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Dear Professor Karen L. Overall,

Enclosed you will find our manuscript entitled “**Potential roles of Selenium and Zinc in the pathophysiology of crib-biting behavior in horses**” to be considered for publication in *Journal of Veterinary Behavior*. The article has not been published elsewhere and is not under consideration by another journal. I have not published or submitted any related papers from this study.

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-The manuscript has not been and will not be published elsewhere or submitted elsewhere for publication.

- There are no conflicts of interest in this study.

I would be thankful if it is processed at your earliest convenience. Thanks in advance for your time and efforts and looking forward to hear from you soon.

Best regards

Arash Omid

Potential roles of Selenium and Zinc in the pathophysiology of crib-biting behavior in horses

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Potential role for selenium in the pathophysiology of crib-biting behavior in horses

Abstract

Crib-biting is repetitive and compulsive behavior that characterized by “grasping a fixed object with incisor teeth and aspirating air with an audible grunt”. Little is known about etiology and pathophysiology of crib-biting behavior in horses. Previously we have shown that oxidative stress is linked to crib-biting, with crib biters showing lower antioxidant capacity than non-crib-biting horses. The aim of the present study was extend our understanding of oxidative stress in crib-biting to determine the serum contents of some mineral trace elements (manganese (Mn), magnesium (Mg), selenium (Se), copper (Cu), and zinc (Zn)), and electrolytes (sodium (Na), potassium (K), calcium (Ca) and phosphorus (P)). Also, activity of enzymes (ALT, AST, ALP and GGT), some hormones (Cortisol, ghrelin, β -endorphin and serotonin) and blood biochemistry values of various parameters were measured to evaluate their possible association with crib-biting behavior in horses. Blood samples were taken from all horses under the following conditions: basal conditions of crib biting horses, during or immediately after crib-biting periods, and from non-crib biting, healthy horses (control group). Serum Se concentration was significantly lower ($P \leq 0.001$) in crib biting horses than in controls, with the lowest levels seen during crib-biting behavior. Other measured parameters did not differ between acute crib biting horses and healthy controls. These observations suggest that alterations in serum Se, an important component of the antioxidant system, may play a role in the pathophysiology of crib-biting behavior in horses, adding further evidence to the theory that crib-biting may be related to increased oxidative stress and alterations in essential trace elements.

Key words: behavior; crib-biting; horses; selenium; trace elements

Introduction

Crib-biting is an abnormal repetitive behaviour observed in horses. During crib biting, horses grasp a fixed object with incisor teeth and aspirating air with an audible

grunt. This behavior is the most prevalent stereotypy in horses which characterized by repetitive and compulsive habit (Sarrafchi and Blokhuis, 2013). There are several consequences of crib-biting, including health problems such as dental disorders (wear of incisors), temporohyoid joint damages, poor performance, weight loss, colic, and diminished learning (Dixon and Dacre, 2005; Grenager et al., 2010; Hausberger et al., 2007; Sarrafchi and Blokhuis, 2013; Archer et al., 2008; Parker et al., 2008a; Parker et al., 2009). In general, crib-biting is seen in stabled horses with suboptimal management and welfare (Cooper and McGreevy, 2007; Parker et al., 2008b).

Little is known about pathophysiology of crib-biting behavior in horses, including basic questions about the biological profiles of crib biting horses. Some authors suggest that crib-biting horses have increased stress sensitivity and lower behavioural flexibility (Bachmann et al., 2003; Parker et al., 2008a; Parker et al., 2009). In a study by Omid et al., (2017), level of antioxidants such as total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), were significantly decreased in crib-biting horses at rest, and further decreased during an acute phase of crib biting. The same findings were observed in humans with various affective disorders such as depression and anxiety (Liu et al., 2014; Xu et al., 2015). Oxidative stress may play a role in the pathophysiology of crib biting (Omid et al., 2017). Trace elements such as selenium (Se), zinc (Zn), manganese (Mn) and copper (Cu) protect the body from oxidative stress. For example, free radical scavenging activity of GPx and the immune system is mediated by Se levels (Leung, 1998). In addition, Zn is present in numerous proteins involved in the defense against oxidative stress (Song et al., 2009). Finally, Cu/Zn -SOD is a cofactor that acts as free radical scavenger (Dancygier and Schirmacher, 2010). What is not clear is the profiles of trace elements in crib biting horses, and how trace elements may contribute to crib biting pathophysiology.

The aim of the present study was to extend our previous assessment of the oxidative stress profiles of crib biters both during an acute crib biting episode and during resting. In particular, we aimed to assess the changes in Mn, magnesium (Mg), Se, Cu, and Zn, sodium (Na), potassium (K), calcium (Ca) and phosphorus (P) levels in crib biters and during crib biting. In addition, we characterized the activity of the en-

zymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ glutamyl transferase (GGT), some hormones (Cortisol, Ghrelin, β -endorphin and Serotonin) and blood biochemistry values of glucose, cholesterol, triglyceride, blood urea nitrogen (BUN), creatinine, urea and albumin.

Materials and methods

Ethics statement

The experiment was performed under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes were considered.

Subjects

Ten crib biting horses (7 stallion, 2 mares, and 1 gelding) of different breeds (cross-breeds, Arabian, Dareshouri), age 2-14 years were used. The animals were single housed in conventional horse boxes in different riding stables in the surrounding of Shiraz. All crib biting horses were current, established crib biters and had been performing the behavior for some time based on owner reports (full history unclear for all horses). Ten age- and sex matched horses with no history of stereotypic behavior, kept under same housing conditions were used as controls.

Protocol

To minimize influences of circadian changes, all studies were carried out between 0930 and 1130 h. Blood samples were taken from all horses under the following conditions: Condition 1: basal conditions of crib biting horses (no stereotypic behavior for at least 30 min); Condition 2: during or immediately after crib-biting periods (crib biting for at least 15 minutes with no interruption longer than 2 minutes); Condition 3: non-crib biting, healthy horses (control group). The order in which crib biting horses (condition 1 and condition 2) were sampled was typically first during acute crib biting, followed by a basal sample. Blood samples were obtained by jugular venipuncture into plain tubes. After centrifugation of blood at 750g for 15 min at room temperature, serum was separated and stored at -80 °C until analysis. The samples with hemolysis were discarded. All blood sampling was carried out by a qualified veterinarian.

Biochemical analysis

Serum samples were treated using combined perchloric and nitric acid. Trace elements including Mn, Mg, Se, Cu and Zn were measured by an atomic absorption spectrophotometer (Shimadzo AA-670, Kyoto, Japan) and were finally presented as ppm. Serum was used for measurement of ALT, AST, ALP, GGT, glucose, cholesterol, triglyceride, BUN, urea, creatinine, total protein, albumin, globulin, calcium and phosphorus. All assays were performed using commercial kits (Pars Azmoon, Tehran, Iran) and biochemical auto analyzer (Alpha Classic, Sanjesh Company, Iran). Measurement of serum sodium and potassium was done using flame photometer (620 Clinical flame photometer, Fater Company, Iran). Horse cortisol, ghrelin, β -endorphin, and serotonin were measured in serum using commercial kits based on sandwich enzyme linked immunoassay (ELISA) (Shanghai Crystal Day Biotech Co., LTD, Shanghai, China).

Statistical Analysis

All descriptive statistics are reported as mean \pm SD. Data were analyzed using IBM SPSS Statistics Version 22.0 for Macintosh. Comparison of physiological parameters was carried out by fitting data to random intercept linear mixed effects models. The fixed factor for all models was 'group', and this had three levels (control, crib biters 'basal' and crib-biters 'acute crib biting'). To account for non-independence of crib-biters (repeated measures), we included subject ID as a random effect. Denominator degrees of freedom were estimated by the Satterthwaite approximation. Post-hoc comparisons were carried out with respect to Bonferroni adjusted alpha values. As there were 26 blood parameters measured we used an adjusted α value of $0.05/26 = 0.002$.

Results

All data were fitted to random intercept linear mixed effects models. There was a main effect of treatment, with cribbers showing lower Se levels than controls at rest, and lower still when cribbing ($F [2,19.3] = 149.8, P = 1.64^{-12}$), (Figure 1). There was also some suggestion of an effect of cribbing status on Zn, with cribbers showing lower Zn levels at basal, but this was normalized during the cribbing behaviour ($F [2,19.9] = 3.7, P = 0.04$) (Table 1). However, this fell above the adjusted α value of 0.002, so this result should be treated with caution. There were no significant differences between the conditions for any of the other parameters (Table 2, 3, 4).

Discussion

In this study, we evaluated the levels of mineral trace elements in crib biting horses 130
in order to examine their potential role in crib biting pathophysiology. We found that 131
crib biters have lower levels of Se at rest, and the Se levels are reduced further dur- 132
ing crib biting. We also found some emerging evidence that there were decreases in 133
Zn at rest, but that the levels were normalized during crib biting behavior, suggest- 134
ing a negative feedback mechanism; however, the result fell short of our corrected α 135
value and should be treated with caution. These data are the first to evaluate the po- 136
tential role of trace elements in crib biting, and together suggest that crib biting may 137
share physiological characteristics with human neuropsychiatric conditions, in 138
which Se and Zn appear to play a role in the pathophysiology. 139

Endogenous cellular function is mediated by trace elements which act as cofactors 141
(Prashanth et al., 2015). For example, Se is an essential component of GPx thus un- 142
derlining its role in anti-oxidative protection against free radical damage to nucleic 143
acids, lipoproteins and cell membranes. It is generally accepted that Se deficiency is 144
linked to adverse mood states (Ferenčík and Ebringer, 2003). Various theories have 145
highlighted putative common mechanisms in captive animal stereotypies and in hu- 146
man disorders in which repetitive or stereotypic behaviors are common, such as 147
schizophrenia and autism. For example, Garner et al., (2003) found that stereotypic 148
behavior in parrots was correlated with behavioral disinhibition. In addition, Omidi 149
et al., (2017) found similar alterations in antioxidant enzymes in crib-biting horses 150
as are observed in patients with schizophrenia. 151

There are various links between Se levels and human psychiatric conditions that are 153
characterized by stereotypic behavior, such as schizophrenia. For example, lowered 154
Se levels have been observed in schizophrenia patients when compared with healthy 155
controls (Vidović et al., 2013; Sharma et al., 2014; Cai et al., 2015). Specifically 156
relating to stereotypic behavior, lowered Se concentration was observed in a 24- 157
year-old man showing involuntary stereotypies of movement and thinking (Davies et 158
al., 2009). Interestingly, schizophrenia has been reported to be more prevalent in ar- 159
eas where the soil contains very low Se (Foster, 1988), suggesting that dietary dep- 160
rivation of Se may be a key risk factor. Indeed, considerable evidence suggests that 161
variation in affective state is related to variation in dietary Se (Sher, 2002). Finally, 162
alteration in the bioavailability of Se may have cytotoxic effects in the brain. Kana- 163

zawa et al., (2008), for example, found enhancement in expression of selenium binding protein (SELENBP1) in schizophrenia and those showing psychotic symptoms. The underlying mechanism by which dietary Se affects behavior, however, is not presently clear. One potential explanation is that Se-GPx interactions may play important roles in anti-oxidant mechanisms (Benton, 2002). Thus, reduced Se may cause oxidative stress, which may in turn increase the risk for mental disorders. In horses, however, the links between dietary Se and oxidative stress may not be so clear. For example, Se-GPX activity in mature horses is not good indicator of dietary Se (Shellow et al., 1985).

In this study, we found that Zn levels were lower in crib biting horses at baseline, but were normalized during an episode of crib biting. As mentioned earlier, owing to our testing of multiple outcome variables, we adopted an adjusted α value for rejection of the null hypothesis. On this occasion, the Zn findings fell short of this and should be treated with caution. Zn is essential for brain development, axonal function and other functions including neuro-transmission at the glutaminergic pathways in the limbic system. Zn is important for normal function of other elements such as Cu co-activate enzymes, SOD (Cu/Zn-SOD isoform) or phospholipase C (Tapiero and Tew, 2003). There is a potential roles of Zn in the pathophysiology and treatment of major depressive disorder (Swardfager, 2013), and, similar to Se, reduced Zn levels have been observed in schizophrenia patients (Cai et al., 2015). It was interesting that here, we found Zn differences were transient, with crib biting horses showing lower Zn at rest, but during the crib biting episode, Zn increased to the levels observed in our control group. The transient nature of Zn levels contingent on crib biting suggests that the act of crib biting may have some functional significance in terms of Zn regulation. This finding requires further investigation, especially in the light of the observed difference falling short of our penalized α value. For example, it may be prudent to replicate the blood work with a larger sample, and to concurrently examine the effects of Zn supplements on crib biting.

We found no differences either in cortisol or β -endorphin levels either at baseline or during crib biting, and this broadly agrees with previous work (Hemmann et al., 2012; Fureix et al., 2013). Previous studies have shown altered HPA activation in response to stressors associated with crib biting, but that was not tested here (Bach-

mann et al., 2003). In addition, we found no significant difference between serum ghrelin concentration for crib-biting horses. The plasma ghrelin profile of crib biting horses is far from clear. For example, Hemmann et al., (2012), observed significantly higher mean plasma ghrelin concentration for crib-biting horses than the control horses, but in a follow up study, the same group (Hemmann et al., 2013) found that although plasma ghrelin concentration was significantly higher before feeding concentrate than before hay feeding or after the concentrate, there was no difference between crib-biting horses and controls. It may be, therefore, that plasma ghrelin concentrations reflect differences in husbandry/feed of the horses instead of being directly linked to crib biting.

Previous work has shown evidence for lower basal serotonin levels in crib biters (Lebelt, et al., 1998), and serotonin reuptake inhibitors have been reported to reduce stereotypic behavior in horses (McDonnell, 1998), suggesting there may be differences in cribber's serotonergic systems. In this study we found no difference between control and crib biting horses in serotonin concentrations at rest, or in crib biters during a crib biting episode when compared with the basal condition.

Conclusion

In conclusion, our observations of blood biochemistry in crib biting horses suggest that alterations in serum essential trace element Se, which is a potent antioxidant in cellular interactions, may play a role in the pathophysiology of crib biting behavior in horses. Further research should now investigate the functional significance of these alterations, perhaps by studying the effects on crib biting of dietary supplements of Se and Zn.

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The idea for the article was conceived by Arash Omid; Arash Omid and Reza Jafari developed the research and managed the literature searches; Matthew O. Parker undertook the statistical analyses; Arash Omid and Matthew O. Parker wrote and approved the final article. Saeed Nazifi measured the laboratory parameters.

Ethics statement

The experiment was performed under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes were considered.

Conflict of interest

The authors declare that they have no conflict of interest.

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- Figure 1:** Crib biting horses showing lower Se levels than controls at rest (Basal), 360
and lower still when cribbing (Cribbing). 361
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Table 1: Serum mineral element changes in control, crib biters (basal) and crib biters (cribbing) horses (Mean±SD).

Variable* (unit)	Control	Basal	Acute Crib Biting	P (LMM)
Mn (µg/ml)	0.008±0.003	0.007±0.003	0.009±0.003	0.66
Mg (µg/ml)	20.042±5.373	19.023±4.504	17.791±6.340	0.65
Se (µg/ml)	0.093±0.005 ^a	0.076±0.004 ^b	0.060±0.003 ^c	P≤0.001
Cu (µg/ml)	2.775±0.598 ^a	2.990±0.399 ^a	3.012±0.536 ^a	0.53
Zn (µg/ml)	1.103±0.191 ^a	0.896±0.171 ^b	1.004±0.152 ^{ab}	0.04
Na (mmol/l)	118.10±21.29	118.10±17.77	120.60±10.36	0.931
K (mmol/l)	3.78±0.75	3.88±0.76	4.10±0.62	0.595
Ca (mg/dl)	13.07±3.59	12.25±2.42	13.30±3.32	0.736
P (mg/dl)	6.36±3.18	5.26±1.61	4.83±1.63	0.313

*Manganese (Mn), magnesium (Mg), selenium (Se), copper (Cu), zinc (Zn), sodium (Na), potassium (K), calcium (Ca) and phosphorus (P); *Note:* For each variable, shared letters indicate post-hoc analyses not significantly different. Different letters indicate $P < 0.05$ or 0.001.

Table 2: Serum enzyme activities in control, crib biters (basal) and crib biters (cribbing) horses (Mean±SD).

Variable*(unit)	Control	Basal	Acute Crib Biting	P (LMM)
ALT (U/L)	10.19±3.62	11.81±3.80	10.55±2.58	0.537
AST (U/L)	3.93±1.72	4.51±1.73	3.74±1.44	0.556
ALP (U/L)	446.03±216.31	412.38±209.45	384.39±108.75	0.759
GGT (U/L)	128.17±41.36	129.21±45.75	105.17±40.72	0.376

*Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ glutamyl transferase (GGT).

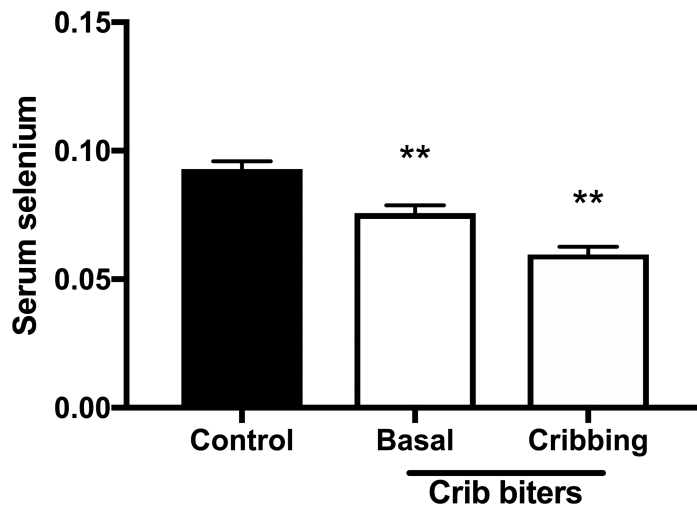
Table 3: Some hormones in control, crib biters (basal) and crib biters (cribbing) horses (Mean±SD).

Variable (unit)	Control	Basal	Acute Crib Biting	P (LMM)
Cortisol(ng/ml)	163.19±95.57	98.53±36.13	121.29±73.40	0.160
Ghrelin(ng/l)	257.17±101.39	254.45±86.38	271.68±71.11	0.894
β-endorphin (ng/l)	756.08±388.58	751.79±277.63	733.46±381.95	0.988
Serotonin(ng/ml)	630.66±229.80	545.29±240.21	620.39±237.77	0.681

Table 4: Blood biochemistry values of some parameters in control, crib biters (basal) and crib biters (cribbing) horses (Mean±SD).

Variable*(unit)	Control	Basal	Acute Crib Biting	<i>P</i> (LMM)
Glucose (mg/dl)	78.97±16.73	72.07±18.74	78.57±9.09	0.540
Cholesterol (mg/dl)	55.40±16.54	57.75±25.28	46.24±6.84	0.330
Triglyceride (mg/dl)	39.18±28.42	35.46±15.41	35.78±18.27	0.912
BUN (mg/dl)	13.58±2.94	15.44±2.69	14.12±3.19	0.364
Urea (mg/dl)	29.09±6.30	33.06±5.76	30.24±6.83	0.364
Creatinine (mg/dl)	1.87±0.37	1.84±0.25	1.97±0.51	0.730
Total Protein (g/dl)	9.13±3.48	8.65±3.09	7.57±1.32	0.450
Albumin (g/dl)	3.22±0.69	3.20±1.10	3.06±0.67	0.899
Globulin (g/dl)	5.66±2.92	5.45±2.82	4.51±1.46	0.552

*Blood urea nitrogen (BUN).



HIGHLIGHTS

- Oxidative stress may play a role in the pathophysiology of crib-biting.
- Serum Se concentration was significantly lower in crib biting horses than in controls, with the lowest levels seen during crib-biting behavior.
- Alterations in the serum essential trace element Se, which is an important component of the antioxidant system, may play a role in the pathophysiology of crib-biting behavior in horses.