Practical Clinical Research Results to Consider When Testing for PPID in Horses

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1. Introduction

Thyrotropin-releasing hormone (TRH) stimulation of adrenocorticotropic hormone (ACTH) has become a common method for diagnosis of pituitary pars intermedia dysfunction (PPID).[1] When testing for PPID, factors to consider include repeatability of testing during the year,[2] time requirements for centrifugation after blood collection,[3] effects of stress on basal ACTH concentrations, the duration of efficacy of thawed TRH after it has been frozen for storage, and the stability of ACTH concentration after freezing. All of these factors potentially may affect test results and were investigated to aid practitioners to obtain consistent reliable results when testing for PPID in horses.

2. Duration of Effectiveness of Frozen/Thawed TRH to Stimulate ACTH Release in Horses

Introduction

Many equine practitioners freeze single doses of TRH following removal of a single dose from a multidose vial. Typically, prior to use, the TRH is thawed and taken to the farm to be used when testing a potential PPID horse. If the horse owner declines testing, the veterinarian has TRH that has been frozen and thawed. Anecdotally, TRH can only be frozen and thawed once for an optimum and consistent response. The potency and stability over time of TRH after one freeze/thaw cycle is unknown. This study was designed to determine the duration of effectiveness of TRH in horses following one freeze/thaw cycle when stored at 5°C over time.

Materials and Methods

The TRH stimulation test used in each of these studies was conducted as follows: blood was collected into a purple top tube[a] for basal ACTH (T0-ACTH) followed by IV administration of 1 mg TRH[b] (1 mL). Blood was then collected exactly 10 minutes post-TRH administration (T10-ACTH). Blood samples were centrifuged and plasma was frozen at −80°C until ACTH analysis using a chemiluminescent immunoassay at the Animal Health Diagnostic Laboratory, Cornell University, Ithaca, NY. Determination of positive, negative, and equivocal status for PPID was according to the 2019 Equine Endocri-

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nology Group recommendations for the diagnosis and treatment of PPID.

Ten horses ($PPID^+$ and $PPID^-$) were enrolled, mean age, 18.8 years (range, 12–25 years). Horses were first paired by PPID status and randomized into 2 groups of 5 horses (each group contained 4 $PPID^-$ and 1 $PPID^+$). Thirty 1-mg/mL doses of constituted TRH were frozen (−20°C) 28 days prior to testing. Fourteen days prior to testing, ten doses of TRH were thawed and kept at 5°C until administration. The remaining 20 doses of TRH were thawed on the first day of testing (Day 0) and stored at 5°C until administration. On Day 0, all horses were TRH stimulation tested using TRH thawed same day. The TRH stimulation procedure was repeated post-thaw on Days 14, 28, 42, and 56. In order to avoid potential carryover effects of multiple TRH stimulation procedures administered every 2 weeks, horses in Group 1 had the TRH stimulation repeated on Days 14 and 42 (post-thaw TRH), whereas Group 2 horses had the TRH stimulation repeated on Days 28 and 56 (post-thaw TRH). All T10-ACTH samples were centrifuged following collection and plasma frozen (−80°C) until analysis at Cornell Animal Health Diagnostic Center. Data were analyzed using a mixed model with repeated measures to compare T10-ACTH and the percent increase (T0-ACTH to T10-ACTH) of ACTH after TRH stimulation, using horse as the subject and day as the repeated effect. Pearson's correlation coefficients were used to examine relationships, and Bland-Altman plots were constructed to compare T10-ACTH on Days 14, 28, 42, and 56 to the T10-ACTH on Day 0.

Results

There was no effect of Group ($P > .25$), so when appropriate, data were combined for analysis. There was no effect of day post-thaw on T10-ACTH ($P = .13$) or the percent increase of T0-ACTH to T10-ACTH after TRH stimulation ($P = .36$). Pearson's correlation coefficients indicated strong relationships between T10-ACTH on day 0 and all other days ($R > 0.98$, $P < .001$). Bland-Altman plots indicated an average day bias of 9.4 pg/mL in all horses compared to day 0, with 95% limits of agreement at −38.8 to 57.4 pg/mL.

Discussion

In this study, the TRH stimulation procedure produced repeatable ACTH concentrations in samples collected 10 minutes after administration of TRH in horses when using TRH that had been frozen, thawed, and stored at 5°C for up to 56 days.

3. The Effect of Trailering and Dentistry on Resting Adrenocorticotropic Hormone Concentration in Horses

Introduction

Several studies have concluded that pain, stress, and concurrent illness were only likely to affect diagnostic usefulness of resting ACTH when severe.[4] The objective of this study was to identify whether trailering or teeth floating (common stressful situations/procedures) increase plasma ACTH levels in horses that might interfere with testing for PPID.

Materials and Methods

Twelve PPID-negative horses were randomized into 3 groups of 4 horses per group. Each group was randomly assigned to an initial treatment: dentistry (DN), trailered (TR), or stabled controls (CN). Following initial treatment, each horse group was randomly assigned to each of the two remaining treatment groups; thereby, each horse group underwent all three treatments. Plasma was collected from all horses prior to each treatment and used as the baseline basal ACTH. The DN horses were placed in stocks, sedated with 0.1 to 0.3 mg/lb xylazine IV, and following mouth speculum placement, teeth were floated[c]. The TR group was loaded on a 6-horse slant trailer and hauled for 40 minutes. Immediately following the dental procedure and trailer ride, post-procedure (P0) plasma samples were collected. Plasma samples were then collected from all horses at 15, 30, 60, and 120 minutes post-procedure. Plasma P0 samples from the CN horses were taken when the TR horses returned. Plasma samples were frozen (−80°C) until analysis at Cornell Animal Health Diagnostic Center. Data were confirmed for normality using the Shapiro-Wilk statistic, and then analyzed using a mixed model with repeated measures (i.e., each horse as its own control), with main effects of treatment (CN, DN, TR) and time, and day × time as the repeated effect. Statistical significance was designated at $P < .05$. Data were summarized as mean ± SE.

Results

No change occurred in ACTH over time in the CN or DN horses ($P = .14$). ACTH was higher in TR compared to CN ($P = .026$) and DN ($P = .016$) horses. In TR horses, ACTH was higher than baseline (PRE) immediately after trailering (T0; $P = .0003$). By 30 minutes post-trailering, as a group, there were no differences in mean basal ACTH compared to PRE concentrations ($P = .55$). One horse in the study maintained elevated ACTH concentrations until the 120-minute time point.

No significant difference in resting ACTH concentrations over time was observed in horses undergoing dentistry procedures compared to baseline. A 40-minute trailer ride resulted in significantly increased basal ACTH concentrations in horses up to 30 minutes post-unloading.

Discussion

In these horses, collecting blood within 30 minutes (in all horses) and up to 120 minutes (in one horse) after trailering resulted in elevated resting ACTH concentrations that could interfere with PPID testing. Based on results of this study, blood should

not be collected for resting ACTH concentration determination for at least 30 minutes after trailering.

4. TRH Repeatability in PPID-Negative and PPID-Positive Horses

Introduction

Even though TRH stimulation of ACTH has been used as a diagnostic test for equine PPID, it is unknown whether the T10-ACTH response to TRH is repeatable in individual horses. The purpose of this study was to conduct TRH stimulation tests at 4-week intervals, beginning in February and ending in June, in horses with and without PPID to determine the repeatability of the T10-ACTH, over time.

Materials and Methods

Twelve horses, 5 PPID positive ($PPID^+$), 5 PPID negative ($PPID^-$), and 2 PPID equivocal with a mean age of 18.8 years (range, 12 to 25 years) were enrolled. Basal ACTH concentration from blood collected in January was used to identify PPID status of each horse. The TRH stimulation procedure was performed on day 0 (February 13) and repeated on Days 28, 56, 84, and 112. The subsequent samples were compared to the T10-ACTH samples collected on Day 0. Data were confirmed for normality using the Shapiro-Wilk statistic, and then analyzed using a mixed model with repeated measures to compare T10-ACTH and the percent increase of ACTH after TRH stimulation, using horse as the subject and day as the repeated effect. Pearson's correlation coefficients were used to examine relationships between T10-ACTH on days 28, 56, 84, and 112 to T10-ACTH on day 0. Bland-Altman plots were constructed to compare T10-ACTH on Days 28, 56, 84, and 112 to the T10-ACTH on Day 0.

Results

The mean basal ACTH in $PPID^-$ horses was 17.6 ± 0.7 pg/mL and 61.4 ± 9.2 pg/mL (T10-ACTH), with a 349 ± 41% increase in T10-ACTH after TRH stimulation. The mean basal ACTH in $PPID^+$ horses was 43.5 ± 3.6 pg/mL (basal) and 410 ± 58 pg/mL (T10-ACTH), with a 948 ± 194% increase in T10-ACTH after TRH stimulation. Repeated measures analysis indicated no effect of day on T10-ACTH ($P = .40$), or the percent increase of T10-ACTH after TRH stimulation ($P = .12$). Pearson's correlation coefficients indicated strong relationships between T10-ACTH on Day 0 and all other days ($R > 0.70$, $P < .01$). Bland-Altman plots indicated an average day bias of 27 pg/mL in all horses compared to day 0, with a day bias of 10 pg/mL in $PPID^-$ (with 95% limits of agreement at −101 to 122 pg/mL) and 43 pg/mL in $PPID^+$ (with 95% limits of agreement at −493 to 581 pg/mL) horses. The Immulite intra-assay CV was 9.3%, which accounts for most of the observed day bias.

Discussion

The TRH stimulation procedure produced repeatable ACTH concentrations in samples collected 10 minutes after administration of TRH in horses collected at 4-week intervals over 112 days from February through June. Observed variation of T10 ACTH over the duration of the study resulted in 2/5 PPID negative horses classified as positive, once and twice, respectively. Additionally, the T10 ACTH in 1/5 PPID positive horses tested equivocal at one time point. The results of this study stress the importance of using the combination of owner history, clinical signs, and laboratory data when determining the proper diagnosis of PPID.

5. Effect of Delayed Plasma Centrifugation on Equine ACTH Concentration

Introduction

If stored at room temperature (21°C), ACTH level in blood decreased soon after collection.[6] Multiple recommendations exist for the timing of centrifugation of chilled samples from sample collection. In the United States, a discordance exists between laboratory recommendations concerning centrifugation time from sample collection. Realistically, many equine ambulatory practitioners do not have the ability to centrifuge the sample within 2 to 4 hours. This study was conducted in order to determine the length of time whole blood can be stored refrigerated prior to centrifugation and maintain accurate ACTH concentration.

Materials and Methods

On day 0, 5 mL of whole blood from each of 10 horse (Five PPID positive and 5 PPID negative) was collected into each of 6 ethylenediaminetetraacetic acid (EDTA) tubes and immediately placed in a refrigerator at 7°C. One tube from each horse was centrifuged within 15 minutes of collection, followed by centrifugation of one tube from each horse at 4, 8, 12, 24, and 36 hours following collection. At each time, centrifuged plasma was pipetted into 1.5 mL polypropylene tubes and stored at −80°C. None of the plasma samples were turbid, hemolyzed, or icteric. Plasma was shipped frozen with cold packs overnight to the Animal Health Diagnostic Center of Cornell University in Ithaca, NY for analysis. The percent change from baseline (PCFB) was reported to standardize the data given that baseline values differed.

Results

The absolute changes over time revealed no pattern of variation. The mean PCFB was 2.8% ± 7.96% (95% CI,: –2.9%, 7.0%). There was no evidence of significant time effect from the repeated measures model with a *P*-value of .5056. There was no evidence of significant time effect on the level of ACTH in PPID positive or PPID negative horses. Three of 10 enrolled horses exhibited variation in ACTH

concentration at 1 time point that changed the diagnostic interpretation of PPID status from PPID negative to equivocal, PPID positive to equivocal and PPID negative to equivocal, respectively. In 2 of the 3 horses, the variation was less than the intra-assay variability (9.3%) whereas the ACTH was increased 16.7% in the third horse.

Discussion

This work demonstrated that refrigeration (4°C) of whole blood for up to 36 hours prior to centrifugation and freezing did not significantly affect plasma ACTH concentrations. Laboratory diagnostic results alone should not be utilized to classify a horse as PPID positive or negative. The history and clinical signs in conjunction with laboratory diagnostic results should always be utilized for the diagnosis of PPID.

6. Effect of Various Freezing Protocols on ACTH Plasma Concentration

Introduction

ACTH has been reportedly understood to be fragile in whole blood samples and is affected by heat and time spent on erythrocytes prior to centrifugation.[7,8] Equine ACTH has been shown to be stable without centrifugation for up to 8 hours stored at 21°C or 4°C[6] and was stable in plasma stored at −20°C and −80°C for 30 days.[6] If plasma samples cannot be shipped the day of collection to the respective laboratory, plasma should be frozen until shipment. It is imperative for the veterinarians to understand if freezing plasma has any negative effects on the stability of equine basal ACTH concentration. The objective of the study was to determine the stability of ACTH in plasma after freezing for different lengths of time prior to determination of basal ACTH concentration.

Materials and Methods

Twelve horses (5 mares, 6 geldings, and 1 stallion) ranging in age from 14 to 29 years from the Middle Tennessee State University herd were screened for ACTH in May with levels found to range from 12.4 pg/mL to 62.0 pg/mL (10 horses < 30 pg/mL = negative, >2 horses 50 pg/mL = positive). In September, 1.0 mg of TRH[a] suspended in 1 mL of saline was administered intravenously to the same 12 horses. Ten minutes later, blood samples were collected in EDTA tubes and refrigerated at 5°C until centrifuged at 1000×*g* for 10 minutes within 2 hours of collection. Plasma was stored in microcentrifuge tubes and frozen for variable lengths of time and conditions. Basal ACTH concentrations were measured at day 0. Plasma samples were stored at −80°C for 3, 7, 30, 60, and 90 days, or stored at −20°C for 3, 7, 30, and 60 days, or stored between ice packs in a freezer to mitigate fluctuation of temperature due to opening and closing of the freezer door at −20°C for 3 and 7 days prior to determination of basal ACTH concentration. Plasma was shipped frozen with cold packs overnight to the Animal Health Diagnostic Center, Cornell University, Ithaca, NY. Samples were batch analyzed for plasma ACTH concentration determined by chemiluminescent immunoassay previously validated for horses. ACTH concentrations were compared to baseline (non-frozen day 0 plasma) for each storage method using a mixed model with repeated measures in which each horse served as its own control and day was the repeated effect. Statistical significance was set at $P \leq .05$.

Results

Mean basal ACTH concentration on day 0 for plasma stored at −80°C was 392.2 pg/mL that declined 6.9% to a low of 365.0 pg/mL by day 90 ($P = .047$). Through day 60, the PCFB never varied more than 2% and was not different ($P > .62$) from day 0. On day 90, the PCFB was −6.9% and different from baseline ($P = .030$). Across the 90-day storage, overall degradation was observed ($P = .034$).

Mean basal ACTH concentration on day 0 for plasma stored at −20°C was 392.2 pg/mL that declined 5.3% to 371.4 pg/mL by day 60. The ACTH concentrations at day 0 and day 60 were not different ($P = .18$). The PCFB was lower by day 60 ($P = .035$). Across the 60-day storage, degradation was observed at −20°C ($P = .004$).

Mean basal ACTH concentration at day 0 for plasma stored between ice packs at −20°C was 392.2 pg/mL that declined 1.1% to 387.9 pg/mL by day 7. No degradation of basal ACTH was observed by day 7 in either the ACTH concentrations ($P > .36$) or the PCFB ($P > .24$).

Discussion

In a practice situation, it is unlikely that samples would be held for more than just a few days. This work shows that keeping frozen plasma stored in an ordinary household refrigerator freezer without keeping it between ice packs to reduce temperature fluctuation is sufficient to preserve testing reliability. For research or epidemiologic purposes, it should be considered that after 60 days ACTH levels declined when stored at −80°C.

7. Conclusions

TRH that has been frozen and thawed (once) was effective for at least 56 days when TRH was kept refrigerated after thawing. Trailering horses for 40 minutes increased basal ACTH for at least 30 minutes (and up to 120 minutes in one horse) post trailering. Results of T10 ACTH from TRH stimulation testing was repeatable from January through early June in the United States (Latitude 35° 50′ 44” North). Basal ACTH concentration determination is reliable from whole blood that has been refrigerated for up to 36 hours prior to centrifugation. Plasma samples for ACTH determination can be

frozen in a refrigerator freezer at −20°C, for up to 30 days or up to 60 days at −80°C with no decrease in ACTH concentration.

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Declaration of Ethics

The Authors have adhered to the Principles of Veterinary Medical Ethics of the AVMA.

Conflict of Interest

Authors S.T. Grubbs, K.N. Shepard, and D.L. Neal are employees of Boehringer Ingelheim Animal Health USA, Inc.

Authors J.C. Haffner and R.M. Hoffman are employees of Middle Tennessee State University.

Animal Care and Use

These studies were conducted by permission of Middle Tennessee State University Institutional Animal Care and Use Committee by protocols 19-2005, 19-2008, 19-2004, 17-2002, and 17-2013.

References and Footnotes

1. Horn R, Bertin FR. Evaluation of combined testing to simultaneously diagnose pituitary pars intermedia dysfunction and insulin dysregulation in horses. *J Vet Intern Med* 2019;33: 2249–2256.
2. Copas VE, Durham AE. Circannual variation in plasma adrenocorticotropic hormone concentrations in the UK in normal horses and ponies, and those with pituitary pars intermedia dysfunction. *Equine Vet J* 2012;44:440–443.
3. Shepard KN, Haffner JC, Neal DL, et al. Effect of delayed plasma centrifugation on equine adrenocorticotropic hormone concentration. *J Vet Diagn Invest* 2019;31:585–587.
4. Durham AE. Endocrine disease in aged horses. *Vet Clin Equine Pract* 2016;32:301–315.
5. Rosner B. Guidelines for judging the significance of a *P*-value. *Fundamentals of biostatistics*. 8th ed. Boston, MA: Cengage Learning; 2016, 219.
6. Prutton JSW, Kass PH, Watson JL, et al. Pre-analytical stability of adrenocorticotrophic hormone from healthy horses in whole blood, plasma and frozen plasma samples. *Vet J* 2015; 204:123–124.
7. Reisch N. Preanalytical stability of adrenocorticotropic hormone depends on time to centrifugation rather than temperature. *Clin Chem* 2007;53:358–359.
8. Rendle DI, Litchfield E, Gough S, et al. The effects of sample handling and N-phenylmaleimide on concentration of adrenocorticotrophic hormone in equine plasma. *Equine Vet J* 2015; 47:587–591.

[a]Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ 07417-1880.

[b]Thyrotropin-releasing hormone, Sigma-Aldrich, Inc., Bellefonte, PA 16823-0048.

[c]PowerFloat®, Calgary, AB T2C 5S7, Canada.