

Association of Adrenocorticotrophin and Cortisol Concentrations with Peripheral Blood Leukocyte Cytokine Gene Expression in Septic and Nonseptic Neonatal Foals

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Background: The hypothalamic-pituitary-adrenal (HPA) axis is influenced by the proinflammatory cytokines IL-6, IL-1 β , and TNF- α in critically ill humans. Information about the association of cytokines with the HPA axis in neonatal foals is lacking.

Hypothesis/Objectives: The objectives were to describe for hospitalized septic and nonseptic foals (1) temporal changes in blood concentrations of ACTH, and cortisol, and leukocyte cytokine gene expression, and (2) coassociation of these HPA axis hormones with blood leukocyte cytokine gene expression.

Animals: Hospitalized septic foals (N = 15) and hospitalized nonseptic foals (N = 11).

Methods: Blood samples, obtained from study foals at admission (T = 0), and 24 (T = 1), 48 (T = 2), 72 (T = 3), and 96 (T = 4) hours after admission, were processed to isolate RNA from leukocytes and to harvest plasma and serum for hormone assays. Plasma ACTH and serum cortisol concentrations were determined by radioimmunoassay. Leukocyte mRNA expression of IL-1 β , IL-6, IL-8, IL-10, and TNF- α was determined using RT-PCR.

Results: Cortisol concentrations were greater ($P < .05$) in foals at admission than at other time points. The expressions of IL-8 and IL-10 mRNA were lower ($P < .05$) at each time point in septic than in nonseptic foals. Among septic foals, ACTH was positively associated ($P = .0026$) with IL-6 mRNA expression.

Conclusions: Sepsis influences secretion of the HPA axis hormones and expression of cytokines in foals. A positive association with the HPA axis and IL-6 expression was detected. The clinical importance of these findings requires additional study.

Key words: Cytokines; Foals; Gene expression; Hormones; Sepsis.

Sepsis is an important problem of foals and is frequently cited as a leading cause of morbidity and mortality in neonatal foals less than 7 days of age.^{1–3} Case fatality proportions for septic foals may be as high as 70%, despite improvements in diagnostic and therapeutic methods.^{1–3} The normal host response to an infectious agent includes production of proinflammatory cytokines to promote defense mechanisms and anti-inflammatory cytokines to downregulate inflammatory mechanisms and maintain homeostasis.^{4,5} Peripheral blood concentrations of cytokines have been measured to evaluate their diagnostic and prognostic value in septic infants and neonatal foals.^{6–13} Persistent increases in interleukin (IL)-6 in septic human infants have been associated with increased duration of hospitalization and increased risk of death.^{6–9}

The hypothalamic-pituitary-adrenal (HPA) axis is the primary neuroendocrine pathway mediating host responses to the stress of infection. In critical illness such as sepsis, inflammatory cytokines, principally IL-1 β , IL-6, and tumor necrosis factor- α (TNF- α), act on the HPA axis to increase concentrations of cortico-

Abbreviations:

HPA axis	hypothalamic pituitary adrenal axis
ACTH	adrenocorticotrophin
AVP	arginine vasopressin
CRH	corticotrophin-releasing hormone
EDTA	ethylenediaminetetraacetic acid
RAI	relative adrenal insufficiency
CIRCI	critically ill-related cortisol insufficiency
RT-PCR	reverse transcriptase polymerase chain reaction

trophin-releasing hormone (CRH), arginine vasopressin (AVP), adrenocorticotrophin (ACTH), and cortisol.¹⁴ The HPA axis has been examined in both healthy and diseased foals in recent years.^{24–27} These studies demonstrated that ACTH and cortisol were increased in critically ill foals.^{24,26,27} The magnitude of the increase in hormone concentrations was associated with survival. Nonsurviving foals had peripheral blood concentrations of ACTH and cortisol, and ACTH:cortisol ratios, that were higher than those of foals surviving to discharge.^{24,26,27}

Hart et al assessed the HPA axis in healthy and septic foals using a paired low/high-dose ACTH stimulation test,^{25,28,29} and Wong et al evaluated a low-dose ACTH stimulation test in sick foals.³⁰ These studies demonstrated that sick foals, including those with sepsis, have some degree of HPA axis dysfunction.^{25,30} However, in contrast to previous studies,^{24,26,27} differences were not identified in the basal ACTH : cortisol ratios between surviving and nonsurviving hospitalized septic foals.^{25,30} One explanation for these different findings is that a great deal of variability exists in ACTH and cortisol concentrations

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in each foal, thus basal ACTH : cortisol ratios may not be fully reliable.

To the authors' knowledge, potential links between the HPA axis and cytokine production by leukocytes have not been described in neonatal foals, although cytokine profiles have been described in foals and humans.^{6-9,12,13,31-34} The purpose of this study was 3-fold: (1) to determine whether there was an association between HPA axis and cytokine expression in hospitalized full-term, septic and nonseptic neonatal foals, (2) to compare temporal changes in secretion of ACTH and cortisol between septic and nonseptic foals, and (3) to compare temporal changes in expression of cytokines among septic and nonseptic foals of the same ages. The hypotheses were that a positive association would exist between proinflammatory cytokine mRNA expression and blood concentrations of HPA axis hormone in septic foals, and that temporal changes in each hormone concentration and mRNA gene expression of cytokines would differ between septic and nonseptic foals.

Materials and Methods

Animals

This prospective clinical study was performed at Texas A&M University's Veterinary Medical Teaching Hospital (VMTH) between January 2009 and October 2009. Full-term (>330 days gestation) foals ≤ 7 days of age of both sexes and any breed were eligible to be included in the study. Two groups of foals were involved in the study. Septic foals (Group 1) were admitted to the VMTH and were considered septic based upon a sepsis score³⁵ of >11 at the time of admission, a positive result of blood culture obtained at time of admission, or both. Each foal included in this study had a sepsis score calculated by the attending veterinarian.

The nonseptic foals (Group 2) were admitted to the VMTH for reasons other than sepsis. Specifically, the nonseptic foals either accompanied their sick dams (eg, a foal accompanying a mare with colic), were born at the hospital (eg, a mare that gave birth at the teaching hospital), or were presented for evaluation of musculoskeletal disease. All nonseptic foals were free of systemic illness, as determined by physical examination, blood culture, CBC, and serum biochemistry profile. Additionally, the nonseptic foals included healthy foals from the Texas A&M Horse Center; these foals were sampled on site and were not transported to the VMTH for sampling. The Texas A&M Horse Center foals were kept in a 10-acre pasture and were stressed before sampling by being caught, restrained, and having venipuncture performed.

Blood samples were obtained from the septic foals at admission (T = 0), and 24 (T = 1), 48 (T = 2), 72 (T = 3), and 96 (T = 4) hours after the initial blood sampling (T = 0). Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes for isolation of white blood cells (WBCs) and measurement of plasma ACTH concentration, and in clot tubes for measurement of serum cortisol concentrations. Plasma and serum were collected by centrifugation of blood samples at $1,200 \times g$ at 4°C and then stored at -80°C until assayed for ACTH and cortisol concentrations. Total leukocyte populations were isolated using a commercial leukocyte total RNA isolation system^a; isolated leukocyte mRNA was frozen at -80°C until analysis.

All experimental procedures were approved by the Clinical Research Review Committee of the College of Veterinary

Medicine and Biomedical Sciences, Texas A&M University and the Institutional Animal Care and Use Committee of Texas A&M University. For the foals admitted to the Texas A&M University Veterinary Medical Teaching Hospital, owner consent authorizing the investigators to collect blood samples for the study was obtained.

Endogenous ACTH and Cortisol Concentrations

Plasma concentrations of ACTH were determined by radioimmunoassay (RIA) of 100- μL duplicate aliquots of samples determined within a single assay.^{b,37,38} In this double-antibody RIA, the primary antiserum was rabbit antiporcine ACTH, the standards were made in triplicate from synthetic ACTH¹⁻³⁹ (ranging from 2.5 to 1000 pg), the radiolabeled tracer was ACTH-¹²⁵I, and the secondary antiserum was goat antirabbit gamma globulin. Counts per minute obtained with an automatic gamma counter (Micromedics Systems Inc)^c and Assay Zap software^d were used to construct the standard curve to calculate ACTH concentrations. The ACTH antiserum cross-reacts 100, 100, 0.8, and 0.1% with ACTH¹⁻³⁹; ACTH¹⁻²⁴ β -lipotropin, and α -lipotropin, respectively. The minimum detectable concentration of ACTH was 5 pg/mL. The intra-assay coefficient of variation was 7%, based on use of pooled quality control stallion plasma samples known to have low or high concentrations of ACTH. In addition, assays of stallion plasma spiked with known amounts of ACTH demonstrated $>98\%$ recovery of exogenous ACTH. Assays of dilutions of the plasma pool demonstrated parallelism of the assay.

Serum concentrations of cortisol were determined in 25- μL duplicate aliquots of samples, and all samples were tested within 1 assay by a single-antibody RIA that utilized polypropylene tubes coated with the rabbit-derived cortisol antiserum.^b The cortisol standards (ranging from 0.5 to 50 $\mu\text{g/dL}$) were made in triplicate. The radiolabeled tracer was cortisol-¹²⁵I. The cortisol antiserum cross-reactivity with steroids was 0.03% for aldosterone, 0.94% for corticosterone, 0.26% for deoxycorticosterone, 0.02% for progesterone, and 0.01% for estradiol. Counts per minute obtained with an automatic gamma counter (Micromedics Systems)^c and Assay Zap software^d were used to construct the standard curve to calculate cortisol concentrations.^c The minimum detectable concentration was 1.2 ng/mL and intra-assay coefficient of variation was 5% based upon the use of pooled quality control stallion serum samples known to have low or high concentrations of cortisol. In addition, assay of stallion serum spiked with known amounts of cortisol demonstrated $>96\%$ accuracy in recovery of exogenous cortisol. Assays of the diluted plasma pool demonstrated parallelism of RIA.

Gene Expression of Cytokines

A commercial kit^a was used to isolate total mRNA from peripheral blood leukocytes. Purity and concentration of the RNA were determined fluorometrically, and isolated RNA was treated for DNA contamination using DNase I.^f cDNA was subsequently synthesized using the Superscript III First-Strand synthesis system for real-time reverse transcriptase (RT)-PCR.^f Real-time RT-PCR was used to quantify mRNA expression of IL-1 β , IL-6, IL-8, IL-10, and TNF- α .^{39,40} Intron-spanning equine $\beta 2$ -microglobulin was employed as the endogenous control.³⁰⁻⁴⁰ Real-time RT-PCR was performed using a GeneAmp 7500 Sequence Detection System.^g The relative quantification of cytokine gene expression was calculated using the $\Delta\Delta\text{C}_T$ method, and the fold-changes in mRNA expression were calculated⁴¹ as $2^{\Delta\Delta\text{C}_T}$, using the admission sample as the reference value for a given foal. Because the admission sample for the septic foals and the initial blood sample obtained from the nonseptic foals were used as the

calibrator for each individual foal, it was not possible to compute fold-change values at admission because these values were, by definition as the reference, assigned a value of 1.

Statistical Analysis

Categorical variables were compared between septic and nonseptic foals using Chi-squared analysis or Fisher's exact tests. The association between ACTH and cortisol concentrations and fold-changes in cytokine expression was analyzed using linear mixed-effects modeling by S-PLUS statistical software.^h The ACTH and cortisol data were log₁₀-transformed to meet distributional assumptions of modeling and for graphic representation. The individual foal was modeled as a random effect (to account for repeated measures on individual foals) and day of hospitalization was modeled as a fixed, ordered, categorical effect, and the correlation structure for mixed-effects modeling was that of compound symmetry. Pair-wise comparisons between hormone concentrations or mRNA fold-changes between septic and nonseptic foals within time point or among time points within either the septic group or nonseptic group were performed using the multiple comparisons method of Sidak.⁴² Significance for all analyses was set at $P < .05$.

Results

Study Population

A total of 26 eligible foals were included in this study; no foals were excluded from the study. Of the 26 foals, 20 were male and 6 were female. There were 15 septic foals and 11 nonseptic foals. Among the nonseptic foals, 8 were admitted to the VMTH and 4 were from Texas A&M Horse Center. The proportion of males did not differ significantly between the septic (80%; 12/15) and nonseptic (73% 8/11) groups. Thoroughbred (65%; 17/26) was the predominant breed of foals followed by Quarter Horse (23%; 6/26), Paint (8%; 2/26), and Arabian (6%; 1/26). There were significantly more Thoroughbreds (91%; 10/11) ($P = .036$) among nonseptic foals than among septic foals (47%; 7/15). Alternatively, there were significantly ($P = .007$) more Quarter Horse-type (Paint or Quarter Horse) horses among septic foals (53%; 8/15) than among nonseptic foals (0%; 0/11).

Sepsis Score

The sepsis score for all nonseptic foals was 0. The sepsis score for the septic foals ranged from 11 to 23 with a median of 16. Of the 15 septic foals, blood culture results were obtained from 13 foals. Two foals did not have blood cultures submitted due to financial constraints. Nine of the 13 (69%) septic foals had positive blood cultures yielding gram-negative bacteria, 2 out of 9 foals had gram-positive bacteremia, and 1 of the 9 foals had mixed (ie, concurrent gram-negative and gram-positive) bacteremia. For the nonseptic foals, blood cultures were submitted on 7 foals, all of which yielded negative results. For those foals for which blood culture data were available, survival was less likely among those that were blood culture-

positive (67%; 6/9) than blood culture-negative (92%; 11/12), but the difference was not significant ($P = .277$). Among septic foals, survival was not different for those that were blood culture-negative (75%; 3/4) than those that were blood culture-positive (67%; 6/9). Although not statistically significant ($P = .277$), septic foals were less likely to survive (67%; 10/15) than were nonseptic foals (100%; 11/11).

Age

The median age at admission of the foals was 2 days (range, 0.16–5 days). The nonseptic group of foals tended to be younger (median, 0.75 days; range, 0.33–5 days) than the septic foal group (median, 2 days; range, 0.16–4 days), but this difference was not statistically significant. The median age at admission of foals that survived (2 days; range, 0.33–4 days) did not significantly differ from that of foals that did not survive (3 days; range, 0.16–5 days).

Effect of Time on ACTH and Cortisol Concentrations

There were no significant differences in ACTH concentrations among time points for either the septic or nonseptic foals (Table 1a). Within each time point, however, ACTH concentrations were significantly ($P < .05$) higher among septic foals than nonseptic foals (Table 1a). For both septic and nonseptic foals, concentrations of cortisol at admission were higher ($P < .05$) than at other times (Table 1b). Within each time point, cortisol concentrations were higher ($P < .05$) among septic than nonseptic foals (Table 1b).

Association of Concentrations of ACTH and Cortisol

Using linear mixed-effects regression, concentrations of ACTH were significantly ($P < .0001$) and positively associated with cortisol concentrations among the total population of foals (ie, combining both septic and nonseptic foals; Fig 1) included in this study. For each ng/mL increase of cortisol concentrations, it was estimated that ACTH concentration was increased by 1.01 pg/mL (95% confidence interval [CI], >1.00–1.02 pg/mL). The association between ACTH and cortisol concentrations remained significant and of similar magnitude to that observed for the total population when analyzing the data from the septic and nonseptic foals as separate groups. Among septic foals, ACTH was significantly ($P < .0001$) and positively correlated with cortisol concentrations. For each ng/mL increase in cortisol, it was estimated that ACTH was increased by 1.02 pg/mL (95% CI, 1.01–1.03 pg/mL). Among nonseptic foals, ACTH concentration was significantly ($P = .0014$) associated with cortisol concentration. For each ng/mL of cortisol, it was estimated that ACTH concentration was increased by 1.01 pg/mL (95% CI, >1.00–1.03 pg/mL).

Table 1. Concentrations of ACTH and cortisol during the first 4 days of hospitalization among 15 septic foals hospitalized at the Veterinary Medical Teaching Hospital, Texas A&M University and 11 nonseptic foals either hospitalized at the Veterinary Medical Teaching Hospital or at the Texas A&M University Horse Center during 2009; data in the table represent mean values (95% CI).

Group	Admission	24 Hours	48 Hours	72 Hours	96 Hours
a. ACTH concentrations (pg/mL)*					
Septic	31.0 ^{a,1} (23.9–40.3)	19.1 ^{a,1} (14.7–24.8)	27.4 ^{a,1} (21.1–35.7)	34.4 ^{a,1} (24.3–27.2)	24.1 ^{a,1} (18.5–31.3)
Nonseptic	20.7 ^{b,1} (14.3–29.9)	12.7 ^{b,1} (8.7–18.5)	18.3 ^{b,1} (13.2–25.3)	14.0 ^{b,1} (10.8–18.0)	16.0 ^{b,1} (10.9–23.7)
b. Cortisol concentrations (ng/mL)*					
Septic	58.9 ^{a,1} (48.8–68.9)	39.8 ^{a,2} (29.8–49.8)	40.3 ^{a,2} (30.3–50.3)	34.4 ^{a,2} (24.3–44.4)	35.1 ^{a,2} (25.1–45.1)
Nonseptic	38.3 ^{b,1} (28.6–48.0)	19.2 ^{b,2} (8.7–29.8)	19.8 ^{b,2} (8.7–30.8)	13.8 ^{b,2} (2.4–25.2)	14.6 ^{b,2} (3.0–26.2)

*Values represent back-transformed means of log₁₀-transformed data.

Values within rows that have different numbers differ significantly ($P < .05$).

Values within columns that have different letters differ significantly ($P < .05$).

Effects of Time and Sepsis on Cytokine mRNA Expression

No significant effects of time, sepsis status, or their interaction were noted in mRNA expression for IL- β , IL-6, or TNF- α . Age and survival also were not significantly associated with cytokine mRNA expression. At each time point (ie, 24, 48, 72, and 96 hours), expression of IL-8 was significantly lower ($P < 0.5$) among septic foals than nonseptic foals (Table 2a), but there were no significant difference in fold-changes of IL-8 mRNA expression among time points for either septic or nonseptic foals (Table 2a). Among septic and nonseptic foals, survival was not significantly associated with IL-8 mRNA expression. Relative to nonsurvivors, the fold change in IL-8 mRNA was 1.3 (95% CI, 0.3–5.4; $P = .7014$). At each time point, expression of IL-10 was significantly ($P < .05$) lower among septic foals than nonseptic foals (Table 2b), but there was no

significant difference in mRNA expression of IL-10 among times for either septic or nonseptic foals. Among septic and nonseptic foals, survival was not significantly associated with IL-10 mRNA expression. Adjusted for time point, the fold change among survivors relative to nonsurvivors was 1.0 (95% CI, 0.2–5.0; $P = .9870$).

Association of Cytokine mRNA Expression with Concentrations of ACTH and Cortisol

Among the total study population (ie, septic and nonseptic foals combined), there were no significant associations of either plasma concentrations of ACTH or serum concentrations of cortisol with mRNA expression of any of the cytokines examined. Among septic foals, plasma ACTH concentration was significantly and positively associated with IL-6 mRNA expression (Fig 2). For each 10-fold change in IL-6 expression, linear mixed-effects modeling estimated that ACTH concentration was increased by 50.1 pg/mL (95% CI, 20.0–251.2 pg/mL; $P = .0026$). Concentration of ACTH was not significantly associated with mRNA expression of IL-1 β , IL-8, IL-10, or TNF- α . Cortisol concentration was not significantly associated with any cytokines evaluated (Fig 3).

Discussion

Results of this study indicate that ACTH and cortisol concentrations were higher among septic foals compared to nonseptic foals. These observed increases in hormone concentrations in critically ill foals represent an expected and appropriate response to sepsis.^{24–27} The finding that cortisol concentrations at admission were significantly higher than at other times in all foals was not expected and has not been demonstrated previously. Higher initial cortisol concentration with a subsequent decrease could suggest dysregulation of the HPA axis at a variety of levels, such as ACTH resistance, downregulation of cortisol receptors, or diminished cortisol synthesis with prolonged critical illness in the neonatal foal. This temporal pattern did not

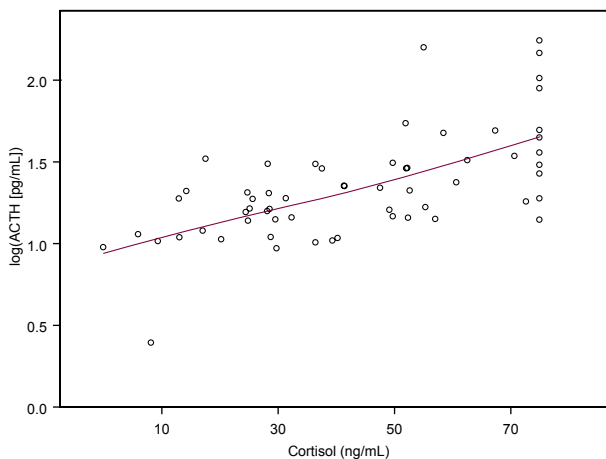


Fig 1. Association of ACTH and cortisol concentrations among 15 septic foals and 11 nonseptic foals admitted to a referral hospital; concentrations were determined serially (see text for details). The concentration of ACTH was significantly ($P < .0001$) and positively associated with cortisol. The line represents the fitted slope of the association determined using linear mixed-effects regression; the slope of the fitted line was 0.007 (95% CI, 0.005–0.009), indicating that ACTH increased by approximately 1 pg/mL for each ng/mL of cortisol.

Table 2. Fold-changes (relative to admission) in mRNA expression for cytokines during the first 4 days of hospitalization among 15 septic foals at Texas A&M University Veterinary Medical Teaching Hospital and 11 nonseptic foals hospitalized at the Veterinary Medical Teaching Hospital or the Texas A&M University Horse Center during 2009; data in the table represent mean values (95% CI).

Group	24 Hours	48 Hours	72 Hours	96 Hours
a. IL-8 mRNA expression*				
Septic	0.3 ^{a,1} (0.1–0.9)	0.4 ^{a,1} (0.1–1.0)	0.2 ^{a,1} (0.1–0.5)	0.2 ^{a,1} (0.1–0.7)
Nonseptic	1.4 ^{b,1} (0.6–3.4)	1.6 ^{b,1} (0.8–3.1)	0.8 ^{b,1} (0.4–1.6)	1.0 ^{b,1} (0.5–2.1)
b. IL-10 mRNA expression*				
Septic	0.2 ^{a,1} (0.1–0.6)	0.3 ^{a,1} (0.1–0.8)	0.2 ^{a,1} (0.1–0.4)	0.2 ^{a,1} (0.1–0.5)
Nonseptic	1.6 ^{b,1} (0.7–3.8)	2.1 ^{b,1} (0.7–6.1)	1.1 ^{b,1} (0.7–1.7)	1.3 ^{b,1} (0.6–3.1)

*Values represent back-transformed means of \log_{10} -transformed data.

Values within rows that have different numbers differ significantly ($P < .05$).

Values within columns that have different letters differ significantly ($P < .05$).

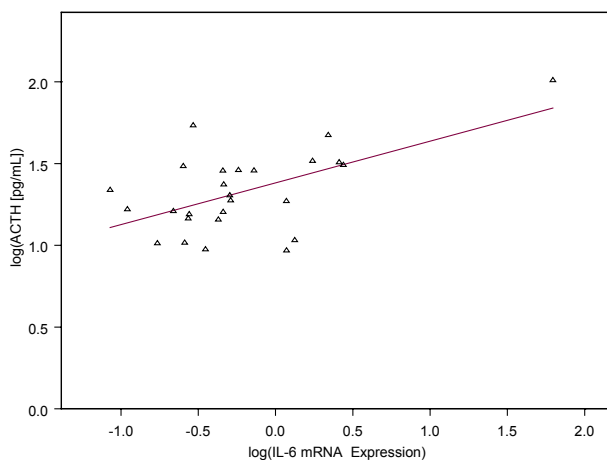


Fig 2. Association of plasma ACTH concentration and mRNA expression of IL-6 by peripheral blood leukocytes among 15 septic foals. Plasma ACTH concentration and IL-6 mRNA fold-changes were \log_{10} -transformed for purposes of statistical analysis and graphic representation. Plasma concentration of ACTH was positively and significantly associated ($P = .0026$) with IL-6 mRNA expression among septic foals. The horizontal line is the mean fit estimated using linear mixed-effects modeling to account for repeated measures on individual foals; after back transformation, for each 10-fold increase in IL-6 expression, ACTH concentration was increased by an estimated 50.1 pg/mL (95% CI = 20.0–251.2 pg/mL; $P = .0026$).

appear to impact survival in the study population, but may help to characterize HPA axis dysfunction in critically ill foals. The higher cortisol concentrations and the decreasing concentrations over time could be due to improvement in HPA axis function resulting from treatment.⁴³ Additional studies are warranted to demonstrate the consistency and elucidate the mechanism of this finding. The finding in this study that ACTH and cortisol concentrations in blood samples from neonatal foals were correlated is consistent with previous reports from human^{44,45} and veterinary medicine.^{46,47,48} Although this finding is not original, the consistency with previous results lends credence to our findings and substantiates what others have observed.

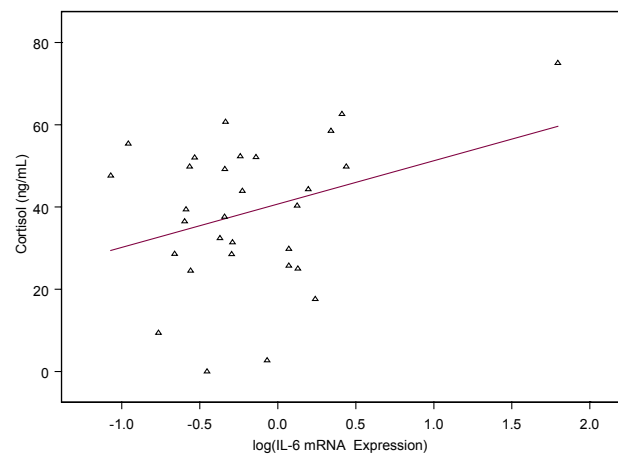


Fig 3. Association of plasma cortisol concentration and mRNA expression of IL-6 by peripheral blood leukocytes among 15 septic foals. Fold-changes in IL-6 mRNA expression were \log_{10} -transformed for purposes of statistical analysis and graphic representation. Although plasma cortisol concentrations tended to be positively associated with increasing IL-6 mRNA expression, this difference was not significant ($P = .0660$). The horizontal line is the mean fit estimated using linear mixed-effects modeling; for each 10-fold increase in IL-6 expression, ACTH concentration was increased by an estimated 11.9 pg/mL (95% CI = -0.9 – 24.7 pg/mL).

Cytokines are produced during activation of the innate and acquired immune responses. They help initiate the inflammatory response and characterize the magnitude and type of the acquired immune response. In critically ill human patients, response to pathogens or injuries is in large part dependent upon the profile of the cytokines produced.^{31,49,50} The response of critically ill patients can vary from a predominately pro-inflammatory response characterized by increased production of TNF- α , IL-1 β , IFN- γ , and IL-12, to a response of anergy, exemplified by production of anti-inflammatory cytokines, viz., IL-10 and IL-4.^{31,49,50} Septic human infants have increased serum concentrations of both IL-4 and IFN- γ after diagnosis and initial treatment of sepsis.³² The response of septic

equine neonates differs from septic human infants because foals fail to develop an increase in mRNA expression of IFN- γ and IL-4.^{12,33,34} Why this failure to develop an appropriate inflammatory response occurs is unclear, but it is likely multifactorial.

In this study, septic foals had decreased IL-8 and IL-10 mRNA expression relative to nonseptic foals at each time point. These results differ from those of previous studies with respect to cytokine gene expression. Some of the differences may be attributable to the cell populations studied (total white cells versus peripheral blood mononuclear cells) and to different outcome measures (mRNA expression versus protein concentrations). However, Gold et al¹² observed that IL-6 concentrations were 15 times higher in nonsurviving septic foals compared to surviving septic foals. Pusterla et al¹³ found that IL-10 mRNA expression was higher in nonsurviving foals compared to surviving foals and that IL-8 was higher in septic foals. The results in our study could indicate that septic foals have a potential imbalance in their inflammatory response and in neutrophil chemotaxis, along with a decrease in anti-inflammatory response. The reduction in expression of both of these cytokines could result in a more pro-inflammatory response (from decreased IL-10 expression) and decreased ability of the immune system to respond appropriately to sepsis (from decreased expression of the chemokine IL-8). However, how these changes might ultimately affect the ability of the foal's immune system to respond to the challenge of sepsis is unclear. Results of this study await replication in studies of larger populations of foals.

Another unexpected finding in this study was the lack of temporal changes in cytokine mRNA expression among septic foals. Pusterla et al¹³ found that septic and sick nonseptic foals had higher IL-8 concentrations than did control foals. Gold et al¹² found that IL-4 gene expression initially was decreased in septic foals compared to control foals, but increased with time to equal the expression in control foals whereas IL-6 gene expression decreased 4-fold over the same time period.¹² Why cytokine gene expression did not change over time in the present study is unclear. One possible explanation is that the comparisons for foals were made relative to their own results at admission rather than to normal values for expression (ie, the septic foals may have arrived with deranged cytokine expression that did not improve with time). The fact that there was a difference between the septic and nonseptic foals for mRNA expression of IL-8 and IL-10 at each time point provides evidence for dysregulation between proinflammatory and anti-inflammatory cytokines among septic foals. Additional work is needed to establish the consistency and elucidate the clinical relevance of these findings.

Our study demonstrated a significant positive association between ACTH and IL-6 mRNA expression in septic foals, and a possible association between cortisol and expression of IL-6 mRNA in septic foals. These findings indicate a relationship between the HPA axis and IL-6 mRNA in septic foals. Whether these associa-

tions are causal and how they contribute to the response of the HPA axis or the foal's ability to respond to sepsis have not been determined. Additional studies of these associations are needed to better characterize the clinical importance of the association of IL-6 mRNA expression and the HPA axis during sepsis.

This study had several limitations. First, the number of foals included in the study was smaller than we had expected, and this decreased the power of the study for comparisons of either septic and nonseptic foals or surviving and nonsurviving foals. Nevertheless, we believe that some meaningful results were derived from the study despite the limited power. Second, septic and nonseptic foals were not matched exactly for age because it was not always possible to precisely match ages of foals in the small population of foals admitted to the hospital. However, because the ages of the foals were fairly similar despite exact matching, we do not believe that substantial bias attributable to age occurred.

Third, the use of total peripheral WBCs as a source of mRNA for analysis of cytokine gene expression rather than a specific subpopulation of leukocytes (eg, neutrophils) could be considered a limitation. Both the magnitude and temporal pattern of mRNA expression of various cytokines may differ among leukocyte subpopulations, and illness might influence cytokine profiles differently among leukocyte subsets. Conversely, studying gene expression of total leukocytes may have been an asset because it may have more accurately reflected the milieu in which systemic inflammatory responses occurred among septic and nonseptic foals. Fourth, although the nonseptic groups were free of infectious diseases, they were heterogeneous with regard to place of birth (ie, at the TAMU Horse Center or other premises) and health status (ie, healthy or with a noninfectious health disorder). It is not possible to know the impact of including some foals that were not completely healthy in the nonseptic group. It is plausible that their inclusion may have biased results away from finding differences between the septic and nonseptic group in cytokine expression and HPA axis activation, if these processes could have been activated by the primary disease problem.

A fifth limitation was that nonseptic and septic foals differed with regard to medical management. Specifically, none of the control foals received medical treatment or transfusion of plasma whereas septic foals required medication, transfusion, and supportive therapy such as administration of IV fluids. This limitation was unavoidable because septic foals required treatment but nonseptic foals did not. Thus, it is not possible to differentiate the impact of sepsis from treatment for sepsis on the parameters evaluated in this or any other observational study of septic foals.

In summary, it appeared that the HPA axis in foals was able to respond appropriately to sepsis. There may however be some degree of dysregulation of the HPA axis among neonatal foals on the basis of the observation that serum cortisol concentrations were higher at admission and subsequently decreased among

foals without concomitant changes in plasma ACTH concentrations. The observation that mRNA expression of IL-8 and IL-10 was decreased in septic foals relative to nonseptic foals suggests dysfunction of the immune system among septic foals. Another key finding was an association between the HPA axis and IL-6 mRNA expression among septic foals. How cytokines and the innate immune system interact with the HPA axis to impact the clinical response to sepsis remains to be elucidated.

Footnotes

- ^a Leukolock, Applied Biosystems, Life Technologies Inc, Carlsbad, CA
^b MP Biomedicals, LLC Diagnostic Division, Orangeburg, NY
^c Micromedics Systems Inc, Horsham, PA
^d Assay Zap Software, Biosoft Ltd, Cambridge, UK
^e Siemens Diagnostic Product Corporation, Deerfield, IL, CAT NO: TKCO5
^f RNeasy mini kit; Qiagen, Inc, Valencia, CA
^g DNase I Invitrogen, Life Technologies Inc
^h Version 8.1; TIBCO, Inc, Seattle, WA

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