Measurement of cortisol concentration in the tears of horses and ponies with pituitary pars intermedia dysfunction

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OBJECTIVE
To compare tear cortisol concentrations between horses and ponies with pituitary pars intermedia dysfunction (PPID) and healthy nonaged (≤ 15 years old) and aged (≥ 20 years old) horses and to determine whether serum and tear cortisol concentrations were correlated.

ANIMALS
11 horses and ponies with PPID and 20 healthy control horses and ponies (11 nonaged and 9 aged).

PROCEDURES
Paired tear and serum samples were obtained from PPID and control animals. All animals were free of active ocular disease. Tear and serum cortisol concentrations were measured with an ELISA and chemiluminescent assay, respectively. Groups were compared with Kruskal-Wallis and Mann-Whitney *U* tests, and Spearman correlation analysis was used to examine relationships between tear and serum cortisol concentrations within groups.

RESULTS
Median tear cortisol concentration was significantly higher in PPID animals than in aged control animals, despite comparable serum cortisol concentrations in PPID and aged control animals. Median tear-to-serum cortisol concentration ratios were also significantly higher in PPID animals than in aged control animals. Serum and tear cortisol concentrations were not significantly correlated in PPID or control animals.

CONCLUSIONS AND CLINICAL RELEVANCE
Some horses and ponies with PPID had increased tear cortisol concentrations, compared with concentrations in healthy aged animals. Localized cortisol production in the tear film or altered cortisol binding dynamics could have contributed to this increase. Further studies are warranted to evaluate these mechanisms and to determine whether increased tear cortisol concentrations are associated with delays in corneal wound healing in horses and ponies with and without PPID. (*Am J Vet Res* 2016;77:1236–1244)

Corticosteroids can inhibit wound healing in the corneal epithelium of humans and other animals through a variety of cellular mechanisms.[1–4] For this reason, topical application of corticosteroids is generally considered contraindicated in horses with infectious keratitis and corneal epithelial defects.[5,6] However, the relative presence of endogenous corticosteroids (eg, cortisol) in the tear film of healthy or diseased eyes and the impact of these hormones in corneal wound healing are not well understood.

Cortisol is produced by the adrenal cortex in response to activation of the HPA axis by environmental or physiologic stress. Activation of the HPA axis results in increased release of ACTH, which then upregulates the synthesis and secretion of cortisol by the adrenal glands. Cortisol is detectable in the tears of healthy horses and dogs, and tear cortisol concentrations increase with systemic administration of ACTH in both species.[7,a]

Pituitary pars intermedia dysfunction is an age-related progressive neurodegenerative disorder that results in the loss of dopamine inhibition of the pars intermedia portion of the pituitary gland.[8] This loss of inhibition leads to overproduction of peptides from the pars intermedia, which results in increased circulating concentrations of ACTH as well as α-melanocyte–stimulating hormone and other related peptides. Interestingly, despite extremely high plasma ACTH concentrations, consistent increases in total serum cortisol concentrations are not commonly found in equids with PPID.[8–10] However, increases in the percentage of free (unbound and biologically

ABBREVIATONS

CBG	Cortisol-binding globulin
CV	Coefficient of variation
HPA	Hypothalamic-pituitary-adrenal
PPID	Pituitary pars intermedia dysfunction

active) cortisol in horses and ponies with PPID, compared with results for clinically normal age-matched animals, have recently been detected,[11] which suggests that plasma cortisol binding capacity (and potentially tissue cortisol activity) may be altered in animals with PPID. This increase in plasma free cortisol concentration might also permit increased diffusion of lipid-soluble free cortisol across the blood-aqueous barrier and subsequently into the tears, which could result in increased tear cortisol concentrations in the tear film of PPID animals.

Clinical manifestations of PPID include coat abnormalities (delayed shedding and hypertrichosis), muscle atrophy, laminitis, hyperhidrosis, and delayed wound healing. Specific mechanisms resulting in these clinical abnormalities have not been fully elucidated but generally have been attributed to physiologic effects of increased concentrations of pars intermedia peptides and excessive activity of the HPA axis. One manifestation of delayed wound healing in aged humans[12] and other animals (anecdotally reported in horses with PPID[8,10]) is a propensity for the development of nonhealing corneal ulcers. Mechanisms that might contribute to corneal ulcers in PPID animals are not fully characterized, although reduced corneal sensation has recently been reported in horses with PPID[10] and could contribute to initial ulcer formation and delayed healing. Increased cortisol activity in the tear film, if present in PPID, could theoretically further impair corneal healing and contribute to persistent nonhealing corneal ulcers in some horses with PPID.

The objectives of the study reported here were to compare tear cortisol concentrations between horses and ponies with PPID and healthy nonaged and aged horses and ponies and to determine whether tear and serum cortisol concentrations were correlated in these groups. We hypothesized that tear cortisol concentrations would be increased in equids with PPID, relative to concentrations in healthy animals, and that serum total cortisol concentrations would be similar between PPID and healthy animals.

Materials and Methods

Animals

Three groups of animals were evaluated: horses and ponies with PPID, healthy nonaged (≤ 15 years old) control horses and ponies, and healthy aged (≥ 20 years old) control horses and ponies. An a priori sample size analysis[b] based on the ability to detect a 3.5-fold difference in tear cortisol concentrations as significantly different (P = 0.05; power = 80%) indicated that 9 horses/group would be required. A 3.5-fold difference among groups was used for this sample size calculation on the basis of 2- to 3-fold increases in tear cortisol concentrations detected in healthy horses following ACTH-stimulation testing in another study[7]; we presumed that a clinically important increase in tear cortisol concentration in PPID animals would be at least as high or higher than that physiologic response in healthy horses. We elected to enroll ≥ 11 horses/group to allow for variation in observed values from hypothetical values used for calculations and potential loss of animals attributable to failure to meet inclusion criteria.

The PPID group consisted of a convenience sample of 14 client- and university-owned horses and ponies with confirmed or suspected PPID, as determined by staff veterinarians at the University of Georgia College of Veterinary Medicine. All animals were ≥ 16 years old and had clinical signs consistent with PPID, including 1 or more of the following: altered shedding patterns, hypertrichosis, laminitis, generalized muscle atrophy, or weight loss. The diagnosis of PPID was confirmed in all animals on the basis of a plasma endogenous ACTH concentration ≥ 50 pg/mL during November through July.[13,14] Although a plasma endogenous ACTH concentration > 27 to 35 pg/mL during this period (November through July) has been described as consistent with a diagnosis of PPID,[14,15] we elected to exclude animals with slight increases in ACTH concentrations (plasma ACTH concentration, 36 to 49 pg/mL) to minimize the likelihood of including animals with a false-positive PPID diagnosis. Because treatment for PPID with pergolide meyslate[c] had commenced in some PPID animals before the onset of sample collection, plasma ACTH measurement was repeated at the time of sample collection and also during November through July, and animals were included in the study only if the plasma ACTH concentration remained ≥ 50 pg/mL at the time of concurrent tear sample collection.

A convenience sample of 32 client- and university-owned horses and ponies with no clinical signs of PPID was also included to provide a healthy cohort for comparison with PPID animals. These animals comprised 11 nonaged and 21 aged control horses and ponies.

Prior to study inclusion, each animal was screened for active systemic or ocular disease; a physical examination, CBC, serum biochemical analysis, and complete ophthalmic examination were performed by a board-certified veterinary ophthalmologist (KEM). Ophthalmic examination consisted of a Schirmer tear test,[d] slit lamp biomicroscopy,[e] fluorescein staining,[f] and direct ophthalmoscopy of the optic nerve and peripapillary region.[g] Intraocular pressures were obtained with rebound tonometry[h] with or without topical application of corneal anesthetic.[i] Animals were excluded from the study if evidence of systemic disease was detected during physical examination (except for clinical signs of PPID described previously for the PPID group) or hematologic analysis or if ophthalmic examination revealed active ocular disease or signs of previous equine recurrent uveitis (synechiae, cataracts, or peripapillary depigmentation). All control group animals were screened for PPID by measurement of resting plasma ACTH concentrations during May or June and were excluded if the plasma ACTH concentration was > 35 pg/mL.[13,16]

All animal use and sampling protocols for university-owned animals were conducted under the guidance and approval of the University of Georgia Institutional Animal Care and Use Committee. Informed written consent was obtained for client-owned animals prior to sample collection; approval of sample collection protocols and consent documentation for client-owned animals was performed by the University of Georgia College of Veterinary Medicine Clinical Research Committee.

Sample collection

Samples were collected from all animals between January and June. Plasma samples were used for measurement of ACTH concentrations and verification of PPID status. In addition, a blood sample (10 mL) was collected via jugular venipuncture into a glass tube without anticoagulant and permitted to clot at ambient temperature. These blood samples were centrifuged, and serum was harvested and frozen at –80°C within 2 hours after collection. Tear samples (approx 100 µL) were collected by atraumatically introducing a glass microcapillary tube into the ventral fornix, as previously described.[7] Tears were collected from both eyes of each animal; samples were pooled for each animal, frozen, and stored at –80°C until analysis.

Validation of ELISA for measurement of tear cortisol concentration

A commercial ELISA[j] was validated for use with equine tears. Tears were collected as described previously on 2 days from 6 healthy horses (approx 2 mL/horse). Samples from all 6 horses were pooled. Pooled tears were diluted 1:2 with assay buffer and divided into equal aliquots. Known concentrations of cortisol were added to each aliquot to achieve added cortisol concentrations within and at the limits of the reported linear range of the assay (0, 0.04, 0.1, 0.2, 0.4, 1, and 2 ng/mL). Cortisol was measured in each spiked tear sample. Briefly, samples were diluted 1:2 in assay diluent and placed in wells of plates; assay standards and nonspecific binding control samples were also included in the assays. Diluted enzyme conjugate (15 µL/24 mL in assay diluent) was added to each well, and plates were incubated for 60 minutes at 22° C before washing to remove unbound enzyme conjugate. Substrate was added, and plates were incubated for 30 minutes. Qualitative results were obtained at an absorbance of 450 nm by use of a microplate reader.[k] Samples were assayed in duplicate. Percentage recovery of expected cortisol concentration was calculated for each sample as follows: (ELISA cortisol concentration/expected cortisol concentration) X 100. The mean value was calculated to determine mean percentage recovery for the assay. To assess precision, pooled tears were again diluted 1:2 with assay buffer and divided into 20 equal aliquots; assay standard (1 ng/mL) was then added to each aliquot. Aliquots were assayed in duplicate, and mean and SD for each aliquot were determined to calculate the intra-assay CV. To determine interassay CV, pooled tear samples were spiked with cortisol at 0 ng/mL, 0.04 ng/mL (low concentration), and 2 ng/mL (high concentration) and assayed in duplicate on 6 plates over multiple days. The CVs were calculated for each concentration as described, and the mean was then calculated to determine overall interassay CV.

Sample analysis

Plasma ACTH concentration of all PPID and control animals was measured by use of a validated chemiluminescent immunoassay (interassay and intra-assay CV was 7% to 9% and 9%, respectively).[15,l] Serum total cortisol concentration was measured by use of a validated chemiluminescent immunoassay (interassay and intra-assay CVs were < 20%).[17,l] Assays were performed by personnel at the Animal Health Diagnostic Center at the Cornell University College of Veterinary Medicine.

Tear total cortisol concentration was measured with the commercial ELISA[j] used in accordance with the manufacturer's protocol. Briefly, tear samples were thawed at room temperature (22°C), diluted 1:2 in assay diluent, and placed in wells of plates; assay standards and nonspecific binding control samples were also included in the assays. Samples were analyzed as described previously. Qualitative results were obtained at an absorbance of 450 nm by use of a microplate reader.[l] All samples were assayed in duplicate, and mean of the results was calculated. Samples with concentrations outside the linear range of the assay were subsequently diluted 1:5, 1:10, or 1:25 in assay diluent and reanalyzed. Standard curves were calculated per manufacturer instructions by the use of linear regression.

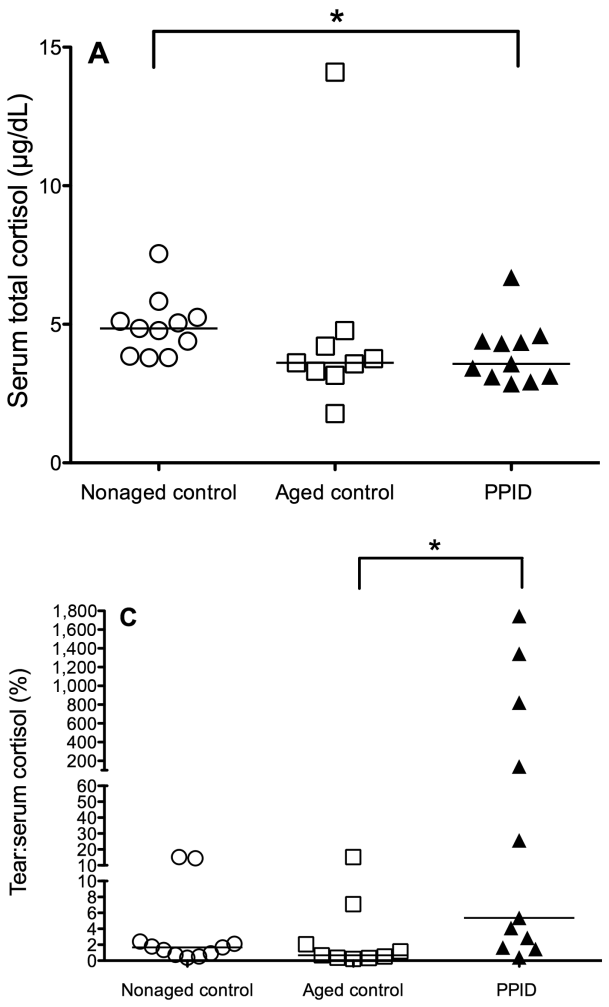
Data analysis

Data distribution was assessed with Shapiro-Wilk tests. Results indicated that most of the data were not normally distributed, so nonparametric analysis methods were used. Median age, serum and tear total cortisol concentrations, and tear-to-serum cortisol concentration ratios among PPID, nonaged control, and aged control animals were compared with Kruskal-Wallis tests, with the Dunn multiple comparison test used for post hoc analysis when appropriate. Within each group, serum and tear cortisol concentrations and ratios were compared between geldings and mares and between treated and untreated PPID animals by use of Mann-Whitney *U* tests. Spearman correlation analysis was used to determine correlations between serum and tear cortisol concentrations in PPID and control groups. Data were analyzed with commercial statistical software,[m] and significance was set at $P < 0.05$ for all analyses.

Results

Animals

Eleven of 14 PPID animals met the inclusion criteria (1 PPID horse was excluded because of major abnormalities on the biochemical profile, and 2 PPID animals were



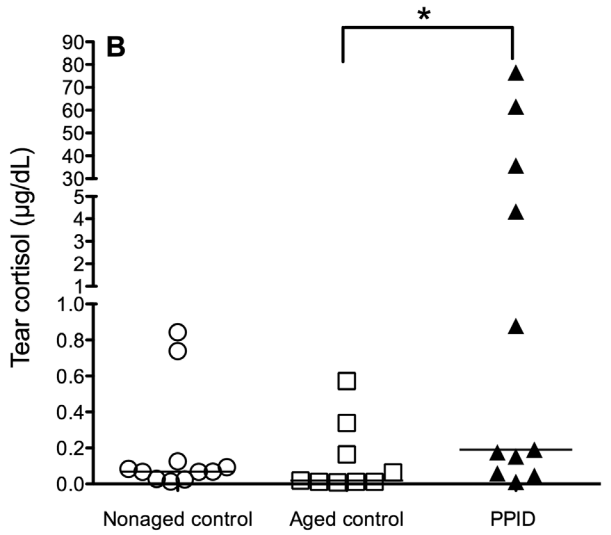


Figure 1—Serum total cortisol concentration (A), tear total cortisol concentration (B), and tear-to-serum cortisol concentration ratio expressed as a percentage (C) for 11 healthy nonaged (≤ 15 years old) control horses (white circles), 9 aged (≥ 20 years old) control horses and ponies (white squares), and 11 horses and ponies with PPID (black triangles). Each symbol represents results for 1 animal. The horizontal line depicts the median for each group. *Value differs significantly ($P < 0.05$) between groups.

excluded because of a plasma ACTH concentration < 50 pg/mL at the time of tear sample collection, most likely attributable to use of pergolide mesylate treatment to control PPID). All 11 healthy nonaged control animals met the inclusion criteria. Twelve of 21 aged control animals were excluded (2 because of abnormalities suggestive of PPID [eg, delayed shedding] detected during physical examination, 1 because of abnormalities detected in results of the CBC, 4 because of abnormalities detected during ocular examination, and 5 because of a plasma ACTH concentration > 35 pg/mL). Thus, data for 11 PPID animals, 11 nonaged control animals, and 9 aged control animals were used for analyses. No mares in any group were pregnant or being used for breeding at the time of sample collection.

The PPID group included 9 geldings and 2 mares (4 Quarter Horses, 2 ponies, 2 warmbloods, 1 Appaloosa, 1 Shire, and 1 Tennessee Walking Horse). Median age of animals in the PPID group was 28 years (range, 16 to 36 years). Four animals were currently being treated with pergolide (0.002 to 0.004 mg/kg/d for ≥ 60 days), and 7 animals had not been treated.

The healthy nonaged control group comprised 6 geldings and 5 mares (6 warmbloods, 2 Thoroughbreds, 2 Quarter Horses, and 1 Appaloosa). Median age was 12 years (range, 7 to 15 years). The healthy aged control group comprised 5 geldings and 4 mares (5 Quarter Horses, 1 Thoroughbred, 1 Quarter Horse–Thoroughbred cross, 1 Paso Fino, and 1 pony). Median age was 21 years (range, 20 to 23 years).

Median age differed significantly ($P < 0.001$) among groups, with aged control animals and PPID animals significantly older than nonaged control animals. There was no significant difference in median age between aged control animals and PPID animals. Median plasma ACTH concentration was significantly higher ($P < 0.001$) in PPID animals (median, 79.5 pg/mL; range, 50 to 421 pg/mL) than in nonaged (median, 22.7 pg/mL; range, 15.8 to 32.4 pg/mL) and aged (median, 27.9 pg/mL; range, 18.8 to 33.4 pg/mL) control animals.

Validation of ELISA for measurement of tear cortisol concentration

Intra-assay and interassay CV for the cortisol ELISA with equine tears was 3.63% and 14.36%, respectively. Mean percentage recovery of cortisol in spiked equine tear samples was 81.82%.

Serum and tear cortisol concentrations for control and PPID animals

Serum and tear cortisol concentrations and the tear-to-serum cortisol concentration ratios were determined for control and PPID animals **(Figure 1)**. Serum cortisol concentration, tear cortisol concentration, and tear-to-serum cortisol concentration ratio were not significantly different between nonaged and aged control

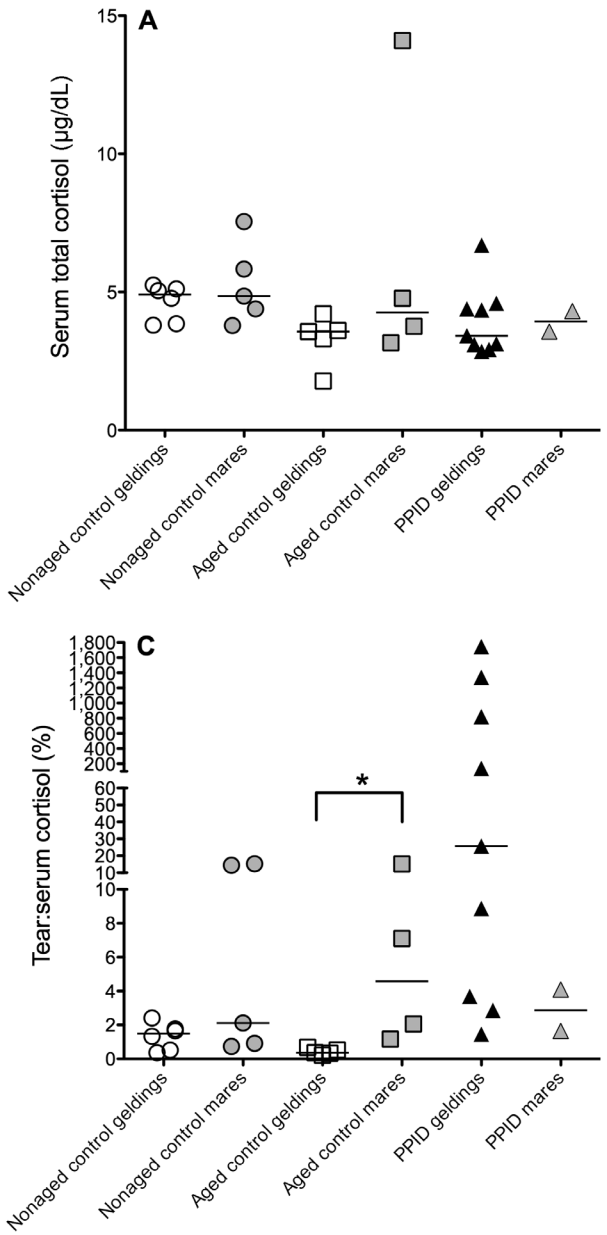


Figure 2—Serum total cortisol concentration (A), tear total cortisol concentration (B), and tear-to-serum cortisol concentration ratio expressed as a percentage (C) for 6 healthy nonaged control geldings (white circles), 5 healthy nonaged control mares (gray circles), 5 healthy aged control geldings (white squares), 4 healthy aged control mares (gray squares), 9 geldings with PPID (black triangles), and 2 mares with PPID (gray triangles). **See** Figure 1 for remainder of key.

animals. There was a significant difference in serum cortisol concentration (P = 0.019), tear cortisol concentration (P = 0.041), and tear-to-serum cortisol concentration ratio (P = 0.021) between PPID and control animals. Specifically, serum cortisol concentration in PPID animals was comparable to that of aged control animals and significantly lower than that of nonaged control animals, whereas tear cortisol concentration and tear-to-serum cortisol concentration ratio were significantly higher in PPID animals than in aged control animals.

Effect of sex on serum and tear cortisol concentrations within control and PPID groups

Serum and tear cortisol concentrations and tear-to-serum cortisol concentration ratio in mares and geldings were determined for each group **(Figure 2)**. Serum cortisol concentration was not significantly different between mares and geldings in nonaged control animals (P = 0.792), aged control animals (P = 0.286), or PPID animals (P = 0.909). Tear cortisol concentration and tear-to-serum cortisol concentration ratio were significantly (P = 0.018) higher in mares than geldings of the aged control group, but no sex differences in tear cortisol concentration or tear-to-serum cortisol concentration ratio were found for the nonaged control animals (P = 0.170) or PPID animals (P = 0.927).

Effect of pergolide treatment in PPID animals

Results were compared between the 4 pergolide-treated PPID animals and the 7 untreated PPID animals. There was no significant difference in serum cortisol concentration (P = 1.000), tear cortisol concentration (P = 0.927), or tear-to-serum cortisol concentration ratio (P = 0.927) between treated and untreated PPID animals **(Table 1)**.

Correlation between serum and tear cortisol concentrations

Correlations between serum and tear cortisol concentrations were assessed. Serum and tear cortisol concentrations were not significantly correlated in nonaged control animals (r = 0.606; 95% confidence interval, −0.011 to 0.889 [P = 0.052]), aged control animals (r = 0.407; 95% confidence interval,

Table 1—Median (range) serum and tear total cortisol concentrations and tear-to-serum cortisol concentration ratio (expressed as a percentage) for horses and ponies with PPID that were or were not treated with pergolide for ≥ 60 days before onset of sample collection.

Variable	Untreated (n = 7)	Treated (n = 4)
Serum total cortisol concentration (μg/dL)	4.3 (2.9–6.7)	3.5 (3.1–4.4)
Tear total cortisol concentration (μg/dL)	0.19 (0.01–61.60)	0.47 (0.05–76.60)
Tear-to-serum cortisol concentration ratio	5.40 (0.40–1,342.00)	13.70 (1.50–1,745.00)

Values did not differ significantly ($P \geq 0.05$) between untreated and treated animals for any variable.

not determined because n < 10 [$P = 0.270$]), or PPID animals ($r = 0.609$; 95% confidence interval, −0.006 to 0.890 [$P = 0.052$]).

Discussion

Results for the present study supported the hypothesis on the basis of significantly increased tear cortisol concentrations in horses and ponies with PPID, compared with results for healthy nonaged and aged control animals. This was despite comparable or lower total cortisol concentration in PPID animals versus healthy control animals of both age groups, which is similar to previous reports[18,19] and suggests that increased tear cortisol concentration in horses and ponies with PPID does not result from general upregulation of the HPA axis and global increases in circulating cortisol concentrations. Indeed, results of a recent study[n] suggest that increased ACTH concentrations released from the diseased pars intermedia in horses and ponies with PPID might consist of, at least in part, a less bioactive isoform or isoforms of ACTH, which helps explain the lack of an increased serum total cortisol concentration in animals with PPID in the study reported here and in other studies.[18,19]

Thus, these data provided evidence that tear and serum cortisol concentrations in horses and ponies with PPID were not directly and systematically associated. This was supported by the fact that 4 PPID animals in the present study had tear cortisol concentrations 1.50- to 17.45-fold as high as serum cortisol concentrations. Increased tear cortisol concentration in animals with PPID could be explained by local ocular production of cortisol in some horses with PPID, as has been described for human corneal epithelial cells in culture.[20] Alternatively, tissue cortisol metabolism could be altered in ocular tissues of horses with PPID and might result in persistence of cortisol in the tear film. Altered expression of the enzyme 11-α-hydroxysteroid dehydrogenase, which has multiple isoforms responsible for the conversion of cortisol to and from its inactive metabolite cortisone in tissues, has been described in horses with laminitis[21] and in humans with keratitis attributable to *Pseudomonas* spp.[20] To our knowledge, expression of 11-α-hydroxysteroid dehydrogenase has not yet been examined in ocular tissues obtained from equids with PPID.

Altered diffusion or partitioning of plasma cortisol into the tear film could also result in increased tear cortisol concentrations in horses with PPID. Plasma cortisol circulates in both free and protein-bound forms in horses and other species, but it is the free form that is generally bioavailable to diffuse across plasma membranes and enter cells.[22] Theoretically, an increase in the amount of free cortisol could result in increased diffusion of free cortisol into the tear film in animals with PPID. Indeed, both increasing age and Cushing's disease in humans are associated with a decrease in availability or binding affinity of the cortisol-binding protein CBG, which results in an increase in the free cortisol concentration.[23,24] An increased fraction of free, but not total, serum cortisol has been detected in horses and ponies with PPID, compared with results for healthy aged horses.[11]

However, when serum total and free cortisol and tear cortisol concentrations were measured in healthy horses at rest and after ACTH stimulation, tear cortisol concentrations were consistently higher than serum free cortisol concentrations,[7] which suggests that both bound and free cortisol are present in equine tear film. In fact, cortisol bound to binding proteins such as CBG serves as a plasma cortisol reservoir by protecting cortisol from metabolism or excretion. It is possible that concentrations of CBG and other cortisol-binding proteins are increased in the tears of horses and ponies with PPID and that they effectively retain cortisol in the tears, which results in increased tear cortisol concentrations regardless of plasma cortisol concentrations. Measurement of both total and free cortisol concentrations and concentrations of cortisol-binding proteins in serum and tears would have been beneficial to help clarify these complex relationships in healthy and PPID animals, but methodological and sample volume constraints precluded including this analysis in the present study.

In the study reported here, significant correlations between tear and serum total cortisol concentrations were not detected for healthy or PPID animals, which suggested that equine serum and tear cortisol concentrations likely were regulated by distinct factors. This result is in contrast to that of another report[7] in which investigators detected significant positive correlations between tear cortisol and serum total and free cortisol concentrations in 4 healthy adult horses by use of repeated sampling over a 6-hour period during an ACTH stimulation test. However, for healthy nonaged control animals and PPID animals in the present study, there was a pat-

tern of higher tear cortisol concentrations in animals with higher serum cortisol concentration ($P = 0.052$ for both groups). Further studies with a larger number of animals are needed. It would have been ideal to measure tear and serum cortisol concentrations with identical methods to better clarify the relationship between these variables. However, this was not possible for the present study. Sample volume limitations precluded analyzing tear cortisol concentrations with the chemiluminescent immunoassay used for serum, and an extraction step yielded inconsistent results for the ELISA used for serum samples. Further refinement of tear cortisol measurement techniques with alternate methods may be helpful for future studies.

An important limitation of the study reported here was the small sample size and diverse characteristics of the PPID group. Although we were able to obtain samples from an adequate number of animals that met inclusion criteria for each group (as determined by a priori sample size calculations), substantial variation in tear cortisol concentrations among horses and ponies with PPID was evident, with extreme increases in tear cortisol concentrations in some PPID animals and tear cortisol concentrations similar to those of healthy nonaged and aged control animals in other PPID animals. This interanimal variation in tear cortisol concentrations might be explained, at least in part, by differences in disease severity or duration because PPID is a chronic and progressive disease.[8] We attempted to minimize the effect of disease severity by limiting sample collection to animals with both clinical signs of PPID and a substantial increase in resting ACTH concentrations or confirmatory testing with ACTH stimulation testing to select for more advanced disease and eliminate animals with potential false-positive results, but animals with both mid- and late-stage PPID were included.

Breed, sex, and age differences among groups might also have impacted the findings. In particular, ponies were included in the aged control group (1/9 animals) and PPID group (2/11 animals), but there were no ponies in the nonaged control group (0/11 animals). Variation in endocrine function in general and the HPA axis in particular has been described for ponies[13,25–27]; similar differences in tear cortisol dynamics between horses and ponies, if present, could have been a potential confounder in the present study. However, repetition of the analysis with data for these 3 ponies excluded did not alter the pattern or significant differences among control and PPID animals (data not shown). Furthermore, sex differences in plasma cortisol binding dynamics have been described for humans,[28–30] and significantly higher tear cortisol concentrations and tear-to-serum cortisol concentration ratios in mares, compared with results for geldings, in the aged control group were identified in the present study. However, it is unlikely that such sex differences (higher tear cortisol concentrations in mares) would explain the differences among PPID and control groups in this study because the PPID group contained only 2 mares, and those 2 mares had among the lowest tear cortisol concentrations in the PPID group (data not shown).

Median age for the PPID group (28 years) was greater, but not significantly so, than the median age for the aged control group (21 years). A previous small study[12] did not identify an effect of age on serum free cortisol concentration in horses. However, given the aforementioned association between increasing age and increased free plasma cortisol concentration in humans,[23,24] it is possible that some of the differences in tear cortisol concentration observed for the PPID group reflected an effect of age rather than an effect of endocrine disease on plasma cortisol dynamics. We attempted to account for this by initially screening a larger number of animals in the aged control group, but eventually we had to exclude 12 of the original 21 aged control animals because of evidence of PPID (7 animals) or other ocular or systemic disease (5 animals). This highlighted the difficulties in identifying an appropriate healthy geriatric control group for large-scale studies conducted to investigate a disease of aging such as PPID.

Alternatively, the substantial variation in tear cortisol concentration detected among PPID animals could have been an effect of medical treatment for PPID because samples were obtained from 7 untreated PPID animals and 4 animals receiving pergolide treatment for PPID. These 4 treated animals were retained in the study because their continued clinical signs of PPID with concurrent plasma ACTH concentrations > 50 pg/mL suggested that the disease was not fully controlled despite initiation of pergolide mesylate treatment ≥ 60 days prior to onset of sample collection. In contrast, 2 additional treated PPID animals were excluded from the analysis because their plasma ACTH concentrations at the time of tear sample collection did not remain higher than the cutoff of 50 pg/mL. Furthermore, repetition of the analysis with exclusion of the data for the treated animals did not change the significant findings (data not shown). Data for the study reported here did not support an effect of pergolide mesylate treatment on serum or tear cortisol concentrations because no differences in these variables were found between treated and untreated PPID animals, although this study was almost certainly underpowered to detect such a treatment effect. Further studies are warranted to determine the manner in which factors such as breed, sex, age, disease duration and severity, and medical treatment impact tear cortisol concentrations of horses and ponies with PPID.

Findings of the present study also illustrated that cortisol can be measured in equine tears via an ELISA and that the commercial ELISA used in this study detected cortisol concentrations in equine tears with reasonable accuracy and precision. This assay also had minimal to no cross-reactivity with other compounds such as progestagens, androgens, and the inactive cortisol metabolite cortisone, which sug-

gested that measured increases in tear cortisol concentrations detected in this study were truly a result of increased cortisol concentrations in the tear film and not increased concentrations of other related compounds. It would have been ideal to additionally measure both serum and tear cortisol concentrations with the chemiluminescent immunoassay to further support this, but tear sample volume limitations precluded this approach.

For the study reported here, results indicated that some horses and ponies with PPID had greatly increased tear cortisol concentrations despite comparable or lower serum total cortisol concentrations, compared with concentrations for healthy nonaged and aged control horses. A significant correlation between single-sample tear or serum cortisol concentrations was not detected in healthy or PPID animals. There was no effect of pergolide treatment on tear or serum cortisol concentrations in this small group of animals with PPID, although this finding should be interpreted with caution given the probable lack of statistical power in this study. The clinical importance of the increased tear cortisol concentrations with regard to ocular disease in horses with PPID cannot be determined from these data; animals with current or historical ocular disease were purposely excluded from the study because it would have been impossible to determine the relative contribution of ocular versus endocrine disease to differences in tear cortisol concentrations among groups. Further studies are needed to investigate mechanisms that result in increased cortisol concentrations in equine tear film and factors associated with increased tear cortisol concentrations in animals with PPID and to determine whether increased tear cortisol concentrations impact corneal wound healing in horses and ponies with PPID and those without PPID.

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Footnotes

a. Wynne BT, Hart KA, Norton NA, et al. Endogenous cortisol concentration in canine tears and serum at rest and after a simulated stress event (abstr). *Vet Ophthalmol* 2015:18:E28.
b. SigmaPlot 11.0, Systat Software Inc, San Jose, Calif.
c. Prascend, Boehringer Ingelheim Vetmedica Inc, St Joseph, Mo.
d. Schirmer tear test standardized sterile strips, Merck Animal Health, Baton Rouge, La.
e. SL-15 portable slit lamp, Kowa American Corp, Torrance, Calif.
f. FUL-FLO fluorescein sodium sterile ophthalmic strips, Akorn Inc, Lake Forest, Ill.
g. Heine Optotechnik, Herrsching, Germany.
h. Tonopen, Reichert Technologies, Depew, NY.
i. Proparacine hydrochloride ophthalmic solution, USP 0.5%, Alcon Laboratories and Sandoz Inc, Duluth, Ga.
j. Salimetrics LLC, Carlsbad, Calif.
k. Epoch micro-volume spectrophotometer system, BioTek Inc, Winooski, Vt.
l. Immulite, Diagnostics Product Corp, Los Angeles, Calif.
m. GraphPad Prism, version 5.0, La Jolla, Calif.
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