Evaluation of dynamic testing for pituitary pars intermedia dysfunction diagnosis in donkeys

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Summary

Background: Endocrine disorders are common in donkeys. Pituitary pars intermedia dysfunction (PPID) is thought to be a frequent disturbance in donkeys due to their longevity. However, information on PPID dynamic testing in donkeys is lacking.

Objectives: The objective of this study was to evaluate the previously described guidelines for PPID diagnosis in horses with suspicion of PPID.

Study design: Prospective experimental study.

Methods: Eighty donkeys were evaluated for PPID suspicion based on clinical signs and baseline adrenocorticotropic hormone (ACTH) concentrations. Six mix-breed donkeys (one jack and five non-pregnant jennies) fulfilling inclusion criteria were subjected to dexamethasone suppression test (DST), thyrotropin-releasing hormone stimulation test (TRH) and combined DST–TRH challenge. Tests were interpreted according to guidelines for PPID diagnosis in horses.

Results: Donkeys fulfilling inclusion criteria were diagnosed with PPID by TRH stimulation test (six of six). Both DST (three of six) and DST–TRH (4/6) challenges failed to detect those animals and showed conflicting results. Similarly, cortisol basal concentrations were not consistent with PPID suspicion.

Main limitations: Characterisation of seasonal and geographical location effect on baseline ACTH concentrations and response to TRH is compelling in this species. Further studies with a larger number of donkeys are needed.

Conclusions: This is the first study in donkeys to evaluate common dynamic tests used for PPID diagnosis in horses. Preliminary results agree with the guidelines for PPID diagnosis in horses and support the use of TRH challenge. This study suggests that a DST–TRH test could be a more appropriate test in donkeys than a DST alone.

Keywords: horse, combined dexamethasone-TRH test; dexamethasone suppression test; donkeys; PPID; TRH stimulation test

Introduction

Although metabolic and endocrine disturbances, such as pituitary pars intermedia dysfunction (PPID), insulin dysregulation, metabolic syndrome and dyslipidaemias, are common in donkeys [1,2], epidemiological and clinical information concerning these diseases are scarce in this species. This lack of species-specific studies constrains clinicians to extrapolate data previously reported for horses or make proper interpretation of results.

Physiological differences have been demonstrated between donkeys and horses, for example, in haematology, biochemistry and haemostatic variables [3–6], and also regarding metabolic and endocrine hormonal regulation [7–9]. Thus, extrapolating data and diagnostic protocols between species could lead to misdiagnosis and subsequent inappropriate management in false-negative cases as well as unnecessary treatments and financial expenses in false-positive cases.

PPID is considered a frequent neuroendocrine disorder in geriatric donkeys, partially due to their longevity [1,2,10]. In horses, this disturbance is more frequent in older animals [11]. Hypertrichosis, polyuria/polydipsia, lethargy, decreased athletic performance and muscle atrophy are typical signs of PPID in horses [12,13]. Unfortunately, many of these signs are difficult to evaluate in donkeys due to breed or species-specific idiosyncrasies (long hair coat, absence of athletic use or calm behaviour) and lack of frequent monitoring. Since clinical findings lack specificity for this disease in donkeys, diagnostic testing is required in those donkeys suspected of having PPID.

Several PPID diagnostic methods have been described in horses and ponies, from baseline adrenocorticotropic hormone (ACTH) concentration measurement to dynamic tests such as the dexamethasone suppression test (DST), the ACTH stimulation test (ACTH-ST), the thyrotropin-releasing hormone (TRH) stimulation test, the combined DST–TRH test, the domperidone stimulation test (DomST), and loss of cortisol circadian rhythm [12,14–16]. Recent guidelines for PPID diagnosis in horses discourage the use of the DST, DomST or ACTH-ST [17]. Considering that endocrine differences between donkeys and horses have been recently described [9], it is reasonable to assume that differences could also exist for PPID diagnosis. Our hypothesis was that recent guidelines reported for PPID diagnosis in horses could also be used in donkeys. Therefore, the main objective of this work was to evaluate different dynamic testing of PPID in geriatric donkeys with PPID suspicion.

Materials and methods

Animals and inclusion criteria

Eighty donkeys from two donkey sanctuaries were evaluated for initial inclusion criteria: older than 12 years old, evidence of endocrinopathic disease in the form of hypertrichosis, polyuria/polydipsia, lethargy, decreased athletic performance, and muscle atrophy. Blood samples were drawn from those animals fulfilling the initial inclusion criteria (n = 22) and ACTH measurements were performed at the Animal Health Diagnostic Center of Cornell University’s College of Veterinary Medicine, using a chemiluminescent immunoassay (Immulite). Animals with baseline ACTH concentrations higher than 50 pg/mL (n = 6) were considered suspected of having PPID and enrolled in the study according to the guidelines for PPID diagnosis in horses [17]. Donkeys were housed in a semi-intensive farm in a shared moderately sized sand paddock with mates for walking, but without access to a large-scale yard for exercise, with free access to drinking water and pasture.
Donkeys were considered healthy based on clinical history and physical examination (heart and respiratory rates, temperature, mucus membranes, colour, capillary refill time, intestinal motility and digital pulse). Donkeys were under a regular deworming and vaccinations programmes.

**Body morphometric measurements**

The morphometric variables used in this study were calculated body weight, body mass index, body condition score, neck score, neck circumference and neck circumference to height ratio (NCHR) following the formulas previously described for donkeys and horses [8,18].

**Testing protocols and interpretation**

Donkeys were housed overnight (2200–0800) with a flake (approximately 1–1.5 kg) of coastal Bermuda grass hay and free access to water [19]. Dynamic diagnostic methods for PPID diagnosis evaluated in this study included the DST, the TRH stimulation test and the combined DST–TRH, and they were performed as described for horses [12,14,15]. For the DST, 40 μg/kg bwt of dexamethasone was administered intramuscularly the day before (1600–1800), and blood samples collected at baseline (predexamethasone injection), and 15, 18 and 20 h post dexamethasone for DST. For the TRH, 1 mg of TRH (Protirelin) was administered intravenously, with blood samples collected at baseline, 10, 20, 30, 45 and 60 min post-TRH. For the DST–TRH, dexamethasone was administered intramuscularly (40 μg/kg bwt) followed by 1 mg intravenously of TRH 3 h later. Blood samples were collected at baseline, and 24 h post dexamethasone and 15, 30, 45 and 60 min post-TRH. Test was carried out on all animals with 1-week washout period between tests. All tests were performed in summer time.

Donkeys were considered as PPID positive if they met the criteria for dynamic testing in horses [14,15]. That is, a plasma cortisol concentration higher than 27.6 nmol/L at 19 h post dexamethasone for DST, plasma ACTH concentration higher than 110 pg/mL 10 min post-TRH for TRH administration and plasma cortisol concentration higher than 66% of the baseline value at 195-min post dexamethasone for the combined DST–TRH test.

**Sample handling and measurement**

A catheter was placed in the left jugular vein the day before each experiment in all donkeys. Blood samples were collected into K2-EDTA tubes for cortisol and ACTH, into heparin-containing tubes for insulin and into oxalate tubes for glucose measurement for testing insulin dysregulation. After collection, blood samples were chilled immediately on ice. Samples were centrifuged at 1500 g for 10 min at 4°C, aliquoted and stored at −20°C until analysis.

Every parameter was determined using test and protocols previously validated for donkeys and horses [8,9,20,21]. Plasma ACTH concentrations were measured with a human-specific immunoradiometric assay [9], plasma cortisol concentrations by radioimmunoassay (mmol/L), insulin with a human-specific immunoradiometric assay and glucose by spectrophotometry (Heska Dry-Chem 7000) [9].

**Data analysis**

Normality was assessed with the Shapiro–Wilk test. Data were not normally distributed. Results are expressed as median and the interquartile range (IQR: 25th percentile–75th percentile). Percentiles were calculated using the Tukey’s Hinges test. Friedman’s test was performed to determine differences over repeated measures, followed by Wilcoxon’s test to assess where differences occurred. P ≤ 0.05 was considered statistically significant. Statistical analysis was performed using a commercial statistical software (IBM SPSS Statistics 24).

**Results**

**Basal hormone concentrations and morphometric variables**

Six mix-breed (one jack and five non-pregnant jennies), weighing 173.7 (61.1) kg bwt, geriatric donkeys (15.5 [7] years old, range: 12–30 years old) fulfilled both clinical and endocrine inclusion criteria (ACTH: 79.2 [65.8] pg/mL, Table 1). Cortisol concentrations (68.9 nmol/L, [24.8] nmol/L) were within the reference range for values reported for donkeys and horses (Table 1) [22]. Based on resting insulin concentrations, the six donkeys included in this study were considered as normoinsulinaemic (Table 1). Morphometric variables are presented in Table 1.

**Dexamethasone suppression test (DST)**

Dexamethasone administration induced a twofold significant decrease in cortisol concentrations at 15 and 20 h (Fig 1a; P ≤ 0.05), with no differences in the interval 15–20 h. ACTH concentrations decreased steadily from baseline until 20 h post dexamethasone. Although statistical differences were not detected, a decrease in ACTH concentrations was appreciated 20 h after dexamethasone administration (Fig 1a). Under diagnostic guidelines for PPID in horses [17], three donkeys were PPID positive for the DST (Fig 2).

**Thyrotropin-releasing hormone stimulation test (TRH)**

TRH administration increased both plasma ACTH and cortisol concentrations (Fig 1b). Cortisol concentrations peaked 10–20 min post-TRH in all donkeys, returning to baseline by 30 min (Fig 1b). A rebound effect was observed at 45 and 60 min, with ACTH concentrations significantly lower (P ≤ 0.04 and P = 0.03, respectively) than baseline (Fig 1b). For this test, all donkeys in the study were considered positive for PPID (Fig 3) [17].

**Combined DST–TRH test**

Dexamethasone induced a significant decrease (P = 0.03) in cortisol concentrations at 180 min. TRH administration led to a significant increase (P = 0.05) in cortisol concentrations at 195 min in relation to pre-TRH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Donkey 1</th>
<th>Donkey 2</th>
<th>Donkey 3</th>
<th>Donkey 4</th>
<th>Donkey 5</th>
<th>Donkey 6</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BWT (kg)</td>
<td>181.4</td>
<td>131.3</td>
<td>231.4</td>
<td>149.9</td>
<td>194.9</td>
<td>119.9</td>
<td>173.7 (61.1)</td>
</tr>
<tr>
<td>BCS [range: 1–9]</td>
<td>6</td>
<td>7</td>
<td>7.5</td>
<td>7</td>
<td>6</td>
<td>7.5</td>
<td>7 (1.1)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>145.4</td>
<td>100.5</td>
<td>149.5</td>
<td>109.9</td>
<td>155.9</td>
<td>129.8</td>
<td>137.6 (43.6)</td>
</tr>
<tr>
<td>NC (cm)</td>
<td>72.1</td>
<td>91.4</td>
<td>94.4</td>
<td>83.3</td>
<td>74.1</td>
<td>71.1</td>
<td>78.7 (20.3)</td>
</tr>
<tr>
<td>NS [range: 0–4]</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>NCHR (cm/m)</td>
<td>64.6</td>
<td>79.9</td>
<td>75.9</td>
<td>71.3</td>
<td>65.7</td>
<td>62.9</td>
<td>68.5 (12.7)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.6</td>
<td>3.7</td>
<td>4.1</td>
<td>4.2</td>
<td>4.1</td>
<td>4.2</td>
<td>4.05 (0.4)</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>6.9</td>
<td>15.8</td>
<td>15.4</td>
<td>12.9</td>
<td>15.1</td>
<td>10.5</td>
<td>14.0 (5.9)</td>
</tr>
<tr>
<td>ACTH (pg/mL)</td>
<td>79.1</td>
<td>79.4</td>
<td>105</td>
<td>67.9</td>
<td>64.9</td>
<td>217</td>
<td>79.2 (65.8)</td>
</tr>
<tr>
<td>Cortisol (nmol/L)</td>
<td>38.6</td>
<td>63.4</td>
<td>57.9</td>
<td>74.5</td>
<td>71.7</td>
<td>93.8</td>
<td>68.9 (24.8)</td>
</tr>
</tbody>
</table>

Data are expressed as median (IQR). IQR, interquartile range; BW, body weight; BCS, body condition score; BMI, body mass index; NC, neck circumference; NS, neck score; NCHR, neck circumference to height ratio.
values, returning to pre-TRH concentrations at 240 min. Despite TRH administration, cortisol concentrations continued decreasing significantly until 24 h ($P = 0.03$). ACTH concentrations decreased significantly ($P = 0.03$) at 180 min and, albeit a punctual elevation in response to TRH administration, returned to pre-TRH levels at 210 min ($P = 0.03$). Dexamethasone suppressed ACTH concentrations until 225 min, returning to baseline concentrations at 24 h (Fig 1c). With this test, four donkeys were diagnosed of PPID (Fig 4) [17].

**Discussion**

The diagnosis of PPID in donkeys using dynamic tests has not been evaluated using criteria developed for horses. The TRH stimulation test identified as positive all suspect animals, while the DST and the combined DST–TRH tests yielded several false-negative results.

Using test criteria developed for PPID diagnosis for horses [12,14,15,17], all donkeys were positive using the TRH test (Table 2). In contrast, 50% (3/6)
and 67% (4/6) of donkeys were positive for the DST and the combined DST–
TRH tests respectively (Table 2). Moreover, agreement between tests was
poor, with the same animal having different results between tests. In view
of these results, caution must be taken when interpreting dynamic tests for
PPID in donkeys, where the TRH stimulation test was the most consistent
test in this species.

ACTH concentrations’ reference values for healthy donkeys and
donkeys with laminitis have been previously published and appear to be
slightly higher than horses [22–24], but lower than 50 pg/mL, the
recommended cut-off value for the diagnosis of PPID in horses [17].
However, these studies [22–24] did not take into consideration factors
such as season, geography, breed, aged donkeys or included donkeys
without PPID. For the study reported here, ACTH resting concentrations
were used as inclusion criteria, and all donkeys had ACTH
concentrations >50 pg/mL. Nonetheless, the rest of donkeys tested in
both farms and considered healthy (without PPID and not included in

Fig 2: Blood adrenocorticotrophic hormone (continuous line) and cortisol (dashed line) concentrations for dexamethasone suppression test in each donkey.
the study) had ACTH concentrations lower than the cut-off value and those previously described [22–24].

Breed, species, age, geographical location, starch-content diet and feeding status, and seasonal or diurnal circadian rhythm have been shown to influence baseline ACTH concentrations in horses [25–32]. Regarding the diet, higher ACTH values have been demonstrated in aged horses feeding a high-starch diet [25]. This issue is important when interpreting ACTH concentrations (both basal and after TRH stimulation test) for PPID diagnosis. Although baseline biochemical parameters in this study were within reference ranges described for donkeys and donkeys were fed with coastal Bermuda grass hay (low starch-content diet), the rest of factors cannot be discarded and additional studies evaluating the influence of these conditions on ACTH and cortisol concentrations are compelling.

Analytic measurement techniques, especially in autumn months, have been shown to influence ACTH results, due to cross-reaction with other plasma pituitary peptides [33,34]. Tests in this study were performed in summer months and ACTH concentrations were measured using a valid method for all samples (chemiluminescent immunoassay) including samples measured at Cornell’s laboratory, making these factors unlikely.

Fig 3: Blood adrenocorticotropic hormone (continuous line) and cortisol (dashed line) concentrations for thyrotropin-releasing hormone stimulation test in each donkey.
to have influenced the results. In addition, this test has good repeatability and paired measurement offers no advantage vs. single measurement [35].

Dynamic tests used in this study were carried out according to protocols previously described for horses [12,14,15], however, it remains unknown whether the dexamethasone or TRH dose or blood sampling time points could have influenced the results. Although TRH dose was extrapolated from horses and, despite the lower bodyweight of the donkeys used in this study, it has been reported that TRH dose did not influence the test results in horses [36,37].
The ACTH response after TRH stimulation is influenced by several factors, including geography and season. The ACTH response after TRH administration is exacerbated in healthy horses closer to the Equator in autumn months [29]. Furthermore, it has been described that ACTH response to TRH in horses varies throughout the year [38,39], suggesting that seasonally adjusted values are also needed for this test in donkeys. In order to reduce variation, dynamic tests were performed in the summer and the same time. In case a seasonal effect, a different cut-off value or a different ACTH response to TRH or dexamethasone challenges were described in this species in the future, the repetition of this study on PPID donkeys in autumn would be imperative in order to confirm the diagnostic power of every dynamic challenge.

Although adrenal gland response has not been studied in donkeys suffering PPID, hypercortisolaemia and/or adrenal gland hyperplasia do not appear to be a common feature in donkeys as previously described in horses [11,12]. PPID donkeys in this study had cortisol concentrations within reference range described for this species [22]. Although further studies are necessary, cortisol measurement is likely not a reliable tool for PPID diagnosis in this species, similarly as previously described for horses [40], where its use is no longer recommended [17].

In conclusion, this study is the first to evaluate commonly used and recommended dynamic tests for PPID diagnosis in donkeys. Our preliminary results are in agreement with protocols published for horses, where baseline ACTH concentration and the TRH stimulation test are recommended screening tests for PPID diagnosis. Similar to horses, the DST and combined DST–TRH tests should be avoided in this species. Further studies taking into account the factors described above are necessary in order to clarify any species-specific difference and elucidate the pathophysiologic mechanisms involved in this disorder in donkeys.

Authors’ declaration of interests

No competing interests have been declared.

Ethical animal research

This study received approval from Welfare Committees (2015PI/04 and 19-03-15-214). Animals’ owners gave consent for their inclusion in the study.

Sources of funding

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TABLE 2: Pituitary pars intermedia dysfunction (PPID) status with different methods

<table>
<thead>
<tr>
<th>Test</th>
<th>Donkey 1</th>
<th>Donkey 2</th>
<th>Donkey 3</th>
<th>Donkey 4</th>
<th>Donkey 5</th>
<th>Donkey 6</th>
<th>Criteria for positive diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline ACTH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&gt;50 pg/mL</td>
</tr>
<tr>
<td>DST</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>Cortisol at 19 h &gt;27.6 nmol/L</td>
</tr>
<tr>
<td>TRH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACTH at 10 min &gt;110 pg/mL</td>
</tr>
<tr>
<td>DST–TRH</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Cortisol at 195 min &gt;66% basal</td>
</tr>
</tbody>
</table>

DST, dexamethasone suppression test; TRH, thyrotropin-releasing hormone stimulation test; DST–TRH, combined dexamethasone and TRH test; +, PPID positive; –, PPID negative.

Authorship

S. Mejia-Pereira contributed to study design and study execution. A. Perez-Ecija, R. Toribio and F. Mendoza contributed to study design, data analysis and interpretation and preparation of the manuscript. B. Buchanan contributed to study design, data analysis and interpretation and preparation of the manuscript. All authors gave their final approval of the manuscript.

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