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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

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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

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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).