



Original Research

Overnight dexamethasone suppression and cortisol index tests in clinically healthy horses and with crib-biting

J.J. Osorio-Cardona, V.M. Usuga-Moreno , J.R. Martínez-Aranzales *

Equine Medicine and Surgery Research Line (LIMCE), Centauro Research Group, School of Veterinary Medicine, Faculty of Agricultural Sciences, University of Antioquia, Medellín 050010 Colombia

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ABSTRACT

Background: The dexamethasone suppression test (DST) has been used in humans for the diagnosis of psychiatric problems such as depression, anxiety and Cushing's syndrome, and in horses for the *ante mortem* diagnosis of dysfunction of the intermediate part of the pituitary.

Aims/objectives: This study aimed to examine the functionality of the hypothalamic-pituitary-adrenal axis through the DST and the cortisol index (CI), to evaluate the rhythmicity of the circadian cycle of cortisol in healthy horses with crib-biting or windsucking.

Methods: A total of 20 Colombian Creole horses of both sexes, under similar management conditions and a complete stabling system, formed two study groups: crib-biting horses (G1) ($n = 10$) and non-crib-biting horses (G2) ($n = 10$). Blood samples were taken in the morning and afternoon to determine the CI, and immediately after collection in the afternoon, the DST protocol was established. The serum cortisol concentration was determined through sandwich ELISA at all established times and after dexamethasone treatment for each group.

Results: Serum cortisol concentrations were equal in both groups (G1: $9.52 \pm 7.01 \mu\text{g/dl}$; G2: $8.4 \pm 5.30 \mu\text{g/dl}$), with no difference in CI, and DST was positive in all animals regardless of their clinical condition.

Conclusion: The DST and the CI did not distinguish horses with and without crib-biting. It is necessary to implement techniques with greater precision in longitudinal studies and establish specific reference values and cutoff points for the interpretation of cortisol suppression for cases of crib-biting, accompanied by other hormones such as adrenocorticotrophic hormone and corticotropin-releasing hormone, owing to their multifactorial nature and high cortisol variability.

1. Introduction

Crib-biting or wind sucking is a classic and oral stereotypy of high presentation in horses under different management systems; it is characterized by its multifactorial nature and still unknown etiology [1,2]. Therefore, several hypotheses have been described within its pathophysiology, which involves neuroendocrine physiology and brain function with alterations in the concentrations of endogenous serotonin, dopamine, cortisol, and opioids [1–4]. Additionally, low levels of endogenous antioxidants and enzymatic cofactors have been reported in horses with crib-biting [5,6].

Recently, differences in the composition and structure of the fecal microbiome have been described between horses with and without crib-biting, indicating the possible participation of the bacteria–intestine–brain axis, which is associated with functional profiles and the

generation of specific active [7,8], as described in humans with behavioral alterations [9,10]. However, more studies are needed because of the variability in intestinal bacterial populations in response to various factors.

Regardless of the cause of crib-biting, it harms the health of equines, as it is associated with various types of colic of varying severity [11–13], gastric ulceration [14], wear of incisor teeth and temporohyoid osteoarthropathy [15,16], decreased fertility in females [17], weight loss and body condition [18], lack of concentration, low performance [4], and commercial devaluation of animals [19].

Among the various predisposing factors of crib-biting, stress has been described as a common element in various situations related to stereotypies, where the concentration of cortisol plays a fundamental role [20, 21]. However, this analyte is characterized by high variability in the different species of Equidae, management methods, environmental

* Corresponding author.

E-mail address: jose.martinez@udea.edu.co (J.R. Martínez-Aranzales).<https://doi.org/10.1016/j.jevs.2025.105628>

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conditions, and zootechnical purposes [22–25]. Therefore, the baseline determination and dynamics of cortisol in many horses do not reflect the intensity of stress, since high concentrations are not constant in patients with crib-biting [26].

The dexamethasone suppression test (DST) has been used in veterinary medicine for the *ante mortem* diagnosis of pituitary pars intermedia dysfunction (PPID) in horses [27] and in humans for the diagnosis of Cushing's syndrome and psychiatric disorders such as depression and anxiety [28,29], by indirectly evaluating the integrity of the hypothalamic-pituitary-adrenal (HPA) axis through changes in the concentration of cortisol.

Given the variability of cortisol concentrations in response to stressors and in horse patients with crib-biting, this study aimed to evaluate this analyte, with morning baseline and afternoon baseline measurements, to determine the cortisol index (CI) and DST to determine its participation or dynamics in horses with crib-biting.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee for Animal Experimentation (CEEa) of the University of Antioquia, Medellín (Colombia) (Approval number 24-27-962; Approval date, 13 August, 2024).

2.2. Horses of study

A total of 20 Colombian Creole horses of both sexes, with similar handling and feeding conditions, under a complete stabling system and originating from the same location, were selected and divided into two groups. Group 1 consisted of 10 horses with crib-biting (9 females and 1 male), with mean values were weight, 288 ± 25 kg; age, 5.9 ± 3.6 years; and body condition score, $5.9 \pm 0.7/9$. Group 2 included 10 horses without crib-biting (5 females and 5 males), with mean values were weight, 293 ± 35 kg; age, 6.4 ± 2.5 years; and body condition score, $6.5 \pm 0.7/9$. Weight was determined using a weight tape, age was estimated by dental chronometry, and body condition score was assessed according to Henneke et al. [30]. All horses were fed hay *ad libitum* with *Dichanthium aristatum* or commercial balanced feed (2 kg/d) (Campeón Dorado®, Solla, Itagui, Colombia) in two rations, approximately 60 g/d of a mineral supplement (Brío salts®, Italcol, Girardota, Colombia), and free access to water.

All the selected horses had a complete and updated health plan and a periodic dental care program. As an exclusion criterion, the animals diagnosed and treated for gastrointestinal disorders in the previous 6 months were not included. Horses with crib-biting (Group 1) were identified and verified by caretakers and researchers, respectively. Horses without crib-biting (Group 2) were defined as clinically healthy,

caregivers and verified for the researchers by direct observation.

2.3. Blood sample collection

After calm handling and previous antiseptic preparation of the puncture site of the jugular vein, blood samples were collected in vials without anticoagulant and subsequently centrifuged at $201 \times g$ for 10 min, after which the serum was collected and kept frozen (-20°C) until analysis. Samples were taken from each animal in each group at 07:00–08:00 and 16:00–17:00, and a third sample was taken 19 to 24 h after the second and following the administration of dexamethasone. Finally, the horses did not undergo exercise routine during the blood collection periods.

2.4. Dexamethasone suppression test (DST)

A standardized TSD protocol was followed. In summary, a blood sample was collected between 16:00 and 17:00, followed by an intramuscular injection of dexamethasone at a dose of $40 \mu\text{g/kg}$. A second blood sample was obtained 19–24 h after dexamethasone administration [27].

2.5. Determination of the serum cortisol concentration

To determine the serum cortisol concentration, a competitive ELISA commercial kit (AccuBind, Monobind, Inc., Lake Forest, CA 92630, USA) was used. The ELISA dishes were analyzed on a UV/VIS spectrophotometer with a wavelength range of 200–1000 nm (MULTISKAN GO with Skanlt Software 6.1.1 for Multiskan GO). The total samples and the calibrators were analyzed in duplicate, and for the validation of the obtained results, the following quality control parameters were considered: the absorbance (OD) of the calibrator “0” $\mu\text{g/dl}$ was ≥ 1.8 , and the concentrations of the two standards used as controls in the assembly were close to the concentration reported by the kit manufacturer. The serum cortisol concentration was expressed in $\mu\text{g/dl}$. The AccuBind ELISA Test System test kit has a sensitivity of 91.5 pg, as reported by a commercial company and verified by the manufacturer; the variability of the 0 ng/ml calibrator was determined, and the 2σ statistic (95 % certainty) was used to calculate the minimum dose. Additionally, duplicate determinations of serum cortisol were considered in the evaluated periods of each test and in both groups of horses to determine the intra-assay and interassay coefficient of variation.

2.6. Determination of the serum cortisol index (CI)

The CI was calculated to assess the rhythmicity of the circadian cycle for each horse in both study groups. The CI was determined using the following formula:

$$CI = \frac{(\text{Highest serum cortisol concentration} - \text{Lowest serum cortisol concentration})}{\text{Highest serum cortisol concentration}}$$

without other oral or locomotor stereotypies. To avoid errors in the identification of stereotypies, the interviewees received prior training to identify such behaviors, through oral description of crib-biting and presentation of videos. The occurrence of a crib-biting event in a horse was characterized by neck muscle flexion and contraction, along with a noise produced by the suction of air, either with (crib-biting) or without (wind sucking) the contact of the upper incisor teeth against surfaces [20–22]. Information on the type of crib-biting, daytime behavior, and years of occurrence was obtained through surveys completed by

A CI value greater than 0.30 was considered “normal,” as described by Douglas [31].

2.7. Statistical analysis

The results were analyzed via descriptive statistics, including means, standard deviations, and percentages. To determine the normality of the data, the Shapiro–Wilk test was used. For the comparison of means with

Table 1

Description, type, time of day and time of completion of crib-biting for each of the 10 selected horses.

Horse	Type crib-biting	Time of day	Time (years)
1	With and without support	Any	3.5
2	With and without support	After eating	0.5
3	With support	Any	8
4	With support	Any	1
5	With support	Any	6
6	With support	Any	5
7	With support	Any	3.5
8	Without support	Any	4
9	With support	After exercise	2
10	With support	Any	3.5

a normal distribution in each group and between groups, Student's t-test was used for related and independent samples, respectively. For variables without a normal distribution, the Mann–Whitney U test was used. To determine a statistically significant difference, $p < 0.05$ was considered.

3. Results

A description of crib-biting events of the selected equines is shown in Table 1. Most equines performed crib-biting with support, and a minority did so without support or both. The approximate time of completion ranged between 6 months and 8 years, with most cases showing without time of preference for its execution.

Intra-assay and interassay coefficients of variation for the serum cortisol samples were 7.2 % and 12.1 %, respectively. The baseline values of the morning and afternoon serum cortisol concentrations and the CI for each group are presented in Table 2. Cortisol concentrations, although tending to be higher in the morning than in the afternoon in both groups, were not statistically different. Additionally, the comparison of CI means between groups showed lower values in crib-biting horses, although without significant differences. Regardless of the condition, the overall CI meaning for both groups was below 0.30.

The baseline values (afternoon) of serum cortisol and post-dexamethasone cortisol concentrations, because of the DST, together with the estimation of the suppression percentage for both groups, are presented in Table 3. A comparison of the pre- and post-dexamethasone means in each group revealed significant differences; however, the mean values of the percentages between each group were similar. No adverse effects were reported for the application of dexamethasone in either group of horses.

Table 2

Serum cortisol concentration in ug/dl obtained in the morning (SCC-AM) and afternoon (SCC-PM), and cortisol index (CI) of non-crib-biting horses and crib-biting horses.

Non-crib-biting horses				Crib-biting horses			
Horse	SCC-AM	SCC-PM	CI	Horse	SCC-AM	SCC-PM	CI
1	6.61	2.77	0.58	1	14.13	11.70	0.17
2	4.29	2.82	0.34	2	19.11	17.08	0.10
3	4.32	2.74	0.36	3	9.89	5.31	0.46
4	6.16	4.89	0.20	4	4.81	2.22	0.53
5	17.74	10.40	0.41	5	23.02	11.62	0.49
6	4.17	4.70	0.11	6	4.41	4.35	0.01
7	10.41	9.68	0.07	7	6.89	7.15	0.03
8	6.85	10.72	0.36	8	6.85	15.41	0.55
9	6.06	5.08	0.16	9	4.020	3.74	0.06
10	18.11	15.46	0.14	10	2.122	1.70	0.19
Mean (± SD)	8.4 ± 5.30	6.92 ± 4.35	0.28 ± 0.16	Mean (± SD)	9.52 ± 7.01	8.03 ± 5.54	0.26 ± 0.22

SD: Standard deviation.

Table 3

Serum cortisol concentration in ug/dl obtained in the afternoon (SCC-PM), serum cortisol concentration in ug/dl obtained 19–24 h post-dexamethasone (SCC-DEX) and suppression percentage (Suppr %) of non-crib-biting horses and crib-biting horses.

Non-crib-biting horses				Crib-biting horses			
Horse	SCC-PM	SCC-DEX	Suppr. (%)	Horse	SCC-PM	SCC-DEX	Suppr. (%)
1	2.77	0.81	71	1	11.71	3.31	72
2	2.82	0.53	81	2	17.09	2.59	85
3	2.74	1.00	63	3	5.31	2.10	61
4	4.89	0.95	81	4	2.23	0.90	60
5	10.40	1.03	90	5	11.62	2.83	76
6	4.70	1.28	73	6	4.35	1.35	69
7	9.68	1.46	85	7	7.15	2.15	70
8	10.72	1.00	91	8	15.41	0.75	95
9	5.08	1.57	69	9	3.75	0.68	82
10	15.46	1.76	89	10	1.71	0.70	59
Mean (± SD)	6.9 ± 4.3 ^A	1.14 ± 0.35 ^B	79 ± 10	Mean (± SD)	8.03 ± 5.26 ^a	1.73 ± 0.93 ^b	73 ± 12

Columns of means with superscript letters different, with a significant difference at the Student's T-test ($p < 0.05$).

4. Discussion

Because that cortisol is considered the hormone of adaptation to different stress-generating activities stress [25,32–34], significant changes are expected in horses that exhibit stereotypical abnormal behaviors, such as crib-biting, once they are interpreted as a lack of well-being or response to activities as opposed to natural behaviors [35]. However, several studies have reported different trends than expected concerning the dynamics of cortisol and other hormones in horses with crib-biting [6,19,36]. This behavior was similar in the animals of this study, where changes occurred in all animals, regardless of whether they were with or without crib-biting.

Despite the variability of cortisol reported in previous studies, it is still considered one of the hormones with a central role in crib-biting since it is related to stress caused by several predisposing factors, such as social isolation and housing or the absence of grazing [21,36]. In addition, a decrease in CI indicates chronic stress, since the disruption of the circadian rhythm reflects the inability to cope with stress [37], which could generate stereotypies [32,34]. However, both groups in the present study were similar in terms of CI, although horses without crib-biting presented with chronic stress and horses with crib-biting without alterations in the circadian rhythm, indicating the involvement of other factors, which may not have been controlled for in both populations.

Therefore, the CI can predict chronic stress in equines [31,38]; however, the alteration of the circadian rhythm is not an indicator of the presence of crib-biting, as has been reported in previous studies [39,40], and in the present study, where the general mean CI in both groups was similar and less than 0.30. However, the presence of stress in both groups was due to activities related to the housing system [21,36]. The CI was variable in several individuals independent of their clinical condition, and the concentration of cortisol in the morning was greater than the concentration in the afternoon in both groups of horses, a behavior expected and considered normal for the circadian rhythm of this hormone [22,41,42].

The DST has been used for the diagnosis of depression and anxiety in humans since these behavioral alterations are related to high concentrations of cortisol [43,44]; however, its use has decreased in the clinic due to its low sensitivity and predictive specificity. This has led to the rethinking of other diagnostic techniques to replace immunoassays and the development of reference intervals and specific cutoff points for each method [45,46]. The present study sought to determine the applicability of DST in the response of hypercortisolemia in crib-biting patients;

however, the suppression was similar to that in the group of healthy horses, and all the horses responded positively to the test, indicating an HPA axis without apparent alterations in both groups.

The lack of difference in the DST response between the groups in this study can be explained by several factors, which involve aspects of the cortisol measurement technique, the substrate used, and the absence of cutoff values, as mentioned above. Differences in the specificity of immunoassays have been reported [47], where overestimation due to cross-reactions with other steroids and low selectivity of protein-bound fractions has encouraged the implementation of methods with greater precision and independence of the type of sample, such as liquid chromatography, combined with mass spectrometry [48,49]. Therefore, it is necessary to implement techniques of greater precision in horses with crib-biting for the determination of new reference values and cutoff points for specific clinical conditions since existing methods were established for the diagnosis of PPID.

Recent pharmacokinetic and pharmacodynamic studies of the response of cortisol and the interaction with dexamethasone have indicated adjustments in the DST to reduce the number of false positives by establishing the dose and time of sampling with greater accuracy [50, 51]. Aspects of relevance for future studies, the interpretation parameters of positive cortisol suppression for PPID ($> 1 \mu\text{g/dl}$) were established with cutoff points without considering the variability of the wide range of cortisol values, as in the case of the equines in this study, which—under tropical conditions, presented high reference values [25], which exceeded those of seasonal countries.

Additionally, factors inherent to each management and environmental condition [25,52,53], possibly influenced the comparison of the basal cortisol concentration of the studied groups, although the management, design, and material conditions of the facilities were similar; however, the frequency and time of execution of the crib-biting episodes were heterogeneous since their execution works as a mechanism to balance cortisol concentrations as part of the process of adaptation to stress [19,33,34,40,54]. This could explain the normal cortisol concentrations reported in some horses with crib-biting.

In cases of PPID, normal blood cortisol levels are described due to alterations in peripheral metabolism and increased urinary excretion of glucocorticoid metabolites, indicating a possible increase in cortisol clearance in this clinical condition [55,56]. However, it is not known whether this same behavior occurs in the hypercortisolism of horses with crib-biting, which explains the normal concentration of this analyte in horses with stereotypy. Additionally, the parameters for the interpretation of the DST were established for the diagnosis of PPID, and the establishment of cutoff points and reference values for the condition is imperative since there are differences in the pathophysiological pathways of both clinical conditions.

5. Conclusions

Under the conditions of this study, CI and DST failed to demonstrate differences in cortisol dynamics between horses with and without crib-biting. In addition, the basal cortisol concentrations were similar in both groups; therefore, it is necessary to implement new techniques of greater precision to determine reference values and cutoff points that allow the establishment of suppression percentages for crib-biting, since the existing ones are for PPID, which despite compromising the HPA axis in both conditions, a particular metabolism of glucocorticoids has been described in the latter. The foregoing guarantees the development of longitudinal studies with a greater number of animals, different sampling times related to the performance of the crib-biting event, and higher precision techniques that involve other hormones, such as ACTH and CRH, once the variability of the cortisol concentration confirms the multifactorial nature of crib-biting.

Ethics in publishing statement

This research presents an accurate account of the work performed, all data presented are accurate and methodologies detailed enough to permit others to replicate the work.

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All authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

CRediT authorship contribution statement

J.J. Osorio-Cardona: Data curation, Formal analysis, Investigation, Methodology. **V.M. Usuga-Moreno:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **J.R. Martínez-Aranzaes:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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