IDENTIFYING HORSES WITH PPID: PART ONE – SAMPLE PROCESSING

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DAVID RENDLE discusses factors relating to sampling and processing, citing various studies as evidence to help in areas that can be, at times, confusing

Summary

Interest in the diagnosis of pituitary pars intermedia dysfunction (PPID or equine Cushing's) has never been higher and the licensing of pergolide mesylate has been accompanied by promotions to encourage testing for the disease by measurement of adrenocorticotropic hormone (ACTH). While ACTH is now accepted as a reliable means of identifying horses with PPID, different laboratories are offering conflicting advice on how samples should be collected and processed, and even on how results should be interpreted. The following articles review available evidence in an attempt to resolve areas of confusion. In part one, factors relating to sampling and processing are discussed. In part two, laboratory factors that may affect results are reviewed and advice is offered on the interpretation of results.

Key words

equine Cushing's, pituitary pars intermedia dysfunction, adrenocorticotropic hormone, laminitis

PITUITARY pars intermedia dysfunction (PPID) is being diagnosed increasingly in horses that do not have the clinical signs formerly considered to be characteristic of equine Cushing's disease.

As our understanding of the disease has increased, it has become apparent those cases demonstrating hirsutism, chronic infections, polyuria, polydipsia, weight redistribution and so on, represent only the tip of the proverbial iceberg of horses with pituitary dysfunction. PPID may result in laminitis before other clinical signs are observed[1] and, as a result, there has been a drive toward identifying and treating pituitary dysfunction when horses first develop laminitis, or preferably before.

The prevalence of PPID in two studies of horses with laminitis seen by referral and ambulatory veterinarians was 31 per cent and 70 per cent respectively[1,2]. The majority of cases not suffering from PPID were identified as having resting hyperinsulinaemia and clinical signs consistent with equine metabolic syndrome, resulting in 90 per cent of laminitis cases being diagnosed with underlying endocrine disease. While it is often dietary factors that tip these animals over the edge, these studies highlight the fact it is underlying endocrine dysfunction that renders them susceptible to laminitis. Any treatment that merely addresses the laminitis and does nothing to investigate and treat the underlying cause is doomed to failure.

How young is too young to test for PPID?

While testing for endocrine dysfunction in horses with laminitis is essential, justifiable concerns have been raised that risk of false-positive diagnosis increases with increased testing frequency, especially in populations with low disease prevalence.

Studies performed using necropsy as the gold standard indicate a high adrenocorticotropic hormone (ACTH) result has a high positive predictive value for PPID (false-positive results are uncommon[7,8]). Furthermore, recent evidence indicates plasma ACTH concentration (and other endocrine tests of pituitary dysfunction) only detect horses that have advanced histopathological changes within the pars intermedia and we are more likely to miss horses with early disease than falsely diagnose horses that do not have it (Dianne McFarlane, personal communication).

However, no test is perfect and the lower the prevalence of any disease in a population, the lower the positive predictive value of any test for it (for example, there is less chance a horse with a positive result actually has the disease). It is, therefore, important to appreciate the prevalence of a disease when performing and interpreting any diagnostic test. Epidemiological surveys of older horse populations have identified coat changes characteristic of advanced PPID in 17 per cent to 19 per cent of horses above 15 years of age, 22 per cent to 30 per cent in horses aged 20 or older and 45 per cent in horses above 30 years of age[3,4,5,6,7,8] (McGowan et al, 2007 – randomly selected 339 aged horses aged more than 15 years from a larger population and identified endocrine changes consistent with PPID in 15 per cent).

Until recently, there were few reports of PPID in horses 10 years or younger[1,9,10] and it has been a matter of debate whether it is appropriate to test younger horses. As PPID has been regarded as a disease of geriatrics, few young horses have historically been tested, which may be the reason why

few cases have been detected.

In the spring Talk About Laminitis initiative sponsored by Boehringer Ingelheim, 33 per cent of horses aged 10 to 15 that were tested had a high ACTH concentration ([Figure 1]). Positive results, though less frequent, were also identified in horses of 10 years or younger. Histopathological changes have been identified in horses of less than 10 years of age11, so positive endocrine tests in younger animals cannot simply be dismissed as erroneous.

Clearly, the sample of horses tested as part of the campaign included owner or vet-suspected PPID cases and the results should not be taken as an indication of PPID prevalence in the general equine population.

However, the results do demonstrate the importance of considering PPID as a cause of laminitis in younger animals. Further supportive evidence is provided by another investigation in which the median age of laminitic horses diagnosed with PPID was 15.5 years, with 18 per cent of PPID cases having no clinical signs other than laminitis[1]. Testing for PPID should be considered, therefore, in all horses with laminitis unless there is good reason not to. Horses less than 10 years of age should be no exception, although testing should be based on genuine clinical suspicions to minimise the false-positive results that would inevitably occur with blanket screening.

Does it matter when testing is performed?

A number of factors related to the timing of sampling have the potential to influence ACTH concentration, and some have been cited as possible causes of false-positive or negative results when testing for PPID. Cortisol is recognised as having a diurnal rhythm, hence it has been suggested ACTH concentration may also vary with time of day.

An investigation of seven horses with PPID and 12 control horses tested at different times of day throughout one year in the UK failed to demonstrate a diurnal rhythm for ACTH in either the healthy or PPID horses[12]. In horses and ponies in Australia, a statistically significant difference in ACTH concentration was identified through each day in healthy horses with a similar, but less profound, trend in horses with PPID[13]. Concentrations of ACTH were consistently highest at 8am and showed a progressive decrease through the day.

Although the differences were statistically significant, in absolute terms the differences in concentration were small and unlikely to influence clinical interpretation. In a further study performed in the US, there was little change in ACTH concentration across a 24-hour period (Dr McFarlane, personal communication). Therefore, although ACTH concentrations may be slightly higher first thing in the morning than at other times of the day, the differences are minimal and are unlikely to be of clinical relevance.

Can testing be performed in the face of concurrent disease?

During periods of stress, corticotropin- releasing hormone from the hypothalamus stimulates the release of ACTH from the pars distalis of the anterior pituitary, which in turn stimulates the release of glucocorticoids from the adrenal cortex. Increased production of ACTH from the pars distalis may have the potential to confound diagnosis of Cushing's in horses that are stressed or systemically unwell.

In hospitalised horses, increases in ACTH concentration were proportional to the severity of clinical illness. However, in all but the most severely compromised patients, ACTH concentrations returned to within reference range within 24 hours of admission[14]. Increases in ACTH concentration have also been observed in horses with grass sickness and colic[15] although other investigations have failed to identify significant increases in ACTH in response to laminitis[1], experimentally induced foot pain[16] and hospitalisation for various diseases[17].

A recent study of 119 horses identified statistically, and potentially clinically, significant increases in ACTH concentration in horses with laminitis, colic and acute illness, but not in horses that had been castrated or were suffering from chronic disease[18].

In horses undergoing veterinary treatment, the effects of sedation should also be considered as ACTH concentration in pituitary venous blood decreased below baseline for 20 minutes following sedation with an alpha-2-agonist in one study[19]. The evidence for the effects of stress on ACTH concentration is, therefore, mixed, but on balance it would be prudent to consider horses with severe pain or illness may have clinically relevant increases that may confound diagnosis of PPID. Chronic laminitis is likely to have little effect, but if borderline results are obtained, it is worth considering further testing when the laminitis is better under control.

Is a single measurement of ACTH reliable?

Some investigators have reported marked variation in resting ACTH concentrations in horses with PPID[20],[21] and this has been attributed to pulsatile release from the pituitary gland[22],[23],[24]. Calculating the average of two measurements of ACTH concentration from samples taken five to 20 minutes apart has been proposed as a means of reducing such variation and increasing the accuracy of diagnosis. An investigation of eight healthy and eight PPID horses, repeatedly tested over a 25-minute period, found ACTH levels varied by up to 9pg/ ml and 57pg/ml in the healthy and PPID groups respectively. There was no evidence of cyclicity of ACTH production, with peaks and troughs occurring at random[13].

When paired samples were collected 10 to 15 minutes apart from 212 horses suspected of having PPID, the median difference for each pair of results was 3pg/ml. Although the median difference was very small, in isolated cases there was a large discrepancy between results and, in a handful of cases, the diagnosis was altered as a result of taking the second sample[13].

Paired samples were also collected from 90 horses with PPID being treated with pergolide and the

median difference between paired results was 4.8pg/ml with the maximum difference being 68pg/ml[25]. Therefore, in a very small proportion of cases, having a second ACTH result a few minutes after the first does alter clinical interpretation. However, if the trouble is being taken to collect paired samples then there is greater benefit (and minimal extra expense) in performing a thyroid-releasing hormone (TRH) stimulation test (see part two).

How quickly after testing do I need to separate the plasma?

Recommendations on the handling of samples prior to measurement of ACTH have gradually relaxed from the days when samples were placed into dry ice immediately on collection. However, ACTH remains susceptible to degradation by proteolytic enzymes in whole blood or plasma at a range of temperatures[26]. ACTH concentration was not significantly different when equine whole blood in ethylenediamine tetraacetic acid (EDTA) was stored for up to three hours at either 1°C or 19°C [17].

Unpublished data from the Liphook Equine Hospital indicates ACTH concentration drops at a similar rate in whole blood or plasma in EDTA over 24 hours, although small numbers have been investigated and, until further work is performed, it is recommended samples should be separated by centrifuging or by gravity within three hours in accordance with the findings of Couetil et al (1996).

In one investigation, ACTH concentrations in samples separated by gravity and stored at 22°C decreased significantly over 24 hours while ACTH concentration in centrifuged samples did not decrease significantly[27]. In some samples separated by gravity, ACTH concentration increased markedly, which may be the result of cross-reactivity with intracellular proteins released from platelets or leucocytes not removed from the sample. Failure to separate samples using a centrifuge may be less important if samples are transported at lower temperatures, so it is recommended samples separated by gravity are sent chilled. Freezing gravity-separated samples (or whole blood) should be avoided as it may result in lysis of remaining cellular components. Care should be taken that gravity-separated or whole blood samples are not placed in direct apposition with an ice pack, which could result in cell lysis.

Chilled or frozen samples?

In centrifuged plasma stored at room temperature, ACTH concentration decreases by around 10 per cent in 24 hours. However, there is considerable variation between individual samples, with some decreasing by as much as 30 per cent and some not decreasing at all[27]. Rapid degradation of ACTH occurs above 30°C [28], and in samples stored at 22°C the variability of results increases [27], so transit with an ice pack is recommended to ensure samples arrive chilled and the accuracy of results is optimised. Freezing centrifuged samples may be worthwhile to ensure they arrive at the laboratory chilled – there is probably little advantage of freezing over chilling.

Are protease inhibitors of benefit?

Protease inhibitors, such as aprotinin and N-phenylmaleimide have been used to stabilise human plasma samples prior to measurement of ACTH concentrations[26],[29]. Addition of protease inhibitors to whole blood may induce haemolysis, precluding their use until after samples are centrifuged as haemoglobin may cross-react with ACTH. In dogs, the addition of aprotinin resulted in a paradoxical concentration dependent decrease in ACTH concentration and the authors suggested it might interfere with the assay[30].

Addition of N-phenylmaleimide to equine plasma did not prevent a reduction in ACTH concentration when samples from eight healthy and eight PPID horses were stored at room temperature[27] and aprotinin was of no benefit when equine plasma was stored at a range of temperatures[31]. Therefore, protease inhibitors have been demonstrated to be of no benefit and, if used with whole blood or as a substitute for careful sample handling, they may actually result in reduced accuracy of ACTH measurement.

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MEASURING ACTH CONCENTRATION:

DO:

• Collect a single blood sample into an **EDTA** tube.

• **Chill** the sample within **three hours** of collection.

• **Separate plasma** from the cell fraction prior to posting, either by **centrifugation or by gravity*.**

• **Freeze or chill**** the sample and place it in a **chiller pack** for transit to the laboratory. I Send the sample to a laboratory that has a validated method and laboratory-specific seasonally adjusted reference ranges.

*Separation should be performed as soon as possible, timing is less critical if the sample is kept chilled.

**Freezing is unnecessary though beneficial especially if the delivery is delayed.

DO NOT:

• Use anything other than EDTA tubes for sample collection.

• Freeze samples that have not been centrifuged.

• Add whole blood to tubes containing aprotinin or N-phenylmaleimide.