mg/L, 0.8 $\pm$ 0.2 g/L, 24.23 $\pm$ 1.32 mg/L, 18.41 $\pm$ 1.03 μmol/L, and 14.2 $\pm$ 0.42 μmol/L, respectively). In conclusion, SAA, Hp, Cp, copper, and zinc were useful prognostic biomarkers for the diagnosis of ER in Arabian horses.

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1. Introduction

Exertional rhabdomyolysis was known as Monday morning disease, a disease related to work horses that were given a day of rest after a week of hard work. The affected horses developed stiffness and pain in the hindquarter musculature [1]. The first form of the disease was described in coldblood horses, whereas the second form was described as postexercise in race horses [2]. Later on the disease was known as tying-up [3]. Due to confusion arising from the terminology regarding equine exercise-induced rhabdomyolysis, scientists all over the world accepted the term equine exertional rhabdomyolysis (ER) to describe such problem in horses. This term indicates muscle disorder in the equine resulting from exercise. Therefore, tying-up, azoturia, setfast, Monday morning disease, Kreuzslag, and coupe de sang are considered synonyms for ER. Arabian horses were affected by rhabdomyolysis [4-6], and the cause is thought to involve carbohydrate metabolism [7]. Diagnosis of rhabdomyolysis depends greatly on

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history, clinical signs, and biochemical analysis of horse blood. Clinical signs differ in mild cases compared with those in advanced rhabdomyolysis, which are characterized by shortened gait, muscle stiffness, and bad performance in racing horses [8,9]. Differential diagnosis is of great importance to exclude colic, laminitis, and aortoiliac thrombosis. Biochemical evaluation of creatine kinase (CK) and aspartate transaminase (AST) activities is a diagnostic tool for muscle disorder. An elevation of their activities indicates muscle damage and myolysis. Recently, proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interlukein-6 (IL-6), and prostaglandin were introduced as effective biomarkers for diagnosis of ER in Arabian horses [6]. Acute phase protein (APP) responses were monitored extensively in animals for clinical purposes in the last 2 decades [10,11]. Therefore, many quantitative APP assays have been established. Acute phase proteins constitute negative (albumin and transferrin) and positive (haptoglobin [Hp], C-reactive proteins, serum amyloid A [SAA], ceruloplasmin [Cp], fibrinogen [Fb], and α-1-acid glycoprotein) proteins whose activity levels decreased or increased, respectively, in response to stimuli [12]. Acute phase proteins are synthesized in liver, and their synthesis is mediated by IL-6, TNF-α, and IL-1, which are released mainly by macrophages. However, extrahepatic synthesis was also reported in mammalian species [13]. Stimuli such as inflammation, infection, or tissue damage trigger cytokine release by defense-oriented cells, thereby inducing APP synthesis. Induction of positive APP in hepatocytes by cytokines [14] was associated mainly with reduction in negative APP biosynthesis [10]. Few publications demonstrated that SAA concentration and other APP were increased in blood of horses suffering from bacterial or viral infection, neoplasm, trauma, and surgery [15]. Earlier research reported that SAA was a major APP in horses, whereas both Hp and Fb act as moderate APPs [16]. Iron is a negative acute phase reactant in horses and other species [17,18]. Abnormally low iron values were associated with different diseases and tissue injury [19]. A defense mechanism of the body against bacterial infection was encountered by decreasing the availability of iron to bacteria via sequestering of iron in mononuclear phagocytes, inducing bacterial growth inhibition [17,18,20]. Ceruloplasmin is one APP that has the ability to scavenge toxins, free oxygen radicals produced during inflammation, and to protect the host against tissue damage [21]. In addition, Cp allows binding between iron and transferrin. Copper is the rate-limiting element in the biosynthesis of Cp. Correlation between copper and Cp was reported in humans and animals [22-24].

The antioxidant effect of zinc is documented [25]. The concentration of zinc is always associated with high cytokine and peroxidation levels [26,27]. It is well known that AST and CK are imperfect indicators whose values are not correlated with the severity of clinical signs, but they are the only criteria available to determine the severity of the disease and to decide when to return the horse to work. Although, cytokines and oxidative stress parameters were shown to be useful biomarkers for diagnosis of ER, additional biomarkers of the disease may be of interest to indicate the severity and prognosis of the disease.

Therefore, the present study investigated APP and trace elements as additional biomarkers of ER.

2. Material and Methods

2.1. Animals and Sampling Protocol

A total of 60 male horses (4-6 years old) were used in our previous work [6]. They were divided into 2 equal groups. Horses of the first group (n = 30) were clinically healthy, with no history of ER, and served as the control group. Horses of the second group (n = 30) were ER-diseased horses. They were selected on the basis of clinical examination and history of overexercise after a period of rest and overfeeding of nonstructural carbohydrates (grains). In addition, biochemical analyses of CK, AST, and lactate dehydrogenase LDH indicated a significant increase in the activities of these enzymes in ER-diseased horses. Blood samples were collected from the jugular vein from all horses in both groups (samples from the second group were collected shortly after exercise) in fresh plain and heparinized vials for serum and plasma collection, respectively. Part of the serum samples were used in our previous work for estimation of proinflammatory cytokines and oxidative stress biomarkers. The remaining serum samples and plasma were kept frozen at −20°C until their use in the current experiment for determination of SAA, HP, Cp, iron, copper, and zinc, whereas plasma samples were used for determination of Fb.

2.2. Determination of Enzymes Activities

Enzymatic methods were used for colorimetric determination of serum AST, CK, and LDH (Bio-diagnostic, Cairo, Egypt) according to manufacturer's instructions.

2.3. Determination of APP

Fibrinogen concentration in plasma was determined [28]. Serum Hp was determined using the hemoglobin binding assay described previously [29]. Serum amyloid A was measured with a commercially available enzyme-linked immunosorbent assay kit (Phase SAA kit; Tridelta Ltd, Ireland). Moreover, a commercially available kit (catalog no. 4096-1000; Biovision Inc, CA) was used for determination of serum Cp by p-phenylenediamine oxidization [30].

2.4. Determination of Trace Elements

Serum iron concentration was determined by colorimetric spectrophotometry using commercially available kit (catalog no. EZ-0067; Assay Biotechnology Co, CA) [31]. Commercially available kits were used for determination of serum copper (catalog no. DICU-250; BioAssay; Quantichrom, CA) [32] and zinc (catalog no. K387-100; Biovision) [33].

2.5. Statistical Analysis

Data obtained for APP and trace elements were compared between the control group (n = 30) and diseased horses (n = 30) by using Student *t*-test. All tests were performed using computer software of the statistical analysis system [34].

3. Results

The observed clinical signs were in the form of pronounced sudden muscular weakness and stiffness, depression, reluctance or inability to move, colic, anorexia, muscle tremors, and myoglobinuria. All clinical signs were recorded shortly after exercise. In addition, rectal palpation of the diseased group revealed highly distended bladders in 19 horses. The onset of symptoms and the severity of ER symptoms in different cases are shown in Table 1.

Data summarized in Table 2 include the activities of CK, AST, and LDH in control and diseased horses. The present findings (Table 2) revealed a significant ($P \leq .05$) increase in the activities of CK, AST, and LDH in ER-diseased horses compare to those in control healthy horses.

The present findings as shown in Table 3 revealed a significant increase of SAA (162.6 ± 5.32 mg/L), Hp (3.6 ± 0.54 g/L), and Cp (39.32 ± 2.31 mg/L) values in ER-diseased horses compared with those in control, healthy horse (11.3 ± 2.2 mg/L, 0.8 ± 0.2 g/L, 24.23 ± 1.32 mg/L), respectively. In addition, findings presented in the Table 3 reveal a significant decrease in the levels of iron (9.32 ± 0.23 µmol/L) in ER-diseased horses compared to that in control, healthy horses (14.2 ± 0.42 µmol/L). As shown in Table 4, the values for zinc (8.65 ± 1.02 µmol/L) were significantly decreased in ER-diseased horses compare to those in control, healthy horses (14.08 ± 3.57 µmol/L). However, Fb (3.4 ± 0.36 g/L) values in ER-diseased horses remained comparable to that in control, healthy horses (3.2 ± 0.54 g/L), respectively (Table 1).

4. Discussion

Equine rhabdomyolysis was reported previously [35,36] as a disorder of glucose metabolism ending in the formation of lactic acid and subsequent acidosis and muscle damage. However, the levels of lactic acid reported in the studies by Carlström [35,36] represented only a fraction of the levels found in exercising muscle of healthy horses. Therefore, a big question arose: is lactic acid the cause of muscle damage or just a result of muscle damage? Interestingly, in our previous research in Arabian horses affected with ER [6], the animals were not worked and were kept on

Table 1
Onset of symptoms and severity of ER symptoms in different cases (n = 30)

Case No.	Interval Sample and Onset of Symptoms (hr)	Severity of ER Symptoms
19	4.34 ± 0.24	Severe
11	5.12 ± 0.16	Moderate

ER, exertional rhabdomyolysis.

Table 2
Selected enzymes activities in control and diseased horses

Parameter	Control	Diseased
CK (IU L^{-1})	202.6 ± 9.90	$15450.23 \pm 86.76*$
AST (IU L^{-1})	275.3 ± 6.60	$20990.00 \pm 69.60*$
LDH (IU L^{-1})	501.5 ± 8.90	$24540.00 \pm 58.6*$

AST, aspartate transaminase; CK, creatine kinase; LDH, lactate dehydrogenase.
* Means are significantly different at *P* value of $\leq$ .05.

a full feed high in soluble carbohydrates, which might be accumulated in muscle. One possibility is when a sudden demand for work was required, the body failed to remove the rapidly accumulated lactic acid in muscle efficiently. Therefore, vasospasm and ischemia of surrounding blood vessels were the expected results; hence, lactic acid was not removed; intracellular pH dropped; and crampy muscle also were observed during tying-up [6]. Creatine kinase, LDH, and AST were used as conventional biomarkers for diagnosis of ER. The significant increases in serum CK, AST, and LDH values observed in the present study suggested ER with expected muscle damage [4]. The present results are in accord with those of previous studies [4,6].

There are three main reasons behind the provision of the current experiments. First, we [6] discovered a significant increase in proinflammatory cytokines in ER-diseased horses that allows assumption of the existence of high APP levels in the same horses due to the well-documented close correlation between APP biosynthesis and cytokines secretion. The lack of studies investigating diagnostic benefit of APP for ER was the second reason for this experiment. Although, many studies demonstrated the role of trace elements as biomarkers for different inflammatory diseases, their biochemical picture in ER was not available, and this was the third reason for conducting the present experiment. The present findings revealed a significant increase in the values of SAA, Hp, and Cp in ER-affected horses compared to those in control, healthy animals. The significant increase in SAA of ER-diseased horses that was observed in the present study concurs with that reported by others [16]. Those authors reported that SAA is a so-called major APP, which is virtually absent from the sera of healthy horses, and its concentration can increase by several orders of magnitude in response to inflammation. In addition, the concentration of SAA starts to increase soon after an injury has occurred or an infection has been established [37]. Moreover the present results are in agreement with previous results [38-40]. Those authors [38-40] mentioned that serum concentrations of SAA were low in healthy horses but increased rapidly to very high levels in response to inflammation and tissue damage.

Table 3
Acute phase response in control healthy and Arabian horses with exertional rhabdomyolysis

Variable	Healthy Horses	ER Horses
SAA (mg/L)	11.3 ± 2.2	$162.6 \pm 5.32*$
HP (g/L)	0.8 ± 0.2	$3.6 \pm 0.54*$
Fb (g/L)	3.2 ± 0.54	3.4 ± 0.36
Cp (mg/L)	24.23 ± 1.32	$39.32 \pm 2.31*$

Cp, ceruloplasmin; Fb, fibrinogen; HP, haptoglobin; SAA, serum amyloid A.
* Means are significantly different at a *P* value of $\leq$ .05.

Table 4
Trace elements in healthy control Arabian horses and those with exertional rhabdomyolysis

Parameters	Healthy Horses	ER Horses
Cu (μmol/L)	18.41 ± 1.03	28.36 ± 1.23*
Zn (μmol/L)	14.08 ± 3.57	8.65 ± 1.02*
Iron (μmol/L)	14.20 ± 0.42	9.32 ± 0.23*

ER, exertional rhabdomyolysis.
* Means are significantly different at P level of ≤.05.

Serum amyloid A (SAA) is the major APP in equines. SAA is participating in the Acute phase response (APR) as a major APP, whereas both Hp and Fb act as moderate APPs. Similar to its mechanism in other mammalian species, albumin is considered a negative APP [12]. Interestingly, the significant increase of Hp in ER-diseased horses, compared to that in controls, which was documented in the present study, can be considered the first report dealing with Hp as biomarker of this disease. However, increased serum or plasma Hp concentration in horses has been observed following surgery [41,42] and experimental inflammations [40,42-44] and during natural diseases [41,44,45]. One report showed Hp to be a useful indicator of viral infection in stabled thoroughbreds [41], as the concentration increased. No such increase in equine serum Hp concentration was observed following vaccination against influenza and tetanus. Noninfectious arthritis [40] and carbohydrate-induced laminitis [43] has also been shown to induce increased concentrations of Hp. Also, increased serum Hp concentration has been reported in horses suffering from grass sickness (equine dysautonomia) [45]. Following castration complicated by postoperative scrotal infection, two peaks in serum Hp concentration were observed [41]. No increase was observed in horses with colic [45].

Iron concentration, either in plasma or serum, decreased as a result of inflammatory processes in human and animals [46,47]. This decrease in iron concentration acts as defense mechanism against bacteria that require iron for their virulence and replication. Few publications describe the iron concentration of hospitalized horse [19,48]. Lower iron concentration was associated with tissue injury and inflammatory processes [19]. In addition, plasma iron concentration was a better inflammatory biomarker than Fb in horses [48]. The significant decrease in iron levels observed in the present study in horses with ER is in agreement with that in previous work [48], which indicated that low iron concentration was a sensitive test for systemic inflammation and was highly specific when clinically healthy, transported horses were used to establish the specificity. Other studies [46,47] also mentioned that plasma or serum iron concentration rapidly decreases in response to inflammation in both humans and animals. Moreover, a significant decrease in iron concentration in horses within 24 hours of experimentally induced inflammation has been reported [18,49]. Plasma or serum iron concentration rapidly decreases in response to inflammation in both humans and animals [46,47]. The nonsignificant changes in Fb observed in ER-affected horse compared to controls is in accordance with those in an earlier study [48] that demonstrated that measurement of plasma iron concentration is a sensitive method for detecting systemic inflammation in horses compared with measurements of plasma Fb. Because of Fb values might not peak for 2 to 3 days [50,51], unchanged values were recognized in both healthy and diseased groups of animals. In addition, there are also a wide range of Fb concentration values in healthy horses, and the standard heat precipitation method used for Fb measurement is insensitive to small changes [52,53]. The role of zinc in modulating oxidative stress has been reviewed [25]. The antioxidant effect of zinc is exhibited via different mechanisms. Zinc inhibits NADPH oxidases, which catalyze the production of superoxide radical. In addition, the dismutation of superoxide radicals to hydrogen peroxide is catalyzed by the enzyme superoxide dismutase, which contains copper and zinc. Moreover, zinc is known to induce the production of metallothionein, which is very rich in cysteine and is an excellent scavenger of hydroxyl radicals in human [25]. In the present study, the significant decrease in zinc concentration in ER-diseased horses compared to that in controls concurs with decreases observed in previous research in humans [26,27]. Increased cytokine secretions were associated with deceased zinc status in patients with cutaneous leishmaniasis [26], and lipid peroxidation also was associated with decreased zinc status in children with giardiasis [27]. Our previous findings [6] combined with our current findings are in accordance with those in previous research in humans [26,27]. The present study demonstrated positive correlation between copper and Cp, as both parameters were increased in ER-affected horses compare to controls. The close correlation between copper and Cp is similar to that reported in previous research in humans and animals [19,22-24].

5. Conclusions

Acute phase proteins (SAA, Hp, and Cp) and trace elements (copper and zinc) were shown to be useful biomarkers for the diagnosis of ER in Arabian horses.

Acknowledgments

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