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DOES ROAD TRANSPORT INFLUENCE PLASMA LEPTIN CONCENTRATIONS IN HORSES? PRELIMINARY STUDY*

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Abstract

Transport is one of the most common stressors for horses leading to an increase in cortisol secretion. Cortisol promotes leptin synthesis and release. The aim of the study was to evaluate the effect of short transport on circulating leptin and cortisol concentrations. A total of 16 crossbred naïve horses (7 geldings, 9 mares) aged 2–11 years, and weighing 530–680 kg were included in the study. The horses were transported in a commercial horse-truck to an unknown holding pen for temporary housing. To measure plasma leptin and cortisol concentrations, three blood samples were collected from each horse: before transport, immediately after unloading from the truck, and nine hours after transport at the arrival point. Transport caused a significant increase in mean plasma cortisol concentration determined at unloading, and after nine hours of unloading, in comparison to values obtained before loading. Plasma leptin concentrations did not change during the study. In conclusion, transportation procedures did not influence plasma leptin concentration in horses, despite significantly increased cortisol release.

Key words: cortisol, horses, leptin, stress, transport

Since thousands of years, horses have been used used by human for transportation. In the 20th century, they started to be animals that are transported. However, each phase of transport might be stressful for horses (Padalino, 2015). Transport is one of the most common stressors for horses since it induces hypothalamus-pituitary-adrenal axis activation with the consequent cortisol secretion (Foreman and Ferlazzo, 1996; Schmidt et al., 2010 a; b; c; Tateo et al., 2012; Fazio et al., 2016). Depending on transport conditions and the method used to measure cortisol levels,

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elevated cortisol concentrations can return to baseline values within a period lasting from 30 minutes to 24 hours after the end of transport (Stull and Rodiek, 2000; Stull et al., 2008; Fazio et al., 2008 b; Schmidt et al., 2010 a; b; c; Padalino et al., 2017). It is known that cortisol is a multifunctional hormone, which stimulates i.a. adipose tissue to leptin synthesis and release (Fain and Bahouth, 2000).

Leptin is an adipocyte derived protein adipokine that most of all decreases hunger and increases energy expenditure (Ramsay, 2001; Dotsch et al., 2003; Buff et al., 2002). Moreover, changes in plasma leptin concentration can influence the horse's mood, food intake, energy balance and breeding efficiency (Hemmann et al., 2013; Kędzierski, 2016 b). Generally, in horses, the plasma leptin concentration corresponds positively with body fat mass (Kearns et al., 2006; Gordon et al., 2007 b; Suagee et al., 2013; Fradinho et al., 2014; Selim et al., 2015). However, short-time changes in the circulating leptin level in response to food deprivation without any changes in body fat mass have been observed (Buff et al., 2005; Gordon and McKeever, 2005; Van Weyenberg et al., 2008; Glunk et al., 2015). On the other hand, feed intake and inflammation increased both cortisol and leptin release (Huff et al., 2010; Van Weyenberg et al., 2013). Intravenous infusion of corticosteroids increased plasma leptin concentration in horses within 12 hours (Cartmill et al., 2003 a; b; 2005; 2006). Moreover, exercise lasting at least 45 min resulted in elevated leptin concentration immediately after the end of exercise (Kędzierski, 2014; 2016 b). Therefore, it seems that changes in cortisol concentrations may naturally induce similar changes in leptin release. It is known that in sheep, plasma leptin concentrations correlate positively with the levels of anxiety and fear during transport (Wickham et al., 2015). It can be hypothesized that in horses, road transport may induce an increase in plasma leptin concentration associated with an increase in cortisol release.

The aim of the study was to test the hypothesis that short transport may increase the plasma leptin concentration as a response to elevated cortisol release in horses.

Material and methods

This study was part of a larger project designed to investigate how procedures associated with the use of horses affect the plasma leptin concentration in horses. The study was conducted according to European Community regulations concerning the protection of experimental animals and was approved by the II Local Ethic Review Committee for Animal Experiments of the University of Life Sciences in Lublin, Poland (46/2017).

A total of 16 crossbred horses (7 geldings, 9 mares) aged 2–11 years, and weighing between 530 and 680 kg were included in the study. Prior to the study, the horses had never been transported. They had been pastured and brought to stables at night during the grazing period. In stables, they were kept in box stalls measuring 3 × 4 m with straw bedding. On the day of the study, the horses were loaded onto a horse-truck and transported to a holding pen for temporary housing, which the horses had not previously encountered. For transportation, a commercial horse-truck with trailer

was used with a capacity for 24 horses in individual stalls. Loading proceeded as follows: each horse was led individually to the horse-truck by one person and prompted to move ahead by another person. If the horse was standing in front of the truck, the handler led it away, in a circle and then back to the trailer. All horses were loaded within one hour. None of the horses refused or bucked up at loading, which would have prevented them from continuing loading. The horses were cross-tied individually in stalls during transport. Transport lasted about one hour. Food and water were not available during transport. The horses were transported at night at a mean temperature of 11ºC. After unloading, the horses were kept in stables, tied in a manner allowing free access to feed and water in individual stalls measuring about 2 × 4 m, near other unfamiliar horses. Hay and water were available *ad libitum*. The horses behaved calmly and from time to time chewed hay.

Blood samples were collected by jugular venipuncture to EDTA K3 tubes, according to the following protocol: 1) at rest, in the stable, before transport, at 11 p.m. 2) immediately after unloading from the track, at 2 a.m. 3) nine hours after transport at the arrival point, at 11 a.m. because researchers' availability to study the horses was time-limited. The obtained blood was immediately centrifuged at 2000 × g for 10 min and plasma was stored at –70ºC until assayed.

Cortisol concentration in plasma samples was measured by enzyme-immunoassay method using CORTISOL ELISA kits (DRG Instruments GmbH, Marburg, Germany). All samples were analyzed in duplicate. The absorbance was measured by Multiscan reader (Labsystem, Helsinki, Finland) using a GENESIS V 3.00 software program. The intra- and inter-assay CV for plasma cortisol determined in the laboratory amounted to 6% and 8%, respectively. For plasma leptin determination, the multi-species leptin RIA kit (Millipore/Linco Research, St. Charles, USA) was used. Samples were counted for one minute in a gamma counter (Packard Instruments Company, USA). Antibodies used in the kit were raised against the human leptin. Therefore, the leptin concentration was expressed as ng/ml human equivalent. The inter-assay coefficient of variability (CV) calculated from two runs was 11%. The median of CVs was 10%. The intra-assay CV calculated from duplicate samples was 12%. The median of all CVs calculated from duplicated samples was 11%.

The results are presented as means ± standard deviation (SD). The data were checked with regard to the normality of distribution using Shapiro-Wilk, Kolmogorov-Smirnov and Anderson-Darling tests. The tests did not reject the normal distribution hypothesis for cortisol values, however, they rejected the normal distribution hypothesis for leptin values. Therefore, a generalized linear model, repeated measure analysis of variance (ANOVA–GLM) was used to compare the data for cortisol. The leptin values were compared using the non-parametric analysis of variance (Friedman Test). Comparisons between the cortisol values obtained before transportation, immediately after unloading, and nine hours after unloading were made by the Tukey's Honest Significant Difference (HSD Tukey's test), as a *post-hoc* test. To evaluate the influence of transport, the differences between results obtained immediately after unloading and resting values, as well as the differences between results obtained nine hours after the end of transport and resting values were calculated. The correlation coefficients between these differences for leptin and cortisol values were

assessed by the Pearson test. Statistical analyses were performed using the statistical software package GraphPad Prism™ (Graph Pad Software, La Jolla, CA, USA). The statistical significance was accepted at $P<0.05$.

Results

The results obtained in the study are presented in Table 1. Plasma cortisol concentrations determined at rest ranged from 35.2 to 115 ng/ml, with the exception of one mare in which it was above 350 ng/ml. This mare was excluded from the study. Transport lasting one hour involved significant increase in the mean plasma cortisol concentration in comparison to basal values. After the nine hour period, the mean plasma cortisol concentration was, generally, the same as immediately after unloading the horses from the truck.

Table 1. Plasma leptin and cortisol concentrations in horses submitted to transportation procedures (means ± SD; n=15)

Samples taken:	At rest	After unloading	9 hours after unloading
Leptin (ng/ml)	10.4±8.77	11.4±8.54	10.2±7.91
Cortisol (ng/ml)	59.0±18.1A	96.1±33.6 B	105±30.9 B

A, B – means with different letters differ significantly, $P<0.001$.

The mean plasma leptin concentration did not significantly change during the study. The correlation coefficient obtained for the differences between leptin and cortisol values determined immediately after unloading and before loading the horses onto the truck, and nine hours after the end of transport and before loading were statistically insignificant ($r=0.09$, $P=0.89$ and $r=0.01$, $P=0.94$; data not tabulated).

Discussion

This study was performed to evaluate the effect of short transportation on cortisol and leptin levels in horses. The obtained results do not support the hypothesis that leptin would have increased due to the transport related peak of cortisol. The plasma cortisol concentration was elevated up to nine hours after the end of transportation because of the new environment and confinement, probably.

Despite transport-related significant increase in plasma cortisol concentrations, which remained elevated up to nine hours after the end of transportation, the plasma leptin concentration was unchanged in the horses under study. This was surprising since numerous studies have reported that increases in corticosteroids induce an increase in circulating leptin concentrations (Cartmill et al., 2003 a; b; 2005; 2006; Huff et al., 2010; Van Weyenberg et al., 2013). On the other hand, other authors report that in some cases, plasma cortisol and leptin levels can change independently

of each other (Van Weyenberg et al., 2008; Gordon et al., 2007 a; Storer et al., 2007). For example, endurance effort lasting about 10 hours induced an almost two-fold increase in plasma cortisol concentration without significant changes in leptin values (Kędzierski and Cywińska, 2014). In Purebred Arabian race horses, plasma cortisol concentration increased eight-fold during the training season whereas leptin concentration remained unchanged (Kędzierski, 2016 a).

Generally, plasma cortisol and leptin concentrations obtained in the studied horses were in the same range as reported by other researchers (Cartmill et al., 2003 a; Buff et al., 2005; Huff et al., 2010; Van Weyenberg et al., 2013, Kędzierski, 2016 a). The study lasted about twelve hours, so the circadian variations in plasma cortisol and leptin concentrations should be taken into consideration. A circadian rhythm in the plasma cortisol concentration, with a peak in the morning and nadir in the evening, has been reported in some but not all studies (Irvine and Alexander, 1994). This rhythm exists in horses accustomed to their environment. The circadian rhythm can be obliterated by changes in daily routine (Irvine and Alexander, 1994). The studied horses had their daily routine interrupted. In the case of leptin, a circadian rhythm did not exist. The plasma leptin concentration decreases in response to fasting, and increases 20 min after feeding (Buff et al., 2005; Gordon and McKeever, 2005). Moreover, the horses studied differ as to age. However, plasma leptin concentration correlates rather with body fat mass than with the age of an animal (Buff et al., 2002).

It is known that in mammals, leptin synthesis is directly promoted by corticosteroids via intra-cellular glucocorticoid receptors and leptin mRNA increase (Lee et al., 2007). However, leptin biosynthesis, storage and release are also regulated by using alternative pathways, including activation of membrane G-coupled receptors, A1 adenosine receptors, regulation of intra-cellular cAMP levels, and p38 mitogen-activated protein kinase (Rice et al., 2000; Kanu et al., 2003; Trujillo et al., 2006). Thus, leptin release from adipocytes is regulated by various pathways controlled by different factors. Increased insulin levels stimulate leptin release, whereas adrenaline plays an opposite role (Meier and Gressner, 2004). It is known that adrenaline secretion increases with fear and/or during exercise (Hada et al., 2001). In turn, adrenalin supresses insulin release. Transport can induce fear in horses (Schmidt et al., 2010 b). Moreover, since transport requires some effort from the horses (e.g. maintaining their balance) during road transport, it can be supposed that during transport, adrenaline release increases, and insulin release decreases.

In this study, in contrast to the results of other studies (Stull et al., 2008; Söder et al., 2012), plasma cortisol concentrations in horses, and thus stress levels, remained elevated nine hours after transport. Cortisol release ratio in transported horses depends on many factors; for example inexperience in being transported, water deprivation and cross-tying in stalls during transportation can increase cortisol release (Friend et al., 1998; Stull, 1999; Stull and Rodiek, 2002; Fazio et al., 2008). Probably, the new environment in the holding pen was stressful for the studied horses, which were still affected by unknown surroundings. It is known that moving horses to an unfamiliar environment increases their adrenergic nervous system activity and catecholamines secretion (Hada et al., 2001). This increased adrenergic response may last at least three days (Janczarek and Kędzierski, 2011 a; b). Thus, despite

nine hours of rest, the studied horses were probably still aroused, and catecholamine release rate was elevated. In this situation, the stimulating effect of cortisol on leptin release from adipose tissue was inhibited by increased adrenergic nervous system activity. Therefore, plasma leptin concentration could remain unchanged. This means that transport procedures did not influence plasma leptin concentrations despite elevated cortisol release. Therefore, the regulation of appetite, food intake and energy expenditure via leptin remained unaffected. This fact can be considered as beneficial for horses. It is known, that elevated levels of leptin inhibit food intake, which can disturb the restoration of energy reserves and the functioning of digestive tract in horses (Husted et al., 2009). On the other hand, it was reported that a low plasma leptin concentration was associated with behavioural disorders in horses (Hemmann et al., 2013). Such findings indicate that in horses, the circulating leptin should have an optimal level.

Limitation of the study was the relative small number of samples which caused that transport-induced cortisol and leptin kinetics have not been tested. The scientific protocol used in this study did not allow differentiating between the transport and the new environmental effects. There were studied only horses which were moved from pasture to stable the day before the transportation, without control group. Finally, the leptin data were analyzed by non-parametric test.

To sum up, this preliminary study suggests that short transport might not influence plasma leptin concentrations in horses, despite significant increase in cortisol release. Further research is needed to ascertain those findings.

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