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Short Communication

Repeatability of the ACTH stimulation test as reflected by salivary cortisol response in healthy horses

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ABSTRACT

The aim of this study was to further characterize the ACTH stimulation test as reflected by salivary cortisol response and to measure the short- and long-term repeatability of it in healthy horses as a tool to assess the capacity of the adrenal cortex to secrete cortisol. Nineteen healthy horses were subjected to 3 ACTH stimulation tests. Intervals were 2 wk and 5 mo between the first and second and the second and third tests, respectively. A dose of 1-μg/kg BW synthetic ACTH was injected intravenously. Saliva samples were collected at baseline and at 30, 60, 90, 120, 150, and 180 min after administration for cortisol measurements using a competitive enzyme immunoassay. A repeated measures ANOVA was used to compare values within and among horses. Mean ± SD total increase in cortisol concentrations integrated over the entire sampling period was 34.5 ± 11.0 ng/mL. The highest measured concentration at a single time point was 9.7 ± 2.7 ng/mL and was reached after 122 ± 22 min. For the short- and long-term repeatability, intraclass correlation coefficient was 0.90 and 0.33, respectively. The 3 ACTH stimulation tests results differed significantly among ($P < 0.00001$) but not within ($P = 0.538$) individual horses. The Freiberger stallions had a higher salivary cortisol baseline concentration and a lower response to ACTH stimulation as compared with Warmblood mares and geldings. The present study confirmed that the administration of ACTH in healthy horses reliably stimulates the salivary secretion of cortisol and shows that the test is repeatable in the short- and long-term.

1. Introduction

The ACTH stimulation test is used for stress and welfare assessment as well as for diagnostic purposes in animals [1]. Synthetic ACTH1-24 is injected intravenously; blood or saliva samples are collected before (baseline) and at defined intervals after injection for cortisol concentration measurements. The increase in cortisol concentration in response to ACTH stimulation allows the evaluation of adrenal cortex function thereby potentially offering an assessment of the residual responsiveness to stressors [2].

The ACTH stimulation test was first developed in human medicine in the 1960s [3]. The range of applications includes diagnosis of adrenal insufficiency [4] and hypothalamo-pituitary-adrenal axis evaluation, for instance in the context of psychological disorders such as depression [5], autism [6], and posttraumatic stress disorder [7].

Modern horses are subjected to various sources of stress such as training and exercise [8,9], racing and competition [10,11], transportation [12,13], isolation [14,15], social interactions [16], sexual excitement [17], hot iron branding, and microchip implantation [18]. It has been suggested that chronic stress can alter adrenal function in animals, but the

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exact mechanisms are not yet understood. Decreased response of the adrenals to ACTH in relation to chronic stress has been described in cattle [19,20]. In contrast, chronically stressed pigs show increased adrenal cortex sensitivity to ACTH [21,22].

Few studies have assessed adrenal function in horses, and the results remain controversial. Several authors demonstrated that hospitalized foals exhibit a blunted cortisol increase in response to ACTH, compared with healthy foals [23–25]. Hedberg et al [26] found similar results in mares with behavioral problems related to estrus. Interestingly, normal responses to ACTH were found in overtrained horses [27], older mares [28], and ovariectomized mares [29]. In contrast, Briefer-Freymond et al [30] found an increased cortisol response to ACTH in horses with stereotypic behavior (crib-biting). Furthermore, horses suffering from pituitary pars intermedia dysfunction also showed hyperreactivity to ACTH [31,32].

The differences between published results suggest that confounding factors other than stress may influence test results. Genetic and external factors, such as housing and environmental conditions, could also affect adreno-cortical sensitivity. Recent studies have attempted to validate the ACTH stimulation test in horses [2,33] but, to our knowledge, the repeatability of the cortisol response to ACTH has not been characterized yet.

The goal of this study was therefore to further characterize the ACTH stimulation test as reflected by salivary cortisol levels and to measure the short- and long-term repeatability in healthy horses to determine if the ACTH stimulation test can be used as a reliable tool to assess the capacity of the adrenal cortex to secrete cortisol.

2. Materials and methods

2.1. Population

This study was performed at the Swiss National Stud Farm and was approved by the cantonal veterinary office of Vaud (VD2660-23529). Nineteen healthy horses were selected including 9 Warmblood (6 mares and 3 geldings) and 10 Freiberger stallions ranging in age from 4 to 20 yr (mean ± SD, 10.8 ± 4.1) and weighing 488 to 645 kg (mean ± SD, 563 ± 44). Horses were housed on straw in their usual individual box stalls and were accustomed to frequent handling. They were fed hay only during the test (approximately 1% BW), but to avoid dilution of saliva, they had no access to water during the sampling procedure.

2.2. ACTH stimulation tests

All horses were subjected to 3 ACTH stimulation tests. To assess the short- and long-term repeatability, the first 2 ACTH stimulation tests were performed within 2 wk of each other in September 2014, and the third one was completed 5 mo later in March 2015. In agreement with Briefer-Freymond et al [30], tests were performed at 1 PM and lasted until 4 PM. Based on Peeters et al [2] and Stewart et al [33], 1-μg/kg BW of synthetic ACTH1-24 (Synacthen tetracosactidum 0.25 mg/mL equivalent to 25 IU/mL, Novartis, Vilvoorde, Belgium) was injected intravenously. Saliva samples were collected using a Salivette (Sarstedt, Nümbrecht, Germany) before (0 min–baseline) and 30, 60, 90, 120, 150, and 180 min after injection. Saliva samples were centrifuged at 529g for 10 min, and then stored at −70°C until assayed.

2.3. Cortisol assay

Salivary cortisol concentrations were determined using a competitive enzyme immunoassay (cELISA, Salimetrics, Newmarket, UK). According to the manufacturer, the sensitivity of the assay was <0.03 ng/mL. Validation tests for use of cortisol in equine saliva were performed in our laboratory with 1 sample each. Recovery was tested with spiked samples. A native sample (0.81 ng/mL) was spiked with different standard concentrations (1.11, 3.33, 10, and 30 ng/mL). The recovery calculated for every concentration ranged from 98% to 104%. Dilution of a sample (1.4 ng/mL; dilution factors: 1:2, 1:4, 1:8, and 1:16) showed full parallelism with the standard curve, and recovery was around 100%. Intra-assay and interassay CVs determined in our laboratory were 6.4% and 4.0%, respectively.

2.4. Statistical analysis

Statistical analysis was performed using the software package NCSS9 (NCSS Statistical Software, Kaysville, UT, USA). Cortisol concentrations of individual horses were described for each ACTH stimulation test by the baseline value (BL), the highest measured concentration and the total increase in cortisol concentration during the entire sampling period (area under the curve corrected for the baseline, dAUC). Check for normal distribution was done by visual assessment of normality plot and Shapiro–Wilk W test. Because cortisol values were normally distributed, they are presented as mean ± SD. Association between the dAUC of the 3 ACTH stimulation tests was assessed by Pearson's correlation coefficient and linear regression. A repeated measures ANOVA was used to compare cortisol concentrations within and among horses. The intraclass correlation coefficient (ICC) was calculated from the output of the ANOVA model to estimate the reliability of the ACTH stimulation test. Calculation of ICC was performed according to the method described by Shrout and Fleiss [34]. Alpha level of statistical significance was set at $P < 0.05$. The sample size was calculated with the software PASS13 (NCSS Statistical Software, Kaysville, UT, USA). According to this analysis, a sample of 19 horses allows us to detect a correlation coefficient of at least $R = 0.6$ with a power of 80%.

3. Results

3.1. Characterization of ACTH stimulation test results

The results are illustrated in Figure 1. In total, 399 saliva samples were collected from 19 horses each tested 3 times. Before ACTH administration, the baseline salivary cortisol concentration ranged from 0.3 to 2.3 ng/mL (mean ± SD, 0.8 ± 0.3). The cortisol concentration was significantly increased 30 min after ACTH administration (repeated measures ANOVA, $P < 0.00001$). The highest measured