Repeatability of TRH Stimulation Test for Equine Pituitary Pars Intermedia Dysfunction during the Months of February through June

By

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Abstract

Pituitary Pars Intermedia Dysfunction (PPID) is the most common endocrine disease among aged horses. Veterinarians remain challenged when diagnosing PPID unless it is an advanced case. Veterinarians rely on advanced clinical signs to be present when diagnosing, but the keys signs like hypertrichosis will not appear until the disease has reached an advanced stage. Currently, the thyrotropin-releasing stimulation test was used to aid in diagnosing early cases of the disease, but it is unknown if the test is repeatable in individual horses. It is known that the test is, however, limited to the months of February to June, as that is when ACTH levels appear to be unaffected by seasonal change. For the study, the horses had a baseline blood sample drawn followed administration of the TRH, and another sample was collected at 10 minutes post administration (T10 ACTH). The purpose of this study is to determine if the thyrotropin-releasing stimulation test T10ACTH level is repeatable during the months of February through June. The results proved the test is repeatable when administered during the months of February to June.

Introduction

Equine Pituitary Pars Intermedia Dysfunction (PPID) is the most commonly diagnosed endocrine disease in horses (Miller et al., 2008). PPID is a disease of the pars intermedia of the equine pituitary gland. The disease is a neurodegenerative disease seen in aging horses. Early signs of the disease appear between 15 to 20 years of age depending on the horse and its breed, though ponies and Morgan horses seem more at risk to develop PPID based on current research (Schott, 2002). However, further research is needed to clarify the role of breed in regard to developing the disease (McFarlane, 2011).

This disease develops from the lack of proper hormone regulation of the pituitary gland. The gland is often described as being located at the base of the brain next to the hypothalamus. The pituitary gland specifically "lies within the sella turcica, separated from the brain by a fold of dura mater known as diaphragma sellae, suspended ventral to the hypothalamus by the infundibular stalk" according to Dianne McFarlane. It is important to know the exact location, to better understand the connection between the pituitary gland, the hypothalamus, and how it effects the disease.

The pituitary is divided into four parts or sections that have their own individual characteristics and functions. The sections are pars distalis, pars intermedia, pars tuberalis, and pars nervosa, although some still refer to the certain sections as adenohypophysis and neurohypophysis instead of the multiple sections. If using the term adenohypophysis, it is understood that it "anatomically subdivided into the pars tuberalis, pars intermedia, and pars distalis, while neurohypophysis is for the pars nervosa" (Schott,

2002, 237-238). While both sets of terms are correct, those currently researching PPID prefer to use the first set of four terms versus the set of two terms.

The pars distalis is comprised of endocrine cells that produce, store, and regulate the release of hormones based on hypothalamic releasing or inhibiting factors. In other words, the pars distalis acts as the warehouse for hormones in the pituitary gland. The pars tuberalis, according to McFarlane, is a thin band of endocrine cells enveloping infundibular stalk. The pars tuberalis purpose follows: it is dense in melatonin receptors and monitors daily melatonin concentrations to regulate levels of reproductive hormones with the seasons. The pars nervosa is made of up from both axons and nerve terminals that specialize in storing and releasing of oxytocin and vasopressin. Finally, the section of the pituitary where PPID stems from, the pars intermedia, consists of melanotropes. The melanotropes are a type of endocrine cell and are the only cells present in the pars intermedia. These cells are directly influenced or innervated by the dopaminergic neurons released by the hypothalamus, which promotes the production of hormones that play key roles in PPID.

While there are many other hormones or hormone precursor proteins produced in the pituitary gland, the focus will be on the hormones that are directly related to PPID. These hormones or hormone precursor proteins include pro-opiomelanocortin (POMC), melanocyte- stimulating hormones (MSHs), adrenocorticotropin (ACTH), and dopamine. POMC is a large hormone precursor protein that is produced by the melantropes of the pars intermedia and from the corticotropes of the pars distalis.

POMC cleavage products vary depending upon the site of production and initiating stimulus. When POMC produced from corticotropes undergoes cleavage by

prohormone convertases1 (PC1), it produces the MSH and ACTH hormones. Whereas POMC produced from melanotropes is cleaved by PC1 and prohormone convertases 2 (PC2) to produce ACTH. The ACTH is further cleaved by the PC2 into α-MSH and corticotropin-like intermediate lobe peptide (CLIP). The differences are due to where and what produced the POMC. This is because the cleaved products affect a horse and its hormone levels differently. The hormone dopamine is produced by the hypothalamus. When produced by the hypothalamus, it is released directly via hypothalamic periventricular neurons directly to the melanotropes of the pars intermedia of the pituitary gland.

When the above-mentioned hormones are functioning properly, the horse can remain in a relatively stable state of homeostasis. To better understand the horse's state of homeostasis, the function behind the previously mentioned hormones must be further explained.

Since POMC can be produced in both the pars distalis and pars intermedia, it is understood to play an important role in the pituitary gland. That role is based on where it is produced even though when both are cleaved, they produce similar products. When produced from the pars distalis, the ACTH from the cleaved POMC is the correct ACTH for a healthy horse. This horse will not show signs of PPID and the other hormones will regulate correctly. If the ACTH is derived from a pars intermedia POMC, the horse will begin to exhibit early signs of PPID. This is because ACTH regulates the amount of secreted cortisol into the horse's body. Cortisol levels are used to measure stress levels, so the amount of ACTH correlates to the amount of cortisol (stress) in the body.

However, this is where research begins to become slightly differentiated on how the ACTH affects the disease because the disease is not fully understood. McFarlane suggests it is because the hypothalamus is not producing the required amount of dopamine, in turn causing the pituitary to become overactive, particularly in the pars intermedia section of the gland. Whereas Schott suggests focusing more on the corticotropes and their production of ACTH and does not mention dopamine as a main cause of PPID but that it does affect the disease.

Most of the more current research is focused on not enough dopamine being produced to regulate the pituitary gland from becoming over active. The over production of hormones can cause the pituitary to become enlarged and to develop adenomas (pituitary lesions). The overproduction and enlarged gland also affect the testing for the disease. This necessitates the use of both diagnostic testing and clinical signs to determine if a horse has the disease.

Currently, clinical signs are the best indicators of the disease; however, appearance of observable signs usually suggests an advanced case. One of the most specific signs of PPID is the presence of hypertrichosis previously reported as hirsutism (long curly haircoat), which has been suggested to be pathognomonic for advanced PPID (McGowan et al, 2013). Other advanced signs include muscle atrophy, weight-loss/lethargy, laminitis, polyuria/polydipsia, hyperhidrosis, abnormal fat distribution, insulin resistance, and susceptibility to secondary infections. For an early stage diagnosis based on clinical signs, it takes hypertrichosis and two other clinical signs to be present (Spelta, 2015).

If advanced signs are not present, then detection of other signs such as decreased athletic performance, change in attitude/lethargy, delayed hair coat shedding, and/or a change in body conformation are indicators that testing should be performed to confirm an early diagnosis. The tests include the dexamethasone suppression test or the thyrotropin-releasing test. The dexamethasone suppression test requires a blood draw for cortisol measurement followed by administration of the dexamethasone. Approximately 19 hours later, another blood sample is then taken and analyzed for cortisol production. If the horse has PPID, then there will be no reduction in cortisol concentrations (AAEP). While this method was once considered the gold standard for diagnosing PPID, the Equine Endocrinology Group now recommends the thyrotropin-releasing test as a more accurate test for early detection and diagnosis of the disease (Frank et al).

The thyrotropin-releasing stimulation test analyzes the adrenocorticotropic hormone concentration in a horse (Beech et al, 2011). To perform the test, a baseline blood sample is drawn, followed by the administration of the TRH. After exactly 10 minutes have passed, a sample will be drawn from the horse. Both samples will then be analyzed for ACTH concentrations by a lab. A healthy horse's ACTH level at rest should be $\leq$ 30 pg/mL and after TRH administration, should be $\leq$ 110 pg/mL (Frank et al). If the horse does have PPID, then its concentrations at rest could be close to that of a healthy horse and then after THR be > 110 pg/mL (Frank et al).

The problem with this method of testing is that it can only be performed from December to June. Around the month of July, ACTH levels start to rise naturally with the seasonal change from summer to fall. Researchers are still working on the understanding as to why production of ACTH rises in the summer and fall months, and levels out again

in the spring. For now, there is no baseline or reference intervals available for July to December.

The pituitary gland of a healthy horse produces the amount of ACTH that the horse needs, whereas a diseased horse's pituitary may produce too much or too little. Normal horses may vary in their concentrations of ACTH production but for now ≤ 30 pg/mL is considered negative. However, some horses may produce more ACTH than the reference interval of 30pg/mL but less than 50pg/mL. If this occurs, then the horse should be evaluated for clinical signs to confirm a diagnosis. The horse may be in the early stages of the disease and may begin to experience lethargy, decrease in performance, or have long hairs starting to grow under its neck.

It is important to keep records of the horse's ACTH concentrations to know the horse's normal range. However, that requires repeatability of the TRH stimulation test through the months of February to June, and there is little research currently on the repeatability on the same group of horses. There has been enough research to establish baseline numbers of reference, but whether it is repeatable is still up for discussion.

Thesis Statement

This study seeks to determine if there is a spring season repeatability of the TRH stimulation test for diagnosing PPID at 4-week intervals in a group of horses.

Methodology

Animal Care and Use. Per the requirements for approval by Middle Tennessee State University Institutional Animal Care and Use Committee (IACUC), the primary investigator and faculty advisor have completed the online Animal Care and Use training through the CITI program. Upon completion of training, The Animal Care and Use proposal was approved by Protocol #19-2004 (see attached).

Materials. The materials to conduct the study included horses, disposable pipettes, microcentrifuge tubes, 1.5-inch 20-gauge vacutainer needles, 10 ml purple top (EDTA) vacutainer tubes, 1 mg Thyroid Releasing Hormone in 1 ml saline for each horse per test were provided by the MTSU Horse Science Center.

Horses. Based on statistical power analysis of previous PPID studies at MTSU, the estimated number of required horses for TRH stimulation response during different months was equal to 11.8. Therefore, 12 horses were used from the MTSU herd. The horses were over the age of 15 and had no changes to their daily routines.

Administration: Once the 12 horses were selected, they were tested for PPID. Any hay and/or remaining feed was removed from the rack and feeder before 10:00 pm the night before testing. The following morning the horses were tested before receiving their morning feed. A 10 ml blood sample was taken through a 1.5-inch 20-gauge vacutainer needle directly into a 10 ml purple top (EDTA) vacutainer tube that was placed in an insulated cooler with frozen freezer packs to cool the samples. 1 mg Thyroid Releasing Hormone in 1 ml saline was then administered intravenously. 10 minutes later, another 10 ml blood sample was taken as described for the first. The samples were then

centrifuged as soon as all samples were collected, and plasma was transferred by disposable pipette into 1.5 ml microcentrifuge tubes and then frozen. One replicate of each sample was stored in the -80º freezer. These procedures were repeated every 4 weeks from the first test in February until the beginning of June. Upon collection of all samples, they were sent frozen to the Cornell Animal Health Diagnostic Center Endocrinology Laboratory in Ithaca, NY. for analysis. Data was 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 the 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:

TRH stimulation of ACTH plasma concentration indicated 5 horses were PPID negative (T-10 <110 pg/mL ACTH) and 5 horses were PPID positive (T-10 >200 pg/mL), with 2 horses PPID equivocal (T-10 110 - 200 pg/mL). The ACTH plasma concentration remained within the category (negative, equivocal or positive) at T-0 and T-10 with few exceptions at each test day. No individual horse changed category from negative to positive or vice versa during the study. There was no effect of Day on T10-ACTH ($P = 0.40$) or the percent increase of ACTH after TRH stimulation ($P = 0.12$). Pearson's correlation coefficients indicated strong relationships between T10-ACTH on Day 0 and all other days ($R > 0.70$, $P < 0.01$). 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-negative and 43 pg/mL in PPID-positive horses.

Discussion:

The research of the TRH test produced consistent ACTH concentrations in samples collected 10 minutes after TRH administration at 4 week intervals over the course of 112 days. The data showed that there were 5 horses classified as negative, 5 horses were positive, and 2 horses were classified as equivocal repeatedly. This means the horses that tested positive or negative on Day 0 tested consistently positive or negative throughout the study, while the equivocal horses fluctuated but did not provide a strong positive or negative diagnosis throughout the study.

Since the T-10 ACTH concentrations remained consistent throughout the study, it indicates the TRH stimulation test is reliable throughout the first half of the year regardless of month. This study demonstrates the TRH stimulation test can be used to diagnose PPID from February to June regardless of the month at that time of year. Beginning in late June to early July, ACTH levels will be elevated until late fall. The TRH stimulation test is not recommended during that time of year due to the elevated levels. Therefore, this type of study has not been performed to determine if the ACTH levels are consistent during the fall period.

Future studies with a greater number of horses will be needed to verify if similar results can be achieved on a larger scale before being considered as reliable in helping to make a diagnosis of PPID. Future studies could also include performing year round testing to see if there is a correlation between the months of July to December similar to

the months of February to June. These studies would be very beneficial since it is known that ACTH levels rise during the summer to winter months and could aid in determining a way of providing testing year round. Year round testing may also aid in determining as to why ACTH levels rise during the summer since that is still unknown to researchers at this time.

Conclusion:

TRH stimulation of ACTH plasma concentration at 10 minutes post injection produces repeatable results in horses during the months of February to June. This indicates that the TRH stimulation test is reliable in the months of February through June.

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Appendix

Appendix A: Glossary

Adenomas: Lesions found on the pituitary during a necropsy

Adrenocorticotropic Hormone (ACTH): hormone produced by the pituitary gland, specifically the pars intermedia, to regulate cortisol production by the adrenal gland.

Cortisol: produced by the adrenal gland in response to pituitary produced ACTH that mediates various metabolic processes, and whose levels in the blood rise in response to stress

Dexamethasone Suppression Test: Diagnostic test previously used to determine if a horse had PPID

Hyperhidrosis: abnormal/ excessive sweating

Hypertrichosis: excessive hair growth and discoloration, such as an overly long hair coat that fails to shed and shows discoloration. Preferred term to hirsutism.

Hirsutism: long curly hair that fails to shed but does not show discoloration

Laminitis: inflammation of the laminae, causing pain, refusing to move, and can lead into foundering.

Muscle Atrophy: partial or complete wasting away of muscle

Pathognomonic: specific characteristic or indication of a disease

Polydipsia: excessive drinking

Polyuria: excessive urination

Appendix B

IACUC

INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

IACUCN001: PROTOCOL APPROVAL NOTICE

Friday, November 30, 2018

Senior Investigator	**John Haffner** (ROLE: Faculty Advisor)
Co-Investigators	Aleana Boudie (Student PI)
Investigator Email(s)	*john.haffner@mtsu.edu; ab2bv@mtmail.mtsu.edu*
Department	Agribusiness and Agriscience
Protocol Title	***Repeatability of TRH stimulation test for Equine Pituitary Pars Intermedia Dysfunction during the months of February through June***
Protocol ID	**19-2004**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Designated Member Review (DMR) mechanism*** and has approved your protocol in accordance with PHS policy. A summary of the IACUC action(s) and other particulars of this this protocol is tabulated as below:

IACUC Action	**APPROVED for one year from the date of this notification**	
Date of Expiration	**12/30/2019**	
Number of Animals	12 (TWELVE)	
Approved Species	**MTSU horse herd**	
Category Subclassifications	☐**Teaching** ☐ Classroom ☐ Laboratory	☒ **Research** ☒ Laboratory ☐ Field Research ☐ Field Study ☒ Handling/Manipulation ☐ Observation
	Comment: NONE	
Approved Site(s)	MTSU Horse Science Center	
Restrictions	**Satisfy DMR requirements AND annual continuing review**	
Comments	NONE	
Amendments		NONE

This approval is effective for three (3) years from the date of this notice. This protocol **expires on 12/30/2021** The investigator(s) MUST file a Progress Report annually regarding the status of this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL BE SENT. A continuation request (progress report) must be approved by the IACUC prior

to **12/30/2019** for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule: Refer tp the following table to request your CR:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	11/30/2019	TO BE COMPLETED
Second year report	11/30/2020	TO BE COMPLETED
Final report	11/30/2021	TO BE COMPLETED

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

Post-approval Protocol Amendments:

Date	*Amendment(s)*	*IRB Comments*
NONE	NONE	NONE

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: iacuc_information@mtsu.edu (for questions) and
Iacuc_submissions@mtsu.edu (for sending documents)

IACUCN001 – Protocol Approval Notice (DMR)