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Plasma levels of heat shock protein 72 (HSP72) and β-endorphin as indicators of stress, pain and prognosis in horses with colic

Kati E. Niinistö [a,*], Raija V. Korolainen [a], Marja R. Raekallio [a], Anna K. Mykkänen [a], Ninna M. Koho [b], Mirja O. Ruohoniemi [a], Juhani Leppäluoto [c], A. Reeta Pösö [b]

[a] University of Helsinki, Faculty of Veterinary Medicine, Department of Equine and Small Animal Medicine, P.O. Box 57, 00014 Helsinki, Finland
[b] Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki, Finland
[c] University of Oulu, Faculty of Medicine, Department of Physiology, P.O. Box 5000, 900014 University of Oulu, Finland

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ABSTRACT

A prospective observational study was performed to evaluate whether the plasma concentration of heat shock protein 72 (HSP72) or β-endorphin is related to clinical signs, blood chemistry, or severity of pain of colic. Seventy-seven horses with colic and 15 clinically healthy controls were studied. The horses were divided into four groups which reflected increasing severity of colic, from normal control horses to horses with mild, moderate and severe colic. Blood samples were collected before any treatment. Packed cell volume (PCV) and plasma HSP72, β-endorphin, cortisol, adrenocorticotropic hormone (ACTH) and lactate concentrations were measured.

Plasma β-endorphin was related with severity of colic and survival, as well as with plasma cortisol, ACTH and lactate concentrations, heart rate, PCV and pain score. High plasma HSP72 concentration may indicate circulatory deficits, but was not associated with clinical signs of colic. Plasma lactate still seemed to be the most useful single prognostic parameter in horses with colic.

Introduction

Equine colic is a disease with a wide range of causes and variable mortality. Although both the timing for a decision to operate and the level of aftercare have improved, the result is still often death or euthanasia. This was confirmed by recent studies where survival rates for surgical colic were as low as 54% (Grulke et al., 2001; Van der Linden et al., 2003). An estimation of the severity and prognosis for equine colic has therefore been of interest to equine clinicians and various physical and biochemical parameters have been screened and statistical models tested.

To predict the outcome of colic, indices of cardiovascular function, such as systolic pressure, blood lactate concentration and oral mucous membrane refill time, have had the best prognostic value (Moore et al., 1976; Parry et al., 1983; Orsini et al., 1988; Morris et al., 1991; Seahorn et al., 1994). Increased activity of serum tumour necrosis factor (TNF) may also be associated with increased mortality (Morris et al., 1991), while colic severity scores based on plasma lactate and other markers have been developed (Orsini et al., 1988; Furr et al., 1995). In horses with >360° colon volvulus, increased plasma lactate concentration alone has a strong association with the outcome (Johnston et al., 2007), and peritoneal fluid lactate has been found to be a useful marker of intestinal ischaemia and as an aid in diagnosing strangulating ischaemic obstruction (Latson et al., 2005). An elevation in activity of alkaline phosphatase in the peritoneal fluid was reported to be a useful predictor for surgery, while serum alkaline phosphatase was not (Saulez et al., 2004). Sandholm et al. (1995) reported that the D-dimer test was useful, in a combined data model, to predict the prognosis in equine colic, although a later study found no significant difference in the median D-dimer concentration between survivors and non-survivors at the time of admission (Stokol et al., 2005). D-dimer analysis is, however, a useful test for the diagnosis of disseminated intravascular coagulation (Stokol et al., 2005).

Unfortunately, none of these parameters, alone or in combination, can satisfactorily predict the need for surgery or the overall outcome. A promising candidate may however be the relationship between cortisol and the severity of colic (Hinchcliff et al., 2005). Secretion of glucocorticoids is stimulated by adrenocorticotropic hormone (ACTH), which in turn is released in response to several factors, such as pain, trauma, hypoxia, hypoglycaemia, surgery, cold, pyrogens and antidiuretic hormone. ACTH is derived from pro-opiomelanocortin (Ferguson and Hoenig, 2001), which also acts as a pro-hormone for β-endorphin. β-endorphin is released in a similar manner to ACTH in response to stress and is an endogenous opioid with analgesic properties. Experimental studies have shown that sheep with endotoxaemia have high β-endorphin

* Corresponding author. Tel.: + 358 40 5740125; fax: + 358 9 19157281.
E-mail address: kati.niinisto@fimnet.fi (K.E. Niinistö).

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concentrations (Hamilton et al., 1986), while marked elevations in immunoreactive β-endorphin concentrations in plasma have been measured in horses with severe colic (McCarthy et al., 1993).

Another marker that may be useful in monitoring colic in horses are the heat shock proteins (HSPs) which are synthesised by cell, either constitutively (Kregel, 2002) or in response to stress, and are useful in maintaining protein homeostasis (Liu and Steinacker, 2001). The most well known is HSP72, which is synthesised in response to several stimuli, including elevated temperature, hypoxia, adenosine triphosphate depletion, metabolic inhibitors, decreased pH, oxidative stress and exercise (Liu and Steinacker, 2001; Kregel, 2002). HSPs are released into blood during tissue injury (Kimura et al., 2004; Lancaster and Febbraio, 2005) and it has been suggested that HSP72 may activate immune defences (Campisi et al., 2003; Asea, 2005) by optimising antigen processing and presentation (Maridonneau-Parini et al., 1988). Serum HSP72 has also been found to be positively related to the concentration of TNF-α and may limit toxic effects in humans with sepsis (Delogu et al., 1997).

Little is known about HSP72 in the horse, although exercise has been reported to increase mRNA and HSP72 protein in skeletal muscle after exercise (Pösö et al., 2002; Kinnunen et al., 2005). Furthermore, elevated temperature induced the synthesis of HSP70 in equine lymphocytes (Gurriero and Raynes, 1990). The aim of the present study was to investigate whether measurable amounts of HSP72 can be found in the plasma of healthy horses and in horses with colic, and to see if plasma concentrations can be used to indicate the severity of colic and/or relate to other stress related hormones, particularly ACTH and cortisol. We also wanted to examine the relationship between β-endorphin and pain during colic, and compare HSP72 and β-endorphin concentrations with plasma lactate concentrations and mucous membrane colour, as markers of tissue perfusion.

Materials and methods

This was a prospective clinical study with equine colic patients at the Helsinki University Veterinary Teaching Hospital and was conducted between March 2001 and May 2004. The study protocol was approved by the ethics committee of the University of Helsinki. Informed written consent was obtained from all owners of horses.

The study material consisted of 77 horses with clinical signs of colic and 15 healthy control horses that were transported to the hospital for blood donation, farriery or castration. Ponies were excluded. Each horse was included only once in the study. Each horse was examined clinically, and any signs of colic and the tentative diagnosis were recorded. Each horse had signalment, heart rate, oral mucous membrane colour and grading of signs of pain recorded on admission before treatment. In many patients, the diagnosis was later confirmed during surgery or post-mortem examination. Diagnosis of medically treated patients was based on clinical findings and response to treatment.

The horses were divided into four groups reflecting increasing severity of colic, as follows: *controls* were normal horses without signs of colic; *mild* were those horses with mild colic and insignificant findings upon clinical examination; *moderate* were horses with medically treated small intestinal disorders that survived and horses with colonic impactions and colonic displacements; *severe* comprised horses with medically treated small intestinal disorders that did not survive and horses with strangulating obstructions of the small or large intestine.

The oral mucous membrane colour was divided into three categories: normal (1, pink), slight change (2, mildly hyperaemic) and severe change (3, dark red/grey). Two veterinarians subjectively estimated the pain score of the horses by inspecting for clinical signs and behavioural changes. Pain was scored into the following categories: none (0), slight (1), moderate (2), severe (3) and extremely severe (4).

Blood was collected from the horses via jugular venepuncture using a 20 G needle before any treatment at the hospital. In some patients, blood was taken with a syringe together with the placement of a jugular catheter. The blood for HPS72, cortisol and lactate analysis was collected into lithium heparin tubes, and blood for β-endorphin and ACTH analysis was placed into plastic EDTA tubes. Blood was centrifuged at 1000 *g* for 15 min immediately upon collection or after refrigeration for a maximum of 1 h. Plasma was decanted into plain tubes, frozen within 90 min of collection, and stored at −70 °C until analysed. Packed cell volume (PCV) was determined immediately after taking the sample by centrifuging the EDTA sample in a routine manner.

Plasma HSP72 concentrations were analysed using enzyme-linked immunosorbent assay (StressXpress; Hsp70 ELISA Kit EKS-700, StressGen Biotechnologies) According to the manufacturer, the sensitivity of the assay was 200 pg/mL and intra- and inter-assay variations were <10%. Antibodies used in the assay were very similar to SPA-810 and SPA-812 (StressGen), which have previously shown to be specific for equine HSP72 (Kinnunen et al., 2005; M. Atalay, personal communication).

Plasma cortisol concentration was analysed by radioimmunoassay (RIA) (Coat-A-Count cortisol, DPC). According to the manufacturer, analytical sensitivity of the assay was 5.5 nmol/L and the intra- and inter-assay variations were <5.1% and 6.4%, respectively.

For the analysis of plasma β-endorphin and ACTH concentrations, 2 mL of EDTA plasma was extracted with cartridges (Sep-Pak C 18, Waters), using an automatic sample preparation system (Gilson ASPEC, Villiers le Bel). ACTH and β-endorphin were then eluted from the cartridges using 80% acetonitrile in 0.1% trifluoroacetic acid. The eluates were evaporated (Speed Vac Concentrator) and reconstituted in the RIA buffer. In our laboratory the recovery of synthetic ACTH 1-39 and β-endorphin from the cartridges were 61%, (±3%) and 68%, (±6%), respectively (SD). The sensitivity of the ACTH RIA was 0.09 pmol/L and that of β-endorphin 0.29 pmol/L. The intra- and inter-assay variations of the RIAs were <10% and <15%, respectively. The plasma lactate concentrations were analysed using a lactate analyzer (YSI 2300 STAT Plus, YSI).

Data were analysed with a commercial statistical program (SPSS). Our primary parameters, HSP and β-endorphin, were not normally distributed so survivors were compared with non-survivors by Mann–Whitney *U*-test. Spearman's rank correlation test was used for non-parametric correlations between the values and the severity of colic. To assess the mutual correlations of the values, we used logistic regression to predict the probability of death. In addition, if the severity of colic correlated with a certain parameter, each category was compared with controls by Mann–Whitney *U*-test. Statistical significance was set at $P < 0.05$. Data were expressed as medians and ranges.

Results

Of the 77 horses with colic, 51 survived and were discharged from hospital and 26 died or were euthanased. The breeds comprised Finn horses ($n = 29$), Warmblood riding horses ($n = 43$), Standardbred trotters ($n = 16$) and other breeds ($n = 4$). The age, sex and breed distribution of horses with different severity of colic are shown in Table 1. The subjects did not represent all colic cases at the equine hospital, but the selection was based on the availability of clinical investigators. Most of the colic cases were referred and had been given first aid, including alleviation of pain, by the

Table 1
Age, sex, breed and medications used on horses with different severities of colic (control and categories from mild to severe). One horse with mild colic had an unknown previous medication history.

Group	Age mean ± SD	Sex S/M/G[a]	Breed WB/CB[b]	Duration of colic median/range	No medication	NSAID	NSAID + α-agonist[c]	NSAID + α-agonist[c] + butorphanol
Controls $n = 15$	8.1 ± 3.5	3/10/2	12/3					
Mild $n = 13$	8.2 ± 3.4	4/6/3	8/5	4/4–96	4	4	4	0
Moderate $n = 39$	8.1 ± 5.0	2/25/12	26/13	13/13–500	6	25	4	4
Severe $n = 25$	9.4 ± 5.4	3/11/11	15/10	8/2–48	3	8	8	6
All horses $n = 92$	8.5 ± 4.9	12/52/28	61/31	10/2–500	13	37	16	10

[a] Stallion/Mare/Gelding.
[b] Warmblood/Coldblood.
[c] α_2-Adrenergic agonist. NSAID, non-steroidal anti-inflammatory drug(s).

referring veterinarian. The proportion of severe cases included in the study was probably higher than in the total number of cases presented to the hospital.

The arrival of the horses at the hospital ranged 2–500 h (median 10 h) from the time when colic symptoms had first been noted (Table 1). The time of arrival did not differ significantly between survivors and non-survivors. The group with mild colic contained 13 patients without specific findings. Thirty-nine horses were scored as having moderate colic, most with medically treated colic. In this group, all but two survived. Twenty-five horses had severe colic and only one of these was subsequently discharged from hospital; two were euthanased because of their age and owners' financial concerns rather that a poor prognosis. These horses were excluded from Table 2.

The pain score and mucous membrane colour varied from mild to severe colic. The median values and ranges of HSP72, lactate, β-endorphin, cortisol and ACTH in different groups are presented in Table 3. Plasma HSP72 concentration did not vary significantly among groups. Increases in plasma β-endorphin, cortisol, ACTH and lactate concentrations were associated with the severity of the colic. Non-survivors had significantly higher plasma β-endorphin, cortisol, ACTH, and lactate concentrations, heart rate, and

Table 2
Serum concentrations (medians and ranges) of HSP72, β-endorphin, lactate, cortisol, ACTH, heart rate and PCV of survivors compared to non-survivors (*P* of non-survivors compared to survivors).

Group	HSP72 μg/L	β-Endorphin pmol/L	Lactate mmol/L	Cortisol mmol/L	ACTH pmol/L	Heart rate beat/min	PCV %
Survivors	3.16	12.29	1.23	300.1	5.1	44.0	35
n = 51	0.00–41.36	3.43–160.0	0.68–4.55	59.4–898.7	0.2–33.6	26–96	28–49
Non-survivors	4.00	34.57	2.42	484.2	12.89	67.0	39
n = 24	0.00–46.71	8.29–122.3	0.73–21.50	78.1–849.0	2.4–132.2	34–100	28–55
P	0.30	0.006	<0.001	0.001	<0.001	<0.001	0.003

Table 3
Serum concentrations (median, range and *P* compared to controls) of HSP72, β-endorphin, lactate, cortisol, and ACTH in control horses and in horses with different severities of colic (*P* compared to controls).

Group	HSP72 μg/L	β-Endorphin pmol/L	Lactate mmol/L	Cortisol mmol/L	ACTH pmol/L
Controls	3.57	5.71	0.82	232.8	5.78
n = 15	0.00–15.4	1.43–15.7	0.50–1.19	123.6–328.5	4.67–10.89
Mild	3.55	11.43	1.11	314.9	4.0
n = 13	0.00–41.36	3.43–45.71	0.85–2.23	88.88–440.00	0.22–26.22
P	0.98	0.09	0.003	0.32	0.10
Moderate	3.14	14.0	1.32	277.2	5.78
n = 39	0.00–41.36	3.71–160.0	0.68–4.55	59.4–898.7	0.44–33.56
P	0.96	0.01	<0.001	0.27	0.51
Severe	4.10	32.29	2.36	442.8	13.56
n = 25	0.00–46.7	8.29–122.29	0.73–21.5	78.12–849.0	2.44–132.22
P	0.49	<0.001	<0.001	<0.001	0.003

Table 4
Correlation coefficients (*r*) of measured clinical and laboratory parameters and their *P* values.

Correlation	HSP72	Lactate	Cortisol	ACTH	β-Endorphin	Heart rate	PCV	Mucous membrane colour
HSP72	1.00							
P								
Lactate	0.387	1.00						
P	0.001*							
Cortisol	0.157	0.466	1.00					
P	0.17	<0.001*						
ACTH	0.069	0.544	0.665	1.00				
	n = 74	*n* = 74	*n* = 74					
P	0.55	<0.001*	<0.001*					
β-Endorphin	0.007	0.388	0.419	0.644	1.00			
	n = 75	*n* = 75	*n* = 75	*n* = 73				
P	0.95	0.001*	<0.001*	0.001*				
Heart rate	0.109	0.569	0.458	0.435	0.293	1.00		
				n = 74	*n* = 75			
P	0.34	<0.001*	<0.001*	<0.001*	0.01*			
PCV	0.202	0.241	0.344	0.272	0.087	0.441	1.00	
				n = 74	*n* = 75			
P	0.08	0.04*	0.002*	0.19	0.45	<0.001*		
MM colour	0.287	0.365	0.25	0.196	−0.011	0.382	0.298	1.00
	n = 74	*n* = 74	*n* = 74	*n* = 72	*n* = 73	*n* = 74	*n* = 74	
P	0.01*	0.001*	0.03*	0.099	0.93	0.001*	0.01*	
Pain score	0.212	0.435	0.564	0.527	0.389	0.396	0.272	0.301
				n = 74	*n* = 75			*n* = 74
P	0.07	<0.001*	<0.001*	<0.001*	0.001*	<0.001*	0.02*	0.01*

* $P \leqslant 0.05$ is considered significant. There were 76 horses in each category, unless otherwise stated.

PCV than the survivors (Table 2). Pain scores of non-survivors were also higher, as well as severity of changes in mucous membrane colour.

The predictive values of different combinations to the probability of survival or death were calculated. When only three traditional values (lactate, heart rate and PCV) were used as explanatory variable, the overall proportion of correctly classified outcome was 81%. Addition of HSP72 and β-endorphin or cortisol into the model only increased the predictive value to 82%. In horses with colic, plasma HSP72 was correlated positively with lactate concentration and changes in mucous membrane colour (Table 4). Plasma β-endorphin, cortisol and ACTH concentrations were positively correlated with each other.

Discussion

This was a prospective observational study and not all cases that were treated in the hospital were included. Of the 77 clinical cases included, only 13 were categorised as mild. As the patients were already selected cases (colic signs severe enough to need treatment in the hospital), it was not relevant to calculate cut-off values. Although we did not perform statistical analysis, the distribution of the cases appeared to reflect the normal throughput in our hospital, as most of our patients with colic are simple obstructions or displacements. The study appeared to involve a larger percentage of severe cases in comparison to previous studies by Johnston et al. (2007) in which 61/73 horses survived and by Hinchcliff et al. (2005) where 26/35 horses survived. In the current study, 51/75 horses survived.

Large variations existed in the parameters within groups. There are several possible explanations for this finding. The duration of colic probably affected the plasma concentrations, as did the age of the horse. Most of the patients had already been given pain medication, mainly flunixin meglumine, before arrival to the hospital. The effect of these medications on our results is uncertain. Butorphanol (Sellon et al., 2004) and detomidine (Raekallio et al., 1991) are known to decrease serum cortisol concentration. On the other hand, transportation of a horse is known to increase plasma ACTH and cortisol concentrations (Fazio and Ferlazzo, 2003) and circadian rhythms also affect the release of ACTH and cortisol. This may explain, at least in part, the differences with previous studies (Santschi et al., 1991; Hinchcliff et al., 2005).

The duration of colic before arrival at the hospital was not associated with the outcome. Our results here differed from those of Grulke et al. (2001), who found that horses with severe surgical colic were more likely to die as the duration of symptoms increased. Long distances and, consequently, long transportation times may have caused bias in our cases, as some of the severely ill horses died during the transport or were euthanased at home. The delays caused by long transport times sometimes hastened the decision to operate, but probably worsened the prognosis of the horse. On the other hand, some milder colic cases took a long time to resolve. Unfortunately transport times were not recorded.

Plasma lactate concentration was the best parameter tested to predict the outcome and to evaluate the severity of colic. This is consistent with results reported by Orsini et al. (1988), Furr et al. (1995) and Latson et al. (2005). Lactate was also highly related to the other indicators of colic in our study and as in previous work, plasma lactate, cortisol and ACTH correlated with colic severity (Hinchcliff et al., 2005).

Both plasma cortisol and ACTH concentrations increased with colic severity, making both of them possible markers. The correlation between plasma cortisol and ACTH concentrations suggested that measuring either cortisol or ACTH was sufficient. However, both measurements using RIA are time-consuming compared, for example, with plasma lactate, and are not practical when a decision to operate must be made quickly.

Plasma HSP72 concentrations were markedly varied in healthy controls and in horses with colic. Since HSP72 is mainly an intracellular protein, part of the variation may be explained by individual differences in the basic expression of the HSP72 gene and the permeability of cell membranes. In a porcine model, earlier induction of HPS72 attenuated the liberation of inflammatory mediators and haemodynamic changes associated with endotoxaemia (Klosterhalfen et al., 1997). If intestinal ischaemia induces high production of HSP72 in horses with colic, it might protect the intestine in the reperfusion phase as HSP72 has been shown to be cytoprotective when induced (Stojadinović et al., 1997). High HSP72 concentrations might actually be an indicator of protective responses in the intestine, but a high concentration in plasma may also be taken as an indication of necrotic cell death (Basu et al., 2000), or at least a change in permeability of the plasma membrane. Moreover, in strangulating patients the cytoprotective effect of HSP72 may be insufficient, as necrosis progresses rapidly.

Plasma HSP72 concentration was associated with plasma lactate concentration and colour changes in the oral mucous membranes of horses with colic, possibly reflecting perfusion and endotoxaemia. Although recent studies have suggested that HSP72 is actively exocytosed into the circulation during stress (Lancaster and Febbraio, 2005), we did not find any association between plasma HSP72 concentration and severity or outcome of colic. This is consistent with another study in which high HSP72 concentrations were unrelated to outcome in human patients with sepsis (Delogu et al., 1997). In humans and pigs, the main source of plasma HSP72 is the visceral organs, especially the intestines (Febbraio et al., 2002; Sepponen and Pösö, 2006), but whether this applies in horses is unknown.

We investigated systemic HSP72 concentrations in samples taken prior to any treatment at the hospital, and in these samples the concentrations did not differ significantly from one group to another. Examining tissue concentrations of HSP72, especially in strangulated intestinal tissue, or systemic concentrations after relieving the strangulation and followed by reperfusion, might have revealed differences among groups, as strangulation of the intestine may have prevented the release of HSP72 into systemic circulation from ischaemic tissue.

β-endorphin may be a good indicator of severe colic as it correlated with pain and heart rate. The mean β-endorphin concentration was significantly higher in horses with more severe colic. It was also a good indicator of survival or death. Horses with colic are often endotoxaemic, and this may contribute to the release of β-endorphin from the pituitary gland in these horses (McCarthy et al., 1993). A rise in β-endorphin is known to have a hypotensive effect, possibly by acting on the serotonergic pathway (Sandor et al., 1987; de Jong et al., 1989). McCarthy et al. (1993) suggested that the enormous rise of β-endorphin in horses with severe colic was a contributor to shock. Similarly, horses undergoing fracture repair surgery or myopathy have high plasma β-endorphin concentrations which correlate with the subjective pain score (Raekallio et al., 1997).

Conclusions

Colic was more severe and the risk of death higher in horses with high plasma β-endorphin, cortisol and ACTH concentrations. Plasma HSP72 concentration was not a useful indicator of colic severity. Further studies are required to determine whether a high plasma HSP72 concentration indicates circulatory deficits, which would be a useful indicator of endotoxaemia. Practically, heart rate, lactate and PCV are still the most useful parameters.

Conflict of interest statement

None of the authors of this paper has a financial of personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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