

HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION
IN HEALTHY AND CRITICALLY ILL NEONATAL FOALS

by

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(Under the Direction of Michelle Henry Barton)

ABSTRACT

The corticosteroid hormone cortisol is released when the hypothalamic-pituitary-adrenal (HPA) axis is activated by stress, and is vital for an appropriate response to critical illness. In many critically ill people, though, the cortisol response is inadequate, a diagnosis termed critical illness-related corticosteroid insufficiency (CIRCI). In people, CIRCI is correlated with increased disease severity and decreased survival, and may be managed with cortisol replacement in the form of low-dose hydrocortisone.

The overall aim of the studies reported herein was to examine the incidence, importance and potential treatment of CIRCI in foals. First, a paired low-dose/high-dose exogenous adrenocorticotrophic hormone (ACTH) stimulation test was developed for use in neonatal foals. The second study characterized HPA axis function during the immediate post-natal period in the healthy full-term foal using this test, to provide reference ranges for HPA axis responses in the healthy foal. Both these studies documented some impairment in cortisol responses during the first week of life in healthy full-term foals that may contribute to the development of CIRCI in ill foals.

Next, the incidence and impact of CIRCI in critically ill neonatal foals was determined in a prospective clinical study. Approximately 40% of ill foals met criteria for CIRCI, comparable to the incidence in septic people. Further, CIRCI was correlated with increased disease severity and decreased survival in foals as in people. Thus, additional studies were undertaken to refine diagnostic criteria for CIRCI and to develop and evaluate a therapeutic regimen for eventual use in foals with CIRCI.

A free cortisol assay was optimized for horses, and free cortisol was measured in healthy adult horses and healthy and septic foals. In contrast to people, free cortisol did not improve diagnostic sensitivity for CIRCI in foals, presumably because foals have much greater proportions of free cortisol than adult horses and people. This increased free cortisol fraction contributed to significant differences in cortisol production and hydrocortisone pharmacokinetics between foals and horses, which were considered during the development of a low-dose hydrocortisone regimen for foals. Finally, this hydrocortisone regimen significantly dampened the pro-inflammatory cytokine responses without impairing neutrophil function in isolated leukocytes from healthy foals.

INDEX WORDS: Equine, Neonate, Cortisol, HPA axis, Adrenal, Stress, Relative adrenal insufficiency, Critical illness-related corticosteroid insufficiency, Steroid, ACTH stimulation test, Free cortisol, Hydrocortisone

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DEDICATION

To my family and friends, for their endless love and support,
and to the foals and horses, the true reason I love my job!

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CHAPTER 1

INTRODUCTION

The studies presented herein were undertaken to contribute to our understanding of hypothalamic-pituitary-adrenal (HPA) axis function during health and illness in the neonatal foal. Specifically, studies were undertaken to determine if critical illness-related corticosteroid insufficiency (CIRCI) occurs in critically ill neonatal foals with similar incidence and importance as is reported in other species.

Chapter 2 contains a review of the literature and is divided into six sections. Section I reviews mammalian HPA axis physiology and pathophysiology, with particular focus on HPA axis hypofunction. Section II discusses current methodology used for diagnosis of HPA axis hypofunction in clinical settings. Section III reviews our current understanding of HPA axis function in the fetal and neonatal foal, and provides evidence that neonatal foals may be at particular risk for development of CIRCI. Section IV contains a review of the incidence and impact of CIRCI during sepsis and septic shock in the human and veterinary medical literature. Section V summarizes current recommendations for the diagnosis and management of CIRCI in critically ill people. Finally, Section VI briefly discusses the rationale for the studies described in this dissertation.

Chapters 3 through 8 present a series of manuscripts describing the results of studies comprising this dissertation research. Chapter 3 describes a dose-response study evaluating cortisol responses to physiologic and supraphysiologic doses of synthetic adrenocorticotropic

hormone (ACTH) in neonatal foals. Chapter 4 contains a report describing hypothalamic-pituitary-adrenal axis assessment in healthy term neonatal foals during the first week of life, utilizing a paired low-dose / high-dose ACTH stimulation test developed from the findings presented in the previous chapter. Chapter 5 describes a prospective clinical study to determine the incidence and impact of CIRCI in hospitalized neonatal foals. Chapter 6 contains a report describing measurement of serum free cortisol fraction in healthy and septic neonatal foals and comparison of free versus total cortisol parameters for predicting disease severity and outcome in septic foals. Chapter 7 presents a study comparing daily endogenous cortisol production and hydrocortisone pharmacokinetics between healthy neonatal foals and adult horses. Chapter 8 contains a report of the immunologic effects of a proposed low-dose hydrocortisone replacement therapy regimen derived from the research described in the previous chapter.

Chapter 9 summarizes the results of the research presented in this dissertation and proposes potential mechanisms contributing to CIRCI in the critically ill neonatal foal.

CHAPTER 2

LITERATURE REVIEW

SECTION I. HYPOTHALAMIC-PITUITARY-ADRENAL AXIS (HPA) PHYSIOLOGY AND PATHOPHYSIOLOGY

HPA Axis Components, Activation and Regulation

The hypothalamic-pituitary-adrenal (HPA) axis (Figure 2.1) plays an integral role in the mammalian endocrine response to stress. The primary function of the HPA axis is to regulate circulating concentrations of the corticosteroid hormone cortisol during both health and disease. Appropriate systemic cortisol concentrations are critical for the maintenance of cellular, organ, and whole body homeostasis.

The HPA axis is activated when physiologic, pathophysiologic, or environmental stressors activate a variety of peripheral and central nervous systems components, whose signals are then interpreted and integrated in the hypothalamus. Activation of the paraventricular nuclei in the hypothalamus culminates in the release of the peptide hormone corticotropin-releasing hormone (CRH) into the hypothalamic-hypophyseal portal vessels. CRH then acts locally in the adjacent anterior pituitary gland to activate type 1 CRH-receptors (CRH-R1) on the cell surface of pituitary corticotroph cells. CRH-R1 is a G protein-coupled transmembrane receptor that, upon binding CRH, increases intracellular cyclic AMP (cAMP) and ultimately induces the release of adrenocorticotrophic hormone (ACTH or corticotropin).¹ ACTH is a peptide hormone

cleavage product of a larger pro-hormone protein called proopiomelano-cortin (POMC). POMC processing is tissue specific, and is catalyzed by the enzymes prohormone convertase 1 and 2 (PC1 and PC2).² In the anterior pituitary, POMC is processed to two peptides: 1) pro-ACTH, which is further cleaved into N-POMC, joining peptide, and ACTH; and 2) β -lipotrophin, which is ultimately processed to γ -lipotrophin and β -endorphin (Figure 2.2).² In some tissues, such as the intermediate lobe (pars intermedia) of the anterior pituitary, N-POMC, ACTH, and γ -lipotrophin are further processed into α -, β -, and γ -melanocyte-stimulating hormone (α -, β -, and γ -MSH).² Details of POMC processing are shown in Figure 2.2.

Upon HPA axis activation by endogenous or exogenous stressors, ACTH is released into the systemic circulation and stimulates the adrenal glands to synthesize and secrete the steroid hormones cortisol (in most mammals) or corticosterone (rodents). Each adrenal gland is divided into an outer cortex that secretes steroid hormones and an inner medulla that secretes catecholamines. While ACTH-receptors (melanocortin 2 receptor, MC2R) are expressed on both cortical and medullary cells, adrenocortical cells are the most sensitive cell type to ACTH in the adrenal gland and in the entire body.³ Five ACTH receptor (also called melanocortin receptor) types have been characterized in humans, the majority of which bind both ACTH and α - and γ -MSH.³ Melanocortin 2 receptor (MC2R) is the only ACTH receptor type that is expressed in the adrenal cortex, and the only type that does not appear to bind any form of MSH.³ MC2R, like CRH-R1, is a G protein-coupled transmembrane receptor that acts via adenylate cyclase to increase cAMP levels, which then serve to activate a variety of enzymes critical for steroid hormone synthesis.³

The adrenal cortex is divided into 3 cellular zones: 1) the outer zona glomerulosa; 2) the middle zona fasciculata, which is the largest zone and comprises 75% of the weight of the entire

adrenal gland; and 3) the narrow inner zona reticularis.⁴ All three adrenocortical zones synthesize and secrete a variety of steroid hormones (corticosteroids). Cells in the zona glomerulosa are primarily responsible for the secretion of aldosterone in response to changes in blood pressure and plasma osmolarity, and play a key role in electrolyte and water balance. These cells require the presence of ACTH to other stimuli to effectively synthesize and secrete aldosterone.⁵ Zona fasciculata cells express MC2R at high levels, and predominantly synthesize and secrete glucocorticoid hormones (cortisol or corticosterone) in response to ACTH.⁴ Cells in the zona reticularis secrete a small amount of glucocorticoids, but predominantly produce adrenal androgens such as androstenedione and dehydroepiandrosterone in response to ACTH in combination with a variety of other regulatory hormones.⁶

All adrenocortical steroid hormones are structurally similar and share a common synthetic pathway (Figure 2.3). All steroid hormones are synthesized from cholesterol, and contain a common sterol backbone with three 6-carbon and one 5-carbon rings. Steroid hormone synthesis occurs in the mitochondria and endoplasmic reticulum, and begins with uptake of cytoplasmic cholesterol into the mitochondrion via the steroidogenic acute regulatory (StAR) transporter protein.⁷ Binding of a regulatory hormone (e.g., ACTH) stimulates cAMP-mediated uptake of circulating plasma lipoproteins and liberation of cholesterol via lysosomal lipases and cholesterol esterase to provide the major source of such cholesterol for steroid hormone synthesis.⁴ *De novo* cholesterol synthesis from acetyl coenzyme A also occurs in the adrenal cortex and can provide an alternative source of cytoplasmic cholesterol for corticosteroid synthesis.^{4,5}

Once cholesterol is taken up into the mitochondrion via StAR, it is converted to pregnenolone via side chain cleavage enzyme (p450_{scc}, cholesterol desmolase). Both

cholesterol delivery to mitochondria via StAR and p450_{scc}-catalyzed pregnenolone synthesis have been proposed as the rate limiting step in corticosteroid biosynthesis.^{8,9} All other steroid hormones are derivatives of pregnenolone; which specific steroids are produced by a particular adrenocortical cell depends on which biosynthetic enzymes are expressed in that cell.^{4,10} The critical enzymes necessary for cortisol synthesis are expressed in zona fasciculata cells, and include 3- β -hydroxysteroid dehydrogenase (3- β -HSD), 17- α -hydroxylase, 21- α -hydroxylase, and 11- β -hydroxylase.⁷ This latter enzyme catalyzes the final step in cortisol/corticosterone synthesis from the 11-deoxycortisol/deoxycorticosterone precursor molecule, and is present only in glucocorticoid-producing cells.⁷

Cortisol is not stored in adrenocortical cells, but rather is secreted into the systemic circulation immediately following ACTH-induced synthesis.⁹ Like all steroid hormones, cortisol is lipophilic and thus is transported in the plasma predominantly bound to plasma proteins. The majority of circulating cortisol is bound to a specific α -globulin, cortisol-binding globulin (CBG), with a small portion bound to albumin.^{11,12} Cortisol-CBG binding is of high affinity but low capacity, while cortisol-albumin binding is the opposite.⁴ In most adult mammals, including horses, approximately 90% of circulating cortisol is bound.^{10,11,13-15} However, as cortisol receptors are located in the cytoplasm of steroid-responsive cells, it is only the free, unbound portion of circulating cortisol that is available to enter cells via diffusion across the plasma membrane and to bind these intracellular glucocorticoid receptors (GRs).^{16,17}

Binding of cortisol to the cytoplasmic GR causes conformational changes that allow dissociation of regulatory heat shock proteins (HSPs), permitting the cortisol-GR complex to dimerize, localize to the nucleus, bind DNA at glucocorticoid-response-elements (GREs), and ultimately up- or down-regulate transcription of glucocorticoid-responsive genes (transactivation

or transrepression).⁴ The GR belongs to the nuclear receptor superfamily and is structurally composed of 3 key domains: 1) the highly variable aminoterminal end, which is critical for mediating specific genomic effects; 2) the central DNA-binding domain, which consists of two highly conserved zinc-fingers; and 3) the carboxyterminal cortisol binding domain, which also contains sequences responsible for HSP binding, nuclear translocation, and receptor dimerization.^{16,17} In humans, there are two GR isoforms, GR α and GR β , but GR β appears to be transcriptionally inactive and may function as an endogenous inhibitor of GR α activity.^{16,17} In contrast, GR α functions as a ligand-dependent transcription factor and regulates gene transcription via: 1) direct protein-DNA interactions at gene promoters containing GRE's, after which a variety of co-activator and chromatin-remodeling molecules are recruited; or 2) indirect protein-protein interactions with other transcriptional factors to alter transcription of genes whose promoters lack a specific GRE.¹⁷

A huge variety of cell types are sensitive to glucocorticoids, permitting cortisol to exert a myriad of systemic effects that are vital for adaptation to stress during both health and disease. Essential physiologic responses to the stress of illness include maintenance of blood pressure, provision of energy to tissues, and control of an appropriate inflammatory response.^{4,5,9,18} Cortisol functions as a positive inotrope by increasing the synthesis of and tissue reactivity to catecholamines.⁴ In addition, cortisol down-regulates the synthesis of the potent vasodilator nitric oxide.¹⁸ These combined effects beneficially impact cardiac contractility and vascular tone, both vital for the maintenance of systemic blood pressure. Cortisol also increases availability of metabolic substrates to tissues by increasing hepatic gluconeogenesis and inhibiting the uptake of glucose by adipose tissue.^{4,5} These effects, in concert with cortisol-induced free fatty acid release from adipose tissue and increased protein catabolism, mobilize

several energy sources needed for host defense, organ function, and repair of damaged tissues.¹⁸ Finally, cortisol exerts varied anti-inflammatory and immunomodulatory effects by decreasing production and activity of various pro-inflammatory cytokines, (e.g., interleukin-1 [IL-1], interleukin-6 [IL-6], and tumor necrosis factor [TNF]), by stimulating the release of anti-inflammatory mediators (e.g. soluble TNF receptor and interleukin-10 [IL-10]), and by inhibiting the activation of pro-inflammatory pathways involving cyclooxygenase-2, inducible nitric oxide synthase, and phospholipase A₂.^{4,18} Cortisol also can directly impair neutrophil chemotaxis and microbial activity, and at high concentrations impairs lymphocyte proliferation and activity and induces leukocyte apoptosis.^{4,17}

The sum of cortisol's systemic effects serves to reduce the physiologic stressors that initially activated the HPA axis, and ultimately reduces subsequent HPA axis tone. In addition, cortisol itself acts via negative feedback mechanisms at hypothalamic, pituitary and adrenal levels to down-regulate both regulatory hormone and cortisol secretion.⁵ Cortisol is metabolized in a reversible fashion in many peripheral tissues via the enzyme 11- β -hydroxysteroid dehydrogenase type 2 (11- β -HSD2), which reduces cortisol to the inactive metabolite cortisone.^{10,19} Glucocorticoid target tissues express an alternative isoform of this enzyme, 11- β -HSD1, which catalyzes the reverse reaction and increases local concentrations, and thus activity, of cortisol.^{10,19} Additional cortisol metabolism and clearance occurs via hepatic and renal biotransformation and excretion. Free cortisol is freely filtered at the glomerulus, where a portion is then subject to tubular reabsorption and a portion is excreted.¹⁰ Circulating cortisol is also transformed to inactive metabolites in both the liver and kidneys, and these metabolites are also renally excreted.^{4,10} All of these metabolic pathways are subject to regulation via inflammatory mediators and other stress-related mechanisms.¹⁹ Thus, with an intact HPA axis,

plasma cortisol concentrations are maintained through a variety of mechanisms at a level that is appropriate for the existing degree of physiologic stress.

General Mechanisms of HPA Axis Hypofunction

Like other endocrine axes, HPA axis dysfunction can be manifest by either abnormal increases or decreases in HPA axis activity. Mechanisms resulting in increased activity of HPA axis components (*hyperfunction*) are varied and result in important disease states in both human and veterinary medicine, but are beyond the scope of this review. Thus, the remainder of this review and the studies presented in this dissertation will focus specifically on mechanisms and consequences of HPA axis *hypofunction* resulting in cortisol insufficiency.

HPA axis hypofunction can result from impairment at one or several levels, and may be transient or permanent.⁹ Permanent HPA axis dysfunction results from destruction of one or more glandular components of the axis, and is infrequent in both human and veterinary medicine. Immune-mediated adrenocortical destruction (e.g., Addison's Disease) is the most common manifestation of permanent HPA axis hypofunction in both human and veterinary patients,^{9,20} but has not been described in horses. Patients with Addison's Disease cannot mount an appropriate cortisol response to stress and thus frequently present with hemodynamic instability and collapse.^{9,20} Aldosterone deficiency, in addition to cortisol insufficiency, is a typical feature of Addison's disease, and results in fluid and electrolyte derangements that contribute to the development of hypovolemia, hypotension and cardiovascular collapse in affected individuals.^{9,20}

Irreversible adrenocortical, hypothalamic or pituitary destruction may also result from neoplastic infiltration, or from invasion of the glands by infectious organisms, but is rare in all

species.^{20,21} Severe adrenocortical hemorrhage and necrosis resulting in adrenal insufficiency – Waterhouse-Friderichsen syndrome – is also described in both people and horses with septic and endotoxic shock, and is believed to result from vascular derangements and ischemia associated with the primary disease.^{22,23} Finally, congenital abnormalities in CRH-R1, MC2R, or GR receptor structure or function and congenital absence of synthetic enzymes necessary for pituitary or adrenocortical hormone synthesis are described in people, and can ultimately result in HPA axis hypofunction.^{9,21}

While irreversible HPA axis hypofunction due to destruction of HPA axis components is uncommon,^{9,24-27} recent evidence suggests that transient HPA axis dysfunction may occur in a substantial number of critically ill people as a direct result of their concurrent illness, a syndrome termed relative adrenal insufficiency (RAI) or critical illness-related corticosteroid insufficiency (CIRCI).^{18,25,28-36} RAI/CIRCI is described in critically ill patients with a variety of conditions, including acute respiratory distress syndrome,^{19,37} major trauma,³⁸ and cardiothoracic surgery,³⁹ but the predominant diseases associated with the development of RAI/CIRCI are sepsis and septic shock.^{25,28,29,31-33,35,36,40} RAI/CIRCI can result from temporary suppression of HPA axis activity at one or more levels, resulting in: 1) inhibition of CRH and/or ACTH secretion, 2) decreased sensitivity to CRH or ACTH at their respective target tissues, 3) exhaustion of adrenocortical cortisol synthetic capacity (“loss of adrenal reserve”), or 4) impaired response to cortisol in the peripheral tissue resistance.^{18,19,32,41}

The specific mechanisms leading to the development of RAI/CIRCI are poorly understood, but may involve a combination of factors, including: 1) direct damage to HPA axis components from the primary disease, 2) inhibition of cortisol production by medications used to treat the primary disease, and/or 3) suppression of activity of one or more components of the

HPA axis by infectious organisms or the host's own immune and inflammatory response.^{19,25,32,42} Periods of hypotension associated with hypovolemic or septic shock can result in decreased adrenal perfusion and ischemic injury to the metabolically active adrenocortical cells.²² If this occurs for prolonged periods or to an extreme degree the damage may be irreversible, as seen in patients with Waterhouse-Friderichsen syndrome described above, but milder circulatory insults may result in transient injury and impaired function. In addition, the anesthetic agent etomidate, which is used frequently in critically ill people and animals, is known to directly inhibit adrenocortical cortisol synthesis by inhibiting 11- β -hydroxylase,⁴³ the enzyme that catalyzes the key last step in the synthesis of active cortisol. Several antimicrobial agents, (e.g., ketoconazole and rifampin), which are often used in septic people and animals, also can inhibit HPA axis activity.¹⁹

It is the patient's own immune and inflammatory response, though, that appears to play the most vital role in the development of RAI/CIRCI during critical illnesses such as sepsis. Bacterial components such as endotoxin (the lipopolysaccharide component of gram negative bacterial cell walls) and host pro-inflammatory cytokines play a role in initiating and maintaining the HPA axis response to sepsis. These factors can directly stimulate HPA axis activity at the hypothalamus and pituitary, resulting in increased production of CRH and ACTH and resultant stimulation of cortisol synthesis and secretion.^{22,32,44,45} In addition, both endotoxin and pro- and anti-inflammatory cytokines have been shown to directly influence cortisol production in adrenocortical primary cell cultures, illustrating the potential for infectious and inflammatory mediators to bypass higher levels of HPA axis regulation to impact cortisol synthesis during profound systemic inflammatory responses.^{46,47}

However, bacterial ligands and inflammatory cytokines may also be capable of *suppressing* HPA axis function at one or more levels, in the face of an overwhelming bacterial infection or excessive host inflammatory response. For example, inducible nitric oxide synthase-mediated death of hypothalamic neurons in cardiorespiratory centers has been described in patients that died of septic shock, and may play a role in HPA axis dysfunction in such patients.⁴⁸ Bacterial endotoxin has been shown to directly decrease pituitary CRH receptor gene expression in both rats and cattle.^{49,50} In addition, TNF- α can directly impair both pituitary ACTH release and adrenocortical cortisol synthesis.¹⁹ Finally, cholesterol availability for corticosteroid synthesis may be limited during sepsis, as several studies have shown decreased levels of plasma high-density lipoprotein (HDL) in critically ill individuals and correlated these decreased HDL levels with blunted cortisol responses to ACTH stimulation testing.^{19,51}

Furthermore, several studies suggest that peripheral tissue cortisol resistance may develop in some critically ill patients. Specifically, pro-inflammatory cytokines such as TNF- α and IL-1 β have been shown to decrease 11- β -HSD1 and 11- β -HSD2 activity in tissues, resulting in decreased availability of active cortisol in peripheral tissues.¹⁹ In addition, impaired GR binding affinity has been documented in an ovine model of acute lung injury⁵² and in a rodent burn model.⁵³ This effect was partially ameliorated in the rodent model when TNF- α and IL-1 β were reduced by neutralizing antibodies.⁵³ Furthermore, in an *ex vivo* study in which leukocytes from healthy people were exposed to plasma from patients with acute respiratory distress syndrome, nuclear localization of cortisol-GR complexes was impaired in cells exposed to plasma from non-survivors as compared to plasma from survivors.⁵⁴ Thus, the complex relationship between endocrine and immune regulation of HPA axis function determines overall cortisol production and activity during sepsis. Any imbalance in these interactions can result in

absolute and/or functional cortisol insufficiency and may play a critical role in the pathogenesis of RAI/CIRCI.

SECTION II: DIAGNOSTIC METHODOLOGY FOR ASSESSMENT OF HPA AXIS HYPOFUNCTION IN A CLINICAL SETTING

Mechanisms used to diagnose HPA axis hypofunction in both human and veterinary medicine fall into two general categories: 1) assessment of endogenous basal HPA axis activity by measurement of circulating or excreted hormone concentrations; and 2) dynamic assessment of HPA axis responsiveness by assessment of hormone production following administration of exogenous regulatory hormones in an attempt to simulate stress-induced HPA axis activation.⁵⁵ A variety of testing methodologies are available in both these categories, though some have more clinical utility than others.

In general, measurement of basal plasma hormone concentrations offers a rapid, easy and safe means of assessing HPA axis activity. In some cases, cortisol may be the only hormone measured, since appropriate cortisol production implies intact function of the higher levels of the HPA axis. Commercial radio- or chemiluminescent immunoassays are readily available for measurement of cortisol in most species and can provide results in a matter of hours.^{10,55} However, cortisol concentrations vary widely during a 24-hour period in both healthy and sick individuals with time of day, season, emotional state, and moment-to-moment changes in physiologic stressors.⁵⁵ Thus, a random cortisol concentration does not always provide an accurate representation of global HPA axis function.

Assessment of cortisol concentrations in conjunction with regulatory hormone (ACTH, CRH) concentrations can provide a more comprehensive picture of HPA axis function. Plasma ACTH concentrations are easily assessed in most species via commercially available immunoassays, but as the majority of CRH is secreted into the hypothalamic-hypophyseal portal vessels rather than into the systemic circulation, accurate measurement of CRH requires sophisticated methodology and is predominantly limited to research settings or extraordinary clinical circumstances. In addition, while in health ACTH and cortisol concentrations are fairly closely correlated, ACTH-cortisol dissociation is not uncommon during illness.⁵⁶ Thus, given the moment-to-moment variation in circulating concentrations of both these hormones, random samples can sometimes be difficult to interpret.

Dynamic tests are designed to circumvent some of these limitations. In such testing, HPA axis activity is manipulated at a specific level and changes in endogenous hormone production are measured as described above. The most commonly used dynamic test for HPA axis assessment is the ACTH stimulation test, which permits assessment of adrenal responsiveness to exogenous synthetic ACTH (ACTH₁₋₂₄, cosyntropin). Typically, in the classic “high-dose” ACTH stimulation test, serum cortisol concentration is measured just prior to and 30-90 minutes after intravenous or intramuscular administration of a supraphysiologic quantity of ACTH (100 to 250 µg, 1.0-2.0 µg/kg). This high-dose ACTH stimulation test is theorized to produce a *maximal* adrenal response, so an inadequate increase in cortisol concentration in this test is commonly used to diagnose both absolute, irreversible adrenal insufficiency (e.g., Addison’s Disease) and RAI/CIRCI.^{9,19,29,37}

However, the high-dose ACTH stimulation test may be less sensitive for diagnosis of the transient, reversible HPA suppression that occurs in RAI/CIRCI. In some patients with

RAI/CIRCI, the adrenal gland fails to produce an appropriate cortisol response to physiologic concentrations of ACTH but may still respond adequately to supraphysiologic ACTH concentrations.^{25,42} Data in humans show that in health, a measurable cortisol response can be produced by administration of much lower doses of ACTH (1-10 μg , 0.01-0.2 $\mu\text{g}/\text{kg}$),^{25,27,42} and such “low-dose” stimulation tests may more accurately diagnose RAI/CIRCI in critically ill patients.^{24,25,27,42} Siraux *et al* compared the low- dose (1 μg) and high-dose (250 μg) ACTH stimulation tests in 46 human patients with septic shock, and determined that the low-dose test identified patients with an inadequate cortisol response that would have been misdiagnosed with the high-dose test alone.³⁵

It is important to note, however, that both the high-dose and low-dose ACTH stimulation tests only evaluate the adrenal component of the HPA axis. Therefore, patients with cortisol insufficiency due to impaired HPA axis function at the hypothalamic and/or pituitary levels may still produce an appropriate cortisol response to exogenous ACTH, and may be falsely interpreted as having intact HPA axis function. The CRH stimulation test is occasionally used to assess function of the higher levels of the HPA axis function, and is most helpful for distinguishing among adrenocortical, hypothalamic and pituitary failure if both ACTH and cortisol concentrations are measured before and after CRH stimulation.⁹ However, this test is expensive and is less sensitive for diagnosis of HPA axis hypofunction than other means,⁵⁷ so it is rarely used as a first-line means of HPA axis assessment in clinical patients.

The overnight metyrapone test may be more useful for assessing the integrity of the entire HPA axis. In this test, metyrapone (30 mg/kg), a specific inhibitor of the adrenocortical steroidogenic enzyme 11- β -hydroxylase, is administered orally at midnight.^{9,58} Because 11- β -hydroxylase catalyzes the conversion of 11-deoxycortisol to cortisol in the last step of

adrenocortical cortisol synthesis, metyrapone administration results in a fall in serum cortisol concentrations. With an intact HPA axis, this drop in plasma cortisol stimulates increased HPA axis activity, resulting in increased CRH and ACTH secretion and subsequent stimulation of adrenocortical cortisol synthesis. However, since the last step in cortisol synthesis is inhibited by metyrapone, cortisol concentrations remain low while concentrations of the precursor 11-deoxycortisol rise. In the overnight metyrapone test, serum cortisol and 11-deoxycortisol concentrations are measured before and 8-10 hours after metyrapone administration. A decrease in cortisol concentrations to below 7.0 µg/dl confirms that 11-β-hydroxylase suppression was adequate. Increases in ACTH and 11-deoxycortisol concentrations confirm adequate HPA axis integrity, while low ACTH and 11-deoxycortisol suggests HPA axis dysfunction at the hypothalamic and/or pituitary levels.^{9,58} However, if ACTH concentrations increase but 11-deoxycortisol remains low, primary adrenocortical dysfunction is suggested. Therefore, the metyrapone test permits more accurate assessment of both adrenal and central levels of the HPA axis than the ACTH-stimulation test does, and appears to be safe for use in most critically ill patients. Unfortunately, 11-deoxycortisol assays are not readily available in all human and veterinary clinical settings, and many assays require a cumbersome extraction step, limiting clinical applications for this test at present.

The insulin tolerance test (ITT) is currently considered the “gold-standard” test for evaluation of HPA axis hypofunction.^{59,60} In the ITT, hypoglycemia is induced by administration of exogenous insulin, and the cortisol response to the physiologic stress of this hypoglycemia is measured. People with intact HPA axis function demonstrate increased serum cortisol concentrations to ≥ 20 µg/dl in response to this induced hypoglycemia. A blunted or absent increase in cortisol following hypoglycemia implies impaired HPA axis integrity. ACTH

can also be measured in conjunction with cortisol to differentiate between adrenal and central HPA axis dysfunction. However, given the risks of collapse and seizure associated with severe hypoglycemia such as is induced in the ITT,^{59,60} as well as the potential for pre-existing hypoglycemia or peripheral insulin resistance during severe illness,⁶¹⁻⁶³ the ITT is contraindicated or difficult to interpret in many critically ill patients. Thus, while the ITT provides comprehensive information regarding HPA axis integrity, its clinical utility for use in this setting is limited.

Finally, as described in Section I above, recent studies suggest that some of the physiologic consequences of CIRCI may be associated with cortisol resistance in peripheral tissues rather than true cortisol insufficiency.^{32,52-54,64} In such cases, the above diagnostic methodologies may suggest intact HPA axis function, with appropriate endogenous hormone concentrations and normal responses to dynamic HPA axis testing, despite clinical evidence of cortisol insufficiency such as persistent hypotension and inflammatory dysregulation. As described above, such peripheral cortisol resistance can be mediated by altered tissue cortisol metabolism, decreases in GR number, or impaired GR activity and signaling. A small number of studies have evaluated GR density, nuclear localization, or expression of cortisol-induced molecules in both experimental and naturally-occurring sepsis,^{32,52-54,64} but unfortunately, methodology for assessing cortisol activity at the tissue level is quite limited and not available for use in clinical patients with suspected RAI/CIRCI.

Thus, at present, measurement of endogenous cortisol and ACTH concentrations and assessment of cortisol responses to low- and high-doses of exogenous ACTH provide accurate assessment of adrenocortical function and offer the most readily available means for assessment of HPA axis function in clinical patients. However, in patients in which adrenocortical responses

appear normal but hypothalamic or pituitary dysfunction is strongly suspected, the metyrapone test may offer a safe and more accurate means for assessing HPA axis integrity. If no diagnostic evidence of HPA axis hypofunction is present but clinical signs consistent with CIRCI persist, the potential for peripheral cortisol resistance should be considered.

SECTION III. HPA AXIS FUNCTION IN THE FETAL AND NEONATAL FOAL

HPA axis function in the fetal and neonatal foal has been investigated in several studies with many of the above tests, and differs from other mammalian species in key ways that may greatly impact the foal's ability to respond to stress in the neonatal period. Compelling evidence shows that maturation of the HPA axis starts during the last four to five days prior to parturition and continues into the first several weeks of life, much later than is described in other species.⁶⁵⁻⁶⁹ For example, premature foals (gestational age < 320 days) have low concentrations of serum cortisol (< 3 µg/dl) in the two hours after birth, as compared to term foals (12 to 14 µg/dl).⁶⁹ Concurrently, premature foals exhibit significantly higher endogenous ACTH concentrations, peaking at 650 pg/ml at 30 minutes postpartum, as compared to a peak of 300 pg/ml in term foals.⁶⁹

This low baseline cortisol and high ACTH peak in premature foals suggest that the problem with cortisol secretion resides at the level of the adrenal gland.⁶⁶ ACTH stimulation tests support this theory, as premature foals show a blunted cortisol response to exogenous ACTH, with only a 28% increase in plasma cortisol 30 to 60 minutes following stimulation, as compared with a 208% increase in normal term foals.⁶⁶ Such impaired endogenous and

exogenous ACTH-induced cortisol secretion in the foal could be associated with either decreased sensitivity to ACTH or impaired cortisol biosynthetic capacity, or both. MC2R density or activity has not been described in foals or horses of any age, but there is some evidence to suggest that the fetal foal's adrenal gland may be incapable of synthesizing cortisol until very late in gestation. Immunohistochemical localization showed that key steroidogenic enzymes necessary for cortisol synthesis (p450scc and 3- β -HSD) are absent or present in very low amounts until just prior to parturition in the foal,^{70,71} much later than is described in other species such as sheep,⁷² pigs,⁷³ or primates.⁷⁴ Thus, maturation of fetal cortisol synthetic capacity seems to occur much later in gestation in foals than in other species.

Furthermore, there is some evidence that adrenocortical function is not fully mature at the time of parturition, even in full-term foals. One study reported that maximal cortisol response to a high-dose ACTH stimulation test in healthy, term foals occurs on the day of birth, with decreasing basal cortisol concentrations and cortisol responses to ACTH in subsequent days after birth.⁶⁶ Furthermore, this decreased adrenocortical responsiveness to exogenous ACTH appears to persist during the first few months of life in the foal, as 12-week-old foals showed significantly greater cortisol responses to a low-dose (0.1 μ g/kg) ACTH stimulation test than younger foals.⁷⁵ In sum, this evidence of fetal and neonatal HPA axis immaturity is certain to impact, and possibly impair, the foal's ability to respond to physiologic stress and disease during the neonatal period.

SECTION IV: THE INCIDENCE AND IMPORTANCE OF RAI/CIRCI DURING SEPSIS AND SEPTIC SHOCK

RAI/CIRCI in Septic Adult Humans

As described above, a large number of studies in people have provided compelling evidence that transient HPA axis dysfunction (RAI/CIRCI) occurs in a substantial number of septic adults.^{25,28,29,31,32,35,36,40} While the reported incidence of RAI/CIRCI varies some with the specific diagnostic criteria used, application of currently accepted diagnostic criteria suggests that RAI/CIRCI occurs in as many as 60-70% of adult humans with sepsis and septic shock.^{29,76}

Given the varied and vital roles that cortisol plays in the regulation of cellular, tissue, and organ function and homeostasis, it seems logical that the concurrent incidence of RAI/CIRCI and sepsis could render a patient incapable of responding appropriately to the pathophysiologic stress of his or her disease, and may contribute to increased disease severity. Indeed, a growing body of evidence suggests that RAI/CIRCI in critically ill patients is associated with an increased rate of shock, multiple organ dysfunction syndrome (MODS), and death.^{24,77} In 41 septic human patients, longer ICU stays and increased severity of multiple organ failure were noted in patients who met criteria for RAI/CIRCI as compared to those with normal HPA axis function.²⁴ Annane *et al* evaluated 189 patients with septic shock, and found an 82% mortality rate in patients who had both inappropriately low serum basal serum cortisol and poor cortisol responses to ACTH stimulation, versus a mortality rate of 26% in patients with intact HPA axis function.⁷⁷

While it is difficult to determine from the above studies if the occurrence of RAI/CIRCI is a direct cause of or simply a marker for increased disease severity and poor prognosis, there is some indirect evidence from recent studies to suggest that cortisol insufficiency may play a

causal role in increasing disease severity. Five comprehensive meta-analyses have evaluated the effects of corticosteroid treatment in patients with sepsis and septic shock, and all demonstrated a significant beneficial effect of low-dose (“physiologic-dose”) corticosteroid supplementation in septic patients with RAI/CIRCI.⁷⁸⁻⁸² In particular, septic adults that met criteria for RAI/CIRCI that were supplemented with low doses of hydrocortisone demonstrated more rapid shock reversal, earlier cessation of vasopressor therapy, and increased survival rates than unsupplemented patients.^{31,83-85}

However, the CORTICUS study, a large multi-center placebo-controlled study of low-dose hydrocortisone in adults with septic shock did not find a beneficial effect with regards to shock reversal or survival, regardless of the presence or absence of RAI/CIRCI.⁸⁶ In addition, this study demonstrated a slight but significant increase in the development of new sites of infection in hydrocortisone-supplemented patients.⁸⁶ Some have questioned, though, whether an inadvertent selection bias for “less-sick” individuals may have impacted these findings, as patient demographics differed in this study from previous recent trials and the study was terminated early due to difficulty with patient recruitment, presumably because many very ill patients had already been placed on low-dose hydrocortisone by their attending physicians.^{19,30,87} Despite the controversy surrounding these findings, the potential that only a subset of septic patients might benefit from low-dose corticosteroid supplementation should be considered, and reinforces the need for more accurate diagnostic criteria to identify such patients.

RAI/CIRCI in Human Pediatrics

Compared to adults, considerably less information is available on RAI/CIRCI in pediatric patients, and specific diagnostic cut-off values for appropriate basal cortisol concentrations and

cortisol responses to ACTH stimulation testing have not been proposed. However, one study that evaluated 57 children with septic shock determined that the incidence of RAI/CIRCI in these children was as high as 44%, depending on the diagnostic criteria applied.³³ Furthermore, a 20% decrease in survival rate was found for septic children that met criteria for RAI/CIRCI (47%), as compared to septic children with intact HPA axis function (67%).³³ A recent study found a similar incidence of RAI/CIRCI, as high as 58%, in 73 critically ill children, though not all of these children were septic.⁸⁸ Finally, in a small-scale study in 22 children with septic shock, the highest mortality rate was found in children with a high basal cortisol and an inadequate cortisol response on a low-dose ACTH stimulation test, consistent with “loss of adrenal reserve.”⁸⁹ In sum, these data suggest that incidence and impact of RAI/CIRCI in critically ill children is similar to that reported in adults.

In addition, there is strong evidence that HPA axis immaturity in both premature and term infants can contribute to an inadequate cortisol response in the face of disease.^{30,34,90,91} While fetal and neonatal HPA axis maturation in the infant is not as delayed as in the foal,^{70,92} both the pre-term and full-term infant do exhibit HPA axis immaturity, manifest by impaired adrenocortical cortisol synthesis and suppression of fetal HPA axis activity by maternal cortisol.^{30,34} One study described low basal serum cortisol concentrations (ranging from 2.0 to 15.4 µg/dl) in 7 critically ill full-term human neonates, with all but one less than the adult diagnostic cut-off for RAI/CIRCI of 10 µg/dl.⁹³ Other studies have confirmed the prevalence of low cortisol concentrations and documented impaired cortisol responses to ACTH stimulation in both pre-term and full-term infants,^{91,93-96} and have shown strong associations between HPA axis dysfunction, increased disease severity and persistent hypotension in critically ill neonates.⁹⁷⁻¹⁰⁰ While a small number of studies report beneficial effects of low-dose hydrocortisone for treating

hypotension in both pre-term¹⁰¹⁻¹⁰³ and full-term^{93,94} infants, an increased risk of spontaneous intestinal perforation in hydrocortisone-supplemented pre-term infants, especially those with high basal cortisol concentrations, has been described.^{103,104} Thus, while there is evidence to suggest that RAI/CIRCI may also occur with substantial frequency in critically ill children and infants, the potential risks and benefits of hydrocortisone supplementation in such patients require further critical evaluation.

RAI/CIRCI in Veterinary Medicine

The incidence of RAI/CIRCI in veterinary critical care has only been examined in a small number of studies. Single cases of suspected RAI/CIRCI have been described in one cat with severe trauma after being struck by a car¹⁰⁵ and in one septic dog.¹⁰⁶ In both cases, the animals had abnormal ACTH stimulation test responses and exhibited clinical signs consistent with CIRCI (persistent hypotension) that resolved rapidly with low-dose corticosteroid therapy.

In addition, three studies in dogs investigated the incidence of RAI/CIRCI during critical illness. Prittie *et al* evaluated basal cortisol concentrations and responses to a high dose ACTH stimulation test in 20 critically ill dogs and did not find evidence of HPA axis dysfunction based on either parameter in this group of dogs.¹⁰⁷ However, only 3 dogs in that study were septic. Two other recent studies, one including 33 septic dogs and one including 31 critically ill dogs (13 of which were septic), did demonstrate evidence of HPA axis dysfunction in 48% and 55% of dogs respectively.^{108,109} In addition, dogs with evidence of HPA axis dysfunction had a significantly higher incidence of persistent hypotension and need for vasopressors as well as decreased survival rates as compared to dogs with intact HPA axis function.^{108,109} While further study in larger groups of animals is clearly needed, these findings suggest that RAI/CIRCI may

be of similar incidence and import in small animal critical care as in the human intensive care unit.

The specific incidence of RAI/CIRCI in any large animal species has not been reported. HPA axis function has been investigated in only a small number of septic neonatal foals, and there is indeed some evidence to suggest that HPA axis dysfunction may occur in this population. One case of transient HPA axis dysfunction, as evidenced by both a low basal cortisol concentration and an impaired cortisol response to a high-dose ACTH stimulation test, has been described in a septic neonatal foal.¹¹⁵ In addition, two independent studies that measured basal ACTH and cortisol concentrations in healthy and septic neonatal foals found significantly increased ACTH:cortisol ratios in non-surviving septic foals.^{116,117} Such high ACTH:cortisol ratios, with high ACTH concentrations and low corresponding cortisol concentrations, suggest HPA axis dysfunction at the level of the adrenal gland may occur in the septic foal. Finally, a recent study by Wong *et al* that demonstrated significantly lower cortisol responses to a low-dose (0.1 µg/kg) of cosyntropin in non-surviving septic foals as compared to survivors provides further evidence for adrenal dysfunction in some septic foals.¹¹⁸ In concert, these studies suggest HPA axis function may be impaired in septic neonatal foals, and also suggest that the incidence and significance of RAI/CIRCI in critically ill foals warrants further investigation.

SECTION V. CURRENT RECOMMENDATIONS FOR THE DIAGNOSIS AND MANAGEMENT OF RAI/CIRCI IN SEPSIS

Diagnosis and Management of RAI/CIRCI in Septic Adults

Unfortunately, despite the substantial incidence and impact of RAI/CIRCI in human critical care, a rapid, practical and comprehensive means of assessing all levels of the HPA axis in critically ill patients is not yet available. Furthermore, specific diagnostic criteria for RAI/CIRCI in septic people are not universally accepted. While the metyrapone test or the ITT may be the best means of comprehensively evaluating HPA axis function, these tests are cumbersome, and potentially dangerous, for use in critically ill patients. Thus, basal total cortisol concentrations and/or total cortisol responses to high- or low-dose ACTH stimulation tests are currently utilized for diagnosis of RAI/CIRCI in human patients with sepsis and septic shock, despite the flaws associated with these methodologies.

In response to experimentally induced stress, healthy people will experience an increase in serum cortisol concentration to 2 to 3 times basal levels, to concentrations $> 18 \mu\text{g/dl}$. Thus, a random basal cortisol concentration of < 18 to $25 \mu\text{g/dl}$ has been used by some to diagnose RAI/CIRCI in septic patients.^{18,25,42} When a basal serum cortisol $< 25 \mu\text{g/dl}$ was used to define RAI in septic patients, a 61% incidence of RAI/CIRCI was detected in 59 adult patients with septic shock.^{18,25} However, since hour-to-hour and day-to-day fluctuations in cortisol concentrations are expected, a lower cortisol cut-off concentration may be more appropriate. One study that compared a variety of diagnostic criteria for RAI/CIRCI against metyrapone test results found that a basal cortisol concentration of $< 10 \mu\text{g/dl}$ was the best predictor of HPA axis dysfunction as diagnosed by metyrapone testing.²⁹

Given the potential for fluctuating cortisol concentrations in critical illness, though, diagnosing RAI/CIRCI on basal serum cortisol levels alone may still be misleading. Some studies suggest that the ACTH stimulation test is a more sensitive method for diagnosing RAI/CIRCI.^{24,27} Annane *et al* performed high-dose ACTH stimulation tests in adult patients with septic shock, and determined that the incremental cortisol response value (delta cortisol) with the smallest P value for survival was <9 µg/dl above the baseline value.⁷⁷ This delta cortisol cutoff value remained an excellent predictor of HPA axis dysfunction as defined by abnormal metyrapone test results in a later study by the same author.²⁹ As discussed in Section II, though, some patients with RAI/CIRCI may exhibit some degree of ACTH resistance, and may be more accurately diagnosed with a low-dose cosyntropin stimulation tests.^{25,35,42}

Also, it is important to note that all of the tests described above are based on *total* cortisol concentrations (bound + free cortisol). However, as described in Section I, it is the *free* circulating cortisol that is biologically available to enter cells and to exert cortisol-specific effects, and is responsible for feedback mechanisms that regulate HPA axis activity. Since both CBG and albumin are negative acute phase proteins that may be substantially decreased in many septic patients by their ongoing and profound systemic inflammatory response,^{11,119,120} plasma cortisol binding capacity and free cortisol concentrations may be quite dramatically altered during critical illness. Thus, free rather than total cortisol parameters may better represent physiologic cortisol status during critical illness. For instance, in hypoproteinemic patients, ACTH-stimulated serum total cortisol concentrations were subnormal, but basal and ACTH-stimulated serum free cortisol concentrations were normal or increased as compared to healthy controls.¹²¹ Even in healthy individuals, significant variation in serum CBG concentrations

between individuals and within the same individual over time has been shown to significantly affect serum free cortisol responses to ACTH-stimulation tests.¹²²

Thus, in septic patients likely to have altered CBG and albumin concentrations, measurement of serum free cortisol concentration may provide a more accurate method for diagnosis of RAI/CIRCI than does basal and ACTH-stimulated serum total cortisol concentration.^{119,121,123,124,125} A recent study comparing free and total plasma cortisol concentrations in people with sepsis and septic shock showed that free cortisol concentrations correlated better with illness severity scores than did total cortisol concentrations.¹²⁵ In addition, wide variation in ACTH-stimulated free cortisol increments among patients with sepsis, septic shock, and healthy controls was noted, despite minimal differences in total cortisol increments between these groups.¹²⁵ Interestingly, in patients with septic shock who also met diagnostic criteria for RAI/CIRCI, both total and free cortisol increases after ACTH stimulation were lower than in patients not meeting criteria for RAI/CIRCI, though no difference in levels of CBG and albumin was present between these two groups.¹²⁵ In addition, when Annane *et al* compared free versus total cortisol parameters to diagnosis of HPA axis dysfunction with a metyrapone test in adults with septic shock, no clear diagnostic advantage of free over total cortisol was identified.²⁹

At present, current recommendations for the diagnosis of RAI/CIRCI in septic adult humans thus include documentation of a basal total cortisol concentration of $< 10\mu\text{g/dl}$ or a total cortisol response to a high-dose cosyntropin stimulation test (delta cortisol) of $< 9\mu\text{g/dl}$.³⁷ The low-dose cosyntropin stimulation test may offer improved diagnostic accuracy in identifying some patients with RAI/CIRCI, but is not well-validated enough to recommend at present. Similarly, diagnostic benefits of free versus total cortisol parameters are not clear at present, and free cortisol measurement is more difficult and not readily available in all settings, so use of free

cortisol parameters is also not currently recommended for diagnosis of RAI/CIRCI in septic patients.

The motivating factor for the above studies, which all sought to determine the ideal diagnostic criteria for RAI/CIRCI in septic patients, is the accurate identification of those patients who might benefit most from cortisol replacement therapy in the form of low-dose hydrocortisone. As described in Section IV above, a number of clinical studies and several meta-analyses have documented a beneficial effect of low-dose hydrocortisone supplementation on both shock reversal and survival in septic patients with RAI/CIRCI.^{31,78-85} However, the CORTICUS study, the largest clinical trial of low-dose hydrocortisone in adults with septic shock to date, did not find a beneficial effect of hydrocortisone treatment on either shock reversal or survival, and documented a slightly increased risk of developing new sites of infection during hospitalization in supplemented patients.⁸⁶ Despite its flaws,^{19,30,87} the CORTICUS study raises important concerns that low-dose hydrocortisone may not be innocuous in septic patients.

Thus, current recommendations for the use of low-dose hydrocortisone in septic adults attempt to limit use to patients in which true cortisol insufficiency may be contributing to increased disease severity. An international task force on the use of corticosteroids in critically ill patients organized by the American College of Critical Care Medicine recommends that adults with vasopressor-dependent septic shock and RAI/CIRCI receive supplemental hydrocortisone at a dose of 200 mg/kg day divided into 4 doses or an infusion of 240 mg/day (10 mg/hr). This dose is approximately 4-5 times the physiologic endogenous cortisol secretion rate of 0.5 mg/kg/day in healthy unstressed people, and maintains plasma cortisol concentrations at a high but physiologically relevant concentration of approximately 18-30 μ g/dl in people.²⁸

Unfortunately, controlled studies to determine if this is the optimal dose and dosing regimen for corticosteroid replacement therapy in septic patients are lacking. A small number of studies in animal models of sepsis, experimental endotoxemia in healthy people, and in clinical patients with sepsis and septic shock have documented desirable anti-inflammatory and hemodynamic effects of similar low-dose hydrocortisone therapy regimens.^{19,28,126-130} However, other studies have actually reported some *pro-inflammatory* and potentially undesirable effects of these hydrocortisone protocols in sepsis models.^{131,132} Thus, the immunologic and physiologic effects of low-dose hydrocortisone, and the contribution of these effects to disease progression and outcomes in septic patients, certainly require further study.

Diagnosis and Management of RAI/CIRCI in Infants

Only a small number of studies have been conducted to evaluate specific diagnostic criteria and management strategies for RAI/CIRCI in infants, and no consensus on diagnosis and management of RAI/CIRCI in critically ill pre-term and full-term human neonates is available to date.^{30,34} Present recommendations for use of low-dose hydrocortisone therapy in critically ill infants are based on clinical evidence of refractory hypotension rather than specific diagnostic criteria for RAI/CIRCI, but evaluation of HPA axis function in critically ill infants with clinical signs consistent with RAI/CIRCI is recommended by measurement of basal total cortisol concentration prior to starting steroid therapy. While a basal cortisol concentration of > 15-20 µg/dl is generally considered appropriate in a critically ill infant,^{30,34} specific cut-off values for diagnosis of RAI/CIRCI are not described. The ACTH stimulation test may be helpful in diagnosis of RAI/CIRCI in pediatric patients, but is not currently recommended until it is better evaluated in this subset of patients.^{30,34} In addition, slightly altered hydrocortisone dosing

protocols are recommended in neonates, as hydrocortisone half-life appears to be longer in this population.³⁰ The recommended low-dose hydrocortisone strategy regimen for use in critically ill pre-term and term infants is 0.5-1.0 mg/kg IV q 8 -12 hours.^{30,34} In a small study in premature baboons, a similar hydrocortisone regimen in 8 animals that had low cortisol production in the 24 hours after birth did result in hemodynamic stabilization,¹³³ but critical evaluation of the hemodynamic, immunologic and clinical consequences of low-dose hydrocortisone is not well described in neonates of any species.

Furthermore, as described in Section IV, low-dose hydrocortisone has been associated with an increased risk of spontaneous intestinal perforation in pre-term infants, particularly if basal cortisol concentrations are normal or high.^{103,104} Thus, hydrocortisone supplementation should be used with caution in infants with adequate basal cortisol concentrations.³⁰ If hydrocortisone therapy is initiated before basal cortisol concentrations are measured, a test dose of 1.0 mg/kg as an IV bolus is recommended. If this results in improved blood pressure and basal cortisol concentrations are low, it may be followed by hydrocortisone at 0.5 mg/kg IV q. 8-12 hours, but a shorter course of treatment is currently recommended in neonates (2-3 days³⁰ vs ~7 days in adults)³⁷ to minimize the risk of adverse effects.

Diagnosis and Management of RAI/CIRCI in Veterinary Medicine

Given key species differences in HPA axis function, diagnostic criteria and management strategies for RAI/CIRCI in people are unlikely to be directly applicable in veterinary critical care. Unfortunately, specific diagnostic criteria for RAI/CIRCI have not been critically evaluated in veterinary patients. Two canine studies found that a delta cortisol cut-off of < 3 µg/dl after a high-dose ACTH stimulation test best predicted hypotension and survival in septic

and critically ill dogs.^{108,109} However, animals were not supplemented with hydrocortisone in either of these studies, and the use of low-dose hydrocortisone is only described in one septic dog.¹⁰⁶ In this case, the dog displayed vasopressor-non-responsive hypotension and an inadequate cortisol response to a high-dose ACTH stimulation test. Shock reversal was achieved within 2 hours of initiating hydrocortisone at 0.5 mg/kg IV q 6 hours in addition to standard therapy for sepsis.

The incidence of RAI/CIRCI in septic foals is unknown, so specific diagnostic criteria have not been proposed. Further, corticosteroid supplementation in transient adrenal insufficiency has also been described in only one septic foal.¹¹⁵ This foal had serially low basal serum cortisol concentrations (0.7-1.2 µg/dl) and blunted cortisol response to a high-dose ACTH stimulation test (11% increase in serum cortisol). Prednisolone supplementation at 1 mg/kg, in addition to standard therapy for sepsis, resulted in resolution of the foal's clinical signs and adrenal insufficiency. In this case, corticosteroid supplementation was necessary to prevent episodes of collapse for several weeks, but after prednisolone was tapered and discontinued, post-treatment ACTH stimulation testing at two months of age revealed normal adrenocortical function (210% increase in cortisol concentration following ACTH stimulation).¹¹⁵ Thus, while corticosteroid supplementation appeared to produce clinical improvement in these two veterinary cases, further study is clearly needed to define optimal diagnostic criteria and management strategies for RAI/CIRCI in septic small and large animals.

SECTION VI. RATIONALE FOR THE STUDIES PRESENTED HEREIN

As discussed above, there is a great deal of evidence that transient HPA axis dysfunction, is of substantial importance in critically ill people and infants, particularly in patients with sepsis and septic shock. Furthermore, this absolute and/or functional cortisol insufficiency (RAI/CIRCI) may be managed successfully with cortisol supplementation in the form of low-dose hydrocortisone treatment in many patients. Recent work also suggests that the incidence and impact of RAI/CIRCI may be comparable in septic dogs, but low-dose hydrocortisone supplementation has not been investigated in veterinary patients with RAI/CIRCI.

Since HPA axis maturation is delayed and cortisol synthetic capacity appears limited in the foal in comparison with other species, the potential for cortisol insufficiency in the critically ill neonatal foal could be quite high. Indeed, as outlined above, several recent studies suggest that some form of HPA axis dysfunction may occur in some septic foals. Therefore, the studies described in Chapters 3 through 8 were conducted to accomplish the following objectives: (1) to fully characterize HPA axis function in the immediate post-natal period in the healthy full-term foal; (2) to develop heretofore unavailable diagnostic methodology for HPA axis assessment in foals; (3) to investigate the incidence and impact of RAI/CIRCI in critically ill neonatal foals; and (4) to develop and evaluate a physiologically-relevant low-dose hydrocortisone therapy regimen for potential use in the management of RAI/CIRCI in critically ill neonatal foals.

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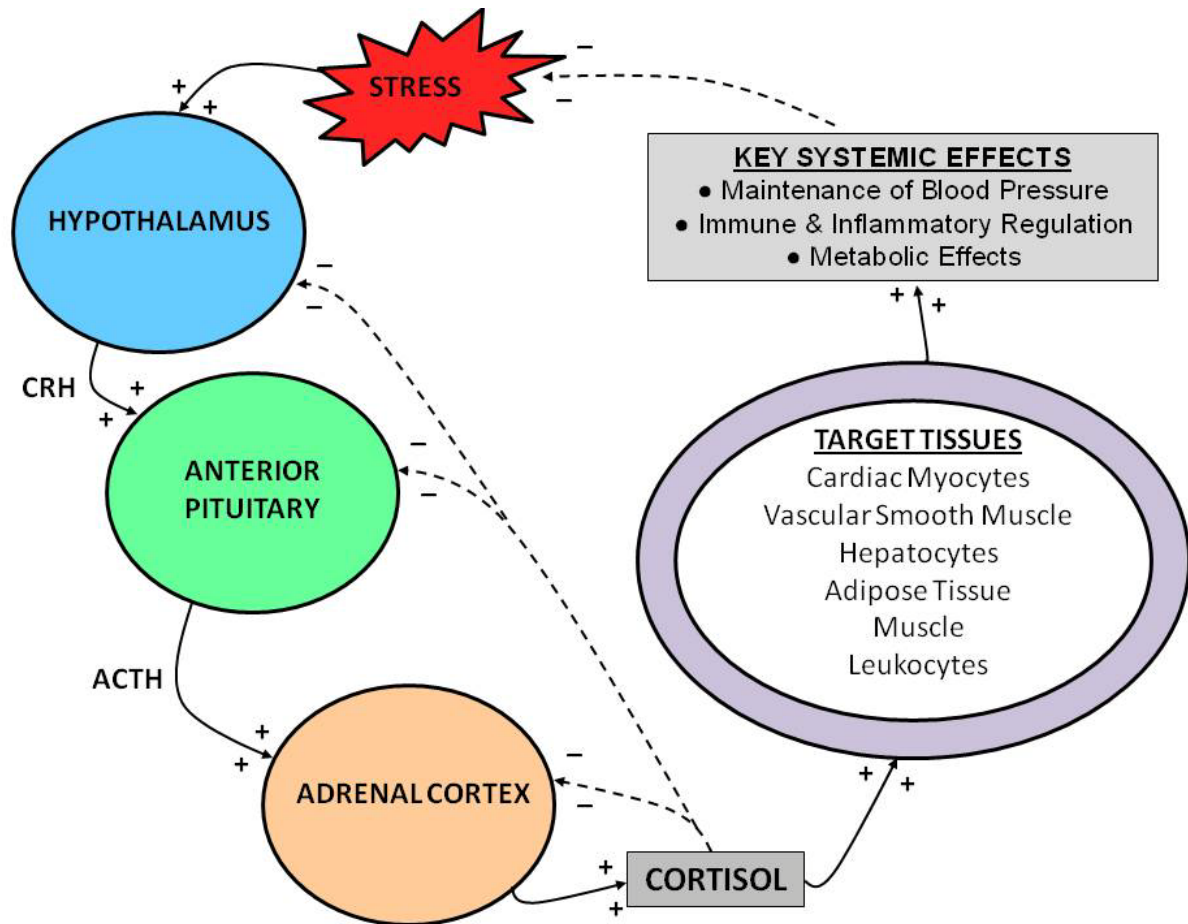


Figure 2.1. The hypothalamic-pituitary-adrenal (HPA) axis. Stimulatory interactions are illustrated with solid arrows and + signs, and inhibitory interactions (negative feedback) are illustrated with dashed arrows and – signs.

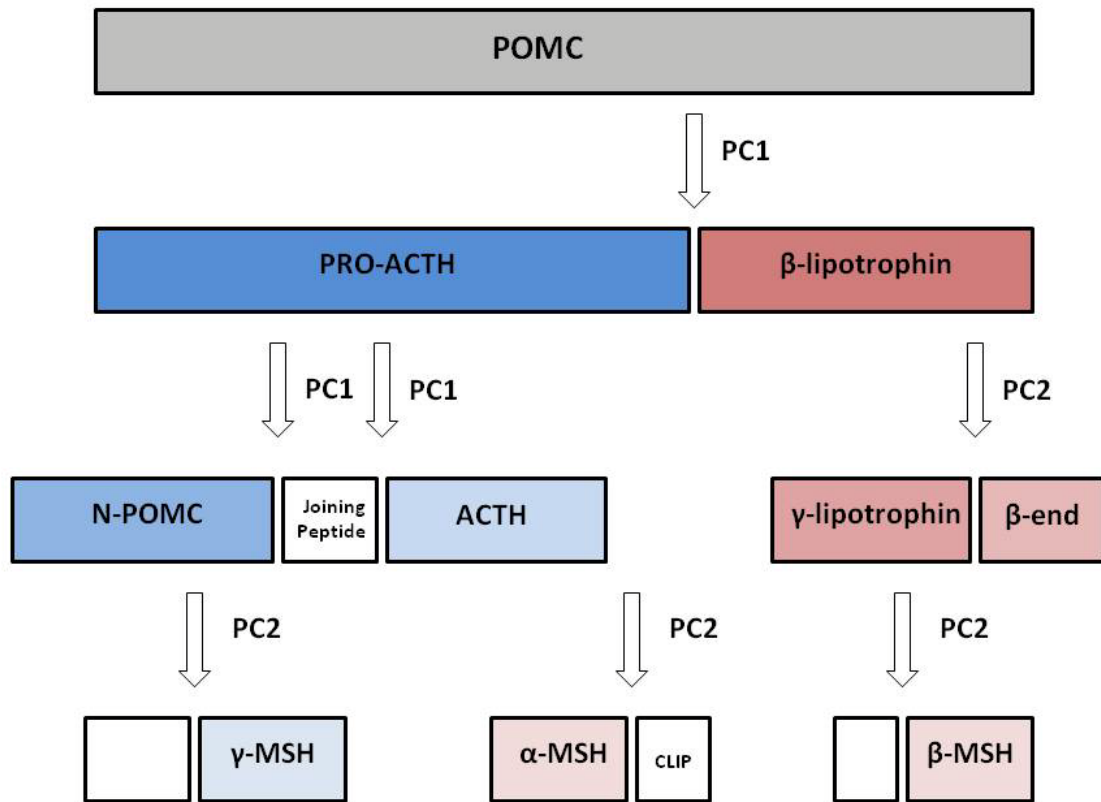


Figure 2.2. Proopiomelano-cortin (POMC) processing. Pro-ACTH is pro-adrenocorticotrophic hormone. N-POMC is N-terminal POMC peptide. ACTH is adrenocorticotrophic hormone. β-end is β-endorphin. MSH is melanocyte stimulating hormone. CLIP is corticotrophin-like intermediate lobe peptide. PC1 and PC2 are pro-hormone convertase 1 and 2 respectively, and are the enzymes responsible for cleavage of precursor peptides at the indicated sites.

Adapted from: White A, Ray D. ACTH precursors in different pathological conditions. Chapter 5 in Gaillard RC, Ed. *The ACTH Axis: Pathogenesis, Diagnosis and Treatment*. Boston: Kluwer Academic Publishers. 2003. p 86.

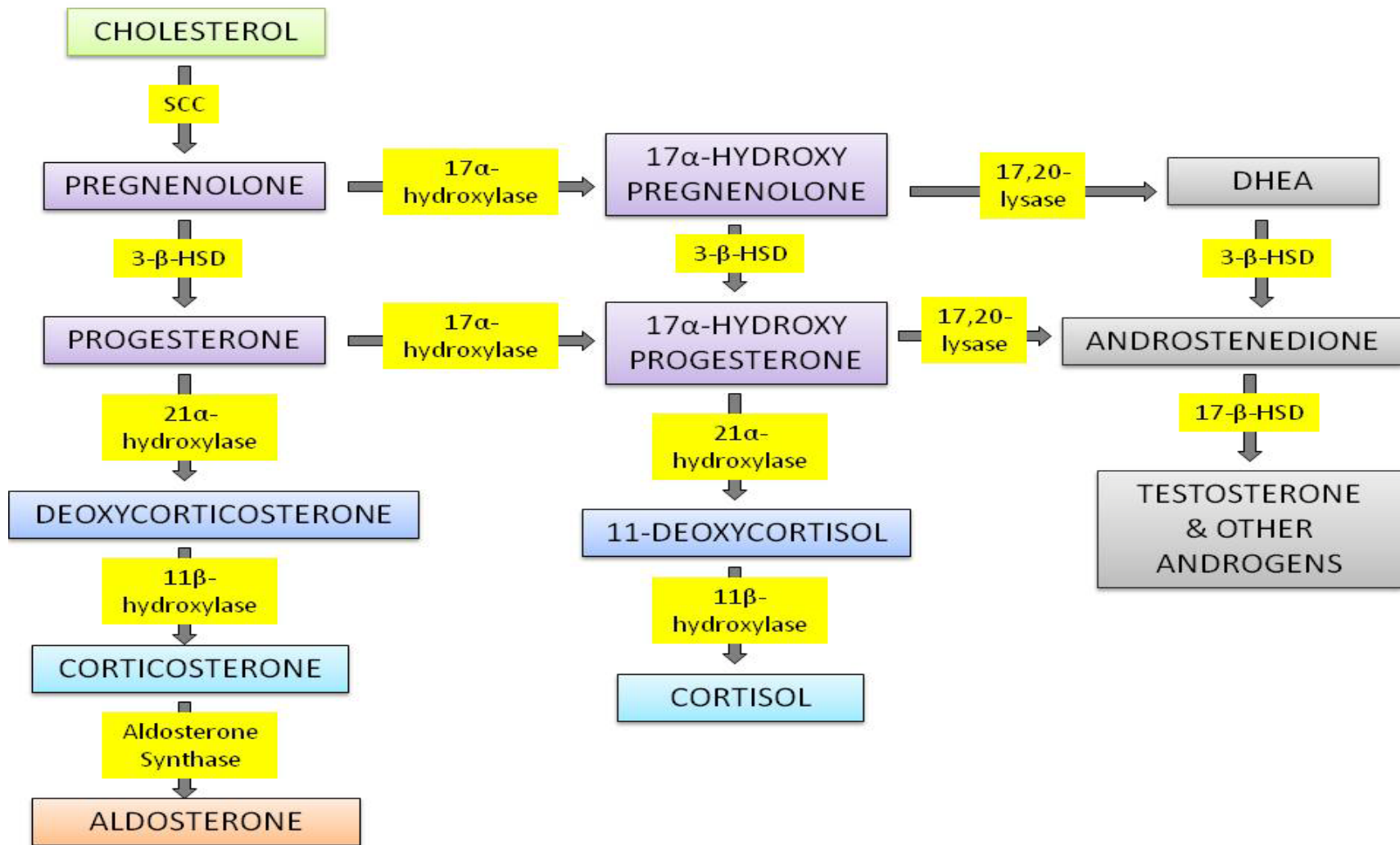


Figure 2.3. Biosynthetic pathway for adrenal corticosteroids. The enzymes responsible for catalyzing each biotransformation are shown in the yellow boxes.

Adapted from <http://en.wikipedia.org/wiki/File:Steroidogenesis.svg> & <http://herkules oulu.fi/isbn951426844X/html/graphic99.png>

CHAPTER 3

SYNTHETIC ADRENOCORTICOTROPIC HORMONE STIMULATION TESTS IN HEALTHY NEONATAL FOALS¹

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ABSTRACT

Background: Cosyntropin (ACTH) stimulation tests are used to evaluate adrenal function. Low dose ACTH stimulation tests are the most accurate method for diagnosing relative adrenal insufficiency in critically ill humans, but have not been evaluated in foals.

Hypothesis: Peak serum cortisol concentrations in healthy foals will not be significantly different after intravenous administration of 1, 10, 100, and 250 μg of cosyntropin.

Animals: 14 healthy neonatal foals

Methods: A randomized cross-over model was used in which cosyntropin (1, 10, 100, or 250 μg IV) was administered on days 3 and 4 of life. Blood samples were collected before and 30, 60, 90, 120, and 150 minutes after administration of cosyntropin for determination of serum cortisol concentration.

Results: Serum cortisol concentrations did not significantly increase after administration of 1 μg cosyntropin. Cortisol concentration peaked 30 minutes after administration of 10 μg cosyntropin and 90 minutes after 100 and 250 μg cosyntropin. There was no relationship between cosyntropin dose and serum cortisol concentration at 30 minutes. Compared to the 10 μg dose, 100 and 250 μg cosyntropin induced significantly greater cortisol concentrations at 90 minutes, at which point the 10 μg cosyntropin dose cortisol values were indistinguishable from baseline. There was no significant difference in the area under the cortisol concentration curve between the 100 and 250 μg doses. No effect of day of testing or foal weight on peak cortisol concentration was detected.

Conclusions and Clinical Importance: The results of this study suggest that 10 and 100 μg doses of cosyntropin would be appropriate for evaluating adrenal function in neonatal foals.

INTRODUCTION

The primary adaptive response to physiologic stress in all mammalian species involves activation of the hypothalamic-pituitary-adrenal (HPA) axis. Both external and internal environmental stressors activate the peripheral and central nervous systems, whose signals are interpreted and integrated in the hypothalamus, culminating in the release of corticotropin-releasing hormone (CRH). CRH acts locally on the anterior pituitary gland, inducing the release of adrenocorticotropic hormone (ACTH), which, in turn, enters the systemic circulation and stimulates the adrenal cortices to release cortisol. Cortisol regulates essential physiologic responses to the stress of illness, including maintenance of blood pressure, provision of energy to tissues, and control of an appropriate inflammatory response.¹ Increased systemic cortisol concentrations also exert a negative feedback effect on both the pituitary gland and hypothalamus, resulting in subsequent down-regulation of both CRH and ACTH secretion. With an intact HPA axis, systemic cortisol concentrations are maintained at a level that is appropriate for the existing degree of physiologic stress in both health and disease.¹⁻³

Given the varied and vital roles that cortisol plays, adrenal dysfunction results in significant pathophysiologic consequences. Absolute disturbances in the HPA axis are less common in horses than in companion animals, though hyperadrenocorticism secondary to pituitary pars-intermedia adenoma is well documented in adult horses.^{4,5} Adrenal exhaustion in adult horses, adrenal insufficiency secondary to long-term anabolic steroid administration in an adult horse, and transient adrenal insufficiency in a septic neonatal foal have also been described.^{4,6,7} There is compelling evidence in human medicine that relative adrenal insufficiency (RAI), an inappropriately low cortisol response for the degree of illness, is

common in critically ill patients, particularly in patients with sepsis, septic shock, and systemic inflammatory response syndrome (SIRS).^{1,8-10} Given that bacterial septicemia is the leading cause of disease and mortality in neonatal foals,¹¹⁻¹⁵ the potential for the existence of similar relative adrenal insufficiency in septic foals warrants further investigation. The literature regarding relative adrenal insufficiency in animals, particularly foals, is scarce, but recent evidence shows that septic foals with relatively greater plasma ACTH and lower serum cortisol concentrations have a higher mortality rate.^a

Thus, accurate assessment of the HPA axis and, in particular, adrenal gland function, is of growing importance in equine medicine. The ACTH stimulation test is the most commonly used diagnostic tool for assessment of adrenal insufficiency in human and veterinary medicine. This test typically is performed using cosyntropin (α 1-24 corticotropin), a synthetic ACTH subunit containing the first 24 of the 39 amino acids of endogenous ACTH. The classic protocol for this test in human and veterinary medicine involves intramuscular or intravenous administration of a single dose of 100 to 250 μ g of cosyntropin per patient, with subsequent measurement of the serum total cortisol concentration from baseline to peak response, typically 30 to 90 minutes following administration of cosyntropin.^{4,16,17} High doses of cosyntropin (i.e. 250 μ g) yield supraphysiologic plasma concentrations of ACTH, and thus produce maximal adrenal gland stimulation and subsequent maximal serum cortisol response.^{1,16,18} Low-dose ACTH stimulation protocols have been evaluated in healthy people and dogs, and have shown that administration of 1 μ g cosyntropin per dog or person also produces a maximal cortisol response.^{16,18-20} Recent reports suggest that low-dose ACTH stimulation tests might be a more accurate test for the diagnosis of relative adrenal insufficiency in septic human patients because a more “physiologic” dose of ACTH is administered.^{17,18,21} This suggestion is based on the finding that people with

RAI often have an appropriate cortisol response to 250 µg of cosyntropin – which results in peak plasma concentrations of ACTH that greatly exceed what is achievable under physiologic conditions of intense stress or illness – but are incapable of producing a cortisol response to lower “physiologically relevant” doses of 1 µg cosyntropin.^{17,18,21}

The efficacy of low-dose ACTH stimulation tests has not been reported in horses or foals. The purpose of this study was to compare the effects of administration of 1, 10, 100, and 250 µg cosyntropin on serum total cortisol concentrations in 3- to 4-day-old healthy foals, in order to determine if a low dose ACTH stimulation test protocol is valid in neonatal foals.

MATERIALS AND METHODS

Animals

Fourteen neonatal foals (12 Quarter Horses, 2 European Warmbloods; 9 males, 5 females) were evaluated on days 3 and 4 of life. Median foal body weight was 50.9 kg (range 43.2 to 65.9 kg). Foals were determined to be healthy prior to inclusion in the study by lack of abnormalities identified on physical examination and a serum immunoglobulin level ≥ 800 mg/dL on day 3 of life. Only full term foals (>330 days gestation) born without assistance were included in the study. On day 2 of life, each mare and foal pair was brought into a stall and allowed to acclimate for 24 hours prior to sample collection. Animals were stabled during the study period, and were cared for according to the principles and guidelines stated in an Animal Use Protocol determined by the University of Georgia’s Department of Animal Resources.

Study Design

A randomized, cross-over, repeated measures design was used to compare serum total cortisol response to 4 different doses of cosyntropin^b (synthetic adrenocorticotrophic hormone, α 1-24 corticotropin) in healthy neonatal foals. Cosyntropin was administered on the morning of day 3 of life, the evening of day 3 of life, and the morning of day 4 of life, a time at which HPA axis function is reportedly stable in neonatal foals.²² Morning tests were performed between 5:00 and 9:00 am, and evening tests between 5:00 and 9:00 pm, with a minimum of 12 hours between serial tests in each individual foal. Eight foals received 1, 10, and 100 μ g of cosyntropin, 2 foals received 10, 100, and 250 μ g doses of cosyntropin, and 4 additional foals were tested only once, with 250 μ g cosyntropin. For the foals that received 3 doses of cosyntropin, the order of administration of each specific dose to each foal was randomized between the three testing times. Similarly, in the 4 foals that received only the 250 μ g dose of cosyntropin, the time of testing was randomized between the 3 testing times specified.

Preparation of Cosyntropin

Cosyntropin^b was supplied as a lyophilized powder in glass vials. Each vial contained 250 μ g of α 1-24 corticotropin which was reconstituted with 1 mL sterile saline (0.9% sodium chloride) according to manufacturer directions.^b The resulting solution was then diluted 10-fold with sterile saline to produce a 25 μ g/mL solution of cosyntropin. The 1, 10, and 100 μ g doses consisted of 0.04 mL, 0.4 mL, and 4 mL of this solution, respectively. The 1 and 10 μ g doses were additionally diluted with sterile saline to a total volume of 4 mL. For the 250 μ g dose, each vial was reconstituted with 1 mL sterile saline and then further diluted to a total volume of 4 mL. Reconstituted and diluted cosyntropin solutions were aliquoted into single dose, sterile glass

vials and stored frozen at -80°C until immediately prior to use (not longer than 4 months).

Reconstituted cosyntropin solutions are stable when frozen in glass vials for up to 6 months.²³

Sample Collection

All blood samples were collected by jugular venipuncture from standing foals, under brief restraint by experienced foal handlers. Prior to administration of each dose of cosyntropin, blood was collected for baseline serum total cortisol measurement. Blood was immediately placed into a glass tube without additives and allowed to clot at room temperature. Prior to withdrawal of the venipuncture needle, the appropriate dose of cosyntropin was administered intravenously as a rapid bolus. Blood was then collected at 30, 60, 90, 120, and 150 (250 µg dose only) minutes following cosyntropin administration and allowed to clot. All blood samples were refrigerated within 30 minutes of collection, centrifuged within 3 hours of collection, and the serum separated and stored frozen at -80 degrees Celsius until assays were performed. Serum total cortisol concentrations have been shown to be stable under refrigeration (2-8 degrees Celsius) for 7 days and frozen for 3 months.^c

Cortisol Assay

Serum total cortisol concentrations (henceforth referred to as cortisol concentrations) were determined on an automated analyzer using a chemiluminescent enzyme immunoassay system^d validated for use in horses.^{24,25} The interassay and intra-assay coefficients of variation are ≤ 20%.²⁵ The limit of detection with this assay is 0.05 µg/dL.²⁴

Statistical Analysis

Peak serum cortisol concentration, time of peak serum cortisol concentration, and delta serum cortisol and peak delta serum cortisol concentrations, were determined for each individual foal for the 10, 100, and 250 μg cosyntropin doses. Peak serum cortisol concentration is defined as the single highest cortisol concentration achieved in each individual foal. Time of peak serum cortisol concentration is the time point (30, 60, 90, 120, or 150 minutes) at which peak serum cortisol concentration was achieved in each individual foal for each dose of cosyntropin. Delta serum cortisol concentration is the cortisol concentration achieved at each time point following administration of cosyntropin (30, 60, 90, 120, and 150 minutes) for each individual foal minus its baseline (time 0) cortisol concentration. Similarly, delta peak serum cortisol is the peak cortisol concentration achieved for each individual foal minus its baseline cortisol concentration.

The mixed procedure for repeated measures^e was used to compare cortisol concentrations at each time point before (time 0, baseline) and after administration of cosyntropin (30, 60, 90 and 120 minutes) for each dose of cosyntropin (1, 10, 100, and 250 μg). Time of peak serum cortisol concentration, peak serum cortisol, delta serum cortisol, and delta peak serum cortisol concentrations, and area under the curve data to 120 minutes were also compared between cosyntropin doses using the mixed procedure for repeated measures. Post-hoc comparisons for significant findings were conducted using a Tukey-Kramer test in all cases, except when comparing time to peak serum cortisol concentration, peak serum cortisol and delta peak serum cortisol concentrations with the 250 μg cosyntropin dose data, in which case the Dunnett-Hsu test was used.

Baseline cortisol concentrations, time of peak serum cortisol concentration, peak serum cortisol and delta peak serum cortisol concentrations were also compared between the three

collection times (i.e., day 3 morning, day 3 evening, day 4 morning) using the mixed procedure for repeated measures. Finally, the exact dose of cosyntropin given per kg of body weight for each foal was calculated for the 10 µg and the 100 µg doses, and Spearman's rank correlation test was used to evaluate for an effect of weight on peak serum cortisol and delta peak serum cortisol concentrations for each dose. P values < 0.05 were considered statistically significant. Data is expressed as mean ± standard deviation.

RESULTS

There were no adverse effects noted after administration of cosyntropin in any foal at any dose. There was an overall effect of time after administration of 1 µg cosyntropin on cortisol concentration (P = 0.003). However, as compared to baseline values, there was no significant increase in cortisol concentration at any time point (30, 60, 90, or 120 minutes) following administration of the 1 µg cosyntropin dose (Table 3.1). The overall effect of time following administration of 1 µg cosyntropin was due to a significant decrease in cortisol concentration at 90 and 120 minutes as compared to the 30 minute cortisol concentration (P = 0.006 and P = 0.01, respectively). Order of cosyntropin dose administration had no effect on response to the 1 µg cosyntropin dose; there was no difference in 30 minute cortisol concentrations between the 3 foals receiving the 1 µg dose at the first sampling time (morning of day 3) versus the 5 foals receiving the 1 µg dose at either the second or third sampling times (evening of day 3, morning of day 4) (P = 0.95).

There was an overall significant effect of time on cortisol concentration after administration of 10 µg, 100 µg, and 250 µg cosyntropin ($P < 0.0001$). Compared to baseline values, serum cortisol concentrations increased significantly ($P < 0.0001$) 30 minutes after administration of the 10 µg, 100 µg, and 250 µg doses of cosyntropin (Table 3.1). There were no significant differences detected in the cortisol concentrations at 30 minutes between the 10 µg and 100 µg doses ($P = 0.17$), the 10 µg and 250 µg doses ($P = 0.57$), or between the 100 µg and 250 µg doses ($P = 0.99$).

For the 10 µg cosyntropin dose, the peak serum cortisol concentration occurred at 30 minutes with a return to baseline value by 90 minutes after administration of cosyntropin (Table 3.1). The two highest cosyntropin doses resulted in a longer duration of cortisol rise, with cortisol concentrations significantly different from baseline values at 30, 60, 90, 120 and 150 minutes after cosyntropin administration ($P < 0.0001$ for all values, except for 100 µg dose 120 minute time point $P = 0.004$; Table 3.1).

There was also an overall significant effect of cosyntropin dose on delta serum cortisol concentration for the 10 µg, 100 µg and 250 µg doses ($P < 0.0001$; Table 3.2). Thirty minutes after administration of cosyntropin, there were no significant differences detected in delta serum cortisol concentrations between the 10 µg and 100 µg doses ($P = 0.15$), the 10 µg and 250 µg doses ($P = 0.15$), or between the 100 µg and 250 µg doses ($P = 0.87$). At 90 minutes, the delta serum cortisol concentrations for the 100 µg dose ($P < 0.0001$) and the 250 µg dose ($P < 0.0001$) were significantly greater than delta serum cortisol concentrations for the 10 µg dose, but there was no difference detected in delta serum cortisol concentration between the 100 µg and 250 µg doses ($P = 0.61$).

Compared to the 10 µg cosyntropin dose, the time of peak serum cortisol concentration was significantly different for the 100 µg ($P = 0.001$) and 250 µg cosyntropin ($P = 0.0005$) doses, and occurred at approximately 90 minutes (Table 3.3). In addition, peak serum cortisol concentrations for the 100 µg ($P < 0.0001$) and 250 µg cosyntropin ($P = 0.0004$) doses were significantly greater than the peak serum cortisol concentration for the 10 µg dose. The time of peak serum cortisol concentration ($P = 0.73$) and peak serum cortisol concentration ($P = 0.81$) were not significantly different between the 100 µg dose and the 250 µg cosyntropin doses. Likewise, when compared to the 10 µg cosyntropin dose, the peak delta serum cortisol concentrations were significantly greater for the 100 µg ($P = 0.0001$) and 250 µg ($P = 0.0006$) cosyntropin doses, and there was no significant difference in peak delta serum cortisol concentration between the 100 µg and 250 µg doses ($P = 0.47$; Table 3.3).

There was an overall effect of cosyntropin dose on the area under the curve ($P < 0.0001$; Figure 3.1). The area under the curve was significantly different between 1 µg and 10 µg cosyntropin doses ($P = 0.022$), and between all other cosyntropin dose comparisons for 1 µg and 10 µg with 100 µg and 250 µg ($P < 0.0001$). There was no significant difference detected in the area under the curves between the 100 µg and 250 µg cosyntropin doses ($P = 0.89$).

There was no effect of test time (i.e. day 3 morning vs. day 3 evening vs. day 4 morning) on baseline cortisol concentration ($P = 0.80$). The baseline cortisol concentration was lower at the day 3 evening test time (1.9 ± 0.9 µg/dL, range 0.7 – 3.8 µg/dL) than the day 3 morning (2.8 ± 0.9 µg/dL, range 1.6 – 4.6 µg/dL) or the day 4 morning (2.7 ± 0.8 µg/dL, range 1.4 – 4.3 µg/dL) test times, though this difference was not significant ($P = 0.06$). Furthermore, there was no significant effect of test time on the time to peak serum cortisol concentration ($P = 0.77$), peak serum cortisol concentration ($P = 0.81$), or peak delta serum cortisol concentration ($P = 0.16$).

Finally, the order of cosyntropin dosing did not have any effect on time to peak serum cortisol concentration ($P = 0.47$), peak serum cortisol concentration ($P = 0.57$), or peak delta serum cortisol concentration ($P = 0.10$).

The dosage of cosyntropin administered to each foal on the basis of weight was $0.192 \pm 0.025 \mu\text{g/kg}$ (range $0.152 - 0.231 \mu\text{g/kg}$) for the $10 \mu\text{g}$ dose and $1.92 \pm 0.25 \mu\text{g/kg}$ (range $1.52 - 2.31 \mu\text{g/kg}$) for the $100 \mu\text{g}$ dose. There was no effect of foal weight for either the $10 \mu\text{g}$ or the $100 \mu\text{g}$ cosyntropin dose on peak serum cortisol concentration ($P = 0.10$ and $P = 0.06$, respectively) or on delta peak serum cortisol concentration ($P = 0.47$ and $P = 0.23$, respectively).

DISCUSSION

The results in this study show that in 3- to 4 day-old foals, the cortisol response to intravenous administration of cosyntropin is dose-dependent, with higher cosyntropin doses resulting in a higher peak and longer duration of rise in serum cortisol. These findings are similar to the reported cortisol response to cosyntropin in people and dogs.^{1,16,18,21,26} The adrenal gland response to cosyntropin is perhaps best evidenced by the significant difference between the area under the curve data for all the doses evaluated, with the exception of the lack of significant difference between the two highest doses ($100 \mu\text{g}$ and $250 \mu\text{g}$; Figure 3.1). Since cortisol concentrations were not measured beyond 120 minutes for the $100 \mu\text{g}$ cosyntropin dose, it is possible that a significant difference in duration of rise in cortisol between the $100 \mu\text{g}$ and $250 \mu\text{g}$ doses was missed. However, in the clinical setting, ACTH stimulation protocols typically aim to evaluate the magnitude rather than the duration of the adrenal gland response. The

results of this study are in agreement with the findings in healthy people and dogs^{16,18,19,21} that despite the duration of the cortisol response, the peak cortisol response to cosyntropin occurs between 30 to 90 minutes and is dose dependent.

The repeated venipuncture required by this study design may have produced a confounding source of environmental stress to the foals in this study, which could have influenced their basal cortisol levels or adrenal response to stimulation with cosyntropin. However, if acclimation to or anticipation of restraint and venipuncture had a significant effect on adrenal function in these foals, one would have expected to see a significant effect of collection time on baseline cortisol concentration over the course of the study period. The lack of a significant effect of test time on baseline or peak cortisol concentrations from day 3 to day 4 implies that there was minimal influence of repeated venipuncture. Furthermore, considering the cosyntropin dose dependency on the cortisol response reported herein, as well as the lack of significant response to the 1 µg cosyntropin dose at any test time, it seems unlikely that any venipuncture-associated stress significantly altered our results. All the foals in the study underwent a similar venipuncture protocol, consistent with the restraint for venipuncture and/or intravenous catheter placement that is inherently necessary for use of ACTH stimulation protocols in the clinical setting; thus, any subtle effect of the blood collection process on adrenal response is likely unavoidable. In addition, with the identification of time to peak cortisol concentration after ACTH administration determined here, future ACTH stimulation test protocols can be streamlined to target only the baseline and peak ACTH-induced cortisol concentrations, to minimize sampling and venipuncture-associated stress.

A longer washout period between sampling periods would have been ideal to eliminate any potential priming or suppression of adrenal responsiveness from administration of a previous

dose of ACTH. A longer washout was not possible here due to the innate fluctuations in adrenal function and responsiveness in the neonatal foal in the first week of life, as dose evaluation needed to be performed in the same foal during a short period when adrenal function is stable.^{22,27} Statistically similar basal cortisol concentrations through the course of the study and the lack of effect of order of dosing (low before high or vice versa) on measures of adrenal response suggests that there was minimal effect of prior ACTH administration with the 12 hour washout period used here. Addition of a negative control group consisting of foals that were subject to repeated venipuncture but received no cosyntropin would have allowed for determination of any priming or suppressive effects, as well as controlling for venipuncture-associated stress as above, but was not possible here due to study constraints.

While the overall response to varying doses of cosyntropin appears to be consistent across species, an important difference elucidated herein is the lack of a detectable adrenal gland response in foals to administration of the lowest dose of cosyntropin (1 µg). The 1 µg dose of cosyntropin has been shown to produce a measurable cortisol response in both healthy people and dogs, resulting in a peak cortisol concentration that is indistinguishable to that produced by administration of the standard high (250 µg) dose of cosyntropin.^{16,18,21} It is especially noteworthy that despite administration of a relatively higher dose of cosyntropin on a µg per kg body weight basis in the foals in this study, as compared to similar studies in infants and people,^{17,18,21} there was not a significant increase in cortisol concentration following administration of the 1 µg cosyntropin dose in foals. It is possible that the 1µg dose did produce a transient rise in cortisol concentration between 0 and 30 minutes that was not detected in the sampling protocol used here; more frequent sampling would have been required to determine

this. Thus, although the general adrenal gland response to cosyntropin appears similar, dose regimens and expected cortisol responses cannot be directly extrapolated across species or ages.

This suggests that the emerging “low dose” (1 µg cosyntropin) ACTH stimulation test, used in people and small animals, is not sufficient to evaluate the adrenal response in neonatal foals. The lack of cortisol response to this low dose of cosyntropin in foals may be attributed to immature adrenal function specific to neonatal foals. Mean basal serum cortisol concentration in neonatal foals in our study was 2.5 ± 0.9 µg/dL, comparable with previous work in foals^{22,a} and notably lower than those reported for healthy 4 to 7 day-old human infants (14.8 ± 1.9 µg/dL).^{28,29} Similarly, cortisol concentrations 30 and 60 minutes after administration of 250 µg ACTH found here in foals (5.5 ± 1.1 µg/dL and 6.9 ± 1.1 µg/dL respectively) were much lower than concentrations achieved in healthy human neonates 30 and 60 minutes after administration of 1 µg/1.73 m² and 250 µg/1.73 m² (38.1 ± 5 µg/dL and 84 ± 6.9 µg/dL).²⁸ In fact, ACTH-induced peak cortisol concentrations achieved here in foals were still lower than *basal* cortisol concentrations in human infants.^{28,29} This suggests that neonatal foals may exhibit decreased adrenal responsiveness as compared to human neonates; all of the healthy foals in this study would be classified with adrenal insufficiency if current human criteria were used.^{2,29,30}

Maturation of the HPA axis and adrenal function have been shown to occur in the immediate periparturient period in foals, beginning several days prior to parturition and continuing through the first week of life,^{22,31-33} much later than it occurs in other species such as lambs and pups.^{34,35} The neonatal foal’s adrenal gland response to administration of ACTH has been shown to be relatively stable on days 3 and 4 of life,²² and is in agreement with the results herein. In addition, our results are consistent with previous work showing a lack of diurnal variation in cortisol secretion in foals in the perinatal period.²⁷ However, a diurnal rhythm in

cortisol secretion, with peak cortisol concentrations in the early morning hours, has been established in adult horses³⁶ and in foals as young as 2 months of age.²⁷ In the study herein, evening basal cortisol concentrations in 3- to 4-day old foals were lower than morning basal cortisol concentrations, though the difference was not statistically significant. It is possible that adrenal cortical sensitivity to ACTH and diurnal cortisol secretion develops as the foal ages, resulting in eventual development of adrenal response to administration of a “low dose” (1 µg) of cosyntropin as the foal ages. Further evaluation of adrenal response to this dose of cosyntropin in older foals and adult horses is necessary to determine if this age-dependent response indeed exists.

Despite the lack of a significant increase in serum cortisol concentration 30 minutes following administration of 1 µg cosyntropin, foals did show a significant cortisol response to administration of 10 µg cosyntropin. At 30 minutes after administration, the cortisol response to 10 µg cosyntropin was indistinguishable from administration to the two higher doses. This suggests that if post-cosyntropin sampling is performed at 30 minutes after administration, the 10 µg dose ACTH stimulation test might be a useful and valid “low dose” ACTH stimulation protocol for assessing adrenal function in the neonatal foal, producing results comparable to the standard 250 µg ACTH stimulation test. Furthermore, the lack of significant differences in the cortisol response between the 100 µg and 250 µg cosyntropin doses suggests that there is no clear advantage to using the 250 µg dose over the 100 µg dose for a “high-dose” ACTH stimulation test in foals.

Little is known about the incidence of adrenal dysfunction in neonatal foals, and primary adrenal insufficiency is rare in both adult horses and foals.⁴ However, there is a growing body of evidence describing the importance of relative adrenal insufficiency (RAI) in critically ill human

patients, both adults and neonates.^{1,8-10} RAI is best described as an inappropriate adrenal response to the severity of disease, and has a reported incidence of as much as 40 to 50% in human patients with septic shock.^{2,8-10} This adrenal insufficiency results in inadequate levels of serum cortisol to meet the physiologic demands brought on by severe illness such as sepsis/septic shock, and has been correlated with prolonged hypotension, systemic inflammatory response syndrome, and decreased survival in people.^{8,9}

The low-dose ACTH stimulation (1 µg) test is currently believed to be the most sensitive test for the detection of RAI in critically ill humans, since recent evidence shows that critically ill people with RAI often have a cortisol response to high (100 to 250 µg) dose ACTH stimulation tests that is indistinguishable from healthy individuals, but fail to appropriately respond to administration of lower doses (1 to 5 µg) of cosyntropin.^{10,17,18,21} A recent study evaluating low- and high-dose ACTH stimulation tests in healthy and septic human neonates found similar results, with a subset of the septic infants showing appropriate cortisol response to the standard 250 µg ACTH stimulation test but decreased cortisol response to the 1 µg dose as compared to healthy infants.²⁸ The septic infants with decreased responses to the 1 µg dose were more likely to be leukopenic and have meningitis, and had decreased survival rates, as compared to infants with appropriate cortisol responses to 1 µg cosyntropin.²⁸

Given the high morbidity and mortality associated with sepsis and septic shock in neonatal foals¹¹⁻¹⁵ and the growing evidence of RAI in septic human patients, development of methods to evaluate the incidence of adrenal gland dysfunction and the potential prevalence of RAI in foals is needed. The results herein suggest that administration of both low (10 µg) and high (100 µg) doses of cosyntropin result in measurable and indistinguishable cortisol responses in healthy neonatal foals 30 minutes after the administration of ACTH. Evaluation of the

cortisol responses to low and high dose ACTH stimulation protocols in healthy foals of various ages, critically ill foals, and healthy and sick adult horses is ultimately needed to further elucidate the significance of RAI in horses and the clinical usefulness of ACTH stimulation tests in equine medicine.

FOOTNOTES

^a Gold J, Divers T, Barton M, et al. ACTH, cortisol and vasopressin levels of septic (survivors and nonsurvivors) in comparison to normal foals. *J Vet Intern Med* 2006; 20:720.

^b Cortrosyn™, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA

^c Immulite™ cortisol assay package insert, Diagnostics Product Corporation, Los Angeles, CA

^d Immulite™, Diagnostics Product Corporation, Los Angeles, CA

^e SAS Statistical Software™ (Version 9.1), SAS Institute Inc., Cary, NC

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Table 3.1. Serum cortisol concentrations ($\mu\text{g/dL}$) before (time 0, baseline) and 30, 60, 90, 120, and 150 minutes after intravenous administration of 1, 10, 100 and 250 μg cosyntropin in healthy 3- to 4-day-old foals.

Cosyntropin dose (μg)	Time (min)					
	Baseline	30	60	90	120	150
1 (n = 8 foals)	2.5 ± 0.6^a (1.7 – 3.3)	2.8 ± 0.8^a (1.9 – 4.6)	2.1 ± 0.6^a (1.3 – 3.2)	1.8 ± 0.7^a (0.7 – 2.7)	1.8 ± 0.7^a (0.9 – 2.8)	Not done
10 (n = 10 foals)	2.6 ± 1.0^a (1.1 – 4.3)	$4.9 \pm 0.9^{*b}$ (3.8 – 6.4)	$3.8 \pm 1.1^{*b}$ (2.6 – 6.4)	2.3 ± 0.8^a (1.0 – 3.8)	2.0 ± 0.6^a (1.4 – 2.8)	Not done
100 (n = 10 foals)	2.6 ± 0.8^a (1.4 – 3.7)	$5.5 \pm 1.2^{*b}$ (3.9 – 7.5)	$7.0 \pm 2.0^{*c}$ (5.1 – 10.8)	$7.4 \pm 2.3^{*b}$ (5.0 – 11.8)	$5.1 \pm 1.6^{*b}$ (2.9 – 8.6)	Not done
250 (n = 6 foals)	2.2 ± 1.3^a (0.7 – 4.6)	$5.5 \pm 1.1^{*b}$ (4.5 – 7.3)	$6.9 \pm 1.1^{*c}$ (5.8 – 8.1)	$8.0 \pm 1.9^{*b}$ (5.5 – 11.0)	$7.7 \pm 2.0^{*c}$ (4.8 – 10.6)	$9.1 \pm 2.6^{*\#}$ (7.3, 10.9)

*Significantly different ($P < 0.05$) from the baseline cortisol value within the same cosyntropin dose. Values with different letter superscripts denote significant differences ($P < 0.05$) between cosyntropin doses within that time point. Data are reported as the mean \pm standard deviation. Numbers in parentheses represent the data range. # Data only available for 2 foals at this time point.

Table 3.2. Delta serum cortisol concentrations in 3- to 4-day-old healthy foals after administration of 10, 100, or 250 µg of cosyntropin.

Cosyntropin dose (µg)	Time (min)				
	30	60	90	120	150
10 (n = 10 foals)	2.4 ± 0.9 ^a (0.7 – 4.4)	1.3 ± 1.6 ^a (-1.2 – 4.7)	-0.3 ± 1.5 ^a (-3.3 – 2.1)	-0.7 ± 1.3 ^a (-2.7 – 1.0)	Not done
100 (n = 10 foals)	2.9 ± 1.0 ^a (1.6 – 4.8)	4.4 ± 1.9 ^b (2.4 – 8.1)	4.8 ± 2.4 ^b (1.5 – 9.1)	2.5 ± 1.8 ^b (-0.8 – 5.9)	Not done
250 (n = 6 foals)	3.3 ± 0.9 ^a (2.2 – 4.3)	4.5 ± 1.2 ^b (3.5 – 6.3)	5.8 ± 2.0 ^b (3.2 – 9.2)	5.5 ± 2.2 ^c (2.5 – 8.8)	7.9 ± 3.1 [#] (6.6, 9.1)

Values with different letter superscripts denote significant differences (P <0.05) between cosyntropin doses within that time period. Data are reported as mean ± standard deviation. Numbers in parentheses represent the data range. [#] Data only available for 2 foals at this time point.

Table 3.3. Peak serum cortisol and peak delta serum cortisol concentrations and estimated time to peak serum cortisol concentration after intravenous administration of 10, 100, and 250 µg cosyntropin in healthy 3- to 4-day old foals.

Cosyntropin dose (µg)	Time to peak cortisol concentration (min)	Peak cortisol concentration (µg/dL)	Delta peak cortisol concentration (µg/dL)
10 (n = 10 foals)	36 ± 12.6 ^a (30 – 60)	5.0 ± 0.9 ^a (3.8 – 6.4)	2.4 ± 1.0 ^a (0.7 – 4.7)
100 (n = 10 foals)	81 ± 14.5 ^b (60 – 90)	7.6 ± 2.1 ^b (5.1 – 11.8)	5.0 ± 2.2 ^b (3.1 – 9.1)
250 (n = 6 foals)	95 ± 12.2 ^b (90-120)	8.1 ± 1.9 ^b (5.5 – 11.0)	5.6 ± 2.1 ^b (3.2 – 9.2)

Values with different letter superscripts are significantly different (P <0.05) between cosyntropin doses. Data are reported as the mean ± standard deviation. Numbers in parentheses are the data range.

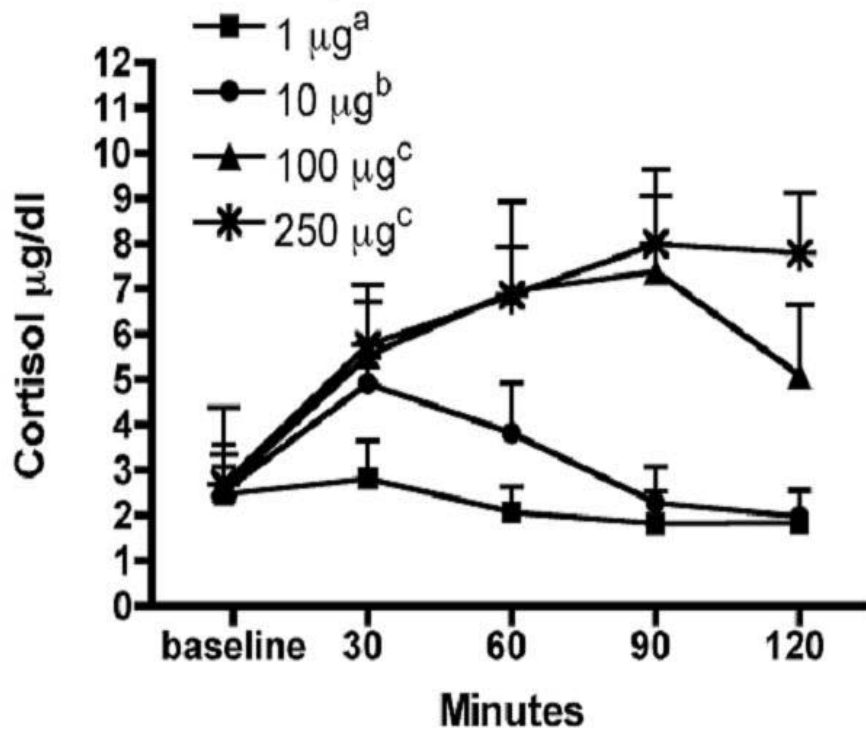


Figure 3.1. Serum cortisol concentration ($\mu\text{g/dL}$) in healthy 3 to 4 day-old foals before and 30, 60, 90, and 120 minutes after intravenous administration of 1, 10, 100, and 250 μg cosyntropin. Cosyntropin doses with different letter superscripts have significantly different area under the concentration curves. Mean \pm standard deviation.

CHAPTER 4

HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ASSESSMENT

IN HEALTHY TERM NEONATAL FOALS

UTILIZING A PAIRED LOW-DOSE / HIGH-DOSE ACTH STIMULATION TEST¹

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ABSTRACT

Background: Hypothalamic-pituitary-adrenal (HPA) axis function is dynamic in the neonatal foal. The paired low-dose/high-dose cosyntropin (ACTH) stimulation test allows comprehensive HPA axis assessment, but has not been evaluated in neonatal foals.

Hypothesis: Foal age will significantly affect cortisol responses to a paired 10 µg and 100 µg dose cosyntropin stimulation test in healthy neonatal foals.

Animals: 20 healthy neonatal foals

Methods: HPA axis function was assessed in 12 foals at birth, 12-24 hours, 36-48 hours, and 5-7 days of age. At each age, basal cortisol and ACTH concentrations were measured and cortisol responses to 10µg and 100µg cosyntropin were assessed with a paired ACTH stimulation test protocol. Eight additional 36-48 hour-old foals received saline instead of 10 µg cosyntropin in the same paired ACTH stimulation test design.

Results: At birth, foals had significantly higher basal cortisol and ACTH concentrations and higher basal ACTH:cortisol ratios compared to foals in all other age groups. A significant cortisol response to both the 10µg and 100µg doses of cosyntropin was observed in all foals. The magnitude of the cortisol response to both doses of cosyntropin was significantly different across age groups, with the most marked responses in younger foals. There was no effect of the paired ACTH stimulation test design on cortisol responses.

Conclusions and Clinical Importance: A paired 10µg and 100µg cosyntropin stimulation test can be used to evaluate HPA axis function in neonatal foals. Consideration of foal age is important in interpretation of HPA axis assessment.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis tightly regulates systemic cortisol concentrations in both health and disease, and thus plays an integral role in the maintenance of cellular, organ, and whole body homeostasis. Hypothalamic integration of input from the peripheral and central nervous systems induced by environmental and endogenous physiologic stressors culminates in modulation of the release of corticotropin-releasing hormone (CRH) into the hypothalamic-pituitary portal circulation. CRH acts on the adjacent anterior pituitary gland to induce the release of adrenocorticotrophic hormone (ACTH or adrenocorticotropin) into the systemic circulation, which ultimately stimulates the adrenal cortices to synthesize and release cortisol, the primary mammalian stress hormone. Increased systemic cortisol concentrations exert a negative feedback effect on both the pituitary gland and hypothalamus, resulting in subsequent down-regulation of both CRH and ACTH secretion, and maintaining systemic cortisol concentrations at a level appropriate for the existing degree of physiologic stress.

Because cortisol is not stored in either the adrenal cortices or peripheral tissues, any disruption in HPA axis activation or cortisol synthesis can rapidly result in systemic cortisol insufficiency. In illness, this systemic cortisol insufficiency is often referred to as Relative Adrenal Insufficiency (RAI) or Critical Illness Related Corticosteroid Insufficiency (CIRCI), and is characterized by transient serum cortisol concentrations inappropriately low for the existing degree of illness and abnormal responses on HPA axis function testing.¹⁻⁶ The clinical impact of RAI/CIRCI in humans is exemplified by recent reports indicating that critically ill patients with concurrent HPA axis dysfunction often have significantly increased disease severity and mortality as compared to critically ill patients with intact HPA axes.¹⁻³ Recent reports in both

dogs^{7,8} and foals^{a,9,10} suggest that these syndromes may be of similar importance in veterinary critical care.

Thus, a means for thorough evaluation of HPA axis function in the neonatal foal is needed for further investigation of HPA dysfunction in foals in a clinical setting.

Comprehensive HPA axis function assessment can include both measurement of basal ACTH and cortisol concentrations, to provide information regarding endogenous HPA axis activation, and determination of cortisol responses to both physiologic and supraphysiologic doses of ACTH to assess adrenocortical sensitivity to ACTH and maximal corticosteroid synthetic capacity.

Because of the dynamic changes observed in HPA axis activity in foals during the first week of life,^{11,12} serial assessment of these parameters in healthy neonatal foals during this period is necessary to determine appropriate age-matched criteria for HPA axis function testing in foals.

Previous studies on ACTH stimulation testing in fetal and neonatal foals utilized 125 to 250 µg of ACTH,¹¹⁻¹³ resulting in supraphysiologic blood concentrations of ACTH that reach almost 300 times those that can be achieved physiologically.¹⁴ Although a supraphysiologic dose of exogenous ACTH is appropriate when testing for absolute adrenal gland insufficiency, lower physiologically relevant doses of ACTH (i.e. 1 to 10 µg) may be more appropriate for investigation of relative, transient dysfunction of the adrenal gland (RAI/CIRCI).¹⁴⁻¹⁷ The timing of serial ACTH stimulation tests also is of clinical importance in the neonatal foal, because HPA axis activation and response to ACTH change rapidly in the first 6-48 hours after birth.¹¹ Thus, a paired low-dose/high-dose ACTH stimulation test design that assesses cortisol response to both physiologic and supraphysiologic doses of ACTH over a short time period (i.e. 2-3 hours) may be the most efficient method for comprehensive HPA axis evaluation in neonatal foals.

The cortisol response to a range of cosyntropin (synthetic ACTH, α 1-24 corticotropin) doses (1 to 250 μ g) recently has been described in healthy 3-4 day old foals.¹⁸ Three-to-four day old foals exhibit a significant dose-dependent increase in cortisol after administration of both low (10 μ g) and high (100 and 250 μ g) doses of cosyntropin, but do not exhibit a significant cortisol response to the 1 μ g cosyntropin dose used for low-dose ACTH stimulation testing in humans.^{14,16,19} Cortisol responses to the 10 μ g and 100 μ g cosyntropin doses have not been evaluated in younger or older foals, and a paired low-dose/high-dose ACTH stimulation test has not been evaluated in foals of any age.

The purpose of this study thus was to compare cortisol responses to a paired low-dose / high-dose ACTH stimulation test (Figure 4.1) in healthy full term foals at 4 time points during the first week of life. We tested the following 3 hypotheses: 1) administration of a low dose of cosyntropin (10 μ g) 90 minutes before a high dose (100 μ g) of cosyntropin in a paired low-dose / high-dose ACTH stimulation test protocol will have no effect on the cortisol response to the high dose of cosyntropin; 2) a low dose (10 μ g) of cosyntropin will be equally effective as a high dose (100 μ g) of cosyntropin in eliciting a cortisol response in healthy neonatal foals within this paired ACTH stimulation test design; and 3) foal age will significantly impact the cortisol response to both the low and the high doses of cosyntropin.

MATERIALS AND METHODS

Animals

The study population (“paired ACTH stim group”) consisted of 12 neonatal Quarter Horse foals (7 males, 5 females). These foals were evaluated at 4 time points during the first week of life: within 1 hour of birth (n=11), between 12-24 hours of age (n=10), between 36-48 hours of age (n=10), and between 5-7 days of age (n=11). Mean foal body weight was 49.9 ± 8.6 kg (range, 38.7 kg to 61.4 kg). A separate group of 8 foals (5 Quarter Horses, 2 Tennessee Walking Horse crosses, and 1 pony) served as a control group to determine if any effects specific to the paired ACTH stimulation test protocol used in the study population were present. The control group consisted of 4 males and 4 females, and mean foal body weight was 46.0 ± 14.2 kg (range 20.5 kg to 65.9 kg). Foals in this group were only available for evaluation at 1 time point, at 36-48 hours of age.

Only full term foals (>330 days gestation) born without assistance were included in the study. Foals were determined to be healthy before inclusion in the analysis and during the study based on a lack of abnormalities identified on serial physical examinations. Adequate passive transfer of immunoglobulin was confirmed by a serum immunoglobulin concentration ≥ 800 mg/dL at 12-24 hours of age in all foals.^b Hematologic analysis and serum biochemical profiles were not routinely performed in foals in either group. Each mare and foal pair in both groups was stabled with daily paddock turnout during the study period, and animals were cared for according to the principles and guidelines in an Animal Use Protocol approved by the University of Georgia’s Department of Animal Resources.

Study Design

In the paired ACTH stim group, a repeated measures design was used to assess HPA axis function at 4 time points during the first week of life: within 1 hour of birth, between 12-24 hours of age, between 36-48 hours of age, and between 5-7 days of age. Time of birth was determined by use of a Foalert™^c system placed on each mare before her anticipated foaling date, and defined as the time at which the Foalert™ alarm and automatic dialer was activated. At each age, blood was collected for measurement of basal serum total cortisol and endogenous plasma ACTH concentrations, and a paired low (10 µg) and high (100 µg) dose cosyntropin^c (synthetic ACTH, α 1-24 corticotropin) stimulation test was performed (Figure 4.1).

Foalings were not attended and Foalert™ systems were not utilized for the control group. The dams of these foals were observed every 2-8 hours from the time at which foaling appeared imminent until parturition occurred, and foaling time was estimated based on these observations to determine foal age. HPA axis assessment was performed once in this group of foals, at 36-48 hours of age. At this time, blood was collected for measurement of baseline serum total cortisol and endogenous plasma ACTH concentrations, and a “sham” paired low-dose / high-dose cosyntropin stimulation test was performed. After collection of basal blood samples, these foals received an equivalent volume of sterile saline (0.9% sodium chloride) instead of the 10 µg dose of cosyntropin, and then received the 100 µg dose of cosyntropin 90 minutes later.

In the paired ACTH stim group, the dosage of cosyntropin administered to each foal on the basis of weight was 0.21 ± 0.03 µg/kg (range, 0.16 to 0.26 µg/kg) for the 10 µg dose and 2.1 ± 0.03 µg/kg (range, 1.6 to 2.6 µg/kg) for the 100 µg dose. In the control group, the dosage of cosyntropin based on foal weight for the 100 µg dose was 2.5 ± 1.1 µg/kg (range 1.5 to 4.9 µg/kg).

Preparation of Cosyntropin

Cosyntropin^d was supplied as a lyophilized powder in glass vials. Each vial contained 250 µg of cosyntropin, which was reconstituted with 1 mL sterile saline according to manufacturer directions. The resulting solution then was diluted 10-fold with sterile saline to produce a 25 µg/mL solution of cosyntropin. The 10 and 100 µg doses consisted of 0.4 mL and 4 mL of this solution, respectively. The 10 µg doses were additionally diluted with sterile saline to an equivalent total volume of 4 mL. Reconstituted and diluted cosyntropin solutions were stored frozen at -80 C in sterile glass vials until immediately before use (not longer than 4 months). Reconstituted cosyntropin solutions are stable when frozen in glass vials at -80 C for up to 6 months.²⁰

Sample Collection

All blood samples were collected by jugular venipuncture under brief restraint by experienced foal handlers. Foals were restrained in sternal recumbency for collection of the basal (time 0) samples at the first sampling age (within 1 hour of birth) if the foal had not yet stood on its own at this time. All other samples were collected from standing foals. Before administration of 10 µg of cosyntropin, blood was collected for measurement of basal serum total cortisol and plasma endogenous ACTH concentrations. Blood was placed into plastic tubes containing 3.6 mg potassium EDTA for measurement of plasma endogenous ACTH concentration. Blood for cortisol concentration measurement was placed into glass tubes without additives and allowed to clot at room temperature for 30-60 minutes.

After collection of basal samples and before withdrawal of the venipuncture needle, 10 µg cosyntropin (paired ACTH stim group) or the equivalent volume of sterile saline (control

group) was administered IV as a rapid bolus. Blood then was collected 30 minutes later for assessment of the peak cortisol response to the 10 µg cosyntropin dose. Ninety minutes after administration of 10 µg cosyntropin, blood again was collected for measurement of cortisol concentration, and before removal of the venipuncture needle, 100 µg of cosyntropin was administered IV as a rapid bolus (both groups). Blood was collected 30 and 90 minutes later for assessment of the cortisol response to the 100 µg cosyntropin dose.

Blood samples were stored at 4 degrees Celsius until centrifugation and separation of the serum or plasma within 3 hours of collection. Serum and plasma samples were stored frozen at -80 degrees Celsius until assays were performed (within 30 days). Plasma ACTH and serum total concentrations are stable frozen at -80 degrees Celsius for 30 and 90 days respectively.^e

ACTH and Cortisol Assay

Plasma endogenous ACTH concentrations (henceforth referred to as ACTH concentrations) and serum total cortisol concentrations (henceforth referred to as cortisol concentrations) were determined on an automated analyzer using chemiluminescent enzyme immunoassays^f validated for use in horses.²¹⁻²³ For the ACTH assay, the interassay and intra-assay coefficients of variation were 7-9% and 9% respectively and the limit of detection was 9 pg/ml.²¹ The interassay and intra-assay coefficients of variation were $\leq 20\%$ ²³ and the limit of detection was 0.05 µg/dL for the cortisol assay.²²

Statistical Analysis

For the paired ACTH stim group, basal ACTH and cortisol concentrations, basal ACTH:cortisol ratio, and cortisol responses to both the 10 µg and the 100 µg dose of cosyntropin

were determined for each individual foal at each of the 4 ages. Low-dose peak cortisol concentration (LD_{peak}) was defined as the cortisol concentration achieved 30 minutes after administration of the 10 µg cosyntropin dose. High-dose peak cortisol concentration (HD_{peak}) was similarly defined as the highest cortisol concentration reached after administration of the 100 µg cosyntropin dose (either 30 or 90 minutes after cosyntropin administration, whichever was higher).

The relative increase in serum cortisol concentrations from basal results also was determined for each cosyntropin dose in each individual foal at each of the 4 ages. The low-dose delta serum cortisol concentration (LD_{delta}) was defined as the peak cortisol concentration reached after administration of 10 µg cosyntropin minus the basal (time 0) cortisol concentration. The high-dose delta cortisol concentrations (HD_{delta}) was defined similarly as the peak cortisol concentration reached after administration of 100 µg cosyntropin minus the basal cortisol concentration. The low-dose fold change in cortisol concentration (LD fold change) and high-dose fold change in cortisol concentration (HD fold change) were calculated by dividing the LD_{peak} and HD_{peak} cortisol concentrations, respectively, by the basal cortisol concentration.

For the control group, basal ACTH and cortisol concentrations and ACTH:cortisol ratios, HD_{peak} cortisol concentrations, HD_{delta} cortisol concentrations, and HD fold change were similarly defined and determined for each individual foal at the 1 sampling age. LD_{peak} and LD_{delta} cortisol concentrations and LD fold change were not determined for this group because these foals did not receive the low dose of cosyntropin.

Basal ACTH and cortisol concentrations, basal ACTH:cortisol ratios, LD_{peak} and HD_{peak} cortisol concentrations, LD_{delta} and HD_{delta} cortisol concentrations, and LD fold change and HD fold change in cortisol concentrations were compared among all foal age groups

using the mixed procedure for repeated measures.^g Multiple comparisons were conducted with Tukey's test. Spearman's rank correlation test^h was used to evaluate for an effect of foal weight and foal sex on cortisol response to cosyntropin (LDdelta and HDdelta cortisol concentrations and LD and HD fold change in cortisol concentrations) for each of the 4 foal age groups. Data also were compared between the control group and the 36-48 hour old foals in the paired ACTH stim group using analysis of variance for repeated measures and Student's t test.^{g,h} Statistical significance was set at $P < 0.05$ for all analyses. Data are expressed as mean \pm standard deviation.

RESULTS

Effect of Foal Age on Basal ACTH and Cortisol Concentrations

An overall effect of foal age on basal ACTH and cortisol concentrations and ACTH:cortisol ratios was present (Table 4.1; $P=0.005$, $P < 0.001$, and $P=0.006$ respectively). At birth, foals had significantly higher basal ACTH ($P=0.002$ to 0.003) and cortisol ($P < 0.001$) concentrations than at any other time point, but no significant differences in either basal ACTH or basal cortisol concentrations were found between any other age group comparisons. An overall effect of age also was present for comparisons of basal ACTH:cortisol ratios ($P=0.006$), but significantly higher ACTH:cortisol ratios only were present in foals at birth as compared to 12-24 hours of age ($P=0.004$).

Cortisol Responses to Cosyntropin in the Paired ACTH Stim and Control Groups

There were no adverse effects noted after administration of either dose of cosyntropin in any foal in either group. Basal ACTH and cortisol concentrations and cosyntropin dosages based on weight were not significantly different between the control group and age-matched foals (36-48 hours old) in the paired ACTH stim group (Figure 4.2; $P=0.995$, $P=0.742$, and $P=0.218$ respectively). In the control group, cortisol concentrations at 30 and 90 minutes after administration of the “sham” low dose were not significantly different from basal cortisol concentration ($P=0.997$ to 0.999). Thirty-minute cortisol concentrations however were significantly higher in the 36-48 hour old paired ACTH stim group than 30 minute cortisol concentrations in the control group ($P<0.001$). There were no significant differences in the response to the high dose of cosyntropin between the 2 groups (HDpeak: $P=0.705$; HDdelta: $P=0.549$; HDfold change: $P=0.900$).

Effect of Cosyntropin Dose on Cortisol Responses Within Age Groups

Data from the paired ACTH stim test group are shown in Table 4.1 and Figure 4.3. Foals in all age groups showed a significant cortisol response to both the 10 μg and 100 μg doses of cosyntropin ($P<0.001$). Cortisol concentrations were significantly increased from basal (time 0) concentrations at 30 minutes after administration of 10 μg cosyntropin at all ages ($P<0.001$ to 0.002). Ninety minutes after administration of 10 μg cosyntropin, cortisol concentrations returned to concentrations statistically indistinguishable from basal concentrations in all foal age groups ($P=0.46$ to 1.0). A significant increase in cortisol concentration from basal values also was observed in all age groups at both 30 and 90 minutes after administration of 100 μg cosyntropin ($P<0.001$). In addition, in all 4 age groups, the HDpeak cortisol concentration was

significantly higher than the LDpeak cortisol concentration ($P < 0.001$ to 0.008). A similar pattern was observed for the delta cortisols, with all age groups showing significantly higher HDdelta than LDdelta cortisol concentrations ($P < 0.001$ to 0.006). There was no effect of foal weight on delta cortisol concentration or fold change in cortisol concentration after administration of either dose of cosyntropin ($P = 0.287$ to 0.924).

Effect of Foal Age on Cortisol Responses to Cosyntropin

An overall effect of foal age also was present for cortisol response to cosyntropin (Table 4.1). LDpeak and HD peak cortisol concentrations were significantly higher in foals at birth than other age groups ($P < 0.001$ to 0.013), and decreased significantly over the first week of life. Similarly, area under the cortisol concentration curve was greatest in foals at birth, and decreased significantly with increasing age (Figure 4.3, $P < 0.001$ for all age comparisons). In addition, the HDpeak cortisol concentration was reached more rapidly in foals at birth, with 6/11 foals reaching the HDpeak at 30 minutes after administration of $100 \mu\text{g}$ cosyntropin. All foals in the 3 older age groups did not attain their HDpeak cortisol concentration until the 90 minute sample, with the exception of 2 foals at 12-24 hours of age and 1 foal at 36-48 hours of age.

LDdelta cortisol was significantly higher at 12-24 hours than at any other age ($P < 0.001$ to 0.038), and significantly different among all foal age groups except between foals at birth and 36-48 hours of age ($P = 0.999$). The HDdelta cortisol showed a similar pattern, but the difference among age groups only reached statistical significance for comparisons between 12-24 hours vs. 5-7 days ($P < 0.001$) and 36-48 hours vs. 5-7 days ($P = 0.038$). LD fold change and HD fold change in cortisol concentration were significantly lower in foals at birth than at 12-24 hours and

36-48 hours ($P < 0.001$ to 0.005). No significant differences in LD fold change or HD fold change between other foal age groups were found.

Effect of Foal Sex on Cortisol Responses to Cosyntropin

There was no effect of foal sex on LDdelta or HDdelta cortisol concentration or LD fold change or HD fold change in cortisol concentration in any age group ($P = 0.370$ to 0.861), except for foals in the oldest age group (5-7 days). In this group, males had a significantly higher LDdelta cortisol ($P = 0.025$), LD fold change ($P = 0.001$), and HD fold change ($P = 0.017$) than did females. HD delta cortisol also was higher in males in this age group, but the difference did not reach statistical significance ($P = 0.063$).

DISCUSSION

The results of this study indicate that a paired low-dose ($10 \mu\text{g}$) / high-dose ($100 \mu\text{g}$) cosyntropin stimulation test may be utilized for evaluation of HPA axis function in the neonatal foal. The lack of significant difference in cortisol response to the $100 \mu\text{g}$ cosyntropin dose between foals in the control group, who received sterile saline instead of the initial $10 \mu\text{g}$ cosyntropin dose, and age-matched foals in the paired ACTH stim group, who received $10 \mu\text{g}$ cosyntropin 90 minutes before the $100 \mu\text{g}$ dose, suggests that this paired ACTH stimulation test protocol does not produce a significant priming or suppressive effect on the cortisol response to the $100 \mu\text{g}$ dose and provides support for our first hypothesis. In addition, the lack of significant increase in cortisol concentrations from baseline concentrations in the foals in the low-dose

saline group during the first 3 sampling points (0, 30, and 90 minutes) suggests that even with brief periodic restraint and direct venipuncture, the test procedure itself did not induce endogenous activation of the foal's HPA axis.

The results of the present study are consistent with previous reports illustrating the dynamic nature of HPA axis function in the neonatal foal.^{11-13,24-26} At birth, basal ACTH and cortisol concentrations were significantly higher than resting concentrations previously reported both in older foals and in adult horses,²⁷⁻²⁹ and were similar to cortisol concentrations recently reported in adult horses with colic ($13.6 \pm 7.6 \mu\text{g/dl}$).ⁱ This provides further evidence that the healthy full term foal is capable of mounting a cortisol response to periparturient stressors.^{12,30} However, by 12 hours of age, basal ACTH and cortisol concentrations decreased significantly in foals in this study as compared to concentrations at birth, reaching concentrations well below reported means in resting adult horses ($5.4 - 6.4 \mu\text{g/dL}$).^{28,29} Resting HPA axis function was consistent among 12-24 hours, 36-48 hours, and 5-7 days of age in the foals in this study, and remained at a level below that described in adult horses. This finding is consistent with previous reports in older foals, aged 3 days to 13 weeks.^{18,27} Thus, whereas the increased cortisol and ACTH concentrations in foals at birth indicate that the neonatal foal's HPA axis is capable of mounting a response to clinically relevant physiologic stress (e.g., the stress of parturition), endogenous HPA axis tone appears to be maintained at a lower level in foals during the first week of life than in adult horses.

Despite the variations in basal HPA axis tone between foals at birth and the older age groups in this study, foals at all ages produced a significant cortisol response to both the $10 \mu\text{g}$ and $100 \mu\text{g}$ doses of cosyntropin, as evidenced by significant increases in cortisol concentrations from basal concentrations after administration of both doses. These findings support previous

reports indicating that the healthy full-term foal is capable of producing a cortisol response to a supraphysiologic amount (1-2 $\mu\text{g}/\text{kg}$, equivalent to the 100 μg dose used in this study) of exogenous ACTH shortly before, at and after the time of parturition.^{11-13,18} In addition, despite high basal HPA axis activation at birth and the subsequent decreased HPA axis tone during the first week of life, healthy full-term foals also are capable of producing a cortisol response to a much lower, more “physiologically relevant,” 10 μg (0.2 $\mu\text{g}/\text{kg}$) dose of exogenous ACTH during this period. Thus, these findings provide support for our second hypothesis, and suggest that this paired ACTH stimulation test protocol, in conjunction with measurement of basal endogenous ACTH and cortisol concentrations, may be utilized to comprehensively evaluate HPA axis function in neonatal foals in a clinical setting.. A paired ACTH stimulation test protocol utilizing standard doses of 10 μg and 100 μg cosyntropin appears to be appropriate for most foals, as evidenced by the lack of a significant effect of foal weight on the cortisol response to either dose of cosyntropin in this study. However, because the majority of the foals evaluated were a similar size and weight, adjustment of the cosyntropin dose on a $\mu\text{g}/\text{kg}$ basis occasionally may be necessary in very small or very large foals. The apparent effect of foal sex on cortisol responses seen in the oldest foals in this study may represent the development of sex-related differences in HPA axis function as foals age. However, further investigation of this potential effect in a larger number of older foals is necessary to determine if foal sex significantly impacts responses to HPA axis function testing in a clinical setting.

The results of the present study also provide support for our third and final hypothesis. Whereas differences in endogenous HPA axis function (as evidenced by baseline cortisol and ACTH concentrations and ACTH:cortisol ratios) and response to exogenous ACTH (peak and delta cortisol concentrations) did not reach statistical significance between all foal age groups for

all results, a significant effect of foal age on the cortisol response to ACTH during the neonatal period was observed. Although the *pattern* of cortisol response to both the low and high doses of cosyntropin was similar across age groups in this study, the *magnitude* of basal HPA axis activation and peak and delta cortisol responses to exogenous ACTH was highest in the 24 hours after parturition, and steadily decreased over the first week. A trend towards lower basal cortisol concentrations and significantly lower delta cortisols was apparent as foals aged to 5-7 days. These findings, and the decrease in the magnitude of both LDpeak and HDpeak cortisol concentrations observed over the first week of age, may represent some degree of limited corticosteroid synthetic capacity in the foal. Induction of the steroidogenic enzyme 17- α -hydroxylase necessary for corticosteroid synthesis occurs just before parturition in the foal.^{31,32} If this enzymatic machinery is not fully mature during the first week of life, the newborn foal may be unable to maintain cortisol synthesis at adult levels much beyond the time of parturition.

This trend towards a decreasing response to cosyntropin over the first week of life, however, could be artifactual, due to some degree of exhaustion of corticosteroid synthetic capacity caused by the serial ACTH stimulation tests utilized in this study design. This seems unlikely, however, because serial ACTH stimulation tests were performed closer together (12-24 hours apart) in these three age groups, versus at least 72 hours apart between foals aged 36-48 hours and 5-7 days. Thus, the oldest foals had the longest time to recover corticosteroid synthetic capacity between serial stimulation tests. Alternatively, serial administration of cosyntropin in this study may have induced the development of tolerance to cosyntropin, perhaps mediated by ACTH receptor down-regulation or production of anti-cosyntropin antibodies, and contributed to the decreased cortisol response to cosyntropin seen with increasing foal age. Evaluation of ACTH stimulation tests at a single time point in individual foals of different ages would have

eliminated any effects of serial testing, but would have required a much larger group of foals and greatly increased inter-horse variability in this analysis.

These findings also provide evidence to support previous work indicating that adrenocortical sensitivity to both endogenous and exogenous ACTH may be generally decreased in some foals,^{9,10} suggesting the observed differences across the first week of life in this study may indeed represent true differences in the neonatal foal. Despite lower basal cortisol concentrations than adult horses, basal ACTH concentrations in the foals in all age groups were notably higher than endogenous ACTH concentrations previously reported in healthy adult horses during the spring (foaling season) (median plasma ACTH in adult horses in January and May = 16.1-17.1 pg/mL)³³ except for foals at 12-24 hours of age. This finding may represent decreased adrenocortical sensitivity to endogenous ACTH in neonatal foals, with higher ACTH concentrations required to produce a comparable cortisol response. Cortisol responses to both doses of exogenous ACTH in the foals in this study (Table 4.1) also were lower than responses reported in human infants (basal cortisol = 14.8 ± 1.9 $\mu\text{g/dl}$; LDpeak = 17.4 $\mu\text{g/dL}$; HDpeak = 24.5 $\mu\text{g/dL}$) receiving comparable doses of ACTH.^{17,34} In addition, except for foals at 12-24 hours of age, the delta cortisol responses to even a supraphysiologic (100 μg , 2 $\mu\text{g/kg}$) dose of cosyntropin observed in foals in this study were substantially lower than the delta cortisol values reported in both adult horses (approximately 9 $\mu\text{g/dl}$) and human infants (approximately 69 $\mu\text{g/dl}$) in response to an equivalent dose of cosyntropin.^{17,35} Thus, both basal HPA axis activation and the cortisol response to both low and high doses of exogenous ACTH appear to be blunted in neonatal foals in comparison to mature horses and human infants. However, considering the large degree of individual variation in endogenous ACTH concentrations and delta cortisol

concentrations in the foals in this study, investigation of these parameters in a larger group of foals would be needed to allow more definitive conclusions.

If some degree of HPA axis immaturity does indeed persist into the postnatal period, the neonatal foal's ability to cope with the substantial physiologic stresses induced by illness may be limited, potentially increasing the risk for development of HPA axis dysfunction (RAI/CIRCI) in critically ill foals. However, the differences in HPA axis function in neonatal foals found in this and other studies¹¹⁻¹³ alternatively may represent beneficial periparturient adaptations unique to the foal or the neonatal period. Comprehensive HPA axis assessment thus should be considered in critically ill neonatal foals to determine if clinically important associations between severe illness, HPA axis dysfunction and decreased survival occur in foals, as in other species.^{3,6,7,9,15}

In summary, these findings suggest that evaluation of basal ACTH and cortisol concentrations, basal ACTH:cortisol ratios, and cortisol responses to a paired low-dose (10 µg) / high-dose (100 µg) cosyntropin stimulation test can provide comprehensive assessment of the HPA axis in the foal. However, due to the unique species- and age-related differences in HPA axis function in the neonatal foal, appropriate interpretation of both basal hormone concentrations and ACTH stimulation testing responses requires careful consideration of foal age. These findings also provide evidence for persistent HPA axis immaturity during the first week of life in the foal, which may have the potential to substantially impact the foal's ability to cope with the stress of illness during the neonatal period.

FOOTNOTES

^a Hart KA, Slovis NM, Barton MH. 2008. Hypothalamic-pituitary-adrenal axis dysfunction in critically ill neonatal foals. Research Abstract, 26th Annual Forum of the American College of Veterinary Internal Medicine, San Antonio, TX. June, 2008.

^b SNAP® Foal IgG Test, IDEXX Laboratories, Inc., Westbrook, ME

^c Foal Alert, Inc., Atlanta, GA

^d Cortrosyn™, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA

^e Immulite™ cortisol assay package insert, Diagnostics Product Corporation, Los Angeles, CA

^f Immulite™, Diagnostics Product Corporation, Los Angeles, CA

^g SAS Statistical Software™ (Version 9.1), SAS Institute Inc., Cary, NC

^h GraphPad Prism (Version 4), GraphPad Software Inc., San Diego, CA

ⁱ Sherlock CE, Mair TS. 2008. Serum cortisol concentrations in horses with colic. Research Abstract, 9th International Equine Colic Research Symposium. Liverpool, UK. June 2008

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Table 4.1. Measures of HPA axis function as determined by a paired low (10 µg) and high (100 µg) dose cosyntropin stimulation test in healthy foals at four time points during the first week of life: at birth, at 12-24 hours, at 36-48 hours, and at 5-7 days of age.

	BIRTH (n=11)	12-24 hours (n=10)	36-48 hours (n=10)	5-7 days (n=11)
Basal Cortisol (µg/dl)	10.2 ± 2.3 ^a (7.6 – 13.5)	3.6 ± 1.6 ^b (2.1 – 6.7)	2.6 ± 1.0 ^b (1.3 – 4.4)	2.0 ± 0.8 ^b (1.0 – 2.7)
Basal ACTH (pg/ml)	285.5 ± 284.8 ^a (19.4 – 968)	19.6 ± 5.3 ^b (13.8 – 30.5)	32.7 ± 26.4 ^b (16.6 – 35.7)	33.8 ± 23.1 ^b (10.3 – 110)
ACTH:Cortisol Ratio	27.4 ± 23.8 ^a (2.6 – 63.4)	6.2 ± 2.0 ^b (2.6 – 8.7)	13.9 ± 7.4 ^{a,b} (5.8 – 23.9)	15.9 ± 5.2 ^{a,b} (9.8 – 30.6)
LDpeak Cortisol (µg/dl)	13.8 ± 3.9 ^a (8.9 – 21.7)	9.1 ± 2.1 ^b (5.2 – 12.3)	6.0 ± 1.6 ^c (3.8 – 8.1)	3.3 ± 0.8 ^d (2.3 – 4.4)
HDpeak Cortisol (µg/dl)	16.6 ± 5.1 ^a (12.1 – 29)	12.9 ± 3.8 ^a (6.8 – 17.5)	9.2 ± 2.9 ^b (5.5 – 14.2)	5.5 ± 1.1 ^c (3.5 – 6.1)
LDdelta Cortisol (µg/dl)	3.6 ± 2.0 ^a (0.4 – 8.2)	5.5 ± 2.1 ^b (2.8 – 9.4)	3.5 ± 1.7 ^a (1.6 – 6.8)	1.3 ± 0.6 ^c (0.6 – 2.6)
HDdelta Cortisol (µg/dl)	6.4 ± 4.1 ^a (0.3 – 15.5)	9.4 ± 3.5 ^b (4.6 – 15)	6.8 ± 3.4 ^{a,b} (3.2 – 12.7)	3.5 ± 1.3 ^c (1.7 – 5.9)
LD Fold Change in cortisol	1.4 ± 0.2 ^a (1.0 – 1.6)	2.9 ± 1.0 ^b (1.4 – 4.2)	2.8 ± 1.6 ^b (1.6 – 6.2)	1.8 ± 0.5 ^{a,b} (1.2 – 2.7)
HD Fold Change in cortisol	1.6 ± 0.4 ^a (1 – 2.1)	4.0 ± 1.7 ^b (1.6 – 6.2)	4.5 ± 2.8 ^b (2 – 9.5)	3.2 ± 1.5 ^{a,b} (1.5 – 5.7)

Within a row, values with different letter superscripts denote significant differences (P<0.05) between age groups. Data are reported as the mean ± standard deviation. Numbers in parentheses represent the data range.

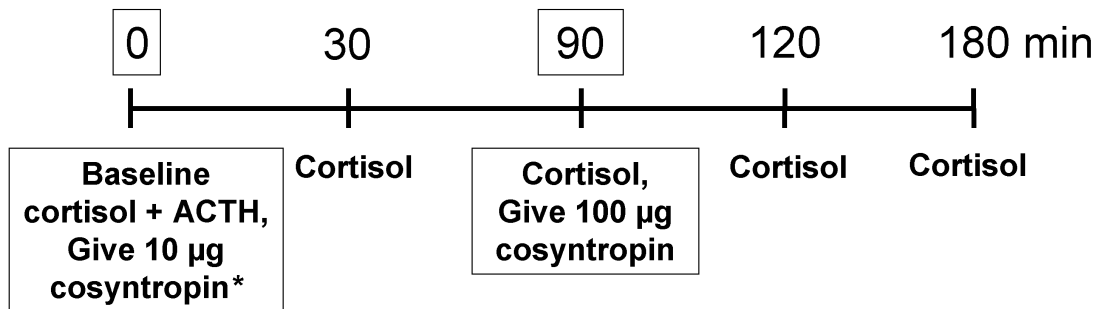


Figure 4.1. Paired low and high dose synthetic ACTH stimulation test design utilized in the study population (paired ACTH stim group). At time 0, blood was collected for measurement of baseline endogenous ACTH and cortisol concentrations, and 10 µg cosyntropin was administered intravenously. Blood was collected 30 minutes later to assess peak cortisol response to the low (10 µg) cosyntropin dose. At 90 minutes, blood was again collected for measurement of cortisol concentration (to ensure cortisol concentrations have returned to baseline levels) and 100 µg cosyntropin was administered intravenously. Blood was then collected 30 and 90 minutes later (at times 120 and 180 minutes) to assess peak cortisol response to the high (100 µg) cosyntropin dose. *Foals in the low dose saline group received an equivalent volume of sterile saline instead of 10 µg cosyntropin at this time point.

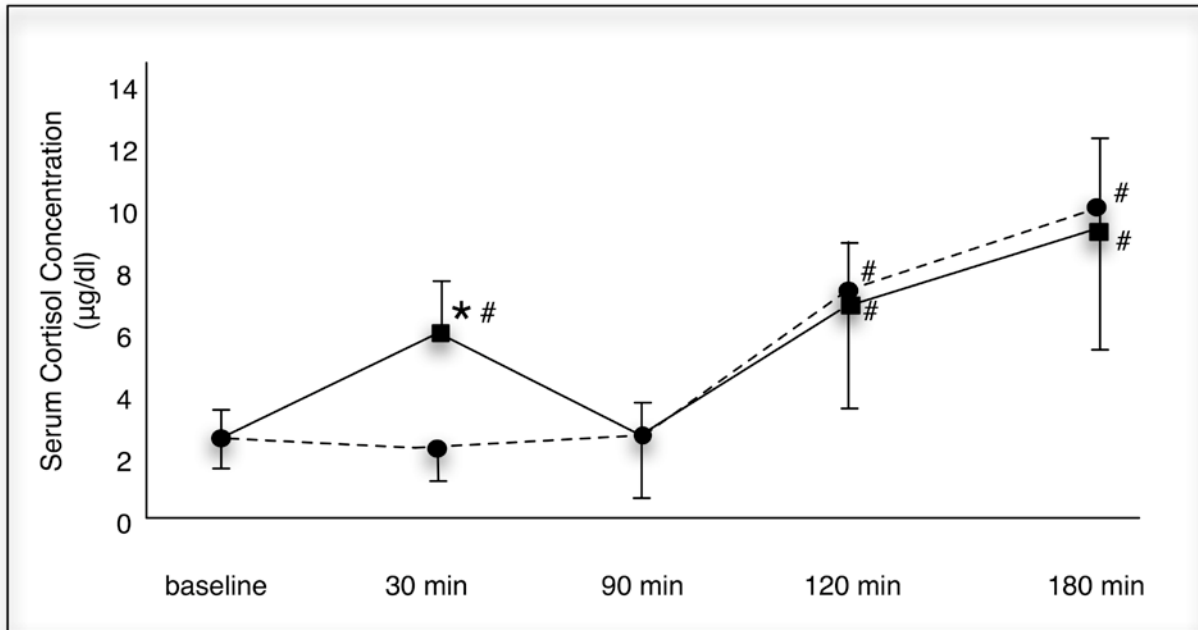


Figure 4.2. Cortisol concentrations over time during a paired low-dose / high-dose cosyntropin stimulation test in 36-48 hour-old foals in the paired ACTH stim group (squares, solid line), and during a “sham” paired cosyntropin stimulation test in foals in the low-dose saline group (circles, dotted line). At time 0, after collection of baseline blood samples, foals in the paired ACTH stim group received 10 µg cosyntropin intravenously, and foals in the low dose saline group received an equivalent volume of sterile saline intravenously. All foals received 100 µg cosyntropin intravenously at 90 minutes, after collection of 90-minute blood samples. *Significantly different between foal groups. #Significantly different from basal cortisol concentrations within each group. Mean ± standard deviation.

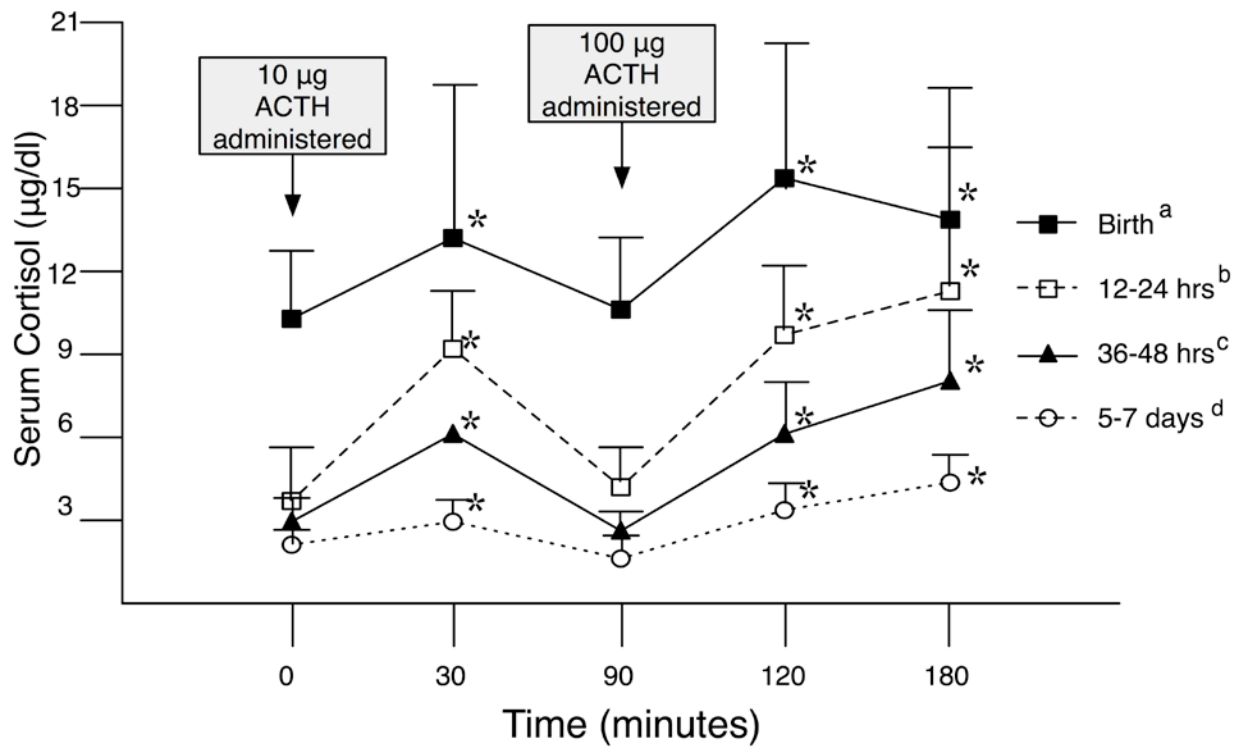


Figure 4.3. Cortisol concentrations over time during the paired low-dose / high-dose cosyntropin stimulation test in 12 healthy foals at 4 age during the first week of life: at birth, at 12-24 hours, at 36-48 hours, and at 5-7 days of age. 10 µg cosyntropin was administered intravenously at time 0 after collection of baseline blood samples, and 100 µg cosyntropin was administered intravenously at 90 minutes after collection of 90-minute blood samples. *Significantly different ($P < 0.05$) from baseline cortisol concentration within each age. Ages with different letter superscripts had significantly different ($P < 0.05$) areas under the cortisol concentration curve. Mean \pm standard deviation.

CHAPTER 5

HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DYSFUNCTION IN HOSPITALIZED NEONATAL FOALS¹

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ABSTRACT

Background: Transient hypothalamic-pituitary-adrenal (HPA) axis dysfunction occurs frequently in critically ill humans and impacts survival. The prevalence and impact of HPA axis dysfunction in critically ill neonatal foals are not well characterized.

Hypotheses: (1) HPA axis dysfunction occurs in hospitalized neonatal foals, and is characterized by inappropriately low basal serum cortisol concentration or inadequate cortisol response to exogenous ACTH; (2) Hospitalized foals with HPA axis dysfunction have more severe disease and are less likely to survive than hospitalized foals with normal HPA axis function.

Animals: 72 hospitalized foals and 23 healthy age-matched foals

Methods: Basal ACTH and cortisol concentrations were measured and a paired low-dose (10 μg) / high-dose (100 μg) cosyntropin stimulation test was performed at admission in hospitalized foals. HPA axis dysfunction was defined as: 1) an inappropriately low basal cortisol concentration or 2) an inadequate increase in cortisol concentration (delta cortisol) after administration of cosyntropin, with cut-off values for appropriate basal and delta cortisol concentrations determined from results obtained in healthy age-matched foals.

Results: Forty-six percent of hospitalized foals had an inappropriately low basal cortisol concentration and 52% had an inadequate delta cortisol concentration after administration of the 100 μg dose of cosyntropin. An inadequate delta cortisol response to the high (100 μg) dose of cosyntropin was significantly correlated with shock and multiple organ dysfunction syndrome in hospitalized foals, and with decreased survival in a subgroup of septic foals.

Conclusions and Clinical Importance: HPA axis dysfunction occurs frequently in hospitalized neonatal foals, and negatively impacts disease severity and survival.

INTRODUCTION

Intact hypothalamic-pituitary-adrenal (HPA) axis function is essential for regulating the physiologic, metabolic, and inflammatory responses to critical illness. Environmental, physiologic, and pathophysiologic stressors activate the HPA axis, resulting in release of adrenocorticotrophic hormone (ACTH) by the anterior pituitary gland, which stimulates the subsequent synthesis and secretion of cortisol from the adrenal cortices. Cortisol exerts a variety of systemic effects that are vital for the response to severe illness, including maintenance of adequate blood pressure, provision of nutrients to tissues, and control of an appropriate inflammatory response.^{1,2}

Despite its vital role in the physiologic response to the stress of disease, transient dysfunction of the HPA axis is well documented in critically ill people, particularly in patients with sepsis and septic shock.³⁻⁷ This dysfunction is often termed Relative Adrenal Insufficiency (RAI) or Critical Illness Related Corticosteroid Insufficiency (CIRCI), and is best defined as an inadequate cortisol response for the existing degree of severe illness.^{2,3,5-14} Although serum cortisol concentrations in patients with RAI/CIRCI often are increased relative to resting concentrations in healthy individuals, the cortisol response remains insufficient for the markedly increased physiologic demands of acute severe illness.^{3,4,7,10} RAI/CIRCI occurs secondary to severe illness or stress (e.g., trauma, surgery), and almost always fully resolves if the patient survives the primary illness. However, due to the vital role the HPA axis plays in the physiologic response to the stress of illness, the occurrence of RAI/CIRCI during critical illness may affect the patient's survival.

An extensive body of evidence in the human medical literature demonstrates a strong association between HPA axis dysfunction and increased incidence of shock, multiple organ dysfunction syndrome (MODS), and death in critically ill patients.^{2,3,5-14} However, a consensus on the diagnostic criteria for HPA axis dysfunction in critical illness has not been fully established in human medicine. Diagnosis of HPA axis dysfunction in septic human patients currently is based on 1 or more of the following findings: 1) a basal serum cortisol concentration < 10 µg/dl;^{4,11,15} 2) a blunted cortisol response (delta cortisol < 9 µg/dl) to administration of a high (supraphysiologic) dose of synthetic ACTH (125-250 µg) in the classical high-dose ACTH stimulation test;^{4,11,16} 3) a blunted cortisol response to administration of a more “physiologic” low dose (1 µg) of synthetic ACTH;^{3,17,18} or some combination of these findings.

Delay in maturation of the HPA axis in the fetal foal as compared to other species¹⁹⁻²⁴ may predispose neonatal foals to development of HPA axis dysfunction during illness. Both premature (gestational age <320 days) and full term neonatal foals appear to lack a fully mature and responsive HPA axis, as evidenced by blunted cortisol responses to both endogenous and exogenous ACTH, as compared to adult horses.^{22,24-28} In addition, recent studies have identified markedly increased ACTH:cortisol ratios (increased plasma ACTH concentration with correspondingly low serum cortisol concentration) in non-surviving septic foals as compared to both healthy foals and surviving septic foals.^{29,30} Low cortisol concentration in conjunction with an increased ACTH concentration suggest that failure of cortisol synthesis is primarily at the level of the adrenal gland, whereas the central portions of the HPA axis appear intact.

This HPA axis immaturity may impair the neonatal foal’s ability to respond appropriately to the physiologic stresses induced by systemic illness. The incidence of sepsis and septic shock, the conditions that are most often associated with RAI/CIRCI in human critical care,^{3,5,7,10,12,14} is

also high in neonatal foals.³¹⁻³³ Transient adrenal insufficiency has been described in 1 neonatal foal with concurrent septicemia and hypovolemic shock,³⁴ and recent evidence suggests that some degree of HPA axis dysfunction may occur in septic neonatal foals.^{29,30,35} To the authors' knowledge, HPA axis function has not been evaluated in hospitalized or septic foals using a paired low-dose / high-dose ACTH stimulation testing protocol.

The objectives of this study were to evaluate HPA axis function in a group of hospitalized neonatal foals utilizing measurement of basal ACTH and cortisol concentrations and cortisol responses to both low and high doses of exogenous ACTH, and to determine associations among HPA axis function, disease severity and outcome in these foals. We hypothesized that: 1) HPA axis dysfunction occurs in hospitalized neonatal foals, and is characterized by an inappropriately low basal cortisol concentration, an inadequate cortisol response to exogenous ACTH as compared to healthy age-matched foals, or both; and 2) hospitalized foals with concurrent HPA axis dysfunction have more severe disease and are less likely to survive to hospital discharge than critically ill foals with intact HPA axis function.

MATERIALS AND METHODS

Animals

This prospective clinical study was performed at the University of Georgia's Veterinary Teaching Hospital and at Haygard Equine Medicine Institute, Lexington, KY between January 2006 and May 2007. Seventy-two hospitalized foals and 23 healthy control foals were evaluated.

The hospitalized group consisted of 72 client-owned neonatal foals ≤ 7 days of age admitted to one of the above referral hospitals for evaluation of any medical or surgical problem, with the exception of primary congenital angular limb deformity. Foals receiving corticosteroids in the 24 hours before admission were excluded from analysis. Clinical and clinicopathologic data were collected from the medical records of hospitalized foals to determine the incidence of sepsis, shock, MODS, and nonsurvival in this population. Sepsis was defined as either a positive blood culture or a sepsis score³⁶ ≥ 11 at hospital admission. Shock was considered present in foals that had at least 2 of the following at admission: (1) mean arterial pressure < 60 mmHg; (2) cold extremities; (3) weak peripheral pulses; (4) capillary refill time > 2 seconds; (5) altered mental status (e.g. marked depression or inability to stand, nurse, or track the mare); (6) rectal temperature < 99.0 degrees Fahrenheit (< 37.2 degrees Celsius); or (7) plasma lactate concentration > 5 mmol/L.³⁷ MODS was defined as at least 2 of the following criteria present at any time during hospitalization: (1) anuria, oliguria or persistent azotemia (serum creatinine concentration > 2.2 mg/dl for ≥ 72 hours) after initial fluid resuscitation and rehydration; (2) clinical diagnosis of perinatal encephalopathy; (3) respiratory dysfunction (hypoxemia, hypercapnia or both) requiring either nasal insufflation of oxygen or ventilation; (4) persistent ileus necessitating withholding of enteral feeding for > 24 hours; (5) > 24 hours of vasopressor therapy; or (6) a clinical diagnosis of disseminated intravascular coagulation. Survival was defined as survival to hospital discharge; nonsurvival was defined as death or euthanasia during hospitalization.

The 23 healthy control foals were divided into 2 groups: 1) a “healthy unstressed group” of 12 neonatal Quarter Horse foals from a herd maintained at the University of Georgia’s equine breeding facility; and 2) a “healthy stressed group” of 11 client-owned foals ≤ 7 days of age.

Healthy unstressed foals were sampled 4 times during the first week of life (within 1 hour of birth, and at 12-24 hours, 36-48 hours, and 5-7 days of age), and remained in their home environment with their dam throughout the study period.²⁷ Healthy stressed foals were subjected either to transportation stress (e.g. travel to a referral hospital with a sick mare or for evaluation of a congenital abnormality), separation stress (separation from the dam for ≥ 2 hours), or both in the 12 hours prior to sampling. All healthy foals were full-term foals and were born without assistance. Foals were determined to be healthy before and during the study by lack of abnormalities identified on physical examination (with the exception of 3 foals with congenital ocular or musculoskeletal abnormalities). In the 3 healthy stressed foals admitted for evaluation of congenital abnormalities, CBC and serum biochemical profile obtained at admission were within reference intervals. Adequate transfer of passive immunity was confirmed by a serum immunoglobulin concentration ≥ 800 mg/dL at 12-24 hours of age or before inclusion in the study in all healthy foals.^a

Study methods were approved by the University of Georgia Institutional Animal Care and Use Committee and the University of Georgia College of Veterinary Medicine Clinical Research Committee, and informed owner consent was obtained before foal enrollment in the sick and healthy stressed groups. Each mare and foal pair in the healthy unstressed group was stabled with daily paddock turnout during the study period, and was cared for according to the principles and guidelines stated in an Animal Use Protocol determined by the University of Georgia's Department of Animal Resources.

Study Design

In all 72 hospitalized foals, blood was collected for measurement of basal serum total cortisol concentration (henceforth referred to as “cortisol concentration”) and plasma endogenous ACTH concentration (henceforth referred to as “ACTH concentration”) after study enrollment at hospital admission. In 58/72 hospitalized foals, a paired low-dose (10 µg) / high-dose (100 µg) cosyntropin^b (synthetic ACTH, α 1-24 corticotropin) stimulation test (Figure 5.1; henceforth referred to as “paired cosyntropin stimulation test”) was performed as described previously²⁷ within the first 12 hours of hospital admission. Briefly, blood was collected for measurement of resting cortisol concentration (time 0) immediately before administration of 10 µg cosyntropin as a rapid IV bolus. Blood was collected 30 minutes later for assessment of peak cortisol response to the 10 µg cosyntropin dose. Ninety minutes after administration of 10 µg cosyntropin, blood again was collected for measurement of cortisol concentration, and 100 µg of cosyntropin immediately was administered as a rapid IV bolus. Blood was collected 30 and 90 minutes later for assessment of the cortisol response to the 100 µg cosyntropin dose. Cosyntropin was administered and all blood samples for the paired cosyntropin stimulation test were collected through the foal’s indwelling jugular catheter.

In the healthy unstressed foals, HPA axis function was assessed at the aforementioned 4 time points during the first week of life to provide age-matched comparisons with the hospitalized foal group.²⁷ Time of birth was determined by use of a Foalert^{TMc} system placed on each mare before her anticipated foaling date, and defined as the time at which the FoalertTM alarm was activated. At each age, blood was collected for measurement of basal ACTH and cortisol concentrations, and a paired cosyntropin stimulation test as described above was performed. Detailed findings from this serial HPA axis assessment in these healthy foals have

been reported previously.²⁷ In the healthy stressed foals, blood was collected for measurement of basal ACTH and cortisol concentrations in all 11 foals and a paired cosyntropin stimulation test was performed as described above in 10/11 foals, within 12 hours of the separation or transportation stress. In all healthy foals, cosyntropin was administered and blood samples were collected by direct jugular venipuncture under brief restraint in standing foals.

Sample Processing

Blood for measurement of ACTH concentration was placed into sterile plastic tubes containing 3.6 mg potassium EDTA anticoagulant and immediately stored at 4 degrees Celsius until processing. Blood for measurement of cortisol concentration was collected into sterile glass tubes without additives, allowed to clot at room temperature for 30-60 minutes, and then stored similarly until processing. All samples were centrifuged and serum or plasma removed within 3 hours of collection. Plasma and serum were stored frozen at -80 degrees Celsius until assays were performed within 30 days.

ACTH and Cortisol Assays

ACTH and cortisol concentrations were measured on an automated analyzer using chemiluminescent enzyme immunoassays^d validated for use in the horse.³⁸⁻⁴⁰ For the ACTH assay, the interassay and intra-assay coefficients of variation were 7-9% and 9% respectively and the limit of detection was 9 pg/ml.³⁸ The interassay and intra-assay coefficients of variation were $\leq 20\%$ ⁴⁰ and the limit of detection was 0.05 $\mu\text{g}/\text{dL}$ for the cortisol assay.³⁹

Definition of HPA Axis Dysfunction

Two definitions of HPA axis dysfunction were adapted from currently accepted criteria utilized in human critical care and applied to the hospitalized foal group: (1) an inappropriately low basal serum cortisol concentration; and (2) an inadequate increase in cortisol concentration (delta cortisol) after administration of cosyntropin in the paired cosyntropin stimulation test.^{1,4,15,41,42} Specifically, an inappropriately low basal cortisol concentration was defined as a basal cortisol concentration less than the lowest cortisol concentration (mean – 1 standard deviation) achieved after administration of a physiologic (10 µg) dose of cosyntropin to healthy age-matched foals.^{27,42} An inadequate delta cortisol concentration was defined as a delta cortisol concentration less than the mean delta cortisol concentration achieved in healthy age-matched foals using the same paired cosyntropin stimulation protocol.

Statistical Analysis

Basal ACTH and cortisol concentrations and basal ACTH:cortisol ratios were determined for all foals. Serum cortisol responses to both the 10 µg and the 100 µg dose of cosyntropin also were determined for all foals that had paired cosyntropin stimulation tests performed. The low-dose delta serum cortisol concentration (LDdelta) was defined as the cortisol concentration reached 30 minutes after administration of 10 µg cosyntropin minus the immediate pre-cosyntropin (time 0) cortisol concentration. The high-dose delta cortisol concentration (HDdelta) was defined similarly as the peak cortisol concentration reached either 30 or 90 minutes after administration of 100 µg cosyntropin minus the time 0 concentration.

Because previous work has shown a significant effect of age on basal ACTH and cortisol concentrations and on cortisol responses to exogenous ACTH in neonatal foals during the first

week of life, all comparisons between foal groups were age-matched. Several independent studies have identified significantly higher basal ACTH and cortisol concentrations in healthy foals at birth than at other time points during the first week of life (at 12-24 hours, 36-48 hours, and 5-7 days of age), and no significant differences in these parameters among foals in these 3 older age groups.^{20,22,27} Thus, for comparison of basal ACTH and cortisol concentrations, foals were divided into 2 age groups based on age at sampling: (1) foals \leq 4 hours of age; and 2) foals $>$ 4 hours of age. However, because previous work in our laboratory has shown that delta cortisol concentrations differ significantly throughout the first week of life in healthy foals,²⁷ for delta cortisol comparisons, hospitalized foals and healthy stressed foals were divided into 4 age groups based on age at sampling: (A) \leq 4 hours; (B) $>$ 4 hrs to $<$ 30 hours; (C) \geq 30 hours to $<$ 3 days; and (D) \geq 3 days to \leq 7 days of age. Data from age groups A, B, C, and D were compared with healthy unstressed foals at birth, 12-24 hours, 36-48 hours, and 5-7 days of age, respectively.

Statistical analyses were performed using SASTM and GraphPad Prism[®] statistical software.^{e,f} Foal weights were compared among the 3 foal groups using an analysis of variance, with multiple comparisons conducted using Tukey's test. Basal ACTH and cortisol concentrations, basal ACTH:cortisol ratios, and LDdelta and HDdelta cortisol concentrations were compared between age-matched healthy unstressed and healthy stressed foals and between age-matched healthy unstressed and hospitalized foals using Student's t tests. Because the variances differed significantly between the healthy unstressed foal group and the hospitalized foal group, Welch's correction was applied to the analyses comparing these groups. Within the sick foal group, the Mann Whitney U test was used to compare basal ACTH and cortisol concentrations, basal ACTH:cortisol ratios, and LDdelta cortisol and HDdelta cortisol

concentrations in hospitalized foals that did and did not meet criteria for sepsis, shock, MODS, and nonsurvival. Chi-square tests were used to evaluate differences in proportions of foals with adverse clinical outcomes (shock, MODS, and nonsurvival) in foals that did and did not meet the aforementioned diagnostic criteria for HPA axis dysfunction. Hypothesis tests were 2-tailed, and statistical significance was set at $P < 0.05$ for all analyses. Data are expressed as mean \pm standard deviation.

RESULTS

Foal Group Characteristics

Breed, sex and gestational age distribution and body weight ranges for control and hospitalized foals are shown in Table 5.1. Body weight was not significantly different among healthy unstressed, healthy stressed and hospitalized foals ($P = 0.179$). Reasons for hospital admission for healthy stressed foals and hospitalized foals also are presented in Table 5.1.

Hospitalized and healthy stressed foals were divided into the previously described age groups for comparison with healthy unstressed foals.²⁷ For comparison of basal ACTH and cortisol concentrations, 12 hospitalized foals fit age group 1 (≤ 4 hours of age) and 60 fit age group 2 (> 4 hours of age). All healthy stressed foals were > 4 hours of age at admission, and thus were placed into age group 2. For delta cortisol comparisons, 9 hospitalized foals fit age group A (≤ 4 hours of age), 18 foals fit age group B (> 4 to < 30 hours of age), 27 foals fit age

group C (≥ 30 hours to < 3 days of age) and 4 foals fit age group D (≥ 3 days to ≤ 7 days of age). No healthy stressed foals fit age group A, 1 fit age group B, 7 fit age group C, and 2 fit age group D.

Basal ACTH and Cortisol Concentrations

Basal ACTH and cortisol concentrations and basal ACTH:cortisol ratios for the 3 foal groups are shown in Table 5.2. There were no significant differences in basal cortisol concentration ($P=0.672$), basal ACTH concentration ($P=0.604$), or basal ACTH:cortisol ratio ($P=0.453$) between age-matched healthy unstressed and healthy stressed foals. Hospitalized foals in age group 1 (≤ 4 hours of age) had similar basal cortisol concentrations ($P=0.917$) but significantly lower basal ACTH concentrations ($P=0.047$) and significantly lower basal ACTH:cortisol ratios ($P=0.022$) than age-matched healthy unstressed foals. Exclusion of 3 premature (gestational age ≤ 320 days) group 1 foals from the analysis did not significantly alter comparisons in basal cortisol concentrations or basal ACTH:cortisol ratios between hospitalized and healthy unstressed foals. Hospitalized foals' basal cortisol concentrations remained similar ($P=0.918$) between hospitalized and healthy unstressed foals, and basal ACTH:cortisol ratios remained significantly lower ($P=0.025$) in hospitalized foals. Basal ACTH concentrations also remained lower in hospitalized foals than in age-matched healthy unstressed foals, but this difference no longer reached statistical significance ($P=0.058$).

In contrast, hospitalized foals in age group 2 (> 4 hours to ≤ 7 days of age) had significantly higher basal cortisol concentrations ($P<0.001$) and significantly higher basal ACTH concentrations ($P=0.015$) than age-matched healthy unstressed foals, but basal ACTH:cortisol ratios were not significantly different ($P=0.156$) between healthy unstressed and hospitalized

foals. Exclusion of 1 premature foal from this analysis did not significantly alter the above findings.

Cortisol Responses to ACTH Stimulation Testing

Delta cortisol concentrations achieved in the paired cosyntropin stimulation test for age-matched hospitalized, healthy unstressed and healthy stressed foals are shown in Table 5.3. Cortisol responses to both the 10 µg and 100 µg cosyntropin doses did not differ significantly between healthy unstressed and healthy stressed foals in age group C (LDdelta: P=0.452; HDdelta: P=0.466). Statistical comparisons between healthy unstressed and healthy stressed foals were not performed in the other 3 age groups due to inadequate foal numbers.

There also were no significant differences in LDdelta cortisol (age group A: P=0.084; age group B: P=0.202; age group C: P=0.154; age group D: P=0.166) or HDdelta cortisol (age group A: P=0.236; age group B: P=0.077; age group C: P=0.348; age group D: P=0.343) concentrations between age-matched healthy unstressed and hospitalized foals. Exclusion from analysis of the 2 premature foals that had paired cosyntropin stimulation tests performed did not significantly alter the above findings. However, all healthy foals (unstressed and stressed) showed a significant increase in serum cortisol concentrations from basal concentrations after administration of both low and high doses of cosyntropin, and a negative delta cortisol (i.e., a decrease in cortisol concentration after administration of cosyntropin) was not observed in any healthy foal.²⁷ In contrast, 9 hospitalized foals had negative LDdelta cortisols and 7 hospitalized foals had negative HDdelta cortisols. Four foals had both negative LDdelta cortisols and negative HDdelta cortisols. Representative data from 3 hospitalized foals are shown in Figure 5.2 to illustrate potential cortisol response patterns to the paired cosyntropin stimulation test

observed in the hospitalized foal group.

Disease Severity and Outcome

Numbers of hospitalized foals meeting criteria for sepsis, shock, MODS, and non-survival for all hospitalized foals (n=72) and for the subset of septic foals from this group (n=54) are shown in Tables 5.4a and 5.4b respectively. Correlations between measures of adrenal axis function and indicators of disease severity and outcome were determined for all hospitalized foals and for the more homogenous subset of septic foals. There was no effect of age group on the incidence of shock, MODS, or on survival status in all hospitalized foals or in the septic subgroup, except for the incidence of shock in hospitalized foals, in which foals in age group C had a significantly lower incidence of shock (P=0.007) than foals in the other age groups. Thus, foal age group was not specifically considered in analyses evaluating disease severity and outcome.

Basal ACTH and cortisol concentrations, basal ACTH:cortisol ratios, and LDdelta and HDdelta cortisol concentrations in hospitalized foals and foals in the septic subgroup meeting criteria for sepsis, shock, MODS, and non-survival are shown in Tables 5.5a and 5.5b respectively. In all hospitalized foals, basal ACTH and cortisol concentrations were significantly higher in foals meeting criteria for sepsis and shock and in nonsurviving foals, but not significantly different in foals meeting criteria for MODS. ACTH:cortisol ratios were not significantly different in hospitalized foals meeting criteria for sepsis, shock, or MODS, or between surviving and non-surviving foals. LDdelta cortisol concentrations also were not significantly different in hospitalized foals with sepsis, shock, or MODS, or in nonsurviving foals. HDdelta cortisol concentrations, however, although not significantly different in

hospitalized foals with sepsis or MODS, were significantly lower in hospitalized foals with shock and in nonsurvivors. Significant findings were identical for the septic foal subgroup (Table 5.5b), with significantly higher basal cortisol and ACTH concentrations and significantly lower HDdelta cortisol concentrations observed in septic foals with shock and in nonsurviving septic foals. Exclusion of 4 premature foals from the analysis did not significantly alter the findings in the hospitalized foals or the septic foal subgroup.

Application of Definitions for HPA Axis Dysfunction

Numbers of hospitalized and septic foals meeting 1 or more definitions of HPA axis dysfunction (an inappropriately low basal cortisol concentration or an inadequate increase in cortisol [LDdelta or HDdelta cortisol] after administration of the low [10 µg] or high [100 µg] dose of cosyntropin compared to age-matched healthy foals) are shown in Figures 5.3a, 5.3b and 5.3c and Table 5.6.

Significant correlations between an inappropriately low basal cortisol concentration and the incidence of shock, MODS, or non-survival were not found for hospitalized foals or for the subgroup of septic foals. In all hospitalized foals, however, a low LDdelta cortisol concentration was significantly correlated with an increased incidence of MODS ($P=0.011$) but not shock ($P=0.792$) or nonsurvival ($P=0.368$). In all hospitalized foals, a low HDdelta cortisol concentration was significantly correlated with both MODS ($P=0.038$) and shock ($P=0.029$), but not with nonsurvival ($P=0.109$). In the subgroup of septic foals, these correlations followed a similar pattern: an inappropriately low LDdelta cortisol concentration was significantly correlated with an increased incidence of MODS ($P=0.003$) but not with shock ($P=0.876$) or nonsurvival ($P=0.293$). In septic foals, an inappropriately low HDdelta cortisol concentration

was significantly correlated with an increased incidence of shock ($P=0.028$) and MODS ($P=0.013$), and with nonsurvival ($P=0.017$) (Figure 5.4). In the entire hospitalized foal group and in the septic foal subgroup, the combination of an inappropriately low LDdelta cortisol and an inappropriately low HDdelta cortisol was no better at predicting survival than an inappropriately low LDdelta cortisol or inappropriately low HDdelta cortisol alone ($P=0.168 - 0.866$). Septic foals that had a normal or high basal cortisol concentration and an inadequate HDdelta cortisol had a significantly higher risk of death than did septic foals with a normal or high basal cortisol concentration and an adequate HDdelta cortisol concentration ($P=0.023$).

DISCUSSION

The above findings strongly support the hypothesis that HPA axis dysfunction occurs in septic foals, with a prevalence similar to the approximate 40-60% prevalence reported in septic people and infants.^{4,12,14,15} In addition, as is reported in people,^{4,12,14,15} septic foals with evidence of HPA axis dysfunction are more likely to have shock or MODS and are less likely to survive to discharge than septic foals with intact HPA axis function.

The significantly higher basal ACTH and cortisol concentrations, with similar ACTH:cortisol ratios, seen in the older hospitalized foals (>4 hours of age) as compared to age-matched healthy foals is consistent with an expected and appropriate cortisol response to illness. However, in the youngest hospitalized foals (≤ 4 hours of age), the lack of a significant difference in cortisol concentrations from basal concentrations observed in age-matched healthy foals suggests that some degree of HPA axis dysfunction may be present in these foals.

Endogenous adrenocortical glucocorticoid synthetic capacity may already be at maximal capacity in these foals due to massive HPA axis stimulation with the concurrent stressors of illness and parturition. Also, inclusion of 3 premature foals (<320 days gestation) may have skewed the findings in this group, because an inadequate cortisol response to both endogenous and exogenous ACTH has been described previously in premature foals.^{22,24} Exclusion of these 3 foals from the analysis, however, did not dramatically alter the findings. In addition, if exhausted endogenous glucocorticoid synthetic capacity or adrenocortical resistance to ACTH is responsible for the lower cortisol concentrations seen in this group of foals, one would expect basal ACTH concentrations and ACTH:cortisol ratios to be higher rather than lower in hospitalized foals as compared to healthy foals.^{21,22,29,30} Low ACTH concentrations in this group of foals thus suggest hypothalamic or pituitary suppression of the HPA axis, perhaps related to the effects of inflammatory cytokines stimulated by the foal's concurrent illness.⁴³⁻⁴⁵

In contrast to previous reports,^{29,30} significant differences in basal ACTH:cortisol ratios between surviving and nonsurviving hospitalized or septic foals were not identified in the present study population. In addition, basal ACTH:cortisol ratio was not significantly correlated with the incidence of sepsis, shock, or MODS in the foals in this study. Inconsistency between this and previous reports likely is related to the extraordinarily wide variation in basal ACTH concentrations observed in critically ill foals.^{29,30} In addition, accurate understanding of the specific temporal relationship of ACTH release, cortisol secretion, and subsequent cortisol-mediated down-regulation of further ACTH secretion in the foal during both health and disease currently is limited. Thus, interpretation of the importance of an altered ACTH:cortisol ratio at any individual time point in a sick foal is difficult.

Although mean basal cortisol concentration was significantly higher in hospitalized foals

> 4 hours of age than in healthy age matched foals, 46% of the hospitalized foal group and 37% of the septic foal subgroup had basal cortisol concentrations less than the concentration achieved in healthy age-matched foals in response to approximated physiologic stress (administration of a 10 µg dose of cosyntropin),²⁷ a definition of HPA axis dysfunction frequently utilized in critically ill infants.^{4,11,12} In contrast to people, however, an inappropriately low basal cortisol concentration was not significantly predictive of shock, MODS, or nonsurvival in the hospitalized or septic foals reported here. In fact, basal cortisol concentration in our study actually was significantly higher in hospitalized foals with sepsis and shock and in nonsurviving hospitalized and septic foals, consistent with the increased HPA axis activation expected with severe illness. The cut-off value for basal cortisol concentration applied in this study may have been inappropriate, or application of this diagnostic criterion to a much larger and more homogeneous group of foals (i.e. foals with septic shock) may identify a correlation between inappropriately low basal cortisol concentration and disease severity or poor outcome. However, although a random basal cortisol concentration < 10 µg/dl currently is utilized to diagnose RAI/CIRCI in human ICUs,^{4,11} the findings reported here do not support use of a basal cortisol concentration cut-off at hospital admission for diagnosis of RAI/CIRCI in critically ill foals.

The alternative diagnostic criteria for RAI/CIRCI utilized in this study (i.e., a delta cortisol concentration lower than the mean delta cortisol concentration achieved in healthy age-matched foals) did identify a population of foals with increasingly severe disease and decreased survival. As in humans and dogs,^{4,46} an impaired cortisol response to a supraphysiologic dose (100 µg) of exogenous ACTH was significantly correlated with an increased incidence of MODS and shock in hospitalized foals, and with decreased survival in septic foals. Thus, these findings suggest that lack of an appropriate cortisol response to a supraphysiologic 100 µg dose of

exogenous ACTH may identify critically ill foals with concurrent HPA axis dysfunction, even if basal cortisol concentrations are appropriately increased. However, further study is required to determine appropriate delta cortisol cut-off values that most accurately identify critically ill foals with clinically important HPA axis dysfunction.

In addition, if applied to a larger, more homogeneous population of foals, the 10 µg cosyntropin stimulation test also may provide useful information regarding HPA axis dysfunction in some foals, with particular regard to ACTH sensitivity, because 5 foals in this study failed to respond to the low dose of cosyntropin but exhibited an appropriate cortisol response to the high cosyntropin dose, results consistent with ACTH resistance at the level of the adrenal gland. At present, however, the low dose (10 µg) cosyntropin stimulation test does not appear to offer substantial advantage over the high-dose (100 µg) cosyntropin stimulation test in identifying HPA axis dysfunction in most critically ill neonatal foals.

Several mechanisms for HPA axis dysfunction in critical illness have been proposed in people, including: 1) inadequate regulatory hormone secretion due to central HPA axis suppression; 2) adrenal resistance to ACTH; 3) impaired or exhausted adrenocortical corticosteroid synthesis (“loss of adrenal reserve”), or 4) impaired tissue response to cortisol (“glucocorticoid resistance”).^{6,44} The results reported here suggest that altered HPA axis function in critically ill foals may be similarly related to several of these mechanisms. The significantly lower ACTH concentrations and lower ACTH:cortisol ratios observed in the youngest hospitalized foals may be due to hypothalamic or pituitary suppression or failure. The lack of significant increases in basal cortisol concentrations, as compared to healthy foals, noted in these youngest hospitalized foals, also suggests that glucocorticoid synthetic capacity may be inadequate in some foals if disease occurs concurrently with the stress of parturition. The

observation of an inadequate cortisol response to a supraphysiologic amount of exogenous ACTH in substantial proportions of hospitalized foals also may indicate loss of adrenal reserve, some degree of adrenocortical ACTH resistance, or both. Finally, correlations between increased basal cortisol concentrations and increased rates of shock may be representative of peripheral glucocorticoid resistance in some critically ill foals. Further investigation into the causative mechanisms of HPA axis dysfunction in critically ill foals, as is ongoing in other species, is warranted.

In summary, comprehensive evaluation of HPA axis function, including measurement of basal cortisol and ACTH concentrations and ACTH stimulation testing, should be considered in critically ill foals, particularly in foals with sepsis and septic shock. An HDdelta cortisol concentration less than the mean HDdelta cortisol concentration achieved in healthy age-matched control foals may be useful in identifying foals with clinically relevant HPA axis dysfunction. The 10 μ g cosyntropin stimulation test does not appear to offer substantial advantage over the 100 μ g cosyntropin stimulation test in identifying HPA axis dysfunction in most critically ill foals. Evaluation of HPA axis function in larger, more homogeneous populations of critically ill foals, such as similar-aged foals with septic shock, is needed to validate specific diagnostic criteria for HPA axis dysfunction and more definitively determine its impact on outcome. At present, the specific role that HPA axis dysfunction plays in the more severe pathology and decreased survival rates in affected foals remains unclear. HPA axis dysfunction and resultant systemic cortisol insufficiency may contribute to a dysregulated immune response and perpetuate systemic hypotension and shock in these foals, or it may simply be a marker of severe disease.

FOOTNOTES

^a SNAP® Foal IgG Test, IDEXX Laboratories, Inc., Westbrook, ME

^b Cortrosyn™, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA

^c Foal Alert, Inc., Atlanta, GA

^d Immulite™, Diagnostics Product Corporation, Los Angeles, CA

^e SAS Statistical Software™ (Version 9.1), SAS Institute Inc., Cary, NC

^f GraphPad PRISM® Statistical Software (Version 4), GraphPad Software, Inc., San Diego, CA

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Table 5.1. Foal group characteristics.

FOAL GROUP	Age	Breed(s)	Gestational Age	Reason for Admission <i>(note: some foals had >1 complaint)</i>	Weight (kg) (range)
Healthy Unstressed (n=12)	7 male 5 female	Quarter Horse (n=12)	≥330 days (n=12)	n/a	49.9 ± 8.6 (38.7 – 61.4)
Healthy Stressed (n=11)	7 male 4 female	<ul style="list-style-type: none"> • Quarter Horse (n=6) • Paint (n=2) • Paso Fino (n=1) • Tennessee Walking Horse (n=1) • Thoroughbred/warmblood cross (n=1) 	≥ 330 days (n=11)	<ul style="list-style-type: none"> • Dam’s colic (n=4) • Dam’s post-breeding exam (n=2) • Dam’s retained placenta (n=1) • Dam’s vaginal tear (n=1) • Congenital abnormalities (n=3) <ul style="list-style-type: none"> • Ocular deformities (n=2) • Angular limb deformity (n=1) 	55.3 ± 6.0 (45.5 – 63.6)
Hospitalized (n=72)	39 male 33 female	<ul style="list-style-type: none"> • Thoroughbred (n=28) • Quarter Horse (n=16) • Warmblood (n=6) • Tennessee Walking Horse (n=5) • Saddlebred (n=4) • Arabian (n=3) • Irish Draft (n=1) • Cleveland Bay (n=1) • Paso Fino (n=1) • Morgan (n=1) • Paint (n=1) • Standardbred (n=1) • Shetland Pony (n=1) • Donkey (n=1) 	305 days (n=1) 315-320 days (n=3) 321-329 days (n=7) 330-345 days (n=41) 346-359 days (n=3) 360-370 days (n=3) Unknown (n=9)	<ul style="list-style-type: none"> • Recumbency (n=16) • Failure to nurse well (n=15) • Weakness/lethargy (n=15) • Colic (n=13) • Diarrhea (n=12) • Post-dystocia or cesarean section (n=7) • Failure of transfer of passive immunity (n=5) • Premature parturition (≤320 days, n=4) • Limb edema/joint swelling (n=3) • Premature placental separation (n=3) • Orphans (n=3) • Agalactia in the dam (n=2) • Seizures (n=2) • Fever (n=1) • Icterus and anemia (n=1) • Inguinal hernia (n=1) • Surviving twin (n=1) • Wry nose/dysphagia (n=1) • Heart murmur (n=1) • Severe extensor tendon contracture (n=1) 	47.5 ± 12.3 (11.8 – 68.2)

Table 5.2. Basal endogenous plasma ACTH concentration, basal serum total cortisol concentration and basal ACTH:cortisol ratio for healthy unstressed foals, healthy stressed foals, and hospitalized foals. The healthy stressed and hospitalized foal groups were divided into two age groups for age-matched comparison with healthy unstressed foals: 1) foals \leq 4 hours, and 2) foals $>$ 4 hours old.

Age Group	Foal Group	ACTH Concentration (pg/ml)	Cortisol Concentration (μ g/dl)	ACTH:Cortisol Ratio
1	Healthy Unstressed (n=11)	285.5 \pm 284.8 (19.4 – 968.0)	10.2 \pm 2.3 (7.6 – 13.5)	27.4 \pm 23.8 (2.6 – 71.7)
1	Healthy Stressed (n=0)	n/a	n/a	n/a
1	Hospitalized (n=12)	90.5 \pm 82.2* (11.9 – 280.0)	9.8 \pm 4.4 (3.3 – 18.7)	8.6 \pm 6.1* (1.7 – 21.9)
2	Healthy Unstressed (n=31)	28.8 \pm 20.9 (10.3 – 105.0)	2.7 \pm 1.3 (1.0 – 6.7)	12.1 \pm 6.7 (2.6 – 26.8)
2	Healthy Stressed (n=11)	25.8 \pm 11.4 (11.7 – 46.5)	2.9 \pm 1.9 (0.8 – 6.2)	11.0 \pm 4.6 (5.4 \pm 19.4)
2	Hospitalized (n=60)	283.1 \pm 777.3* (10.0 – 4,980.0)	11.1 \pm 12.4* (0.5 – 62.0)	15.7 \pm 19.7 (0.6 – 96.5)

* Denotes values significantly different ($P < 0.05$) from healthy unstressed foals within each respective age group. Data are reported as mean \pm standard deviation. Numbers in parentheses represent the data range.

Table 5.3. Delta cortisol concentrations achieved with a paired low (10 µg) / high dose (100 µg) cosyntropin stimulation test in healthy unstressed foals, healthy stressed foals, and hospitalized foals. Healthy stressed and hospitalized foals were divided into four age groups for age-matched comparisons with healthy unstressed foals: A) foals ≤ 4 hours old; B) foals >4 hours to <30 hours old, C) foals ≥ 30 hours to <3 days old, and D) foals ≥3 to ≤ 7 days old.

Age Group	Foal Group	LDdelta Cortisol Concentration (µg/dl)	HDdelta Cortisol Concentration (µg/dl)
A	Healthy Unstressed (n=11)	3.6 ± 2.0 (0.4 – 8.2)	6.4 ± 4.1 (0.3 – 15.5)
A	Healthy Stressed (n=0)	n/a	n/a
A	Hospitalized (n=9)	1.5 ± 3.5 (-3.1 – 7.7)	4.6 ± 6.6 (-3.2 – 16.5)
B	Healthy Unstressed (n=10)	5.5 ± 2.1 (2.8 – 9.4)	9.4 ± 3.5 (4.6 – 15)
B	Healthy Stressed (n=1)	4.9 [#] (n/a)	11.3 [#] (n/a)
B	Hospitalized (n=18)	6.7 ± 5.5 (-0.6 – 23.2)	13.2 ± 10.3 (1.9 – 36)
C	Healthy Unstressed (n=10)	3.5 ± 1.7 (1.6 – 6.8)	6.8 ± 3.3 (3.2 – 12.7)
C	Healthy Stressed (n=7)	3.4 ± 2.1 (1.2 – 7.5)	6.9 ± 3.1 (1.6 – 10.1)
C	Hospitalized (n=27)	4.6 ± 5.0 (-3.5 – 24.8)	7.3 ± 5.1 (-4.0 – 19.2)
D	Healthy Unstressed (n=11)	1.3 ± 0.6 (0.6 – 2.6)	3.5 ± 1.3 (1.7 – 5.9)
D	Healthy Stressed (n=2)	1.4 and 1.6 [#] (n/a)	3.0 and 5.5 [#] (n/a)
D	Hospitalized (n=4)	5.3 ± 6.9 (-1.1 – 15)	2.1 ± 6.2 (-5.3 – 8.4)

Within each respective age group, no significant differences ($P < 0.05$) were found between healthy unstressed foals and healthy stressed foals, or between healthy unstressed and hospitalized foals. [#]Statistical comparisons with healthy unstressed foals were not performed in these groups due to low foal numbers. Data are reported as mean ± standard deviation. Numbers in parentheses represent the data range.

Table 5.4.a. Disease severity and outcome groupings for all hospitalized foals (n=72).

	Number of foals with necessary medical record data available	Number of foals meeting criteria	Percent of foals meeting criteria
Sepsis	69	54	78%
Shock	72	40	56%
MODS	71	22	31%
Nonsurvival	71	25	35%

Table 5.4.b. Disease severity and outcome groupings for septic foals (n=54).

	Number of foals with necessary medical record data available	Number of foals meeting criteria	Percent of foals meeting criteria
Shock	54	36	67%
MODS	53	21	40%
Nonsurvival	54	20	37%

Table 5.5.a. Basal cortisol and ACTH concentrations, basal ACTH:cortisol ratios, and LDdelta and HDdelta cortisol concentrations in all hospitalized foals) meeting criteria for sepsis, shock, multiple organ dysfunction syndrome (MODS), and non-survival as compared to hospitalized foals that did not meet those criteria.

		Basal ACTH (pg/ml) (n=72)	Basal Cortisol (µg/dl) (n=72)	Basal ACTH to Cortisol Ratio (n=72)	LDdelta Cortisol (µg/dl) (n=58)	HDdelta Cortisol (µg/dl) (n=58)
SEPSIS	Yes	313.6 ± 814.2* (10.0 – 4,980.0) <i>P=0.015</i>	12.3 ± 12.1* (0.5 – 62.0) <i>P=0.003</i>	17.7 ± 23.9 (0.9 – 111.7) <i>P=0.449</i>	4.5 ± 5.0 (-3.5 – 23.2) <i>P=0.110</i>	7.6 ± 7.8 (-5.3 – 36.0) <i>P=0.725</i>
	No	47.6 ± 79.5 (10.0 – 323)	5.5 ± 7.7 (0.9 – 32.3)	9.8 ± 8.4 (1.4 – 32.3)	4.4 ± 3.0 (0.4 – 12.7)	9.1 ± 8.1 (-0.9 – 32.5)
SHOCK	Yes	415.5 ± 918.4* (10.0 – 4,980.0) <i>P<0.001</i>	15.4 ± 12.7* (3.3 – 62.0) <i>P<0.001</i>	20.1 ± 27.1 (1.5 – 111.7) <i>P=0.418</i>	3.8 ± 5.6 (-3.5 – 23.2) <i>P=0.073</i>	6.0 ± 8.0* (-5.3 – 36.0) <i>P=0.011</i>
	No	35.5 ± 55.4 (10.0 – 323)	5.4 ± 6.1 (0.5 – 32.3)	9.8 ± 7.9 (0.6 – 32.3)	5.9 ± 4.8 (1.3 – 24.8)	10.3 ± 7.1 (-0.9 – 32.5)
MODS	Yes	440.5 ± 1155 (10.0 – 4,980.0) <i>P=0.105</i>	13.8 ± 15.7 (1.0 – 62.0) <i>P=0.234</i>	19.9 ± 25.9 (0.9 – 96.5) <i>P=0.457</i>	3.9 ± 6.4 (-3.5 – 23.2) <i>P=0.110</i>	5.2 ± 5.7 (-4.0 ± 16.5) <i>P=0.062</i>
	No	158.8 ± 323.8 (10.0 – 1665)	9.5 ± 8.5 (0.5 – 32.3)	13.8 ± 19.1 (0.6 – 111.7)	5.1 ± 4.9 (-3.1 – 24.8)	8.8 ± 8.3 (-5.3 – 36.0)
NON-SURVIVAL	Yes	538.6 ± 1132* (10.0 – 4,980.0) <i>P=0.013</i>	15.6 ± 14.8* (1.6 – 62.0) <i>P=0.005</i>	20.9 ± 26.1 (1.5 – 96.5) <i>P=0.276</i>	3.2 ± 4.7 (-3.5 – 15.0) <i>P=0.163</i>	4.6 ± 6.7* (-5.3 – 16.5) <i>P=0.041</i>
	No	93.3 ± 185.6 (10.0 – 1,128.0)	8.1 ± 8.0 (0.5 – 36.2)	13.0 ± 18.1 (0.9 – 111.7)	4.9 ± 4.5 (-1.9 – 23.2)	9.0 ± 7.9 (-2.3 – 36.0)

* Denotes a significant difference ($P < 0.05$) in the specified parameter in hospitalized foals that met the specific criteria for sepsis, shock, MODS, or non-survival, as compared to hospitalized foals not meeting that specific criterion. Elimination of 4 premature foals from the analysis did not significantly alter the findings. Data are reported as mean ± standard deviation. Numbers in parentheses represent the data range.

Table 5.5.b. Basal cortisol and ACTH concentrations, basal ACTH:cortisol ratios, and LDdelta and HDdelta cortisol concentrations in the septic foal subgroup meeting criteria for shock, multiple organ dysfunction syndrome (MODS), and non-survival as compared to septic foals that did not meet those criteria.

		Basal ACTH (pg/ml) (n=54)	Basal Cortisol (µg/dl) (n=54)	Basal ACTH to Cortisol Ratio (n=45)	LDdelta Cortisol (µg/dl) (n=45)	HDdelta Cortisol (µg/dl) (n=45)
SHOCK	Yes	434.2 ± 939.1* (10.0 – 4,980.0) <i>P</i> <0.001	16.2 ± 13.0* (3.3 – 62.0) <i>P</i> <0.001	21.2 ± 27.8 (1.5 – 111.7) <i>P</i> =0.615	4.2 ± 5.8 (-3.5 – 23.2) <i>P</i> =0.260	6.1 ± 8.3* (-5.3 – 36.0) <i>P</i> =0.021
	No	26.2 ± 12.7 (10.0 – 54.7)	4.5 – 3.6 (0.5 – 11.2)	10.2 ± 7.6 (0.9 – 30.8)	5.2 ± 2.8 (1.3 – 10.9)	10.6 ± 5.8 (1.8 – 26.8)
MODS	Yes	476.5 ± 1205 (10.0 – 4,980.0) <i>P</i> =0.598	14.4 ± 16.3 (1.0 – 62.0) <i>P</i> =1.00	21.0 ± 26.9 (0.9 – 96.5) <i>P</i> =0.751	2.7 ± 4.1 (-3.5 – 10.9) <i>P</i> =0.089	5.4 ± 5.8 (-4.0 – 16.5) <i>P</i> =0.174
	No	215.7 ± 382.3 (10.0 – 1665.0)	11.1 ± 8.5 (0.5 – 30.6)	16.2 ± 22.5 (1.9 – 111.7)	5.3 ± 5.2 (-3.1 – 23.2)	8.6 ± 8.4 (-5.3 – 36.0)
NON-SURVIVAL	Yes	664.9 ± 1238* (10.0 – 4,980.0) <i>P</i> =0.004	18.2 ± 15.5* (3.3 – 62.0) <i>P</i> =0.004	24.4 ± 28.1 (1.5 – 96.5) <i>P</i> =0.182	3.3 ± 5.0 (-3.5 – 15.0) <i>P</i> =0.220	3.8 ± 6.9* (-5.3 – 16.5) <i>P</i> =0.030
	No	106.5 ± 209.2 (10.0 – 1,128.0)	8.8 ± 7.9 (0.5 – 36.2)	13.7 ± 20.5 (0.9 – 111.7)	5.0 ± 4.9 (-1.9 – 23.2)	9.1 ± 7.7 (-2.3 – 36.0)

* Denotes a significant difference ($P < 0.05$) in the specified parameter in septic foals that met the specific criteria for shock, MODS, or non-survival, as compared to septic foals not meeting that specific criterion. Elimination of 3 premature foals from the analysis did not significantly alter the findings. Data are reported as mean ± standard deviation. Numbers in parentheses represent the data range.

Table 5.6. Percentages of hospitalized foals, septic foals, foals with shock, and foals with MODS that met one or more of the definitions of HPA axis dysfunction: 1) an inappropriately low basal cortisol concentration [a basal cortisol concentration less than the lowest cortisol concentration (mean – 1 standard deviation) achieved after administration of a physiologic (10 µg) dose of cosyntropin to healthy age-matched foals]; or 2) an inadequate delta cortisol concentration following administration of the low (10 µg) or high (100 µg) dose of cosyntropin [a LDdelta cortisol or HDdelta concentration less than the mean delta cortisol concentration achieved in healthy age-matched foals using the same paired cosyntropin stimulation test protocol].

	Percent of hospitalized foals meeting criteria (number/total foals)	Percent of septic foals meeting criteria (number/total foals)	Percent of foals with shock meeting criteria (number/total foals)	Percent of foals with MODS meeting criteria (number/total foals)
Inappropriately Low Basal Cortisol	46% (33/72)	37% (20/54)	29% (11/38)	39% (9/23)
Inadequate LDdelta Cortisol	50% (29/58)	44% (20/45)	52% (16/31)	80% (12/15)
Inadequate HDdelta Cortisol	52% (30/58)	51% (23/45)	65% (20/31)	80% (12/15)
Inadequate LDdelta and HDdelta Cortisol	40% (23/58)	38% (17/45)	52% (16/31)	80% (12/15)
Inadequate LDdelta but Adequate HDdelta Cortisol	9% (5/58)	7% (3/45)	0% (0/31)	0% (0/15)
Inappropriately Low Basal Cortisol and Inadequate HDdelta Cortisol	16% (9/58)	11% (5/45)	13% (4/31)	27% (4/15)
Appropriate Basal Cortisol and Inadequate HDdelta Cortisol	34% (20/58)	38% (17/45)	52% (16/31)	53% (8/15)

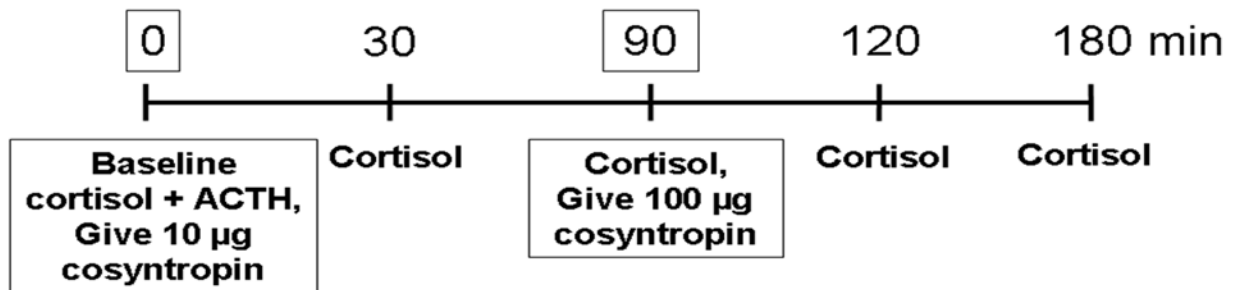


Figure 5.1. Paired low and high dose synthetic ACTH stimulation test design utilized in healthy unstressed, healthy stressed, and hospitalized foal groups. At time 0, blood was collected for measurement of pre-stimulation cortisol concentration, and 10 µg cosyntropin was administered intravenously. Blood was again collected 30 minutes later to assess peak cortisol response to the low (10 µg) cosyntropin dose. At 90 minutes, blood was again collected for measurement of cortisol concentration (to ensure cortisol concentrations returned to basal levels) and 100 µg cosyntropin was administered intravenously. Blood was then collected 30 and 90 minutes later (at times 120 and 180 minutes) to assess peak cortisol response to the high (100 µg) cosyntropin dose.

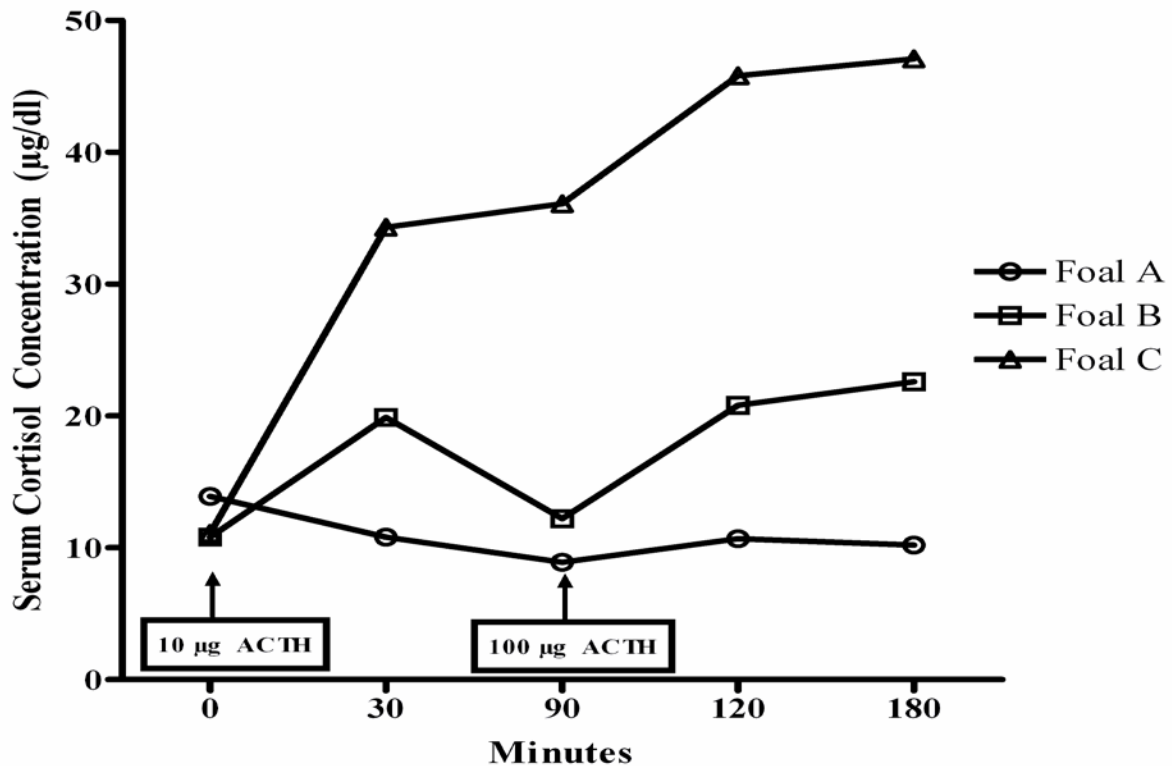
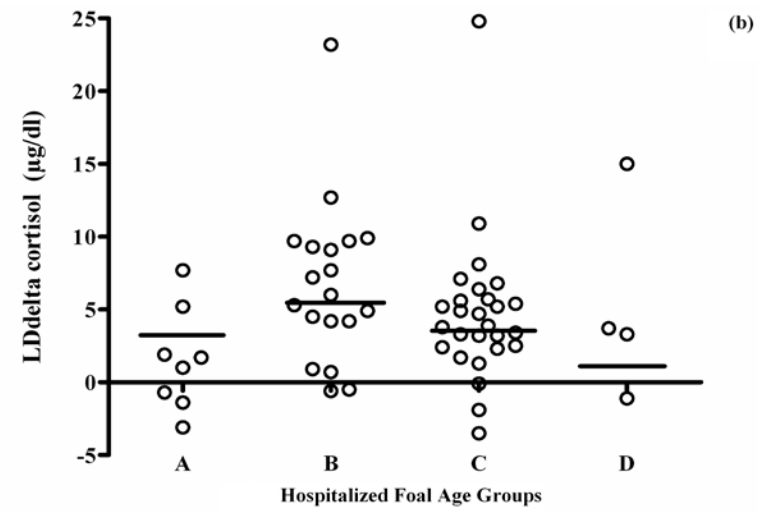
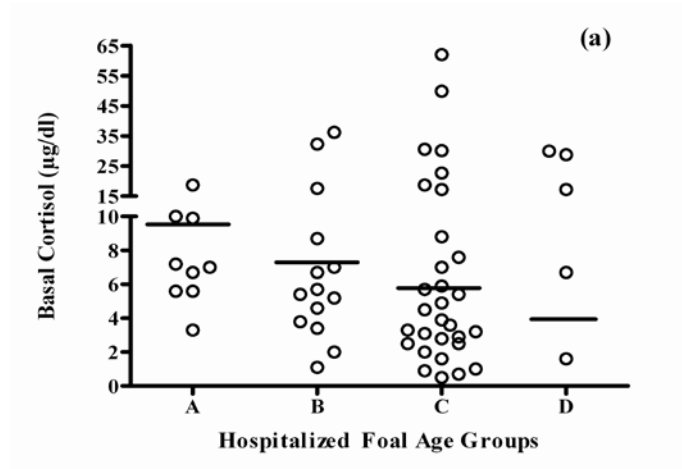


Figure 5.2. Cortisol responses over time during the paired cosyntropin stimulation test observed in 3 individual foals (A, B, and C) in the hospitalized foal group. Foal A illustrates a negative delta cortisol response (decrease in cortisol concentration from basal levels) following administration of both the low (10 µg) and high (100 µg) dose of cosyntropin. Foal B illustrates a significant but similar increase in cortisol concentration following administration of both the low and high doses of cosyntropin. Foal C illustrates a significant increase in cortisol concentration after administration of the low dose of cosyntropin, and an even greater cortisol response to the high dose of cosyntropin with a failure of cortisol concentrations to return to basal levels at 90 minutes. All three foals met criteria for sepsis, and foals A and C also met criteria for shock. Foals B and C survived to discharge, while Foal A did not.

Figure 5.3. Numbers of hospitalized foals meeting proposed definitions of HPA axis dysfunction. Each open circle (\circ) represents an individual hospitalized foal. Hospitalized foals were divided into the following four age groups for age-matched comparisons with healthy foals: (A) ≤ 4 hours of age; (B) >4 hrs to <30 hours of age; (C) ≤ 30 hours to <3 days of age; and (D) ≥ 3 to ≤ 7 days of age. Horizontal black bars represent cutoff values for: (A) basal cortisol concentration in healthy foals, as defined by the mean $- 1SD$ basal cortisol concentration observed in healthy age-matched foals; (B) LDdelta cortisol concentration, as defined as the mean LDdelta cortisol concentration observed in healthy age-matched foals with the same ACTH stimulation test protocol; and (C) HDdelta cortisol concentration, as defined by the mean HDdelta cortisol concentration observed in healthy age-matched foals with the same ACTH stimulation test protocol.

Figure 5.3.



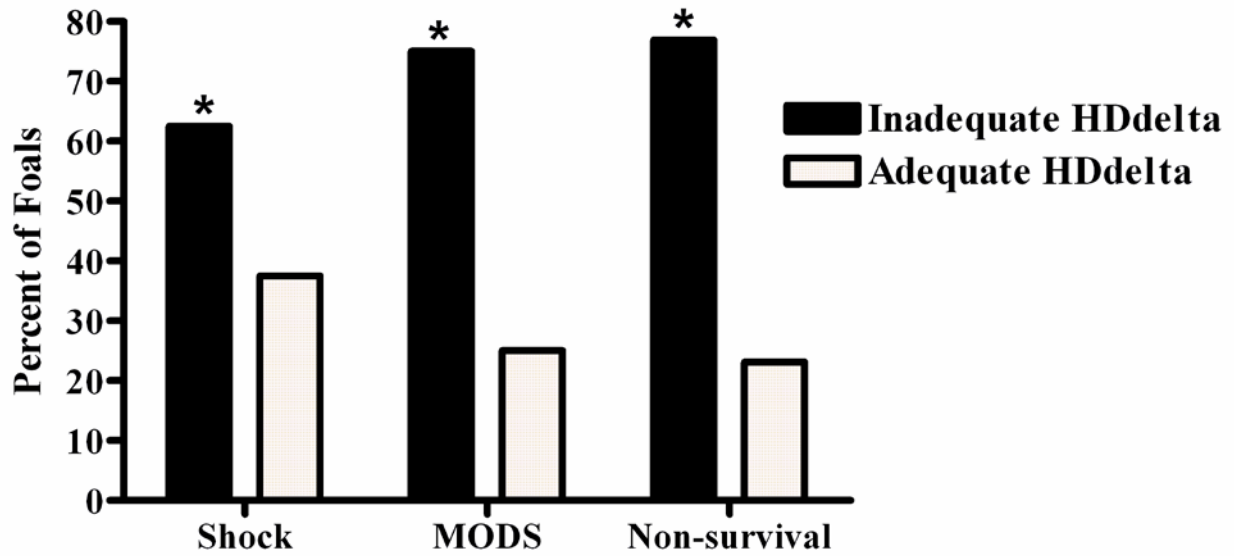


Figure 5.4. Percent of septic foals with inadequate and adequate HDdelta cortisol concentrations that met criteria for shock, multiple organ dysfunction syndrome (MODS), or non-survival. * Denotes significantly increased incidence of shock, MODS, and non-survival in septic foals with inadequate HDdelta cortisol concentration.

CHAPTER 6

SERUM FREE CORTISOL FRACTION IN HEALTHY AND SEPTIC NEONATAL FOALS¹

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ABSTRACT

Background: Relative cortisol insufficiency occurs in septic foals and impacts survival.

Measurement of free (biologically available) cortisol may be a better indicator of physiologic cortisol status than total cortisol, but is not described in foals.

Hypotheses: (1) In septic foals, low basal and cosyntropin-stimulated free cortisol concentration will correlate with disease severity and survival; (2) Free cortisol concentration will more accurately predict disease severity and outcome than total cortisol concentration in septic foals.

Animals: 51 septic foals; 11 healthy foals; 6 healthy adult horses

Methods: Basal and cosyntropin-stimulated total cortisol concentration and free cortisol fraction (FCF) were determined using chemiluminescent immunoassay and ultrafiltration/ligand binding methods respectively. Disease severity and survival data were collected from medical records. Group data were compared using ANOVA, Mann Whitney U tests, and receiver operator characteristic curves.

Results: Basal FCF was highest in healthy foals at birth, and was significantly higher in healthy foals of all ages than in adult horses. Basal total and free cortisol were significantly higher in septic foals than healthy foals. Basal total and free cortisol were also significantly higher, and total and free cortisol responses to cosyntropin significantly lower, in septic foals with shock and in non-surviving foals. Free cortisol offered no significant advantage over total cortisol for predicting disease severity or outcome in septic foals.

Conclusions and Clinical Importance: Serum FCF is impacted by age and illness in the horse. There is no advantage to measuring free over total cortisol for predicting disease severity and outcome in septic foals.

INTRODUCTION

The primary adaptive response to severe physiologic stress such as sepsis or septic shock involves activation of the hypothalamic-pituitary-adrenal (HPA) axis. Hypothalamic integration of input from the peripheral and central nervous systems culminates in the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into the systemic circulation. ACTH then stimulates adrenocortical synthesis and secretion of the essential stress hormone cortisol. Cortisol serves to modulate vital physiologic responses to the stress of illness, by helping maintain blood pressure, provide energy sources to tissues, and regulate the immune and inflammatory response.^{1,2} Increased systemic cortisol concentrations also exert a negative feedback effect on the HPA axis, resulting in subsequent down-regulation of regulatory hormone secretion. Thus, with an intact HPA axis, systemic cortisol concentrations are ideally maintained at a level that is appropriate for the existing degree of physiologic stress in both health and disease.^{1,2}

However, a growing body of evidence suggests that transient HPA axis dysfunction is common in critically ill people and animals, particularly those with severe trauma or sepsis.¹⁻⁷ This diagnosis is often termed relative adrenal insufficiency (RAI) or critical illness related corticosteroid insufficiency (CIRCI), and is best defined as an inadequate cortisol response to the existing degree of illness-related physiologic stress.^{1,3,4,6,8-11} The pathogenesis of this transient HPA axis dysfunction in sepsis is poorly understood, but the consequences are severe; the occurrence of RAI/CIRCI during sepsis is associated with an increased incidence of shock, multiple organ dysfunction syndrome (MODS), and death in both people^{1,3,5,11-15} and foals.¹⁶⁻¹⁸

A number of studies in human critical care have demonstrated a significant beneficial effect of low-dose hydrocortisone supplementation (e.g. “cortisol replacement therapy”) in septic people with RAI/CIRCI, with more rapid shock reversal, shorter intensive care unit stays, and improved survival rates in supplemented patients.^{1,3,8,19-23} However, not all studies documented such a beneficial effect,²⁴ and hydrocortisone replacement therapy in septic shock is not universally accepted in human critical care. To the authors’ knowledge, similar studies in veterinary patients have not been reported to date. However, this controversy illustrates the importance of accurate diagnosis of RAI/CIRCI in septic patients, with the primary goal to provide a rapid and inexpensive means by which to identify the patients that will benefit from therapeutic intervention with physiologic doses of corticosteroids.

Unfortunately, diagnostic criteria for RAI/CIRCI are not fully defined in either human or veterinary medicine. Current recommendations for diagnosis of RAI/CIRCI in septic human patients are based on documentation of 1 or more of the following: (1) an inappropriately low basal serum total cortisol concentration ($< 10 \mu\text{g/dl}$);^{4,6,8} (2) a blunted total cortisol response (Δ cortisol $< 9 \mu\text{g/dl}$) to administration of a high (supraphysiologic) dose of synthetic ACTH (100-250 μg);^{4,8,25} or (3) a blunted total cortisol response to administration of a “physiologic” low dose of synthetic ACTH (1-10 μg).^{3,26,27} Recent work in foals suggests that the second of these criteria, a blunted total cortisol response to a supraphysiologic dose of ACTH (100 μg), best identifies septic foals with clinically relevant RAI/CIRCI.^{17,28}

Some studies in critically ill people, though, suggest that basal or ACTH-stimulated total cortisol concentrations can misrepresent physiologic cortisol status in some patients.²⁹⁻³⁴ In healthy people, approximately 90% of circulating cortisol is bound to plasma proteins, including cortisol binding globulin (CBG, approximately 95%) and albumin ($\leq 5\%$).^{29,30} The biologically

active, and thus more clinically relevant, form of cortisol is the smaller free (ie. non-protein bound) fraction. Since both CBG and albumin levels have been shown to decrease variably but significantly in critical illness,^{31,32} basal and ACTH-stimulated serum total cortisol measurements may not accurately reflect systemic cortisol status in critically ill patients. For instance, in hypoproteinemic patients, ACTH-stimulated serum total cortisol concentrations were subnormal, but basal and ACTH-stimulated serum free cortisol concentrations were normal or increased as compared to healthy controls.³¹ Even in healthy individuals, significant variation in serum CBG concentrations between individuals and within the same individual over time has been shown to significantly affect serum free cortisol responses to ACTH-stimulation tests.³⁰

Thus, in septic patients likely to have altered CBG and albumin concentrations, measurement of serum free cortisol concentration may provide a more accurate method for diagnosis of RAI/CIRCI than basal and ACTH-stimulated total cortisol concentration. A recent study comparing free and total plasma cortisol concentrations in people with sepsis and septic shock showed that free cortisol concentrations correlated better with illness severity scores than total cortisol concentrations.³² In addition, wide variation in ACTH-stimulated free cortisol increments among patients with sepsis, septic shock, and healthy controls was noted, despite minimal differences in total cortisol increments between these groups.³² Interestingly, in patients with septic shock who also met diagnostic criteria for RAI/CIRCI, both total and free cortisol responses after ACTH stimulation were lower than in patients not meeting criteria for RAI/CIRCI, though no difference in CBG or albumin concentration was present between these two groups.³²

To the authors' knowledge, basal or ACTH-stimulated serum free cortisol concentrations have not been evaluated in healthy or septic foals. Given recent evidence suggesting the

usefulness of free cortisol concentrations in determining adrenal function in humans with sepsis and/or RAI/CIRCI,²⁹⁻³⁴ and the lack of universal application of total cortisol parameters used for RAI/CIRCI diagnosis in people to septic foals,^{16-18,28} measurement of free cortisol may offer a more accurate method for assessment of HPA axis function in septic foals. Thus, the primary objectives for this study were: (1) to adapt an ultrafiltration assay for measurement of free cortisol in equine serum; 2) to compare free cortisol concentrations between healthy foals and adult horses, and between healthy and septic foals; (3) to determine relationships between free cortisol concentration and indicators of disease severity and outcome in septic foals; and (4) to compare the predictive value of free cortisol and total cortisol parameters for predicting disease severity and outcome in septic foals. We hypothesized that: (1) in septic foals, low basal and cosyntropin-stimulated free cortisol concentration will correlate with disease severity and survival; and (2) serum free cortisol concentrations will more accurately predict disease severity and outcome than serum total cortisol concentrations in septic foals.

MATERIALS AND METHODS

Animals

Three groups of animals were evaluated. The septic foal group included 51 client-owned neonatal foals that were ≤ 7 days of age at hospital admission and that met criteria for sepsis with either a positive blood culture or a sepsis score³⁵ ≥ 11 at admission. Septic foals receiving corticosteroids in the 24 hours prior to admission were excluded from the study population. Eleven healthy neonatal Quarter Horse foals from a herd maintained at the University of

Georgia's equine breeding facility were also evaluated at four time points during the first week of life to provide age-matched comparisons for the septic foal group. Each healthy foal was sampled within one hour of birth, at 12-24 hours, at 36-48 hours, and at 5-7 days of age.³⁶ All healthy foals were full term foals and were born without assistance. Foals in this group were determined to be healthy prior to and during inclusion in the study by lack of abnormalities identified on physical examination. Adequate transfer of passive immunity was confirmed by a serum immunoglobulin concentration ≥ 800 mg/dL at 12-24 hours of age in all healthy foals.^a Finally, 6 healthy adult horses from the University of Georgia's equine research herd were also evaluated once during the study period, to allow comparisons between healthy foals and adult horses.

Study methods were approved by the University's Institutional Animal Care and Use Committee and the College of Veterinary Medicine's Clinical Research Committee, and informed owner consent was obtained prior to foal enrollment in the septic foal group. Each healthy adult horse and healthy mare/ foal pair was stabled with daily paddock turnout during the study period, and was cared for according to the principles and guidelines stated in an Animal Use Protocol determined by the University of Georgia's Department of Animal Resources.

Study Design

In the 51 septic foals, blood was collected for measurement of basal serum total cortisol concentration (henceforth referred to as "total cortisol concentration") and basal serum free cortisol fraction (henceforth referred to as "free cortisol fraction") after study enrollment at hospital admission. A paired low-dose (10 μ g) / high-dose (100 μ g) cosyntropin^b (synthetic ACTH, α 1-24 corticotropin) stimulation test^{17,36} (henceforth referred to as "paired cosyntropin

stimulation test,” Figure 6.1) was also performed within the first 12 hours of hospital admission in 45/51 septic foals. Blood was collected for measurement of total cortisol concentration and free cortisol fraction before and 30 minutes after intravenous administration of 10 µg cosyntropin to assess the cortisol response to the low dose of cosyntropin. Ninety minutes after administration of the 10 µg dose, 100 µg cosyntropin was administered intravenously, and blood collected 30 and 90 minutes later to assess the cortisol response to the high dose of cosyntropin. Cosyntropin was administered and all blood samples for the paired cosyntropin stimulation test were collected through the foal’s indwelling jugular catheter. Detailed total cortisol findings from this group of septic foals have been reported previously.¹⁷

Clinical and clinicopathologic data were collected from the medical records of septic foals to determine the incidence of sepsis, shock, MODS, and nonsurvival in this population. Shock was considered present in foals that had at least two of the following at admission: (1) mean arterial pressure < 60 mmHg; (2) cold extremities; (3) weak peripheral pulses; (4) capillary refill time > 2 seconds; (5) altered mental status (e.g. marked depression or inability to stand, nurse, or track the mare); (6) rectal temperature < 99.0 degrees Fahrenheit (< 37.2 degrees Celsius); or (7) plasma lactate > 5 mmol/L.³⁷ MODS was defined as at least two of the following criteria present at any time during hospitalization: (1) anuria, oliguria or persistent azotemia (serum creatinine > 2.2 mg/dl for ≥ 72 hours) following initial fluid resuscitation and rehydration; (2) clinical diagnosis of neonatal encephalopathy; (3) respiratory dysfunction (hypoxemia or hypercapnea or both) requiring either nasal insufflation of oxygen or ventilation; (4) persistent ileus necessitating withholding of enteral feeding for > 24 hours; (5) > 24 hours of vasopressor therapy; or (6) a clinical diagnosis of disseminated intravascular coagulation.

Survival was defined as survival to hospital discharge; nonsurvival was defined as death or euthanasia for reasons of worsening disease or poor prognosis during hospitalization.

In the healthy foals, HPA axis function was assessed at the afore-mentioned four time points during the first week of life to provide age-matched comparisons with the hospitalized foal group. Time of birth was determined by use of a Foalert™^c system placed on each mare prior to the anticipated foaling date, and defined as the time at which the Foalert™ alarm was activated. At each age, blood was collected for measurement of basal serum total cortisol concentration and free cortisol fraction, and a paired cosyntropin stimulation test was performed as outlined above. Detailed total cortisol findings from this serial HPA axis assessment in these healthy foals have been reported previously.³⁶ In the 6 adult horses, blood was collected for measurement of basal total cortisol concentration and free cortisol fraction at one time point in each horse. Cosyntropin stimulation tests were not performed in adult horses. In healthy foals and adult horses, blood samples were collected and cosyntropin administered (foals only) by direct jugular venipuncture.

Blood for measurement of total cortisol concentration and free cortisol fraction was collected into a sterile glass tube without additives and allowed to clot at room temperature for 30-60 minutes, and then stored at 4 degrees Celsius until processing. All samples were centrifuged and the serum removed within 3 hours of collection, and stored frozen at -80 degrees Celsius until assays were performed.

Total and Free Cortisol Assays

Total cortisol concentrations were determined on an automated analyzer using a chemiluminescent enzyme immunoassay^d validated for use in the horse.^{38,39} The lower limit of detection of this assay is 0.2 µg/dL.^d

Free cortisol fraction was determined using a modification of an ultrafiltration/ligand-binding assay previously described in humans and pigs.^{40,41} Briefly, 0.1 µCi of [³H]-cortisol^e in ethanol was placed into 2 ml glass tubes and allowed to evaporate to dryness. Then, 400 µl of serum was added to each tube, vortexed and incubated at 37 degrees Celsius for 30 minutes for binding equilibration between labeled and unlabeled cortisol. Each equilibrated sample was then diluted with 400 µl of assay buffer (1X phosphate-buffered-saline + 0.1% gelatin). For measurement of total radioactivity, a 50 µl portion of this diluted serum sample was retained and placed into a scintillation vial. 400 µl of the diluted equilibrated serum was then placed into an ultrafiltration device^f that had been preconditioned with assay buffer, and centrifuged at 14,000 x g for 60 minutes at 25 degrees Celsius. For measurement of ultrafiltrate radioactivity, 50 µl of the ultrafiltrate was retained and placed into a scintillation vial. Radioactivity was measured in 2 ml of scintillation fluid^g in a liquid scintillation counter.^h Since total and ultrafiltrate radioactivity was measured in identical sample volumes within the same vehicle system, counting efficiency was similar for both measurements, so total counts per minute were not corrected for quench. Further, because undiluted serum samples were equilibrated with [³H]-cortisol, and a 1:2 dilution of equilibrated serum in assay buffer was performed immediately before ultrafiltration, dilution would have a minimal effect on cortisol binding kinetics. Thus, no correction of scintillation counts for sample dilution was performed.

Free cortisol fraction (FCF), percent free cortisol (% free cortisol) and estimated free cortisol concentration were calculated using the following formulas:

$$\text{FCF} = \text{ultrafiltrate radioactivity} / \text{total radioactivity}$$

$$\% \text{ free cortisol} = \text{FCF} \times 100\%$$

$$\text{estimated free cortisol concentration } (\mu\text{g/dl}) = \text{FCF} \times \text{total cortisol concentration } (\mu\text{g/dl})$$

Free Cortisol Assay Optimization

Because measurement of free cortisol fraction using ultrafiltration methodology has not been described in the horse, a series of preliminary experiments were carried out to optimize the reported methodology^{40,41} for equine samples. First, filtration efficiency was tested to ensure that significant binding of serum protein to the ultrafiltration device did not occur. Three 400 μl replicates of undiluted equine serum and 1:2, 1:5, and 1:10 dilutions of equine serum with 1X PBS were centrifuged at 14,000 x g for 30 and 60 minutes. Undiluted and 1:2 dilutions of equine serum were also centrifuged for 60 minutes at 10, 15, and 25 degrees Celsius to determine maximal filtration efficiency centrifugation temperature. Sample and ultrafiltrate weights were determined before and after filtration, and the recovery fraction was calculated by subtracting the ultrafiltrate weight from the sample weight. Filtration efficiency was maximized with a 60 minute centrifugation at 25 degrees Celsius and by diluting the serum sample at least 1:2 (data not shown).

Next, serial dilutions of [³H]-cortisol were prepared in assay buffer to ensure that significant binding of [³H]-cortisol to the filter did not occur with the above optimized ultrafiltration protocol. Total radioactivity was recorded in triplicate for each sample as above, and then triplicates of each preparation were placed into the ultrafiltration devices and

centrifuged for 60 minutes at 25 degrees Celsius. Ultrafiltrate radioactivity was determined, and the radioactivity recovery was calculated by dividing the ultrafiltrate radioactivity by the total radioactivity. Radioactivity recovery ranged from 87.3% to 93.3%, and was not significantly different between the various dilutions of [³H]-cortisol (P=0.235, data not shown). Thus, the previously described ultrafiltration protocol for free cortisol fraction determination^{40,41} was modified for use in the horse to include a 60 rather than 30 minute centrifugation at 25 degrees C and a 1:2 dilution of the original serum sample with assay buffer to maximize filtration efficiency.

The modified protocol was then tested using thawed equine serum samples to determine if pre-filtering the serum to remove large molecular aggregates associated with freezing further increased filtration efficiency. Duplicate serum samples were obtained and frozen for at least 96 hours at -80 degrees Celsius and then thawed at room temperature. The ultrafiltration assay was performed as described above, except one of the two samples was filtered through a sterile 22 micron 13 mm membrane filterⁱ prior to equilibration with [³H]-cortisol. The recovery fraction was determined from the sample and ultrafiltrate weights, and the FCF was calculated from the total and ultrafiltrate radioactivity as described above. The recovery fraction and the FCF did not differ significantly between unfiltered and pre-filtered samples (P=0.199 and P=0.841 respectively, data not shown); thus, pre-filtering the serum was not included in the final modified protocol.

Statistical Analysis

Basal total cortisol concentration, free cortisol fraction, and estimated free cortisol concentration were determined for all foals and adult horses. For clarity and consistency, free

cortisol fraction is henceforth expressed as % free cortisol. Serum cortisol responses to both the 10 µg and the 100 µg dose of cosyntropin were also determined for all healthy and septic foals that had a paired cosyntropin stimulation test performed. The low-dose delta total cortisol concentration (LDdelta total) was defined as the cortisol concentration reached 30 minutes after administration of 10 µg cosyntropin minus the immediate pre-cosyntropin (time 0) cortisol concentration. The high-dose delta cortisol total concentration (HDdelta total) was defined as the peak cortisol concentration reached either 30 or 90 minutes (whichever was higher) after administration of 100 µg cosyntropin minus the time 0 concentration. The low-dose and high-dose estimated delta free cortisol concentrations (LDdelta free, HDdelta free) were defined similarly using basal and cosyntropin-stimulated estimated free cortisol concentrations.

Total and free cortisol parameters were compared between healthy adult horses and healthy foals using the Mann Whitney U test. To test for an effect of age on free cortisol parameters in healthy foals, basal % free cortisol and estimated free cortisol concentration were compared among the four healthy foal age groups using a repeated measures analysis of variance, with multiple comparisons conducted using Tukey's test.

Total and free cortisol parameters were compared similarly between healthy and septic foals using the Mann Whitney U test. Because previous work has shown a significant effect of age on basal total cortisol concentrations and on total cortisol responses to exogenous ACTH in neonatal foals during the first week of life,^{36,42,43} all comparisons between the healthy and septic foal groups were age-matched. Based on findings from these previous studies,^{36,42,43} septic foals were divided into 4 age groups: (A) ≤ 4 hours; (B) > 4 hrs to < 30 hours; (C) ≥ 30 hours to < 3 days; and (D) ≥ 3 days to ≤ 7 days of age; data from age groups A, B, C, and D were compared with healthy foals at birth, 12-24 hours, 36-48 hours, and 5-7 days of age, respectively.

Finally, within the septic foal group, data were further stratified using the Mann Whitney U test to compare total and free cortisol parameters between septic foals that did and did not meet criteria for shock, MODS, and nonsurvival. Receiver operator characteristic (ROC) curves were constructed to compare the predictive value of basal total cortisol concentration with basal free cortisol concentration, and LD- and HDdelta total cortisol concentrations with LD- and HDdelta free cortisol concentrations for predicting disease severity and outcome in septic foals.

Hypothesis tests were two-tailed and statistical significance was set at $P < 0.05$ for all analyses. Statistical analyses were performed using commercial statistical software.^{j,k}

RESULTS

Study Population Characteristics

Breed, sex and age/gestational age distribution and body weight ranges for healthy and septic foals and for healthy adult horses are shown in Table 6.1. Body weight was not significantly different between the septic and healthy foals groups ($P=0.498$). Reasons for hospital admission for septic foals are also detailed in Table 6.1.

Septic foals were divided into the previously described age groups for comparison with healthy foals.³⁶ Eleven septic foals fit age group A (≤ 4 hours of age), 12 foals fit age group B (>4 to <30 hours of age), 23 foals fit age group C (≥ 30 hours to <3 days of age) and 5 foals fit age group D (≥ 3 hours to ≤ 7 days of age).

Basal Total and Free Cortisol in Healthy Foals and Adult Horses

Basal total and free cortisol data for healthy foals and adult horses are shown in Table 6.2. A significant effect of age on basal total cortisol concentration, as described previously,^{36,42,43} as well as on % free cortisol and estimated free cortisol concentration, was found in healthy foals ($P < 0.001$ for all analyses), with the highest values for all parameters seen in foals at birth. Total cortisol concentration was not significantly different between healthy foals and adult horses ($P = 0.159$ to $P = 0.428$) except in foals at birth, in which it was significantly higher ($P < 0.001$). However, healthy foals had significantly higher % free cortisol and estimated free cortisol concentration than adult horses at all ages ($P < 0.001$ to $P = 0.004$).

Basal and Cosyntropin-Stimulated Total and Free Cortisol in Healthy and Septic Foals

Basal and cosyntropin-stimulated total and free cortisol data from healthy and septic foals are shown in Table 6.3. Basal total cortisol concentration was significantly higher in septic foals than in healthy foals at all ages except in the youngest foals (≤ 4 hours of age). Basal estimated free cortisol concentration was significantly higher in septic foals in age groups B (> 4 to ≤ 30 hours) and C (> 30 hours to < 3 days) but was not significantly different in the youngest (≤ 4 hours) or oldest (≥ 3 to ≤ 7 days) septic foals. LDdelta and HDdelta total cortisol concentrations and LDdelta and HDdelta estimated free cortisol concentrations were not significantly different between healthy and septic foals in any age groups.

Disease Severity and Outcome in Septic Foals

Basal and cosyntropin-stimulated total and free cortisol data for septic foals that did and did not meet criteria for shock, MODS, and non-survival are shown in Table 6.4. Septic foals that met criteria for shock and for non-survival had significantly higher basal total and free cortisol concentrations, and significantly lower HDdelta total and free cortisol concentrations. Septic foals that met criteria for MODS had significantly lower LDdelta total and free cortisol concentrations, but no significant differences in any other total or free cortisol parameters.

ROC Curve Comparisons in Septic Foals

ROC curves and corresponding areas under the curves (AUCs) used to compare the predictive value of basal and cosyntropin-stimulated total cortisol concentrations and % free cortisols for predicting disease severity and non-survival in septic foals are shown in Figure 6.2 and Table 6.5 respectively. No significant differences in the AUC were found between any total and free cortisol parameters; thus, there was no meaningful predictive advantage of measuring basal or cosyntropin-stimulated free cortisol concentration over total cortisol concentration for predicting shock, MODS, or non-survival in septic foals.

DISCUSSION

The above findings illustrate unique plasma cortisol binding dynamics in foals. While % free cortisol in adult horses, at approximately 5-10%, was consistent with previous reports⁴⁴ and findings in other species,^{29,30,40,41,45} the proportion of cortisol present in the free fraction in

healthy neonatal foals throughout the first week of life was three-to-six-fold higher than in adult horses, ranging from approximately 60% at birth to approximately 30% by 5-7 days of age.

Total cortisol concentrations were significantly higher in foals at birth than in adult horses, but no difference in total cortisol concentration was found between adult horses and foals 12 hours to 7 days of age. Thus, the substantial increases in % free cortisol and estimated free cortisol concentration observed in healthy foals is due to a dramatic reduction in plasma cortisol binding capacity in foals rather than an overall increase in total cortisol concentration.

As described previously, plasma cortisol is primarily bound to cortisol binding globulin (CBG), with a small percentage (<5%) bound to albumin.^{29,30} Given that only a small fraction of cortisol is bound to albumin, the reduction in plasma cortisol binding capacity found in foals in this study is most likely due to decreased levels of serum CBG in foals as compared to adult horses. Unfortunately, to the authors' knowledge, an assay for equine CBG is not currently available to confirm this theory. Reductions in CBG concentrations and concomitant increases in plasma free cortisol fraction from adult levels have also been documented in premature and full-term infants.^{46,47} However, in full-term infants, % free cortisol ranged from 32% at birth to 19% by 3 months of age,⁴⁶ levels approximately half of those found in foals during the first week of life in the study herein. Thus, while a decrease in plasma cortisol binding capacity associated with low plasma CBG concentrations may be common to both human and equine neonates, the plasma cortisol binding capacity in healthy neonatal foals is still substantially less than that of human infants.

In addition, the findings above show that septic foals > 4 hours of age have significantly higher basal total cortisol concentrations and estimated free cortisol concentrations than healthy age-matched foals. Such an increase in total cortisol concentrations is appropriate and expected

with HPA-axis activation by the physiologic stresses of sepsis, and is consistent with previous reports.¹⁶⁻¹⁸ Due to the neonatal foal's limited plasma cortisol binding capacity as described above, this stress-induced increase in cortisol secretion most likely results in saturation of limited plasma protein cortisol binding sites and thus also leads to an increase in estimated free cortisol concentration. Concurrent suppression of CBG production in septic foals due to negative acute phase effects^{31,32} may also contribute to reduced plasma cortisol binding capacity in septic foals.

Despite the above differences in basal total and free cortisol between healthy and septic foals, total and free cortisol responses to cosyntropin were similar between healthy and septic foals. Further stratification of the data in septic foals by disease severity and outcome, though, illustrated higher basal total cortisol and estimated free cortisol concentrations, and lower total and free delta cortisol responses to cosyntropin stimulation in septic foals with shock and non-surviving foals as compared to septic foals that did not have shock and survivors. Thus, low basal free cortisol concentration was not correlated with more severe disease or decreased survival as initially hypothesized. These findings for free cortisol are consistent with total cortisol findings described previously,¹⁷ and these consistencies observed between basal and cosyntropin-stimulated total and free cortisol are likely due to the fact that such a large proportion of total cortisol is present in the free form in foals. As discussed above, the increase in basal total and free cortisol in the sickest and non-surviving foals are consistent with stress-induced HPA-axis activation, and are not unexpected. The lower delta cortisols in the sickest and non-surviving foals may indicate that adrenocortical cortisol synthesis is already at maximal capacity and further trophic hormone stimulation can not increase cortisol concentrations any further (i.e, "loss of adrenal reserve"). Alternatively, adrenal sensitivity to ACTH may be reduced in critical illness, resulting in this limited adrenocortical cortisol response.

The results herein also do not support the second hypothesis, that free cortisol concentration better predicts disease severity and death in septic foals than total cortisol concentration. This was unexpected, and differs from human studies showing better correlations between basal and cosyntropin-stimulated free cortisol concentrations and disease severity and outcome than with total cortisol.²⁹⁻³⁴ Again, this difference between foals and humans is most likely due to the dramatically higher free cortisol fraction in foals. In humans, with $\leq 10\%$ free cortisol, a small increase in the free cortisol fraction is likely to have substantial physiologic effects as more biologically active cortisol is available. In foals, though, with such a large free cortisol fraction even in health, a small increase in this free fraction may have minimal or no physiologic effects if intracellular cortisol receptors are already saturated. Thus, this substantially larger free cortisol fraction in foals as compared to people explains the lack of predictive advantage of measuring free cortisol versus total cortisol in septic foals as compared to septic people.

Given this large free cortisol fraction in foals, one may wonder if the occurrence of CIRCI is then even possible in septic foals, since so much cortisol is theoretically biologically available to cross cell membranes and interact with the cytoplasmic cortisol receptor to induce relevant physiologic effects in response to stress. However, while it initially may seem counterintuitive, the reduced plasma cortisol binding capacity and substantially increased free cortisol fraction in the neonatal foal have several important consequences that may make foals at even greater risk for CIRCI. Circulating free cortisol is preferentially metabolized and eliminated via renal excretion and hepatic conjugation over bound cortisol;⁴⁸ therefore, bound cortisol essentially provides a reservoir of cortisol in the plasma, and decreased plasma cortisol binding capacity may result in more rapid cortisol clearance and could contribute to relative

cortisol insufficiency in illness.⁴⁹ In addition, CBG appears to play a vital role in the mediation of some of cortisol's physiologic effects, by promoting delivery and release of cortisol to specific steroid-responsive target tissues and sites of inflammation and by direct interactions with cell surface receptors that bind CBG-cortisol complexes.⁵⁰⁻⁵² Recent work also documents hyporesponsiveness to glucocorticoids and an aggravated response to septic shock in mice that are genetically deficient for CBG.⁵³ Thus, the substantially decreased plasma cortisol binding capacity observed in septic foals may limit rather than enhance the biologic activity of cortisol in stress responses by increasing cortisol clearance and impairing CBG-cortisol-complex-mediated cellular and physiologic responses. Ultimately, this may impair cortisol-mediated physiologic responses to sepsis and contribute to the pathogenesis of CIRCI in septic neonatal foals.

In sum, the study herein documents substantially greater plasma free cortisol fraction in neonatal foals as compared to adult horses and both adult and neonatal humans. This increased free cortisol fraction is the result of reduced plasma cortisol binding capacity in foals as compared to adult horses, presumably due to decreased CBG concentrations (though further study is needed to confirm this). In septic foals in this study, basal and ACTH-stimulated free cortisol concentrations were no better than total cortisol concentrations for predicting disease severity and outcome. Thus, measurement of basal or ACTH-stimulated free cortisol appears to offer no advantage over measurement of ACTH-stimulated total cortisol for diagnosis of CIRCI or prognostication in septic foals. Further study of cortisol concentrations and cosyntropin-stimulation test responses over time in critically ill foals, and documentation of CBG concentrations and cortisol clearance rates in healthy and septic neonatal foals, is necessary to better understand the relationship between plasma unique cortisol binding dynamics on the pathogenesis of CIRCI in septic foals.

FOOTNOTES

^a SNAP® Foal IgG Test, IDEXX Laboratories, Inc., Westbrook, ME

^b Cortrosyn™, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA

^c Foal Alert, Inc., Atlanta, GA

^d Immulite™, Diagnostics Product Corporation, Los Angeles, CA

^e Amersham Radiochemicals, GE Healthcare Life Sciences, Pittsburgh, PA

^f Millipore, Billerica, MA

^g Scintiverse BD Cocktail, Fisher Scientific, Pittsburgh, PA

^h LS 6500 Multipurpose Scintillation Counter, Beckman Coulter, Brea, CA

ⁱ Acrodisc 0.2µm 12mm Supor Membrane Syringe Filter, Pall, Port Washington, NY

^j GraphPad Prism Statistical Software (Version 4), GraphPad Software Inc., San Diego, CA

^k Stata (Version 11.0), StataCorp LP, College Station, TX

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Table 6.1. Animal group characteristics.

Group	Sex	Breed(s)	Age (horses) or Gestational Age (foals)	Reason for Admission <i>(note: some foals had > 1 complaint)</i>	Weight (kg) (range)
Septic Foals (n=51)	30 male 21 female	<ul style="list-style-type: none"> • Thoroughbred (n=21) • Quarter Horse (n=10) • Warmblood (n=4) • Tennessee Walking Horse (n=4) • Saddlebred (n=4) • QH cross (n=2) • Arabian (n=1) • Irish Draft (n=1) • Cleveland Bay (n=1) • Morgan (n=1) • Shetland Pony (n=1) • Donkey (n=1) 	305 days (n=1) 315-320 days (n=3) 321-329 days (n=4) 330-345 days (n=32) 346-359 days (n=2) 360-370 days (n=3) Unknown (n=6)	<ul style="list-style-type: none"> • Recumbency (n=12) • Failure to nurse well (n=10) • Weakness/lethargy (n=12) • Colic (n=13) • Diarrhea (n=12) • Post-dystocia or cesarean section (n=6) • Failure of transfer of passive immunity (n=5) • Premature parturition (≤ 320 days, n=3) • Joint swelling (n=1) • Premature placental separation (n=2) • Orphans (n=2) • Agalactia in the dam (n=2) • Seizures (n=1) • Fever (n=1) • Inguinal hernia (n=1) • Surviving twin (n=1) • Severe extensor tendon contracture (n=1) 	47.6 \pm 13.1 (11.8 – 79.5)
Healthy Foals (n=11)	6 male 5 female	Quarter Horse (n=11)	≥ 330 days (n=11)	n/a	50.4 \pm 7.5 (38.7 – 61.4)
Healthy Horses (n=6)	4 male 2 female	Quarter Horse (n=6)	2-11 years	n/a	n/a

Table 6.2. Basal total cortisol concentration, basal % free cortisol, and basal free cortisol concentration in healthy foals (n=11) at four ages during the first week of life and in healthy adult horses (n=6).

	Basal Total Cortisol ($\mu\text{g}/\text{dl}$)	Basal % Free Cortisol (%)	Basal Free Cortisol ($\mu\text{g}/\text{dl}$)
Healthy Foals, at birth	$10.2 \pm 2.3^{a*}$ (7.6 – 13.5)	$58 \pm 8^{a*}$ (41 – 66)	$6.0 \pm 1.8^{a*}$ (3.1 – 8.9)
Healthy Foals, 12-24 hours old	3.6 ± 1.6^b (2.1 – 6.7)	$43 \pm 6^{b*}$ (32 – 49)	$1.9 \pm 1.5^{b*}$ (0.9 – 5.5)
Healthy Foals, 36-48 hours old	2.4 ± 1.0^b (1.3 – 4.4)	$46 \pm 10^{b*}$ (32 – 66)	$1.1 \pm 0.5^{b*}$ (0.5 – 2.1)
Healthy Foals, 5-7 days old	2.0 ± 0.8^b (1.0 – 2.7)	$33 \pm 6^{c*}$ (25 – 41)	$0.6 \pm 0.3^{b*}$ (0.3 – 1.2)
Healthy Adult Horses	2.8 ± 1.1 (1.7 – 4.3)	7 ± 3 (5 – 12)	0.2 ± 0.2 (0.1 – 0.5)

Within a column, values with different letter superscripts are significantly different between individual foal age groups ($P < 0.05$). *Denotes values significantly different between foals and adult horses ($P < 0.05$). Data are reported as the mean \pm standard deviation. Numbers in parentheses represent the data range.

Table 6.3. Basal and cosyntropin-stimulated total and free cortisol concentrations in age-matched healthy and septic foals. Cosyntropin-stimulated values (LDdelta and HDdelta parameters) were determined in response to a paired low (10 µg) and high (100 µg) dose cosyntropin stimulation test.³⁶ Foals were divided into four age groups for age-matched comparisons: (A) ≤ 4 hours of age; (B) > 4 to < 30 hours of age; (C) ≥ 30 hours to < 4 days of age; and (D) ≥ 4 to ≤ 7 days of age.

		Healthy Foals (n=9)	Septic Foals (n=11)	P value
Age Group A	Basal Total Cortisol (µg/dl)	10.2 ± 2.3 (7.6 – 13.5)	10.4 ± 4.5 (3.3 – 18.7)	0.849
	Basal Free Cortisol (µg/dl)	6.0 ± 1.8 (3.1 – 8.9)	5.7 ± 2.8 (1.8 – 11.2)	0.621
	LDdelta Total Cortisol (µg/dl)	3.6 ± 2.2 (0.4 – 8.2)	1.5 ± 3.8 (-3.1 – 7.7)	0.133
	LDdelta Free Cortisol (µg/dl)	1.4 ± 1.3 (-0.3 – 4.2)	1.0 ± 2.2 (-1.3 – 4.5)	0.408
	HDdelta Total Cortisol (µg/dl)	6.3 ± 4.3 (0.3 – 15.5)	4.6 ± 7.1 (-3.2 – 16.5)	0.460
	HDdelta Free Cortisol (µg/dl)	2.4 ± 2.8 (-0.9 – 7.3)	3.1 ± 4.7 (-1.9 – 9.8)	0.481
		Healthy Foals (n=10)	Septic Foals (n=12)	P value
Age Group B	Basal Total Cortisol (µg/dl)	3.6 ± 1.6 (2.1 – 6.7)	11.5 ± 8.8* (2.0 – 36.2)	0.003
	Basal Free Cortisol (µg/dl)	1.9 ± 1.5 (0.9 – 5.5)	6.6 ± 6.1* (0.5 – 23.2)	0.006
	LDdelta Total Cortisol (µg/dl)	5.5 ± 2.1 (2.8 – 9.4)	7.3 ± 6.4 (-0.6 – 23.2)	0.337
	LDdelta Free Cortisol (µg/dl)	3.1 ± 1.9 (-1.0 – 5.3)	4.2 ± 3.7 (-0.3 – 13.0)	0.619
	HDdelta Total Cortisol (µg/dl)	9.4 ± 3.5 (4.6 – 15)	12.8 ± 10.2 (1.9 – 36)	0.692
	HDdelta Free Cortisol (µg/dl)	5.6 ± 2.6 (1.5 – 9.7)	7.5 ± 5.3 (0.6 – 18.8)	0.234
		Healthy Foals (n=10)	Septic Foals (n=23)	P value
Age Group C	Basal Total Cortisol (µg/dl)	2.4 ± 1.0 (1.3 – 4.4)	13.4 ± 16.0* (0.5 – 62)	0.006
	Basal Free Cortisol (µg/dl)	1.1 ± 0.5 (0.5 – 2.1)	8.1 ± 10.6* (0.1 – 39.6)	0.021
	LDdelta Total Cortisol (µg/dl)	3.5 ± 1.7 (1.6 – 6.8)	3.9 ± 3.4 (-3.5 – 10.9)	0.688
	LDdelta Free Cortisol (µg/dl)	2.0 ± 1.0 (0.7 – 3.8)	1.9 ± 2.3 (-4.3 – 6.6)	0.916
	HDdelta Total Cortisol (µg/dl)	6.8 ± 3.3 (3.2 – 12.7)	6.8 ± 5.1 (-4.0 – 16.4)	1.00
	HDdelta Free Cortisol (µg/dl)	4.2 ± 2.1 (2.0 – 7.6)	3.9 ± 3.4 (-3.6 – 11.2)	0.425

Table 6.3 continued.

		Healthy Foals (n=11)	Septic Foals (n=5)	P value
Age Group D	Basal Total Cortisol (µg/dl)	2.0 ± 0.8 (1.0 – 3.6)	16.9 ± 12.8* (1.6 – 30)	0.024
	Basal Free Cortisol (µg/dl)	0.6 ± 0.3 (0.3 – 1.2)	9.3 ± 6.9 (0.3 – 16.3)	0.062
	LDdelta Total Cortisol (µg/dl)	1.3 ± 0.6 (0.6 – 2.6)	5.2 ± 6.9 (-1.1 – 15.0)	0.171
	LDdelta Free Cortisol (µg/dl)	0.7 ± 0.5 (0.1 – 1.8)	3.9 ± 3.9 (-0.4 – 8.6)	0.171
	HDdelta Total Cortisol (µg/dl)	3.5 ± 1.3 (1.7 – 5.9)	2.1 ± 6.2 (-5.3 – 8.4)	0.948
	HDdelta Free Cortisol (µg/dl)	2.2 ± 1.7 (1.0 – 6.9)	1.3 ± 4.2 (-3.5 – 5.7)	0.424

Data are shown as mean ± SD (range). *Denotes a significant difference ($P < 0.05$) between age-matched healthy and septic foals.

Table 6.4. Total and free cortisol parameters in septic foals that did or did not meet criteria for shock, MODS, or non-survival. Basal total and free cortisol data were available from 51 foals, and cosyntropin stimulation test results (LDdelta and HDdelta total and free cortisol) were available from 45 foals.

		Basal Total Cortisol (µg/dl)	Basal Free Cortisol (µg/dl)	LDdelta Total Cortisol (µg/dl)	LDdelta Free Cortisol (µg/dl)	HDdelta Total Cortisol (µg/dl)	HDdelta Free Cortisol (µg/dl)
Shock	Yes (n=36)	15.2 ± 13.1* (1.0 – 62.0)	9.2 ± 8.5* (0.7 – 39.6)	4.0 ± 5.7 (-3.5 – 23.2)	2.4 ± 3.5 (-4.3 – 13)	6.2 ± 8.1* (-5.3 – 36)	3.5 ± 4.8* (-3.6 – 18.8)
	No (n=15)	6.5 ± 7.2 (0.5 – 28.8)	2.4 ± 2.1 (0.2 – 6.7)	5.4 ± 2.9 (1.3 – 10.9)	3.1 ± 1.7 (0.8 – 6.6)	10.8 ± 6.0 (1.8 – 26.8)	6.9 ± 3.3 (1.5 – 13.8)
		<i>P</i> = 0.001	<i>P</i> < 0.001	<i>P</i> = 0.170	<i>P</i> = 0.155	<i>P</i> = 0.022	<i>P</i> = 0.009
MODS	Yes (n=18)	11.1 ± 12.4 (1.0 – 49.9)	8.8 ± 11.0 (0.2 – 39.6)	1.9 ± 3.2* (-3.5 – 9.3)	1.3 ± 2.8* (-4.3 – 6.9)	5.4 ± 5.8 (-4.0 – 16.5)	3.5 ± 4.1 (-3.6 – 11.2)
	No (n=29)	11.6 ± 8.6 (0.5 – 30.6)	6.5 ± 5.4 (0.1 – 19.4)	5.7 ± 5.3 (-3.1 – 23.2)	3.3 ± 3.1 (-2.2 – 13.0)	8.6 ± 8.5 (-5.3 – 36.0)	5.0 ± 4.9 (-3.5 – 18.8)
		<i>P</i> = 0.400	<i>P</i> = 0.472	<i>P</i> = 0.010	<i>P</i> = 0.023	<i>P</i> = 0.165	<i>P</i> = 0.262
Non-survival	Yes (n=18)	19.4 ± 15.9* (3.3 – 62.0)	11.5 ± 10.2* (1.8 – 39.6)	3.2 ± 5.2 (-3.5 – 15.0)	1.8 ± 3.3 (-4.3 – 8.6)	3.8 ± 6.9* (-5.3 – 16.5)	1.9 ± 3.9* (-3.6 – 9.8)
	No (n=33)	9.0 ± 8.0 (0.5 – 36.2)	4.9 ± 5.0 (0.2 – 23.2)	4.9 ± 4.9 (-1.9 – 23.2)	2.9 ± 2.9 (-2.2 – 13)	9.1 ± 7.7 (-2.3 ± 36.0)	5.5 ± 4.4 (-1.2 – 18.8)
		<i>P</i> = 0.003	<i>P</i> = 0.001	<i>P</i> = 0.226	<i>P</i> = 0.323	<i>P</i> = 0.030	<i>P</i> = 0.014

* Denotes a significant difference (*P* < 0.05) in the specified parameter in septic foals that met specific criteria for shock, MODS, or non-survival, as compared to septic foals not meeting that specific criterion.

Table 6.5. Areas under the curve (AUCs) for receiver operator characteristic curves comparing the predictive value of basal and ACTH-stimulated total versus free cortisol concentration for predicting shock, MODS, and non-survival in septic foals (n=51).

	Shock (AUC ± SE)	MODS (AUC ± SE)	Non-survival (AUC ± SE)
Basal Total Cortisol (µg/dl)	0.838 ± 0.056	0.425 ± 0.088	0.759 ± 0.072
Basal Free Cortisol (µg/dl)	0.857 ± 0.057	0.441 ± 0.088	0.755 ± 0.071
	P = 0.291	P = 0.413	P = 0.772
LDdelta Total Cortisol (µg/dl)	0.631 ± 0.084	0.696 ± 0.093	0.621 ± 0.103
LDdelta Free Cortisol (µg/dl)	0.638 ± 0.083	0.719 ± 0.091	0.599 ± 0.107
	P = 0.918	P = 0.158	P = 0.626
HDdelta Total Cortisol (µg/dl)	0.707 ± 0.079	0.652 ± 0.096	0.725 ± 0.096
HDdelta Free Cortisol (µg/dl)	0.727 ± 0.076	0.623 ± 0.096	0.753 ± 0.084
	P = 0.425	P = 0.191	P = 0.254

No significant differences ($P < 0.05$) in the AUCs between total and free cortisol parameters were found.

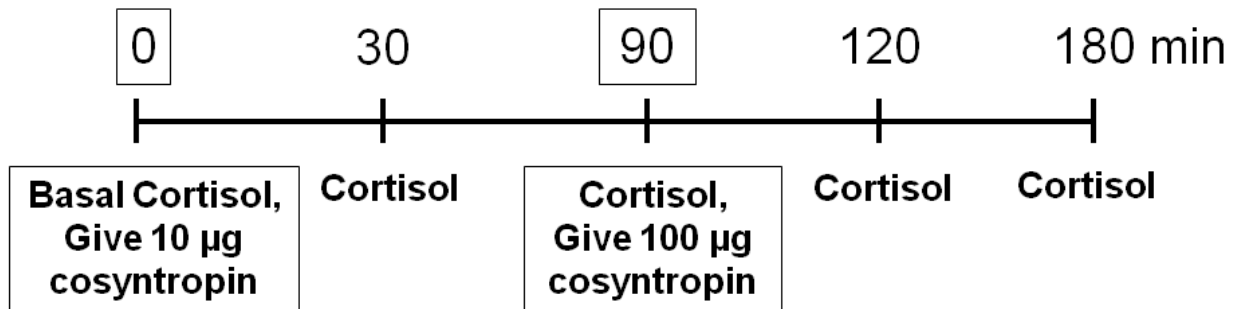


Figure 6.1. Paired low and high dose synthetic ACTH stimulation test design utilized in healthy and septic foals. At time 0, blood was collected for measurement of pre-stimulation cortisol concentration, and 10 µg cosyntropin was administered intravenously. Blood was again collected 30 minutes later to assess peak cortisol response to the low (10 µg) cosyntropin dose. At 90 minutes, blood was again collected for measurement of cortisol concentration (to ensure cortisol concentrations returned to basal levels) and 100 µg cosyntropin was administered intravenously. Blood was then collected 30 and 90 minutes later (at times 120 and 180 minutes) to assess peak cortisol response to the high (100 µg) cosyntropin dose. *[Note, a version of this figure was previously published in J Vet Int Med 2009, 23:901-912.]*

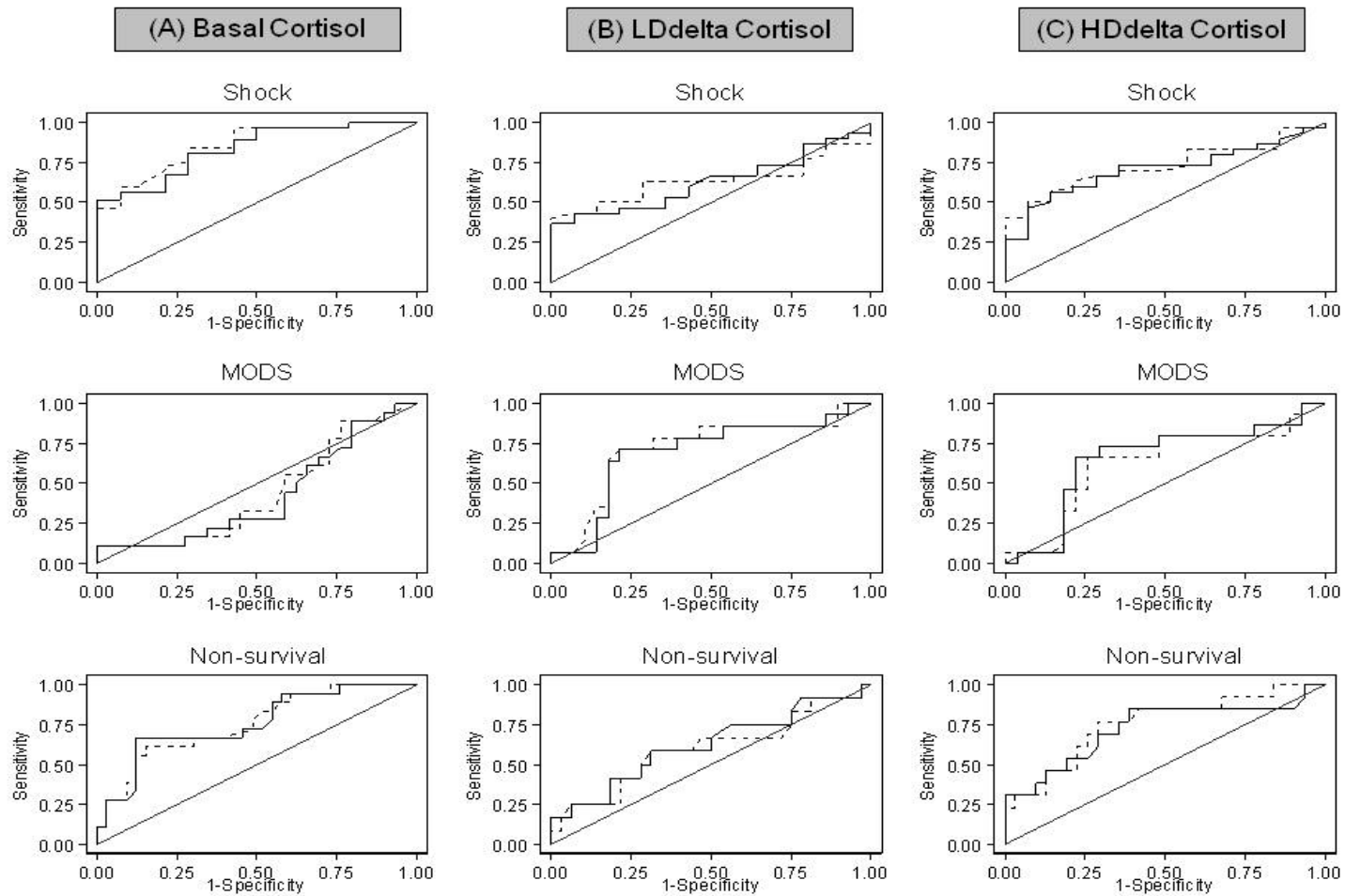


Figure 6.2. Receiver operator characteristic (ROC) curves for (A) basal total and free cortisol concentration, (B) LDdelta total and free cortisol concentration, and (C) HDdelta total and free cortisol concentration for predicting shock, MODS, and non-survival in 51 septic foals. In all curves, total cortisol parameters are represented by solid lines and free cortisol parameters by dashed lines. The diagonal line in each curve represents a hypothetical curve with an area under the curve (AUC) of 0.5.

CHAPTER 7

DAILY ENDOGENOUS CORTISOL PRODUCTION AND HYDROCORTISONE PHARMACOKINETICS IN ADULT HORSES AND NEONATAL FOALS¹

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ABSTRACT

Objective: To compare daily endogenous cortisol production rate (PR) and the pharmacokinetics of an intravenous bolus of hydrocortisone (1 mg/kg) between neonatal foals and adult horses.

Animals: 10 healthy full-term 2-to-4-day-old foals and 7 healthy adult horses

Procedures: Blood was collected every 15-20 minutes for 24 hours from each animal for determination of 24-hour mean cortisol concentration. Then, 1 mg/kg of hydrocortisone was given intravenously 12 hours after suppression of endogenous cortisol production with dexamethasone (0.08 mg/kg IV) and serial blood samples were collected to determine hydrocortisone pharmacokinetics. Cortisol and hydrocortisone concentrations were determined using a previously validated chemiluminescent immunoassay, and daily cortisol production rate (PR) determined with deconvolution analysis. Data were compared between adult horses and foals with Mann Whitney tests (significance $P < 0.05$).

Results: Mean 24-hour cortisol concentration was significantly lower in foals (20 ± 4 ng/ml) than horses (26 ± 6 ng/ml), but daily cortisol PR was significantly greater in foals (6710 ± 320 ng/kg/day) than horses (2150 ± 400 ng/kg/day). Foals had significantly greater hydrocortisone volume of distribution and clearance and significantly lower peak hydrocortisone plasma concentrations ($V_{d_{ss}} = 1.92 \pm 1.11$ L/kg; $Cl_s = 1.39 \pm 0.11$ L/kg/hr; peak = 1051 ± 343 ng/ml) than horses ($V_{d_{ss}} = 0.58 \pm 0.15$ L/kg; $Cl_s = 0.35 \pm 0.07$ L/kg/hr; peak = 8934 ± 3843 ng/ml).

Conclusions and Clinical Relevance: These findings illustrate important differences in cortisol production and metabolism between neonatal foals and adult horses that reflect reduced plasma protein binding of cortisol in foals, and may contribute to cortisol insufficiency during prolonged

critical illness in neonatal foals. In addition, the data herein may facilitate development of a hydrocortisone replacement therapy regimen for use in some critically ill foals.

INTRODUCTION

The appropriate endocrine response to the physiologic stress of acute illness involves activation of the hypothalamic-pituitary-adrenal (HPA) axis and culminates with the synthesis and secretion of the glucocorticoid hormone cortisol from the adrenal glands. Cortisol has a number of diverse systemic effects that collectively serve to decrease physiologic and pathophysiologic stress associated with illness, including maintenance of blood pressure, metabolic effects, and regulation of the local and systemic inflammatory response. Circulating cortisol concentrations increase rapidly in response to stress, and then, via negative feedback mechanisms at hypothalamic and pituitary levels of the HPA axis, also serve to modulate further cortisol production, thus maintaining systemic cortisol concentrations in a range appropriate for the ongoing degree of stress.

However, in many critically ill patients, cortisol concentrations are inadequate for the existing level of pathophysiologic stress, a syndrome termed critical illness-related corticosteroid insufficiency (CIRCI). CIRCI can result from impairment of the HPA axis at any of several levels, including: 1) inadequate regulatory hormone secretion from the hypothalamus and/or pituitary gland; 2) adrenocortical dysfunction resulting in limited cortisol synthetic capacity; or 3) decreased glucocorticoid sensitivity or activity in the peripheral tissues.^{1,2} CIRCI has been described in both humans^{1,3-6} and animals⁷⁻¹⁰ and is documented in approximately 50% of septic people^{2,11,12} and septic foals.¹³ In addition, CIRCI is correlated with increased incidence of shock, multiple organ dysfunction syndrome (MODS), and death in critically ill people, dogs and foals.^{1-3,7,9,13}

A number of studies in people,¹⁴⁻¹⁷ including 3 meta-analyses,¹⁸⁻²⁰ have demonstrated improved outcomes in septic patients with CIRCI when they are provided with glucocorticoid supplementation in the form of low-dose hydrocortisone replacement therapy. The general premise for hydrocortisone replacement therapy during CIRCI is to provide synthetic cortisol in the form of hydrocortisone in an amount comparable to an appropriate cortisol response in a patient with intact HPA axis function.^{2,21-23} However, it is very difficult to predict the specific cortisol response to critical illness that would be ideal for each individual patient.

Hydrocortisone dosage recommendations used in human critical care thus vary widely, though current recommendations for hydrocortisone replacement therapy regimens in septic adults consist of either a 50 mg IV bolus every 6 hours (approximately 2.5- 3 mg/kg/day) or an 100 mg loading dose followed by a continuous intravenous infusion of 10 mg/hr (approximately 4-6 mg/kg/day).^{15,23} Hydrocortisone replacement therapy protocols for use in neonatal and pediatric septic shock are less well defined; current recommendations range from 2-50 mg/kg/day as a continuous infusion or intermittent bolus dosing.²⁴ The evolution of such dosing strategies was based on the maximal cortisol secretory rate in healthy people in response to exogenous HPA axis stimulation with cosyntropin²⁵ or by multiplying the daily endogenous cortisol production rate (PR) in healthy, unstressed individuals by a variable “illness factor” to take into account appropriate increases in cortisol in response to the stress of severe illness.²⁶

While septic neonatal foals with CIRCI may also potentially benefit from hydrocortisone replacement therapy, such therapy has not been critically evaluated in foals. Given the marked differences in HPA axis function and cortisol dynamics in foals as compared to adult humans and human neonates,^{27-31,a} hydrocortisone replacement therapy regimens used in people may not be directly applicable to foals. In addition, to the authors’ knowledge, daily endogenous cortisol

PR and hydrocortisone pharmacokinetics have been investigated in adult horses in just two studies,^{32,33} and have not been evaluated in foals to date.

Thus, the purpose of this study was to determine daily endogenous cortisol production rate and hydrocortisone pharmacokinetics and compare these parameters between adult horses and foals, with the ultimate aim of determining an appropriate hydrocortisone replacement therapy regimen for future study in septic foals with CIRCI. We hypothesized that daily endogenous cortisol production rate and hydrocortisone pharmacokinetic parameters would differ significantly between adult horses and foals.

MATERIALS AND METHODS

Animals

Ten healthy 2-to-5-day-old Quarter Horse foals (4 males and 6 females) from research herds maintained at the University of Georgia's and Clemson University's equine breeding facilities were used in this study. All foals were full term foals (gestational age ≥ 330 days) and were born via unassisted vaginal delivery. Foaling was observed and accurate foal age known in 3 foals. The remaining 7 foals were born during the night to mares kept on pasture, so foal age was estimated within approximately 12 hours for these foals. Foals were determined to be healthy prior to and during inclusion in the study by lack of abnormalities identified on physical examination. Adequate transfer of passive immunity was confirmed by a serum immunoglobulin concentration ≥ 800 mg/dl^b at 12-24 hours of age in all foals. Six foals (3 males and 3 females) were used to determine an appropriate dexamethasone suppression protocol for use in this study,

and 24-hour cortisol production and hydrocortisone pharmacokinetics were determined in the remaining 4 foals (1 male, 3 females).

In addition, 7 healthy adult horses from the University of Georgia's equine research herd were sampled to allow comparisons between foals and adult horses. Four horses aged 5-12 years were used for the determination of 24-hour cortisol production, and included a Warmblood gelding, a Paint gelding, a Quarter Horse stallion, and a Paint stallion. Three of these horses were not available for immediate determination of hydrocortisone pharmacokinetics the day following 24-hour cortisol production assessment as was performed in foals, so three additional Quarter Horse geldings (5-15 years of age) and the Paint gelding described above were used to determine hydrocortisone pharmacokinetics at a later date.

Study methods were approved by the University of Georgia's and Clemson University's Institutional Animal Care and Use Committees. Mare/foal pairs were stabled in a free box stall for at least 24 hours before sampling was initiated and during the entire study period. Adult horses were similarly stalled during the study period, but were acclimatized to the stall for at least 48 hours before sample collection was initiated. At least 12 hours before initiation of sampling, jugular catheters were placed in horses and foals to facilitate blood collection. Catheters were placed in standing foals under brief restraint by experienced foal handlers in the stall with the mare, and sedation was not necessary for catheter placement in any foals or adult horses. Each adult horse and mare/foal pair was cared for according to the principles and guidelines stated in an Animal Use Protocol determined and approved by the Universities' Departments of Animal Resources.

Determination of 24-hour Serum Cortisol Concentration

Sampling Protocol

At 8am the morning following catheter placement, blood sampling to determine 24-hour endogenous cortisol concentration was initiated in 4 two-day-old foals and 4 adult horses. 2 ml of blood was collected via the jugular catheter every 15 minutes (foals) or every 20 minutes (horses) for 24 hours. Blood was placed into glass serum separator tubes and allowed to clot at room temperature. Samples were centrifuged and serum removed within 30-90 minutes of collection. Serum samples were stored at -80°C until analysis (within 30 days).

Cortisol Assay

Serum total cortisol concentration (henceforth referred to as cortisol concentration) was determined on an automated analyzer using a chemiluminescent enzyme immunoassay^c validated for use in horses.^{34,35} The lower limit of detection for this assay was 2 ng/ml.^d

Each animal's 24-hour mean cortisol concentration was determined by calculating the arithmetic mean of all the cortisol concentrations obtained over the 24-hour sampling period in each animal. These individual mean cortisol concentrations were then averaged in the four adult horses and averaged in the four foals to determine a mean 24-hour cortisol concentration in each group of animals.

Assessment of Hydrocortisone Pharmacokinetics

Sampling Protocol

In this portion of the study, the pharmacokinetics of a single intravenous bolus dose of 1.0 mg/kg hydrocortisone was assessed in 4 foals and 4 adult horses. In foals, the hydrocortisone pharmacokinetic study was performed in the same 4 foals used above, and was started 12 hours

after culmination of the sampling for determination of 24-hour cortisol concentrations. As described previously, in adult horses these two portions of the study were not performed in concert or in the same horses (with the exception of one adult horse that was used at different time points for both the 24-hour cortisol concentration and hydrocortisone pharmacokinetic portions of the study).

In all animals, endogenous cortisol production was suppressed with dexamethasone 12 hours prior to the administration of hydrocortisone. Since efficacy of the dexamethasone suppression protocol is known in adult horses {Soma, 2005 #469}³⁶ but has not been evaluated in foals, preliminary studies were performed in 6 additional 2-to-4-day-old foals to determine an appropriate dexamethasone suppression protocol for use in foals. In these 6 foals, 2 ml of blood was collected prior to and every 2 hours after dexamethasone administration for 48 hours. The first foal received 0.04 mg/kg of dexamethasone sodium phosphate^e intravenously at 6am. Suppression of endogenous cortisol production below the assay's lower limit of detection (2 ng/ml) was evident by 6 hours and was maintained until 20 hours after dexamethasone administration (data not shown). Since suppression of endogenous cortisol production for at least 24 hours was necessary for sampling for hydrocortisone pharmacokinetics, the second, third and fourth foals received a second 0.04 mg/kg dose of dexamethasone intravenously 16 hours after the first dose in an attempt to maintain suppression for this 48-hour period. However, adequate cortisol suppression was obtained with this protocol in just one of the three foals (data not shown). Therefore, the dexamethasone dose was doubled to 0.08 mg/kg IV and two doses 16 hours apart were administered to two additional foals. Suppression of endogenous cortisol production to or below the assay's lower limit of detection was obtained in both these foals by 6-8 hours and maintained for 48 hours after initial dexamethasone administration (Figure 7.1).

Thus, 0.08 mg/kg dexamethasone was administered IV to the 4 foals used for the hydrocortisone pharmacokinetics study 12 hours before administration of hydrocortisone (at the culmination of the 24-hour cortisol concentration sampling) and again 16 hours after the first dexamethasone dose to maintain suppression of endogenous cortisol production for the entire sampling period. This same dexamethasone suppression protocol was also used in the adult horses in this study, and suppression of endogenous cortisol to ≤ 2 ng/ml confirmed in each horse before hydrocortisone administration.

Hydrocortisone sodium succinate^e (1.0 mg/kg) was administered as a rapid IV bolus via direct venipuncture in the non-catheterized jugular vein in both foals and adult horses. Two ml blood was collected via the intravenous catheter immediately prior to and then at 6, 9, 12, 15, 30, 45, 60, 75, and 90 minutes and 2, 4, 6, 8, 10, 12, and 24 hours after hydrocortisone administration. Samples were processed and serum collected and stored as above. Serum hydrocortisone concentrations were determined using the same cortisol assay as described above, which demonstrates 100% cross-reactivity with hydrocortisone and 0% cross-reactivity with dexamethasone.^d

Pharmacokinetic Analysis

Pharmacokinetics analyses were performed, using a non-linear regression program.^g The goodness of the fit was evaluated by the Akaike Information Criterion (AIC), residual plots and visual inspection. The data were weighted as $1/(y_{\text{pred}})^2$, where y_{pred} is the model-predicted concentration at the actual time. Area under the curve (AUC) following intravenous administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (β) of the log serum drug concentrations versus time curve was determined by the method of least-squares regression.³⁷

Pharmacokinetics parameters of hydrocortisone were explained by using a two-compartmental open body model with first order elimination process. The compartmental model used for the determination of the pharmacokinetics parameters of hydrocortisone is represented by general equation *a* where C_p is plasma concentration of compound at any time (t), A and B are the Y intercepts associated with distribution and elimination phase, respectively, and α and β represent the rate constant of distribution and terminal elimination phase, respectively.³⁸ The rate constant of distribution (α), and distribution half-life ($t_{1/2\alpha}$) were determined by using the method of residuals.³⁹ The terminal half-life ($t_{1/2\beta}$)⁴⁰ was calculated according to Equation 1.

$$C_p = A \times e^{-\alpha t} + B \times e^{-\beta t} \quad (a)$$

$$t_{1/2\beta} = \ln 2 / \beta \quad (1)$$

Total body clearance (Cl_s) was calculated by use of Equation 2.⁴⁰

$$Cl_s = IV \text{ Dose} / AUC_{0-\text{inf}} (IV). \quad (2)$$

The volume of distribution in central compartment (Vd_c), volume of distribution in terminal elimination phase (Vd_β) and volume of distribution at steady state (Vd_{ss}) were calculated according to Equations 3, 4 and 5, respectively.⁴¹

$$Vd_c = \text{Dose (IV)} / A + B \quad (3)$$

$$Vd_\beta = IV \text{ Dose} / AUC_{0-\text{inf}} \times \beta \quad (4)$$

$$Vd_{ss} = IV \text{ Dose} \times AUMC_{0-\text{inf}} / (AUC_{0-\text{inf}})^2 \quad (5)$$

AUMC is area under the first moment curve and calculated by the trapezoidal method and extrapolated to infinity.³⁹ K_{10} is first order elimination rate constant, which describes elimination of drug from the central compartment. K_{12} and K_{21} are distribution rate constant from central to peripheral and from peripheral to central compartment, respectively. K_{10} , K_{12} , and K_{21} were calculated according to Equations 6, 7, 8, respectively.

$$K_{10} = \alpha \times \beta / K_{21} \quad (6)$$

$$K_{12} = \alpha + \beta - k_{21} - K_{10} \quad (7)$$

$$K_{21} = B \times \alpha + A \times \beta / (A + B) \quad (8)$$

Determination of Daily Endogenous Cortisol Production Rate

Cortisol production rate (PR, ng/kg/hr) was calculated using mean endogenous cortisol concentration for 24 hr and Cl_s following IV bolus dose of 1 mg/kg hydrocortisone (Equation 9).³³

$$PR: Cl_s \times \text{Mean endogenous cortisol concentration} \quad (9)$$

In foals, each individual foal's 24-hour cortisol concentration and hydrocortisone Cl_s were used to determine an individual cortisol PR for each foal. Since the same adult horses were not used to determine 24-hour cortisol concentrations and hydrocortisone pharmacokinetics, the mean 24-hour cortisol concentration for the group of 4 adult horses in the first portion of the study was multiplied by the hydrocortisone Cl_s achieved in each individual horse in the second portion of the study to determine an individual cortisol PR for each horse.

Statistical Analysis

Mean 24-hour cortisol concentration, pharmacokinetic parameters, and daily endogenous cortisol production rate were compared between adult horses and foals with Mann Whitney U tests using commercial statistical software.^g A non-parametric test was selected because the small sample size (n=4) in each group precluded accurate assessment of the distribution of the data. Hypothesis tests were two-tailed, and statistical significance was set at $P < 0.05$ for all analyses. Data are expressed as mean \pm standard deviation (range).

RESULTS

24-hour Cortisol Concentrations

Pulsatile secretion of cortisol was apparent in adult horses and foals, but a circadian rhythm to cortisol secretion with lower concentrations in the afternoon and evening was only apparent in adult horses (representative 24-hour cortisol secretion patterns in two animals shown in Figure 7.2; data from all animals not shown) Mean 24-hour cortisol concentration was significantly lower ($P < 0.001$) in foals (20 ± 4 ng/ml) than in adult horses (26 ± 6 ng/ml).

Hydrocortisone Pharmacokinetics

Hydrocortisone serum concentrations and calculated pharmacokinetic parameters for adult horses and foals are shown in Figure 7.3 and Tables 7.1 and 7.2. Hydrocortisone was detectable in the serum of all foals and adult horses at the first (6 min) sampling point after IV administration. In foals and adult horses, hydrocortisone was not detectable at 10 hours and 24

hours respectively following hydrocortisone administration. Peak serum hydrocortisone concentration was significantly lower in foals than in adult horses ($P=0.029$), and volume of distribution at steady state and hydrocortisone systemic clearance were significantly greater in foals than adult horses (Table 7.3, $P=0.029$ for all comparisons). Serum half-life ($t_{1/2\beta}$) was not significantly different between adult horses and foals ($P=0.886$), but foals exhibited substantially more variability in half-life duration than adult horses (Table 7.3).

Daily Endogenous Cortisol Production Rate

Daily endogenous cortisol PR was significantly higher ($P=0.029$) in foals than in adult horses (Table 7.3).

DISCUSSION

The objective of this study was to determine and compare daily endogenous cortisol production and hydrocortisone pharmacokinetics between adult horses and foals. The results herein demonstrate substantial differences in cortisol homeostasis between foals and adult horses and thus support our initial hypothesis that daily endogenous cortisol production rate and hydrocortisone pharmacokinetic parameters differ significantly between adult horses and foals.

Daily endogenous cortisol PR and hydrocortisone pharmacokinetic parameters in adult horses determined in the study herein are consistent with previous reports.^{32,33} Using similar methodology, Toutain *et al*³³ calculated a clearance rate of 0.609 L/kg/hr following a 1 mg/kg intravenous bolus of hydrocortisone and reported a daily cortisol PR of 7080 ng/kg/day in adult

horses at rest. However, in that study endogenous cortisol production was not suppressed prior to bolus administration of hydrocortisone. In a later study, though, when dexamethasone was used to suppress endogenous cortisol production prior to administration of an intravenous infusion of hydrocortisone at 0.03 mg/kg/hr, cortisol systemic clearance and daily cortisol PR were closer to values found in the study herein, at 0.404 L/kg/hr and 4590 µg/kg/day.³³ Lassourd *et al*³² reported a similar systemic clearance rate of 0.338 L/kg/hr in 6 healthy adult horses at rest following administration of radiolabelled cortisol, and reported a daily cortisol production rate of 1060 ng/kg/day in these horses. Daily cortisol PR in adult horses as reported herein (2150 ± 400 ng/kg/day) is also similar to the daily cortisol PR reported in healthy people (6.1 ± 0.4 mg/m²/day, approximately 1630 ng/kg/day).⁴²

Daily cortisol production and hydrocortisone pharmacokinetics have not been described previously in neonatal foals. The results herein indicate that daily endogenous cortisol PR is significantly higher in foals than in adult horses. It is possible that this increased PR in neonatal foals reflects increased HPA axis stimulation in response to greater environmental or physiologic stress during the neonatal period as foals transition from the intrauterine to the extrauterine environment. However, daily endogenous cortisol production rates in full-term infants are comparable to rates reported in older children and adults.⁴³ In addition, while increased plasma ACTH and cortisol concentrations are consistently observed in neonatal foals immediately after parturition, concentrations of these hormones decrease to or below adult resting concentrations by 6-12 hours of age in healthy foals,^{28,30,31,44} suggesting that the healthy 2-to-3-day-old foal is not undergoing significant HPA axis stimulation as compared to adult horses at rest. Finally, if ongoing stress-related HPA axis activation during the neonatal period were the sole explanation for the increased daily cortisol PR in foals, one would expect mean 24-hour cortisol

concentration to also be increased in foals as compared to adult horses at rest. However, in the study herein mean 24-hour cortisol concentration was actually significantly *lower* in foals than adult horses.

Thus, the increased daily cortisol PR observed in foals in this study is unlikely a simple reflection of increased HPA axis stimulation in response to stress in the neonatal period, but rather is presumably related to the four-fold increase in cortisol clearance in foals as compared to adult horses herein. Specifically, the increased cortisol PR in foals most likely reflects the HPA axis's attempt to maintain appropriate circulating basal cortisol concentrations in foals in the face of this dramatically increased systemic clearance rate.

This significantly increased systemic clearance rate and increased volume of distribution for cortisol/hydrocortisone found in foals as compared to adult horses in this study are best explained by the significantly reduced cortisol binding capacity recently described in foals.^a Cortisol/hydrocortisone and other steroid hormones are lipophilic and are transported in the plasma on specific binding proteins, including cortisol binding globulin (CBG) and albumin. In most species and in adult horses, approximately 90-95% of circulating cortisol is protein bound, with just 5-10% present in the free fraction.⁴⁵⁻⁵⁰ However, it is this small free fraction that is biologically available to enter target cells and bind the cytoplasmic glucocorticoid receptor to exert cortisol-specific tissue effects.⁴⁶

In neonatal foals during the first week of life, a substantially larger proportion of circulating cortisol (30-60%) is present in the free fraction.^a This larger free fraction permits cortisol to more rapidly and extensively enter target tissues, resulting in the larger hydrocortisone volume of distribution observed in foals in the study herein. And, as protein-bound cortisol essentially provides a reservoir of circulating cortisol that is protected from metabolism and renal

excretion,⁵¹ this decreased plasma cortisol-binding capacity in foals most likely results in the more rapid clearance and larger volume of distribution for hydrocortisone observed in this study. It is possible that hepatic and renal metabolism and excretion of cortisol differ greatly between adult horses and foals and thus may also contribute to the more rapid clearance in foals, but the larger volume of distribution in foals remains best explained by decreased cortisol binding capacity in foals. In addition, a similar increase in cortisol clearance and volume of distribution associated with transient exercise-induced increases in free cortisol has been documented in adult horses.³²

The physiologic and clinical consequences of these differences in cortisol/hydrocortisone distribution and metabolism observed in foals may be substantial. Cortisol is synthesized and secreted immediately in response to changes in systemic ACTH concentrations with HPA axis stimulation, and is not stored in the adrenal cortices or peripheral tissues. While the increased daily cortisol PR in foals in this study does demonstrate that the foal's adrenal cortices can increase cortisol production appropriately in health, the neonatal foal's capacity to maintain such increased cortisol production in critical illness is unknown. Cortisol and other steroid hormones are synthesized from cholesterol via a multi-step process requiring a number of specific adrenocortical cellular enzymes; however, in contrast to other species, some of the critical enzymes necessary for cortisol production are not detectable in the adrenal cortices until the immediate peri-parturient period in the foal.⁵² Thus, the foal may be unable to sustain such increased cortisol production for a prolonged period of time. In fact, a number of studies have described decreased cortisol responses to both endogenous and exogenous ACTH in healthy full-term neonatal foals as compared to adult horses,^{27,28,30,44,53} as well as higher ACTH:cortisol ratios in non-surviving septic foals as compared to survivors.^{54,55} These findings may reflect decreased

sensitivity to ACTH, limited glucocorticoid synthetic capacity, or both in neonatal foals as compared to adult horses. When coupled with the increased cortisol clearance rate described in foals herein, this may put foals at increased risk for the development of cortisol synthetic failure and CIRCI during periods of prolonged, intense HPA axis stimulation such as in critical illness.

In addition, these differences in hydrocortisone clearance and distribution must be considered when glucocorticoids are used in foals in a clinical setting. Foals may require higher hydrocortisone doses and more frequent dosing than adult horses to achieve similar plasma concentrations. However, because substantially more cortisol is also biologically available in foals due to increased free fraction, foals may be able to compensate somewhat for this rapid clearance and lower plasma concentrations. Thus, an appropriate hydrocortisone replacement therapy dose for use in septic foals with CIRCI might be in the range of 1.0 to 3.0 mg/kg/day, 1.5 to 4.5 times the mean daily endogenous cortisol PR of 6710 ± 320 ng/kg/day (0.671 ± 0.032 mg/kg/day) found in healthy foals herein. Calculation of this proposed dose was performed as described in people,^{25,26} using an “illness factor” to multiply levels in healthy individuals based on an expected 1.5- to 4.5-fold increase in basal cortisol concentrations as observed in response to physiologic and supraphysiologic cosyntropin stimulation in healthy foals.^{27,28} The high end of this hydrocortisone dose is slightly lower than doses used most often in septic people and infants to date (approximately 2-4 mg/kg/day),^{15,23,24} but a lower dose may be suitable for use in foals due to their significantly increased free cortisol fraction.

However, any such proposed hydrocortisone replacement therapy regimen must be evaluated further before clinical application to sick and septic foals. One important limitation of the study herein is that it was conducted only in healthy foals and adult horses; thus, the effects of illness on daily endogenous cortisol production rate and hydrocortisone pharmacokinetics in

foals are not known. In addition, given the well-described negative consequences of high-dose corticosteroid therapy in septic people,^{18,56} any hydrocortisone replacement therapy regimen for use in septic foals should be evaluated to ensure it does not cause any undesirable effects. Thus, while the results of the study reported herein highlight important differences in cortisol production and metabolism among foals and both adult horses and humans, and provide initial data to determine a potential hydrocortisone replacement therapy regimen for use in foals, further study is necessary before such therapy can be recommended for clinical patients.

FOOTNOTES

^a Hart, K.A., M.H. Barton, D.C. Ferguson, D.J. Hurley. 2008. Serum free cortisol fraction in healthy and septic neonatal foals. Research Abstract, presented at the 14th Annual International Veterinary Emergency and Critical Care Symposium, Phoenix, AZ, September 17-21, 2008. *Journal of Veterinary Emergency and Critical Care*, 18(4): 423.

^b SNAPTM Foal IgG Test, IDEXX Laboratories, Inc., Westbrook, ME

^c ImmuliteTM, Diagnostics Product Corporation, Los Angeles, CA

^d Immulite Cortisol Assay package insert. Diagnostics Product Corporation.

^e Dexamethasone sodium phosphate, Butler Animal Health Supply, Dublin, OH

^f Solu-CortefTM, hydrocortisone sodium succinate, Pfizer Inc., New York, NY

^g WinNonlin, version 5.01, Pharsight Corporation, Cary, NC

^h GraphPad Prism Statistical Software (Version 4), GraphPad Software Inc., San Diego, CA

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Table 7.1. Calculated pharmacokinetic parameters in four adult horses following a single intravenous bolus dose of hydrocortisone (1 mg/kg).

	Horse 1	Horse 2	Horse 3	Horse 4	Mean ± SD
Weight (kg)	604	606	528	594	583 ± 37
A (ng/ml)	13349	7073	4378	9068	8467 ± 3780
B (ng/ml)	542	674	303	347	466.5 ± 173
t_{1/2} K₁₀ (hr)	0.17	0.256	0.318	0.234	0.245 ± 0.061
t_{1/2} α (hr)	0.093	0.088	0.185	0.150	0.129 ± 0.047
t_{1/2} β (hr)	2.08	2.01	2.24	2.43	2.19 ± 0.187
Cl_s (L/kg/hr)	0.290	0.348	0.440	0.318	0.349 ± 0.065
Vd_c (L/kg)	0.072	0.128	0.202	0.107	0.127 ± 0.055
Vd_β (L/kg)	0.364	0.580	0.511	0.361	0.454 ± 0.109
Vd_{ss} (L/kg)	0.435	0.708	0.713	0.468	0.581 ± 0.150
AUC_{0-inf} (ng x hr/ml)	3422	2849	2150	3178	2900 ± 552
MRT (hr)	1.5	2.04	1.62	1.47	1.658 ± 0.263
R²	0.984	0.993	0.988	0.989	0.989 ± 0.004

Table 7.2. Calculated pharmacokinetic parameters in four 3-day-old foals following a single intravenous bolus dose of hydrocortisone (1 mg/kg).

	Foal 1	Foal 2	Foal 3	Foal 4	Mean ± SD
Weight (kg)	50.9	46.8	50.5	42.3	47.63 ± 4
A (ng/ml)	1,303	847	741	977	855 ± 118
B (ng/ml)	236	49	11	38	83.5 ± 103
t_{1/2} K₁₀ (hr)	0.43	0.565	0.595	0.505	0.524 ± 0.073
t_{1/2} α (hr)	0.29	0.475	0.503	0.447	0.429 ± 0.095
t_{1/2} β (hr)	1.03	2.13	7.1	2.02	3.07 ± 2.73
Cl_s (L/kg/hr)	1.305	1.370	1.55	1.35	1.394 ± 0.108
Vd_c (L/kg)	0.808	1.12	1.33	0.986	0.897 ± 0.126
Vd_β (L/kg)	1.94	4.22	15.82	3.95	6.48 ± 6.31
Vd_{ss} (L/kg)	1.18	1.61	3.56	1.33	1.92 ± 1.108
AUC_{0-inf} (ng x hr/ml)	767	730	645	739	720 ± 53
MRT (hr)	0.905	1.175	2.4	0.983	1.37 ± 0.7
R²	0.998	0.998	0.999	0.994	0.997 ± 0.002

Table 7.3. Comparisons in selected pharmacokinetic parameters for a single intravenous bolus dose of hydrocortisone (1 mg/kg) between healthy adult horses and foals.

	ADULT HORSES (n=4)	FOALS (n=4)
Co (ng/ml)	8934 ± 3843 (4681 – 13,891)	1051 ± 343* (752 – 1015)
t_{1/2} β (hr)	2.19 ± 0.187 (2.01 – 2.43)	3.07 ± 2.73 (1.03 – 7.1)
Cl_s (L/kg/hr)	0.349 ± 0.065 (0.290 – 0.440)	1.394 ± 0.108* (1.305 – 1.55)
Vd_c (L/kg)	0.127 ± 0.055 (0.072 – 0.202)	0.897 ± 0.126* (0.808 – 1.33)
Vd_β (L/kg)	0.454 ± 0.109 (0.361 – 0.580)	6.48 ± 6.31* (3.95 – 15.82)
Vd_{ss} (L/kg)	0.581 ± 0.150 (0.435 – 0.713)	1.92 ± 1.11* (1.18 – 3.56)
Daily Cortisol Production Rate (ng/kg/day)	2140 ± 400 (1780 – 2710)	6710 ± 320* (6510 – 7180)

Data are shown as mean ± SD (range). * Significantly different (P<0.05) between adult horses and foals.

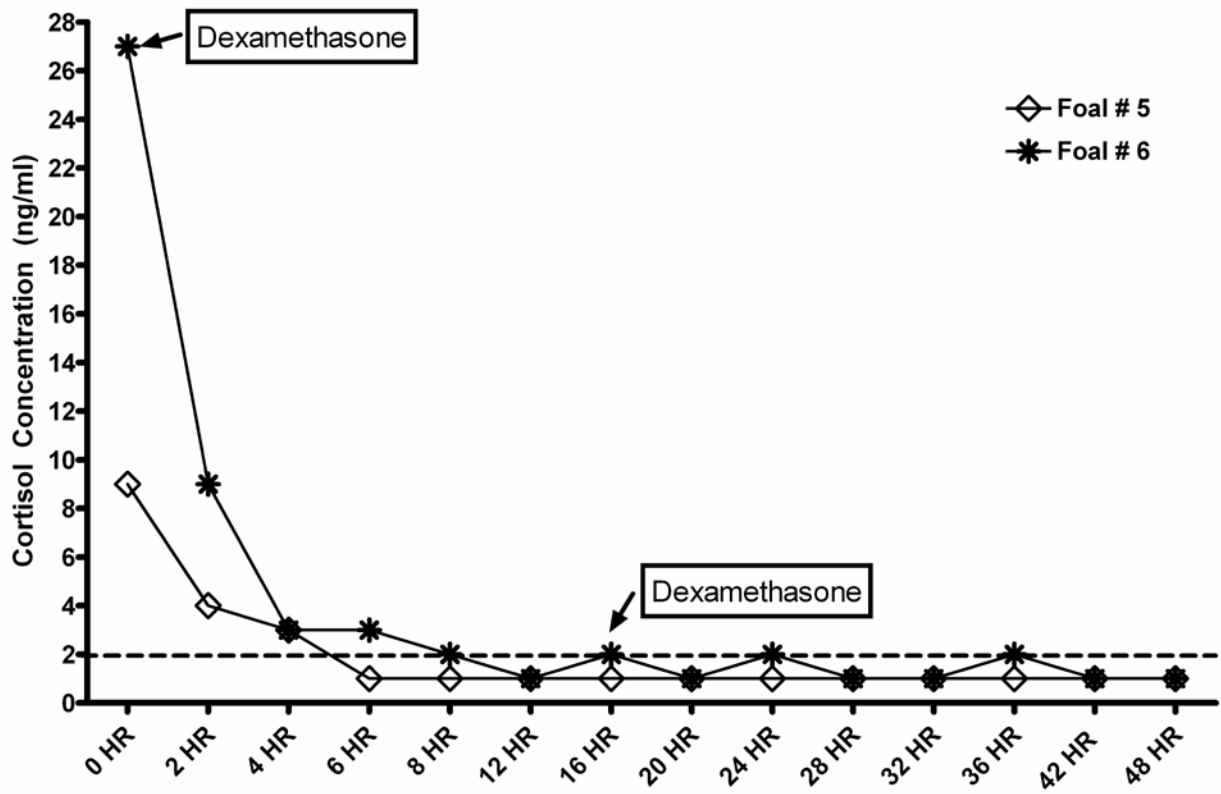
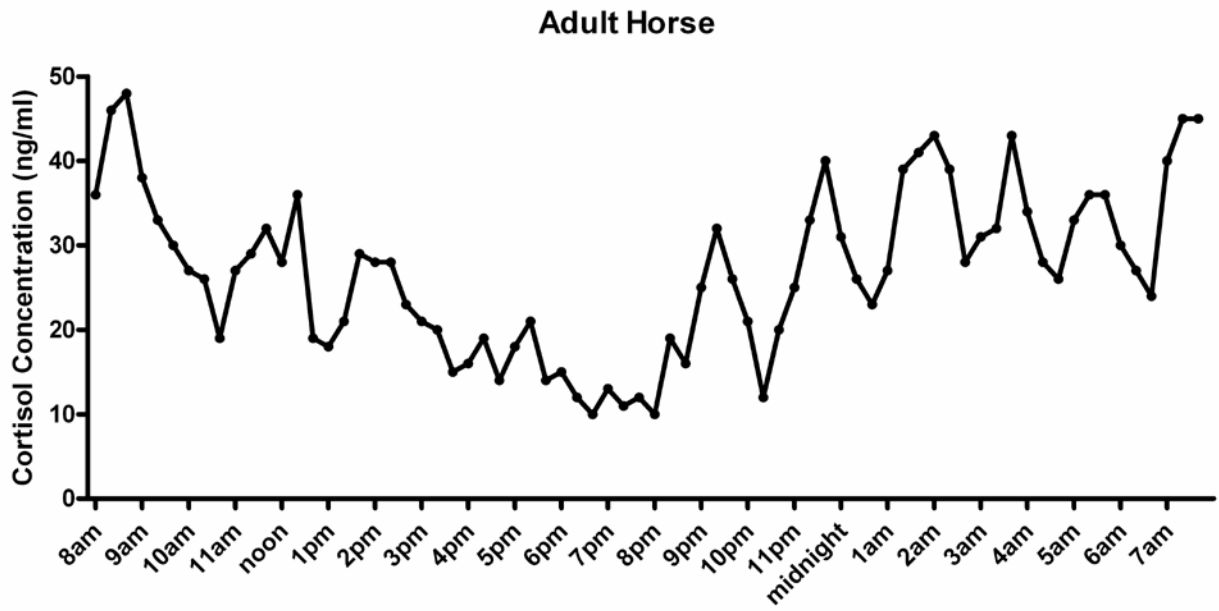


Figure 7.1. Serial serum cortisol concentrations in two 2-day-old foals before and after dexamethasone suppression. Dexamethasone (0.08 mg/kg IV) was administered at T=0 hours and 16 hours. The horizontal dashed line represents the lower limit of detection of cortisol by the chemiluminescent immunoassay (2 ng/ml).

a)



b)

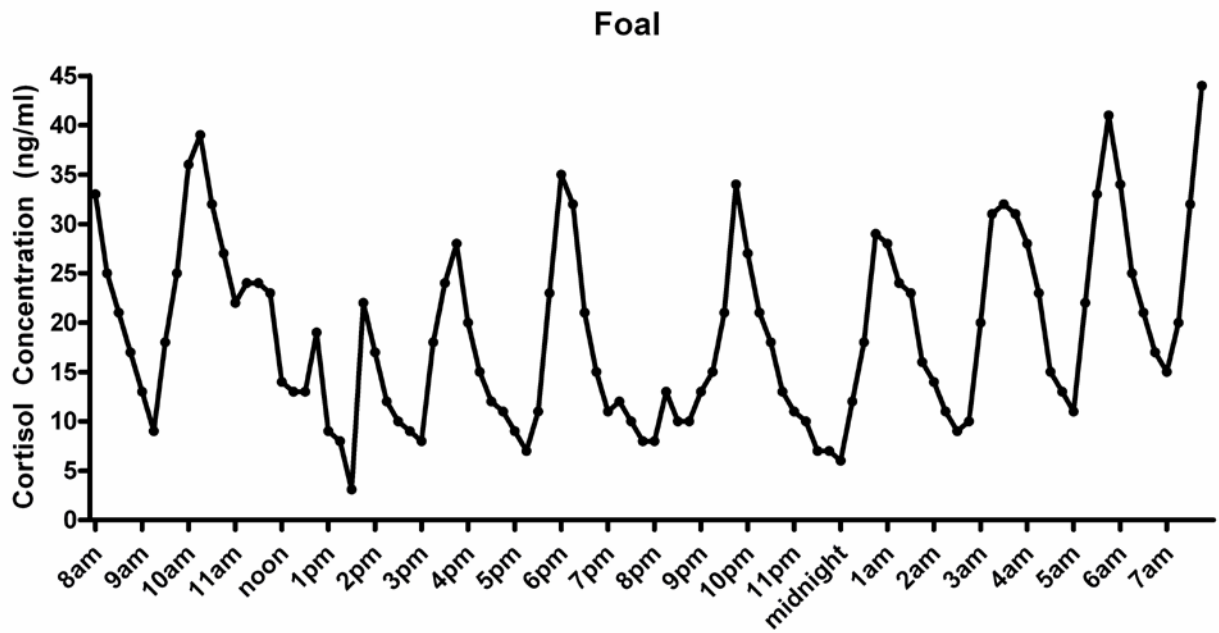


Figure 7.2. Serial (q. 15-20 minute) serum cortisol concentrations over a 24-hour period in a representative adult horse (a) and 2-day-old foal (b).

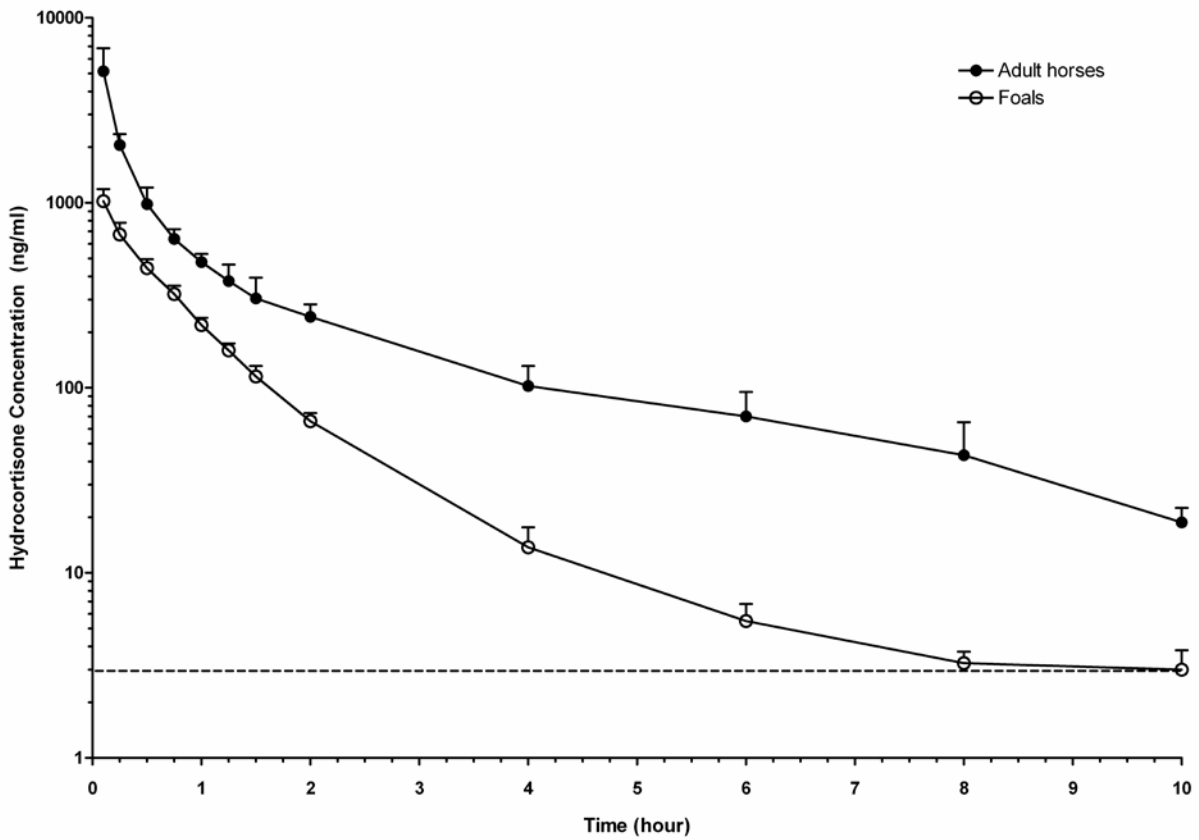


Figure 7.3. Serum hydrocortisone concentrations following 1 mg/kg intravenous bolus administration to adult horses (n=4) and 3-day-old foals (n=4). The horizontal dashed line represents the lower limit of detection of cortisol by the chemiluminescent immunoassay (2 ng/ml).

CHAPTER 8

EFFECTS OF LOW-DOSE HYDROCORTISONE THERAPY ON IMMUNE FUNCTION IN NEONATAL HORSES¹

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ABSTRACT

Low-dose hydrocortisone therapy modulates inflammatory responses in adults and improves outcomes in some septic adults and neonates, but its immunologic effects have not been evaluated in neonates. The objective of this study was to evaluate effects of low-dose hydrocortisone therapy on *ex vivo* immune function in healthy neonatal horses (foals). We hypothesized that low-dose hydrocortisone therapy would dampen pro-inflammatory responses without impairing neutrophil function. Hydrocortisone (1.3 mg/kg/day IV) was administered to foals for 48 hours, then tapered and discontinued over 36 hours. Peripheral blood leukocytes were collected from foals before, during and after hydrocortisone treatment. A separate group of age-matched untreated foals served as the control group. Endotoxin-induced mononuclear cell gene expression of inflammatory cytokines was measured by qRT-PCR. Neutrophils were incubated with labeled, killed *S. aureus* or *E. coli* for assessment of phagocytosis, and with phorbol myristate acetate, zymosan, or endotoxin for measurement of reactive oxygen species (ROS) production. Neutrophil phagocytosis and ROS production were similar in both groups. Foals receiving hydrocortisone had significantly decreased endotoxin-induced production of TNF- α , IL-6, IL-8, and IL-1 β . These data suggest that this hydrocortisone treatment regimen ameliorates endotoxin-induced pro-inflammatory cytokine expression in neonatal foals without impairing neutrophil function necessary to combat bacterial infection.

INTRODUCTION

Cortisol is vital for the stress response to critical illness, and plays an essential role in the maintenance of blood pressure, and in regulation and moderation of the immune and inflammatory response.¹ In some patients, though, the cortisol response to critical illness may be transiently insufficient for the severity of disease, a condition called Relative Adrenal Insufficiency (RAI) or Critical Illness-Related Corticosteroid Insufficiency (CIRCI).²⁻⁶ The specific mechanisms resulting in RAI/CIRCI are poorly understood, but suppression of the HPA axis at one or several levels by infectious organisms or inflammatory cytokines is purported to play a significant role in the pathogenesis of the syndrome in septic patients.^{1,4} In addition, as fetal HPA axis function and adrenocortical steroid synthetic capacity do not mature until late gestation, cortisol insufficiency may be of particular significance in the pre-term infant even in the absence of concurrent infection.^{4,7} Though specific diagnostic criteria for RAI/CIRCI in adult or pediatric patients are not universally accepted, a number of studies document evidence of cortisol insufficiency in septic adult and pediatric patients and in critically ill full-term and pre-term infants^{3,5,8-14}

The physiologic consequences of RAI/CIRCI in the septic patient can be dramatic, including cardiovascular collapse and an excessive, unregulated systemic inflammatory response that can lead to tissue and organ damage, organ failure, and death.⁴⁻⁶ A number of studies have documented a significantly higher incidence of shock, multiple organ dysfunction syndrome (MODS) and death in septic adults and children with RAI/CIRCI, as compared to septic patients with intact HPA axis function.^{3,5,10,15} An exaggerated and unregulated systemic inflammatory response characterized by imbalance in pro- and anti-inflammatory cytokine production is also

documented in both septic people and animals, and is predictive of increased disease severity and death in these populations.^{1,16-29} It has been theorized that cortisol insufficiency during sepsis may contribute to an overwhelming inflammatory response that contributes to this increased disease severity and mortality.^{1,24,29}

Thus, glucocorticoids have been administered to septic patients in varying doses in attempts to quell overwhelming inflammatory responses. There is compelling evidence that *high-dose* corticosteroid therapy can result in suppression of both the HPA axis and the immune response, and such treatment regimens in septic patients are associated with detrimental side effects and decreased survival in both adults and neonates.³⁰⁻³⁴ However, a number of recent studies and several meta-analyses have evaluated the effects of low- and moderate-dose hydrocortisone therapy in septic patients, and have demonstrated improved shock reversal and survival in some groups of septic patients.^{5,6,30,32,33,35-37} The basic premise of such low-dose (“stress-dose”) hydrocortisone therapy is to provide short-term synthetic cortisol supplementation for patients with transient cortisol insufficiency associated with their primary illness. Dosages for such protocols in clinical patients have been extrapolated from daily cortisol production rates and maximal cortisol responses to ACTH in healthy individuals^{2,38} and administered via an intravenous infusion or intermittent intravenous bolus dosing for a short (3-7 day) course.^{2,6}

To the authors’ knowledge, the effects of low-dose hydrocortisone therapy on immune function in neonates of any species are not described. Given the numerous differences in HPA axis and immune function and in hydrocortisone pharmacokinetics between adults and neonates,^{4,7,39} specific evaluation of low-dose hydrocortisone replacement therapy regimens in

the neonate is critical to determine optimal therapeutic recommendations for septic and pre-term neonates exhibiting clinical signs consistent with cortisol insufficiency.

Comprehensive study of low-dose hydrocortisone therapy and of the pathogenesis and management of RAI/CIRCI in neonatal rodents or other small mammals is limited by their small size, and results may be impacted due to the lack of naturally occurring neonatal septicemia and RAI/CIRCI in these species. However, like infants, neonatal horses (foals) exhibit substantial HPA axis immaturity in the perinatal period,⁴⁰⁻⁴⁶ are highly susceptible to the development of sepsis,⁴⁷ and are exquisitely sensitive to gram negative sepsis and endotoxemia.⁴⁷⁻⁵¹ In addition, naturally occurring RAI/CIRCI is described in approximately 40% of septic neonatal foals and is correlated with increased incidence of shock, MODS, and mortality,⁵² similar to reports in septic adults and infants.^{3,5,8-14} Furthermore, septic foals also exhibit inflammatory dysregulation similar to septic infants, characterized by increased expression of pro-inflammatory cytokine genes such as TNF- α and IL-6.^{17,18,51}

Thus, the primary objective of this study was to examine the effects of low-dose hydrocortisone replacement therapy on measures of immune function in neonatal foals in an *ex vivo* model. In addition, effects of low-dose hydrocortisone therapy on *in vivo* HPA axis function were examined. We hypothesized that low-dose hydrocortisone therapy: 1) would not significantly impair the ability of peripheral blood granulocytes to produce reactive oxygen species (ROS) or phagocytose bacteria; 2) would significantly decrease endotoxin-induced expression of pro-inflammatory cytokine genes and significantly increase endotoxin-induced expression of anti-inflammatory cytokine genes in peripheral blood mononuclear cells; and 3) would not significantly suppress HPA axis function *in vivo* after therapy was discontinued.

MATERIALS AND METHODS

Animals

Thirty-eight healthy 2-to-7-day-old full-term foals (gestational age \leq 330 days, weight 40-60 kg) from research herds maintained at the University of Georgia's and Clemson University's equine breeding facilities were used in this study. Foals were determined to be healthy prior to and during the study period by lack of abnormalities identified on daily physical examination. Adequate transfer of passive immunity was confirmed by a serum immunoglobulin concentration \geq 800 mg/dl^a at 24 hours of age in all foals. Eleven foals (4 females, 7 males) comprised the treatment (TREATED) group and received low-dose hydrocortisone as described below, and 28 foals (15 females, 13 males) comprised a control (CONTROL) group that did not receive hydrocortisone. Seventeen CONTROL foals underwent the same sampling protocol as TREATED foals for comparisons in immune function testing (see details below). The remaining 11 CONTROL foal were used for serial HPA axis function assessment during the first week of life, and provided age-matched HPA axis function comparisons for TREATED foals. Detailed results of a serial HPA axis assessment in these foals have been previously published.⁴⁰ Group assignments were randomized based on order of foaling and were known to the investigators during the study period.

Study methods were approved by the University of Georgia's and Clemson University's Institutional Animal Care and Use Committees. Mare/foal pairs were stabled in a free box stall with daily paddock turnout during the study period, and were cared for according to the principles and guidelines stated in an Animal Use Protocol determined and approved by the Universities' Departments of Animal Resources.

Low-dose Hydrocortisone Treatment and Sampling Protocol

Between 36-60 hours of age, all TREATED foals had an intravenous jugular catheter placed under brief standing restraint. Beginning 12-18 hours after catheter placement (thus, at 48-72 hours of age), TREATED foals received a tapering 3.5 day course of hydrocortisone sodium succinate^b as follows: 1.3 mg/kg/day for 48 hours, 0.65 mg/kg/day for 24 hours, and 0.33 mg/kg/day for 12 hours. This total daily dose was determined by multiplying the daily endogenous cortisol production rate in healthy 2-5 day old foals^c by a factor of 2 to approximate an appropriate cortisol response to stress, as described in other species.^{2,38,53} The total daily dose of hydrocortisone was divided into 6 equivalent doses that were administered as an intravenous bolus every 4 hours.

Sixty-five milliliters of blood was collected from the jugular catheter just before the first dose of hydrocortisone (PRE-TREATMENT), after 48 hours of therapy just before the initial dose was halved (DURING TREATMENT), and 12 hours after cessation of hydrocortisone administration (POST-TREATMENT). At each time point, 5 ml of blood was placed into glass tubes without anticoagulant and allowed to clot. Samples were centrifuged within 1 hour of collection and serum stored at -80°C until analysis for serum cortisol/hydrocortisone concentrations. The remaining 60 ml was anti-coagulated with 2 ml 100 µM EDTA for isolation of peripheral blood leukocytes. At the DURING TREATMENT sample, an additional 5 ml of blood was collected 5 minutes after the hydrocortisone IV bolus dose was administered for measurement of peak concentrations.

In the 17 CONTROL foals used for immune function comparisons, blood was collected as above via direct jugular venipuncture on the mornings of day 2, day 4, and day 6 of life to provide age-matched comparisons for the TREATED group.

Twenty-four hours after discontinuation of hydrocortisone therapy (on day 6 of life), post-treatment HPA axis function and responsiveness was assessed in 8/11 TREATED foals. At this time, blood was collected for measurement of basal cortisol concentrations and a paired low-dose (10 µg) / high-dose (100 µg) cosyntropin^d (synthetic ACTH, α 1-24 corticotropin) stimulation test (henceforth referred to as “paired cosyntropin stimulation test”) was performed as described previously⁴⁰. All blood samples were collected by jugular venipuncture under brief standing restraint. Five ml of blood was collected as above for measurement of basal cortisol concentration, then 10 µg cosyntropin was administered intravenously as a rapid bolus. Blood was then collected 30 minutes later for assessment of the peak cortisol response to the 10 µg cosyntropin dose. Ninety minutes after administration of 10 µg cosyntropin, 100 µg of cosyntropin was administered, and blood was collected 90 minutes later for assessment of the cortisol response to the 100 µg cosyntropin dose.

In the 11 CONTROL foals used for HPA axis function comparisons, blood was collected via direct venipuncture on days 2 and 5 of age for measurement of basal cortisol concentrations for comparisons with PRE-TREATMENT and POST-TREATMENT cortisol concentrations in TREATED foals. A paired cosyntropin stimulation test as described above was performed between days 5 and 7 of life in these CONTROL foals for comparison with TREATED foals’ POST-TREATMENT paired cosyntropin stimulation test responses.

Cortisol and Hydrocortisone Assays

Serum total cortisol concentrations (henceforth referred to as cortisol concentrations) were determined on an automated analyzer using a chemiluminescent enzyme immunoassay^e validated for use in the horse.^{54,55} The interassay and intra-assay coefficients of variation for this

assay are $\leq 20\%$ ⁵⁵ and the limit of detection is 0.2 $\mu\text{g/dL}$.^f Serum hydrocortisone concentrations were determined using this same cortisol assay, which demonstrates 100% cross-reactivity with hydrocortisone.^f

***Ex vivo* Immune Function Testing**

Peripheral blood granulocytes and mononuclear cells were isolated within 120 minutes of collection by density-gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) as previously described.^{56,57} Viability of both cell types was greater than 95% as assessed by trypan blue exclusion. *Ex vivo* immune function testing in both groups of foals at all time points included the following assessments: 1) neutrophil production of reactive oxygen species (ROS) mediated by distinct intracellular signaling pathways; 2) neutrophil phagocytosis of gram negative and gram positive bacteria; and 3) basal and endotoxin-induced gene expression of selected pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs). All assays were performed in all 11 TREATED foals at all 3 sampling time points, but due to technical issues, not all assays were performed in all CONTROL foals at each time point. Specific numbers of CONTROL foals with data available for each assay at each time point are shown in the Results section.

Neutrophil ROS production in response to endotoxin (100 ng/ml; *E. coli* 055:B5 LPS, List Biological Inc, Campbell, CA), zymosan (1000 ng/ml; Molecular Probes, Eugene, OR), and phorbol myristate acetate (PMA, 10^{-7} M, Molecular Probes, Eugene, OR) was measured using a fluorometric assay as previously described.⁵⁶ Assessment of ROS production in response to these 3 stimulants permitted assessment of the cells' Toll-like receptor 4 (TLR4)-mediated ROS

production (endotoxin), dectin-1-mediated ROS production (zymosan), and overall capacity to produce ROS in response to protein kinase C activation (PMA).

To determine neutrophil phagocytic function, isolated neutrophils were re-suspended to a final concentration of 3×10^6 cells/ml in RPMI 1640 without phenol red supplemented with 50 μ g gentamicin sulfate, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Bodipy-labelled, inactivated *Escherichia coli* (Molecular Probes®, Invitrogen, Carlsbad, CA) and *Staphylococcus aureus* (Molecular Probes®, Invitrogen, Carlsbad, CA) were diluted to a final concentration of 2×10^6 /ml. 200 μ l of resuspended cells were incubated in duplicate with 50 μ l *E. coli* or *S. aureus* or with media only for 60 minutes at 37°C and 5% CO₂. Cells were then washed with FACS buffer and fixed in 1% formalin. Flow cytometric analysis was conducted within 7 days of fixation on an Accuri C6 Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI). Extra-cellular bacterial fluorescence was quenched with 0.4% trypan blue (Sigma Chemical, St. Louis, MO) and samples were then assessed for the percent of fluorescent-positive cells using CFlowPlus software (Accuri Cytometers, Inc., Ann Arbor, MI).

After isolation, PBMCs were resuspended in RPMI-1640 supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% equine serum (Hyclone, Logan, UT). The cells were equally divided among 6 sterile 60 x 15 mm Petri dishes and incubated for 30 minutes at 37°C in a 5% CO₂ incubator to allow them to stabilize before stimulation. Plates were then treated with 1 ng/ml endotoxin (*E. coli* 011:B4 LPS, List Biological Laboratories, Campbell, CA) or an equivalent volume of media and incubated at 37°C and 5% CO₂ for 1, 4, and 20 hours. [Note: 4-hour incubations were performed in all TREATED and CONTROL foals, but 1- and 20-hour incubations were only performed in 8 foals in each group.] After each incubation, cells

were washed and scraped from the plates in cold phosphate buffered saline, pelleted via brief centrifugation at 14,000 x g, and lysed with RNA cell lysis solution (Qiagen, Valencia, CA) in combination with 10 ul/ml 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and stored at -80°C until RNA extraction. Total RNA was extracted from the cell lysates using the RNeasy mini RNA extraction kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol and treated with DNase I at 25°C for 30 minutes. Only samples having 260:280 nm absorbance ratios between 2.0 and 2.2 as measured on a NanoDrop spectrophotometer (ThermoFisher Scientific, Wilmington, DE) were processed for cDNA synthesis with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 500 ng RNA as template.

Expression of TLR-4, TNF- α , IL-1 β , IL-6, IL-10, IL-4, interleukin-8 (IL-8) and transforming growth factor- β (TGF- β) were quantified using validated two-step real time quantitative reverse transcription-polymerase chain reaction assays (qRT-PCR) with SYBR Green detection in an Applied Biosystems 7900HT sequence detection system (Foster City, CA), with 18S ribosomal RNA used as an endogenous housekeeping control as previously reported.^{58,59} Changes in gene expression were calculated by relative quantification against 18s rRNA using the $\Delta\Delta C_T$ method with plain media controls used as the calibrator. Fold changes in gene expression between endotoxin-stimulated and unstimulated cells from the same foal for each incubation period (1, 4, and 20 hours) at each sampling time point were calculated as $2^{-\Delta\Delta C_T}$.

Data Analysis

Between-group comparisons between age-matched TREATED and CONTROL foals were conducted using Student's t tests for parametrically distributed data and Mann Whitney U

tests for non-parametric data, after normality was assessed with the Kolmogorov-Smirnov test. For inflammatory mediator gene expression, endotoxin-induced fold changes in gene expression ≤ 3 -fold were not considered relevant induction of expression. Thus, if both CONTROL and TREATED foals failed to exhibit an endotoxin-induced change in expression of > 3 -fold for a specific gene, further between-group comparisons for that gene at that day and incubation time point were not conducted. Statistical analysis was performed using commercial statistical software,[§] and statistical significance was set at $P < 0.05$ for all analyses.

RESULTS

No severe adverse effects were noted in any TREATED foals. Two foals developed a partial thrombus at the jugular catheter site during or after the sampling period. In each case, no clinical or clinicopathologic evidence of septic thrombophlebitis was present and the thrombus resolved with warm compresses. One foal developed mild diarrhea 12 hours prior to discontinuation of hydrocortisone therapy, but remained afebrile with an excellent attitude and appetite and a normal leukogram, with resolution of the diarrhea in 48 hours without specific therapy.

ROS Production

Neutrophil ROS production in response to endotoxin, zymosan, and PMA in TREATED and CONTROL foals is shown in Figure 8.1. No significant differences in ROS production in response to any of the three stimulants were found between TREATED and age-matched

CONTROL foals in the PRE-TREATMENT, DURING TREATMENT, or POST-TREATMENT samples. However, while overall ROS production in response to PMA was almost identical between TREATED and CONTROL foals during and after hydrocortisone therapy, increases in ROS production that approached statistical significance were noted in TREATED foals in response to zymosan during hydrocortisone treatment (P=0.058) and in response to endotoxin after hydrocortisone treatment (P=0.067).

Phagocytic Function

Neutrophil phagocytosis of *E. coli* and *S. aureus* in TREATED and CONTROL foals is illustrated in Figure 8.2. No significant differences in phagocytosis of either organism were found between TREATED and age-matched CONTROL foals before, during, or after hydrocortisone therapy.

Inflammatory Molecule Gene Expression

Endotoxin-induced changes in gene expression for TNF- α , IL-6, IL-1 β , IL-8, and IL-10 in TREATED and CONTROL foals are shown in Figure 8.3. Both TREATED and CONTROL foals exhibited a > 3 fold endotoxin-induced change in gene expression for TNF- α , IL-6, IL-1 β , IL-8, and IL-10 for one or more incubation times on each sampling day, permitting between-group comparisons for these genes on each sampling day. However, neither group exhibited a > 3 fold endotoxin-induced change in expression for TNF- α after 4 or 20 hours of incubation or for IL-10 at 1 or 20 hours of incubation on any sampling day, so between-group analysis was not conducted for these genes at these incubation time points. In addition, endotoxin-induced changes in gene expression for TLR-4, IL-4, and TGF- β were < 3 fold for both groups at all

incubation time points on all sampling days (data not shown), so further between-group analysis was not conducted for these genes at any time point.

No significant differences in endotoxin-induced gene expression were found between TREATED and CONTROL foals at the PRE-TREATMENT sample for any genes except IL-8, for which TREATED foals had significantly lower expression than age-matched CONTROL foals ($P = 0.027$) after 4 hours of incubation with endotoxin. IL-8 expression in TREATED and CONTROL foals at this PRE-TREATMENT sample was similar for 1 hr ($P = 0.645$) and 20 hr ($P = 0.879$) incubations.

At the DURING TREATMENT sample, TREATED foals exhibited significantly lower endotoxin-induced expression of IL-6 ($P < 0.001$ for both 4 and 20 hr incubations), IL-1 β ($P < 0.036$ for 20 hr incubation), and IL-8 ($P = 0.004$ for 4 hr incubation, $P = 0.019$ for 20 hr incubation) than age-matched CONTROL foals. In addition, TREATED foals also exhibited lower expression of IL-1 β and IL-10 after 4 hrs of endotoxin incubation that approached statistical significance ($P = 0.053$, $P = 0.050$ respectively).

At the POST-TREATMENT sample, endotoxin-induced expression of IL-6 ($P = 0.023$ for 4 hr incubation), IL-1 β ($P = 0.021$ for 1 hr incubation, $P < 0.001$ for 4 hr incubation), and IL-8 ($P = 0.002$ for 4 hr incubation) remained significantly lower in TREATED foals as compared to age-matched CONTROL foals. Endotoxin-induced expression of TNF- α was also significantly lower in TREATED foals ($P = 0.047$ for 1 hr incubation) at this sample time. Endotoxin-induced expression of IL-1 β and IL-8 after 20 hrs of incubation also was lower in TREATED foals and approached statistical significance ($P = 0.054$, and $P = 0.054$ respectively).

Serum Cortisol and Hydrocortisone Concentrations

Serum cortisol and hydrocortisone concentrations in TREATED foals before, during, and after low-dose hydrocortisone treatment are shown in Table 8.1. Paired cosyntropin stimulation test results in TREATED and CONTROL foals are shown in Table 8.2. There were no significant differences between TREATED and age-matched CONTROL foals in HPA axis function as assessed by basal cortisol concentration before and after hydrocortisone treatment or by peak cortisol responses or delta cortisol (peak – basal cortisol) responses to either the low (10 µg) or high (100 µg) dose of cosyntropin after hydrocortisone treatment.

DISCUSSION

The results herein provide support for our initial hypotheses, and illustrate that a short tapering course of low-dose hydrocortisone dampens the pro-inflammatory cytokine response to *ex vivo* endotoxin exposure in mononuclear cells without significantly suppressing neutrophil function in neonatal foals. Furthermore, HPA axis function, as assessed by resting cortisol concentrations and cortisol responses to cosyntropin, was not significantly different 12-24 hours after discontinuation of hydrocortisone in TREATED foals as compared to age-matched controls. In sum, these findings suggest that a similar low-dose hydrocortisone therapy protocol may be effective at ameliorating a detrimental pro-inflammatory response in septic neonates without impairing the innate immune and endocrine responses necessary to clear the inciting bacterial infection.

Interestingly, while overall granulocyte ROS production in response to PMA was not significantly different between TREATED and CONTROL foals, a trend towards *increased* receptor-mediated ROS production in response to both endotoxin and zymosan was observed in TREATED foals, with some values approaching statistical significance. This finding was unexpected, as several previous studies have documented unchanged or inhibited granulocytic ROS production after treatment with various doses of hydrocortisone (1.4 or 14 mg/kg IV bolus + 0.18 mg/kg/hr IV infusion in rabbits;⁶⁰ 100 mg IV bolus +/- 10 mg/hr IV infusion in people⁶¹⁻⁶⁴). However, a small number of studies have also shown that glucocorticoids can exert some immunostimulatory effects in addition to their well-described anti-inflammatory actions, particularly at low glucocorticoid concentrations such as are observed *in vivo* during unstressed states.^{65,66} It is possible that the trend towards increased receptor-mediated ROS production observed in TREATED foals herein is an example of such an immunostimulatory effect of low corticosteroid concentrations. Such an effect might be explained by steroid-induced up-regulation of the necessary receptors or second messenger molecules needed for ROS production via the TLR-4 and TLR-2/NOD-1 pathways. Such an effect could be beneficial at promoting host bactericidal activity and clearance at sites of local inflammation and infection, but could also have detrimental effects if the oxidative response is unchecked and contributes to overwhelming systemic inflammation. However, as the major effects of hydrocortisone therapy in the study herein were anti-inflammatory, and any differences in ROS production between groups did not reach statistical significance, additional study is needed to clarify if low-dose hydrocortisone truly has any stimulatory effects on receptor-mediated ROS production in neutrophils or other phagocytes.

The finding that low-dose hydrocortisone does not impair phagocytosis of gram positive or gram negative bacteria by isolated foal neutrophils is consistent with previous reports in adults. No impairment in phagocytic function was noted in healthy adult horses⁶⁷ or in bacteremic rabbits⁶⁰ that received hydrocortisone (1 mg/kg IV bolus in horses, 1.4 or 14 mg/kg IV bolus then 0.18 mg/kg/hr IV infusion in rabbits). In addition, neutrophil phagocytosis was either unchanged⁶⁴ or enhanced⁶³ in septic adults who received low-dose hydrocortisone via standard therapeutic protocols. Thus, the results herein support these previous studies and suggest that such innate immune mechanisms remain similarly intact in neonates receiving low-dose hydrocortisone.

The significant reduction in endotoxin-induced gene expression of the pro-inflammatory cytokines TNF- α , IL-6, IL-1 β , and IL-8 in mononuclear cells from TREATED foals was also consistent with previous reports of decreased pro-inflammatory mediators in septic adult humans receiving low-dose hydrocortisone.^{38,63,64,68,69} However, to the authors' knowledge this is the first study to show persistence of these anti-inflammatory effects following discontinuation of hydrocortisone therapy, as evidenced by a significant decrease in TNF- α , IL-6, and IL-1 β expression in TREATED foals 12 hours after the last dose of hydrocortisone was administered. As transcriptional effects of corticosteroids can involve modification of production of other regulatory molecules,⁷⁰ it is not surprising that some of these functional genomic effects might persist beyond exposure to the steroid compound itself. Thus, though further study is needed, it is possible that a short course of hydrocortisone may also result in prolonged anti-inflammatory effects in clinical patients.

In addition to the decrease in pro-inflammatory cytokine expression, *increased* expression of the anti-inflammatory cytokines TGF- β , IL-10 and IL-4 was anticipated in

TREATED foals. However, relevant (> 3 fold) endotoxin-induced changes in expression of TGF- β or IL-4 were not observed in any foals in either group for any incubation time-points, so further between-group comparisons were not possible for these genes in the study herein. Expression of these anti-inflammatory cytokines may not be induced until later in the course of the inflammatory response, and might have been observed if a longer period of endotoxin exposure had been carried out in this study. However, as substantially decreased *ex vivo* PMBC viability was expected with incubations longer than 24 hours, endotoxin-stimulation was limited to 20 hours for this study. Thus, an effect of hydrocortisone on anti-inflammatory cytokine expression could have been missed with this experimental protocol. Alternatively, it is possible that expression of TGF- β and IL-4 is simply not regulated via TLR-4-mediated pathways in foal PBMCs and thus would not be induced by endotoxin exposure.

Expression of the anti-inflammatory cytokine IL-10 was significantly increased after 4 hours of endotoxin exposure in comparison to unstimulated cells in both groups of foals, but expression in TREATED foals was comparable to CONTROL foals. Corticosteroid treatment has been shown to increase production of the predominantly anti-inflammatory cytokine IL-10 from isolated human PBMCs,²⁴ but other studies have documented *suppression* of IL-10 production with low-dose hydrocortisone therapy in septic adult humans⁶⁴ or healthy adult humans exposed to endotoxin.⁶⁶ These seemingly contradictory effects may reflect temporal changes in cytokine production related to the duration of the inflammatory response in clinical sepsis versus experimental endotoxemia. Furthermore, as the study herein examined IL-10 gene expression rather than protein production as in the aforementioned studies, similar corticosteroid-induced changes in IL-10 production in neonatal foals may have been missed in this study if such changes are mediated at the post-transcriptional rather than transcriptional level.

One concern regarding use of corticosteroids in critically ill patients is suppression of the patient's endogenous HPA axis activity through negative feedback by high levels of exogenous corticosteroids. The results herein, though, suggest that significant HPA axis suppression did not occur in TREATED foal with the low-dose hydrocortisone therapy protocol used in this study, as 12- and 24-hour post-treatment basal cortisol concentrations and peak and delta cortisol responses to both low- (10 µg) and high- (100 µg) dose cosyntropin stimulation 24 hours after cessation of hydrocortisone therapy in TREATED foals were not significantly different from responses in age-matched CONTROL foals. And, as peak hydrocortisone concentrations achieved with this low-dose hydrocortisone regimen (range 15 – 44.9 µg/dl) remained within the range of physiologic cortisol concentrations reported in critically ill neonatal foals,^{52,71,72} such dosing protocols seem unlikely to result in substantial or long-term HPA axis suppression.

However, it is important to note that the low-dose hydrocortisone regime used in this study employed a lower daily dose and shorter course of therapy than current recommendations in septic adult humans.⁶ The dose used in our study was based on daily endogenous cortisol production rates in healthy foals^c and derived similarly to low-dose hydrocortisone recommendations in adult humans.^{2,38,53} In addition, given differences in corticosteroid production and metabolism between the neonate and adult,^{4,7,39,73} as well as the greater risks of spontaneous intestinal perforation associated with corticosteroid administration in pre-term neonates,³⁷ a lower dose and shorter course of therapy may be more appropriate for neonates. In addition, this lower-dose/shorter-course hydrocortisone regimen still resulted in suppression of the pro-inflammatory response comparable to the degree described with higher doses in adult animals and people.^{21,24,38,60,64,69}

In addition, the study herein utilized an *ex vivo* model of infection with cells from healthy animals, and the results should be confirmed with further *in vivo* and clinical studies in neonates. As the inflammatory response in clinical patients is likely to be present for a longer duration and to a greater degree than in this experimental model, it is possible that a higher dose or longer course of corticosteroids might still be needed to produce similar pro-inflammatory suppression in critically ill individuals. Some studies also suggest that cortisol resistance in peripheral tissues may be a significant problem in some critically ill patients and may play a role in the pathogenesis of CIRCI;^{1,5,74-76} in such patients, hydrocortisone therapy may be less effective. Furthermore, as the appropriate cortisol response to illness varies between individuals and during different stages of disease, determination of one specific hydrocortisone dosing protocol with global applications to all critically ill patients is likely impossible. In an individual patient, titration of the hydrocortisone dose until the desired hemodynamic and anti-inflammatory effects are achieved may offer the most ideal therapeutic approach. Given the anti-inflammatory effects shown for the protocol used herein, a total daily dose of 1-2 mg/kg divided into several intermittent IV boluses, as recently recommended by Fernandez and Watterberg, appears to represent an appropriate starting place for such titration in the neonate.⁴

In conclusion, the low-dose hydrocortisone therapy protocol employed in the study herein dampened the *ex vivo* pro-inflammatory response to endotoxin in neonatal foals without significantly impairing *ex vivo* innate immune function or endogenous HPA axis activity. The anti-inflammatory effects of this protocol occurred with a lower dose and shorter course than is currently employed in clinical patients with CIRCI, and some anti-inflammatory effects persisted for at least 12 hours after discontinuation of hydrocortisone. Further study is needed to evaluate

immunologic and clinical effects of a similar protocol in *in vivo* experimental models and in critically ill neonates.

FOOTNOTES

^a SNAP™ Foal IgG Test, IDEXX Laboratories, Inc., Westbrook, ME

^b Solu-Cortef™, hydrocortisone sodium succinate, Pfizer Inc., New York, NY

^c Hart KA, Dirikolu L, Ferguson DC, Barton MH. Daily endogenous cortisol production and hydrocortisone pharmacokinetics in healthy neonatal foals and adult horses. Research Abstract, presented at the American College of Veterinary Internal Medicine Forum, Anaheim, CA, June 9-12, 2010.

^d Cortrosyn™, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA

^e Immulite™, Diagnostics Product Corporation, Los Angeles, CA

^f Immulite™ Cortisol Assay package insert. Diagnostics Product Corporation.

^g GraphPad Prism Statistical Software (Version 4), GraphPad Software Inc., San Diego, CA

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Table 8.1. Serum cortisol/hydrocortisone concentrations ($\mu\text{g}/\text{dl}$) in TREATED and age-matched CONTROL foals before, during and after low-dose hydrocortisone treatment. Samples for trough and peak cortisol/hydrocortisone concentrations DURING TREATMENT (after 48 hours of low-dose hydrocortisone administered) were collected immediately before and 5 minutes after IV bolus administration of hydrocortisone respectively.

	Serum Cortisol/Hydrocortisone Concentration PRE-TREATMENT (Day 2 of age)	Serum Cortisol/Hydrocortisone Concentration DURING TREATMENT (Day 4 of age)		Serum Cortisol/Hydrocortisone Concentration POST-TREATMENT (Day 6 of age)
		Trough	Peak	
TREATED Foals (n=11)	2.6 \pm 1.0 (1.6 – 4.7)	2.1 \pm 1.4 (0.4 – 4.9)	22.3 \pm 9.5 (15.0 – 44.9)	2.5 \pm 1.0 (1.2 – 4.6)
CONTROL Foals (n=11)	2.4 \pm 1.0 (1.3 – 4.4)	n/a	n/a	2.0 \pm 0.8 (1.0 – 3.6)

Data shown are mean \pm standard deviation (range). No significant differences ($P < 0.05$) were found between TREATED and CONTROL foals. Cortisol and hydrocortisone are chemically indistinguishable.

Table 8.2. Serum cortisol concentrations ($\mu\text{g/dl}$) before (Basal Cortisol) and after administration of 10 μg cosyntropin (Low-dose Peak Cortisol) and 100 μg cosyntropin (High-dose Peak Cortisol) in TREATED and age-matched CONTROL foals undergoing a paired low-dose high-dose cosyntropin stimulation test. Delta cortisol concentrations were calculated by subtracting the basal cortisol concentration from the peak cortisol concentration reached after 10 μg cosyntropin (Low-dose Delta Cortisol) and 100 μg cosyntropin (High-dose Delta Cortisol). Cosyntropin stimulation tests were performed 24 hours after discontinuation of hydrocortisone therapy in TREATED foals (on day 6 of life) and between days 5-7 of life in CONTROL foals.

	Basal Cortisol	Low-dose Peak Cortisol	High-dose Peak Cortisol	Low-dose Delta Cortisol	High-dose Delta Cortisol
TREATED Foals (n=8)	1.8 \pm 0.9 (0.7 – 3.6)	3.1 \pm 1.0 (1.9 – 4.6)	5.0 \pm 1.7 (3.4 – 7.9)	1.4 \pm 0.7 (0.6 – 2.8)	3.2 \pm 1.4 (1.8 – 6.1)
CONTROL Foals (n=11)	2.0 \pm 0.8 (1.0 – 3.6)	3.3 \pm 0.8 (2.3 – 4.4)	5.5 \pm 1.1 (3.5 – 7.4)	1.3 \pm 0.6 (0.6 – 2.6)	3.5 \pm 1.3 (1.7 – 5.9)

Data are shown as mean \pm standard deviation (range). No significant differences ($P < 0.05$) were found between TREATED and CONTROL foals.

Figure 8.1. Reactive oxygen species (ROS) production in isolated neutrophils from TREATED foals (n=11) before, during, and after hydrocortisone treatment and in age-matched CONTROL foals (n = 13) after stimulation with (A) phorbol myristate acetate (PMA; 10^{-7} M), (B) endotoxin (100 ng/ml), and (C) zymosan (1000 ng/ml). ROS production is expressed as mean (+ standard deviation) corrected arbitrary fluorescent units (AFUs), which were calculated by subtracting background fluorescence in unstimulated control cells from fluorescence in stimulated cells. No significant differences ($P < 0.05$) were found between TREATED and CONTROL foals.

Figure 8.1 (A)

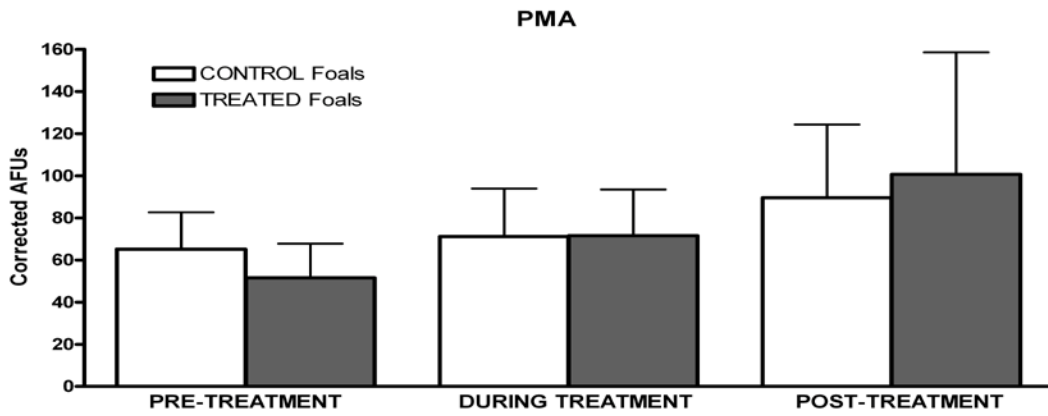


Figure 8.1 (B)

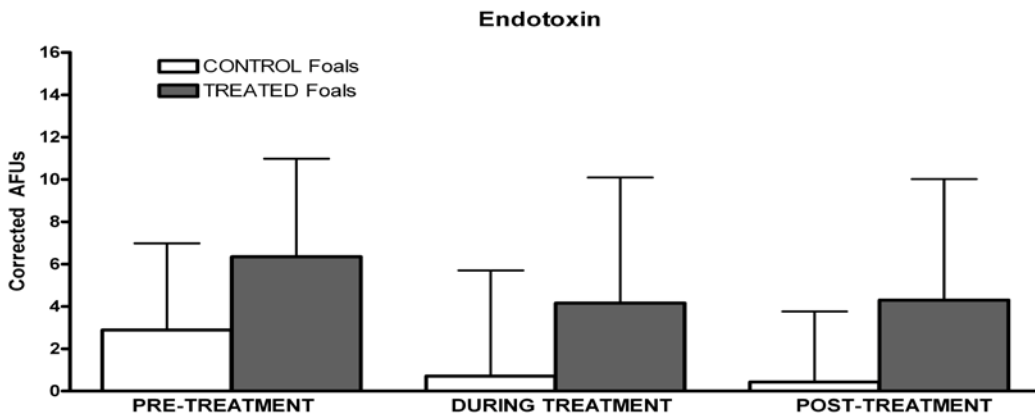
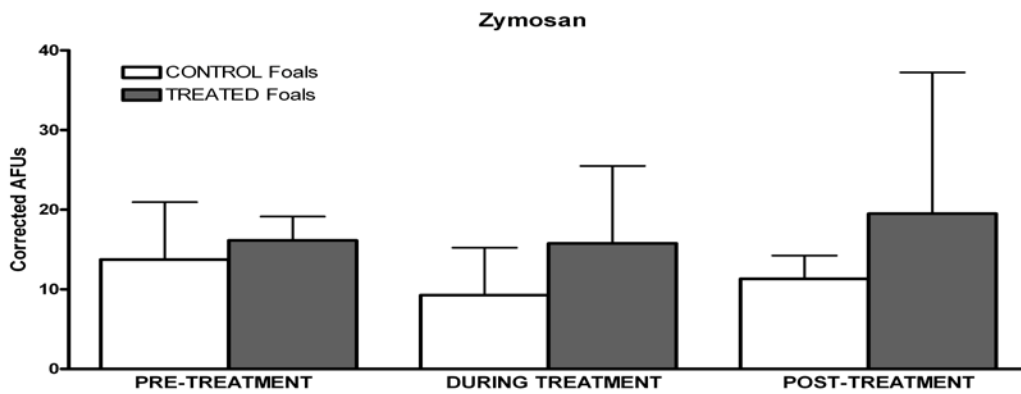
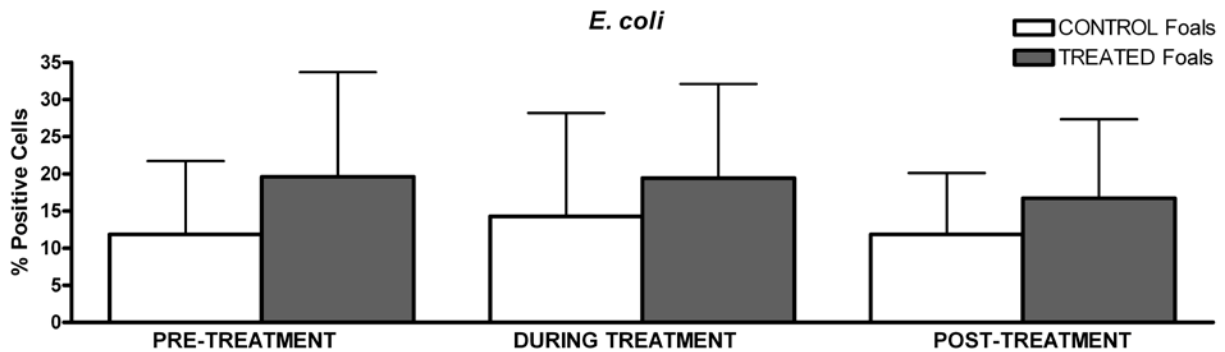


Figure 8.1 (C)



A)



B)

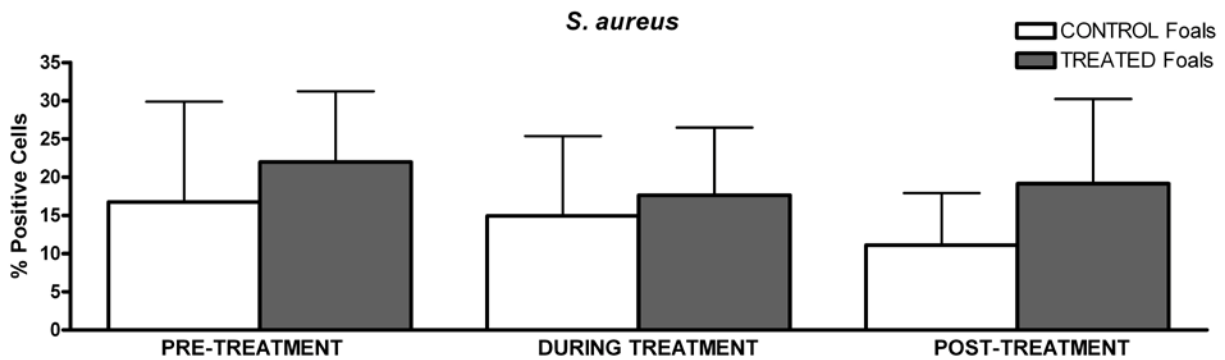


Figure 8.2. Phagocytosis of killed, bodipy-labelled (A) *E. coli* and (B) *S. aureus* by isolated neutrophils from TREATED foals (n=11) before, during, and after treatment with low-dose hydrocortisone and in age-matched CONTROL foals (n=9). Uptake of bacteria is expressed as mean (+ standard deviation) % positive cells. Extracellular fluorescence was quenched with 0.4% trypan blue in all samples before analysis. No significant differences ($P < 0.05$) were found between TREATED and CONTROL foals.

Figure 8.3. Mean (+ standard deviation) fold change in mRNA expression of TNF- α , IL-6, IL-1 β , IL-8, and IL-10 in PBMCs from TREATED foals (n=11) and age-matched CONTROL foals (n= 15) incubated with endotoxin (1 ng/ml) for 1, 4, and 20 hours. Fold change in mRNA expression is relative to unstimulated PBMCs from the same animal incubated for the same 3 durations. Expression before, during, and after hydrocortisone treatment is shown in parts (A), (B), and (C) respectively. *Denotes a significant ($P < 0.05$) difference in endotoxin-induced mRNA expression between TREATED and age-matched CONTROL foals.

Figure 8.3 (A)

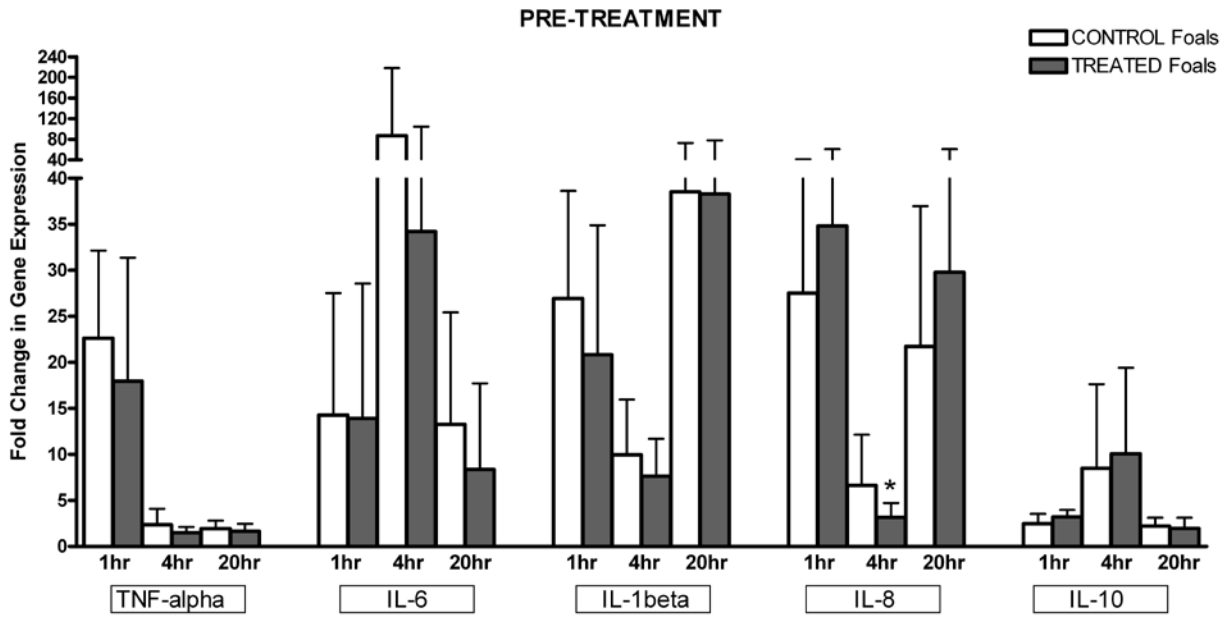


Figure 8.3 (B)

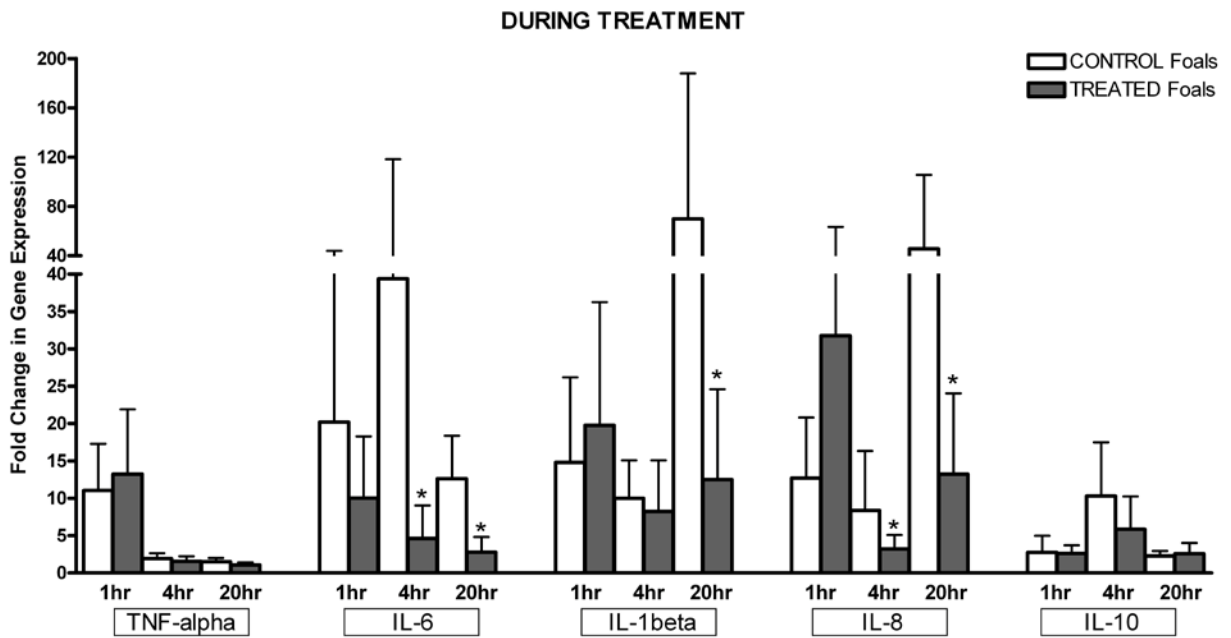
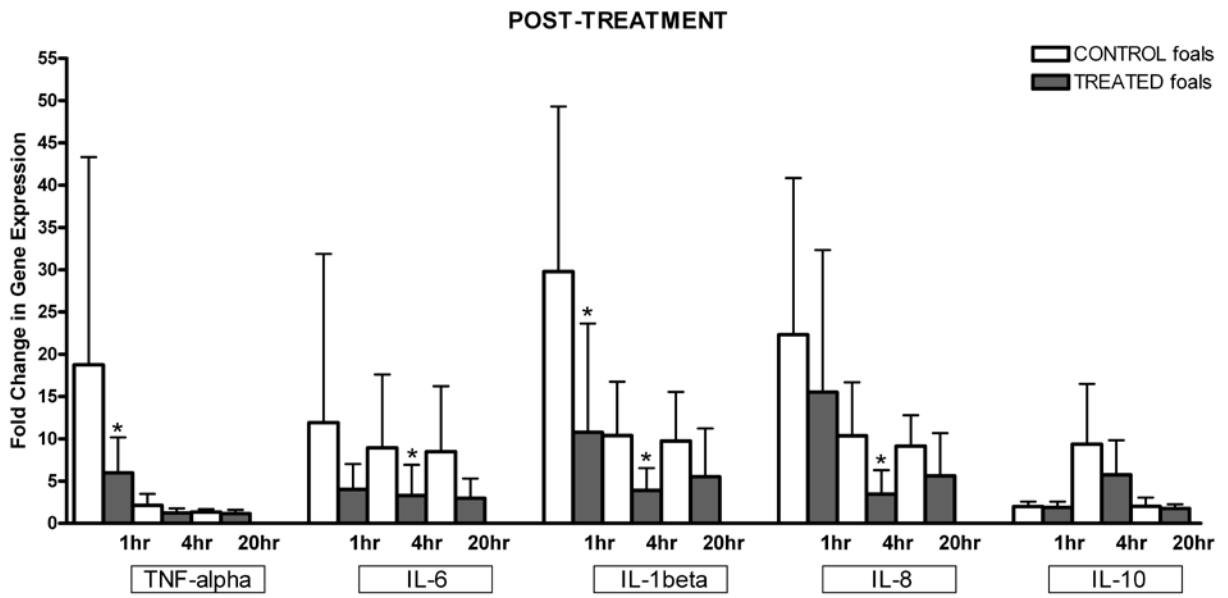


Figure 8.3 (C)



CHAPTER 9

CONCLUSION

A large amount of evidence has been presented in previous chapters to document the frequent occurrence of transient HPA axis dysfunction, resulting in absolute and/or functional cortisol insufficiency (RAI/CIRCI), in critically ill people.¹⁻¹⁶ Further, RAI/CIRCI is correlated with increased disease severity and poor prognosis in people,^{10,13,17,18} and can be managed successfully in many patients with cortisol replacement therapy in the form of low-dose hydrocortisone.^{5,19-26} Furthermore, studies suggesting that RAI/CIRCI occurs with similar prevalence in critically ill infants are presented, along with evidence that illness-induced HPA axis dysfunction may be compounded by HPA axis immaturity in this population.^{4,14} Finally, several recent studies documenting evidence for a comparable incidence and impact of RAI/CIRCI in critically ill dogs are discussed.^{27,28} In concert, these studies indicate that RAI/CIRCI is of significant concern in both human and veterinary medicine.

Prior to the studies conducted during completion of this dissertation, the incidence of RAI/CIRCI had not been investigated in the neonatal foal. However, based on previous work describing delays in HPA axis maturation resulting in limited cortisol synthetic capacity in the fetal and neonatal foal,²⁹⁻³³ foals may be uniquely predisposed to the development of cortisol insufficiency if severe illness occurs during the neonatal period. Further, bacterial sepsis and septic shock – the diseases most often associated with RAI/CIRCI in other species – occur frequently in the neonatal foal, and are the leading cause of mortality in foals less than 7 days of

age.³⁴⁻³⁸ Thus, given the combination of HPA axis immaturity and a high incidence of bacterial sepsis, the potential for RAI/CIRCI in the critically ill neonatal foal was considered great.

Thus, the overall objective for the studies presented herein was to investigate HPA axis function during both health and illness in the neonatal foal. The first two studies attempted to define and characterize normal HPA axis function during the immediate postnatal period in the healthy foal, and develop a paired low-dose / high-dose ACTH stimulation test protocol for use in foals in a clinical setting. The third study sought to determine the incidence and impact of HPA axis dysfunction (RAI/CIRCI) in critically ill neonatal foals utilizing both basal hormone concentrations and this paired low-dose / high-dose ACTH stimulation test. The fourth study optimized a free cortisol assay used in other species for equine use, determined heretofore unreported serum free cortisol fraction in healthy and septic neonatal foals, and investigated the relative diagnostic accuracy of total and free cortisol parameters for assessing HPA axis function and predicting disease severity and outcome in septic neonatal foals. In the fifth study, daily endogenous cortisol production and hydrocortisone pharmacokinetics were compared between healthy neonatal foals and adult horses, and were used to determine a physiologically relevant hydrocortisone dose and dosing strategy for cortisol replacement therapy in neonatal foals with RAI/CIRCI. The final study then evaluated the immunologic effects of this low-dose hydrocortisone replacement therapy regimen in healthy neonatal foals in an *ex vivo* sepsis model to ensure it did not induce an undesirable degree of immunosuppression that could be harmful in foals with naturally-occurring sepsis.

The results of the initial studies herein provide additional evidence that HPA axis immaturity persists into the postnatal period in the foal. First, while 3-4-day-old foals exhibited a dose-dependent response to intravenous cosyntropin as has been reported in people, dogs, and

adult horses,^{11,15,39-42} they did not show a measurable cortisol response to a 1 µg (~0.02 µg/kg) dose of cosyntropin. This finding is in contrast to findings in healthy people, infants and dogs,^{15,41-43} in which a significant increase in cortisol concentrations from baseline concentrations was found after administration of a comparable amount or dose of cosyntropin. This suggests that limitations in adrenocortical sensitivity to ACTH, cortisol synthetic capacity, or both may be present in the immediate postnatal period in the foal.

When HPA axis function was evaluated at four time points during the first week of life in healthy foals in the study discussed in Chapter 4, further support for impaired cortisol responses to both endogenous and exogenous ACTH was found. While both endogenous cortisol and endogenous ACTH concentrations were increased appropriately in foals at birth in response to periparturient stresses, by 12 hours of age cortisol concentrations were substantially *lower* than resting cortisol concentrations reported in healthy adult horses.^{44,45} (Figure 9.1a) Concurrently, resting ACTH concentrations were either comparable to or considerably *higher* than concentrations reported in healthy adult horses at rest during the spring season.⁴⁶ (Figure 9.1b) In addition, by 5-7 days of age, delta cortisol responses to even a supraphysiologic (100 µg, ~2 µg/kg) dose of cosyntropin observed in foals in this study (3.5 ± 1.3 µg/dl) were decreased in comparison to delta cortisol values reported in adult horses (approximately 9 µg/dl) in response to an equivalent dose of cosyntropin.⁴⁰ Again, these findings imply that either adrenocortical ACTH-sensitivity or cortisol synthetic capacity are somewhat limited in the healthy full-term neonatal foal.

The results from the initial two studies in healthy foals presented herein determined normal endogenous HPA axis activity and appropriate cortisol responses to this paired low-dose / high-dose ACTH stimulation test protocol during health in the immediate postnatal period, to

permit interpretation of future HPA axis testing in critically ill neonatal foals in a clinical setting. This work facilitated the adaptation of current diagnostic criteria for RAI/CIRCI utilized in septic people⁹ for application to critically ill foals, and permitted the first study investigating the incidence of RAI/CIRCI in critically ill neonatal foals described in Chapter 5. Results of this study suggest that RAI/CIRCI occurs in approximately 40% of critically ill and septic foals, an incidence comparable to septic people, infants, and dogs.^{2,8,10,18,27,28} In addition, a blunted cortisol response to the high-dose ACTH stimulation test was correlated, as in other species,^{2,8,10,13,18,27,28} with significantly increased disease severity and decreased survival rates. In fact, non-surviving foals had significantly *higher basal cortisol concentrations* but significantly *lower cortisol responses to a high-dose of exogenous ACTH* than surviving foals. Together, these observations provide additional support to the concept that cortisol synthetic capacity may be quickly exceeded (so-called “loss of adrenal reserve”) by high cortisol demand in some critically ill neonatal foals.

Because the findings discussed in Chapter 5 imply a substantial incidence and impact of RAI/CIRCI in the septic neonatal foal, the study in Chapter 6 was undertaken to determine if free cortisol may offer a similar diagnostic advantage in foals with RAI/CIRCI, as it appears to in people.⁴⁷⁻⁵¹ In contrast to these human studies, no predictive advantage of free over total cortisol parameters for predicting disease severity or outcome in septic foals was identified. However, important differences in plasma cortisol binding capacity in foals as compared to adult horses were identified. Specifically, cortisol binding capacity appears to be significantly and dramatically reduced in neonatal foals, as evidenced by a free cortisol fraction of 30-60% in the healthy foal versus approximately 10% in the healthy adult horse. Moderately increased free cortisol fractions are described in neonates of other species,^{52,53} but not to the degree

demonstrated here in the neonatal foal. While free cortisol is important for diffusion into cells to interact with the glucocorticoid receptor and exert cortisol-specific genomic and non-genomic effects, it is also more readily available for metabolism and excretion. Thus, these findings imply that the substantially reduced cortisol binding capacity in neonatal foals may permit more rapid cortisol clearance in the neonatal foal, which has been shown in other species to contribute to cortisol insufficiency during periods of increased cortisol demand such as during severe illness.⁵⁴

The study in Chapter 7 compared daily endogenous cortisol production and hydrocortisone pharmacokinetics between healthy neonatal foals and adult horses to further investigate potential physiologic implications of this increased free cortisol fraction in foals. Two-to-four-day-old foals showed a significantly larger hydrocortisone volume of distribution and more rapid hydrocortisone (cortisol) clearance rate than adult horses. These parameters were increased approximately 3-fold over values in adult horses, consistent with an approximately 3-fold increase in free cortisol fraction in neonatal foals at this age as compared to adult horses. Daily endogenous cortisol production rate was significantly increased in foals as well, probably in an attempt to maintain appropriate physiologic cortisol concentrations. However, despite this increased daily cortisol production rate, mean 24-hour cortisol concentration was slightly but significantly lower in foals than adult horses. Thus, these findings again suggest that either ACTH-sensitivity or cortisol synthetic capacity may be restricted in the neonatal foal. Measurement of ACTH secretion rates in conjunction with cortisol in foals and adult horses in a future study could help differentiate between these two possibilities.

In addition, assessment of daily endogenous cortisol production rate in healthy neonatal foals in this study permitted development of an appropriate hydrocortisone dose for future

cortisol replacement therapy in septic foals with RAI/CIRCI. In people and infants with RAI/CIRCI, a total daily hydrocortisone dose of approximately 2-4 times the endogenous cortisol production rate in unstressed healthy individuals is recommended.^{1,4,8,14,55} Thus, a hydrocortisone dose of 1.3 mg/kg/day (2 times the daily endogenous cortisol production rate in healthy foals) was determined for further evaluation and potential use in septic foals with RAI/CIRCI. Division of this total dose into 4-hour dosing intervals was elected based on pharmacokinetic parameters and endogenous cortisol secretion patterns identified in healthy foals in the previous study, and a tapering 3.5 day course of treatment designed based on recommendations in people and infants.^{4,9}

In the final study presented herein, the immunological effects of this proposed low-dose hydrocortisone therapy regimen were determined in healthy foals. No evidence of impairment in neutrophil function was identified in treated foals as compared to age-matched untreated foals, suggesting innate immune responses integral to antibacterial defense mechanisms remained intact. The endotoxin-induced pro-inflammatory cytokine response in peripheral blood mononuclear cells, though, was significantly decreased during hydrocortisone treatment in treated foals, and this ameliorated pro-inflammatory response persisted for at least 12 hours following discontinuation of hydrocortisone. These results are consistent with findings in other species,^{21,54,56-61} and imply that this proposed low-dose hydrocortisone therapy regimen may have desirable anti-inflammatory effects in septic foals without inducing undue immunosuppression or immunoparalysis that could impair clearance of the initial bacterial infection.

In sum, the results of the studies presented herein provide evidence for persistent postnatal HPA axis immaturity in the full-term foal, and suggest that decreased adrenocortical

sensitivity to ACTH or limited cortisol synthetic capacity are key factors in this impairment that may render the critically ill foal at great risk for development of RAI/CIRCI. In addition, these findings document decreased cortisol binding capacity resulting in more rapid cortisol clearance in foals, which might further contribute to cortisol insufficiency during periods of increased cortisol demand such as critical illness. Finally, this work documents a comparable incidence and impact of RAI/CIRCI in critically ill neonatal foals as is described in other species, and provides initial studies to investigate specific diagnostic criteria and potential therapeutic interventions for RAI/CIRCI in foals.

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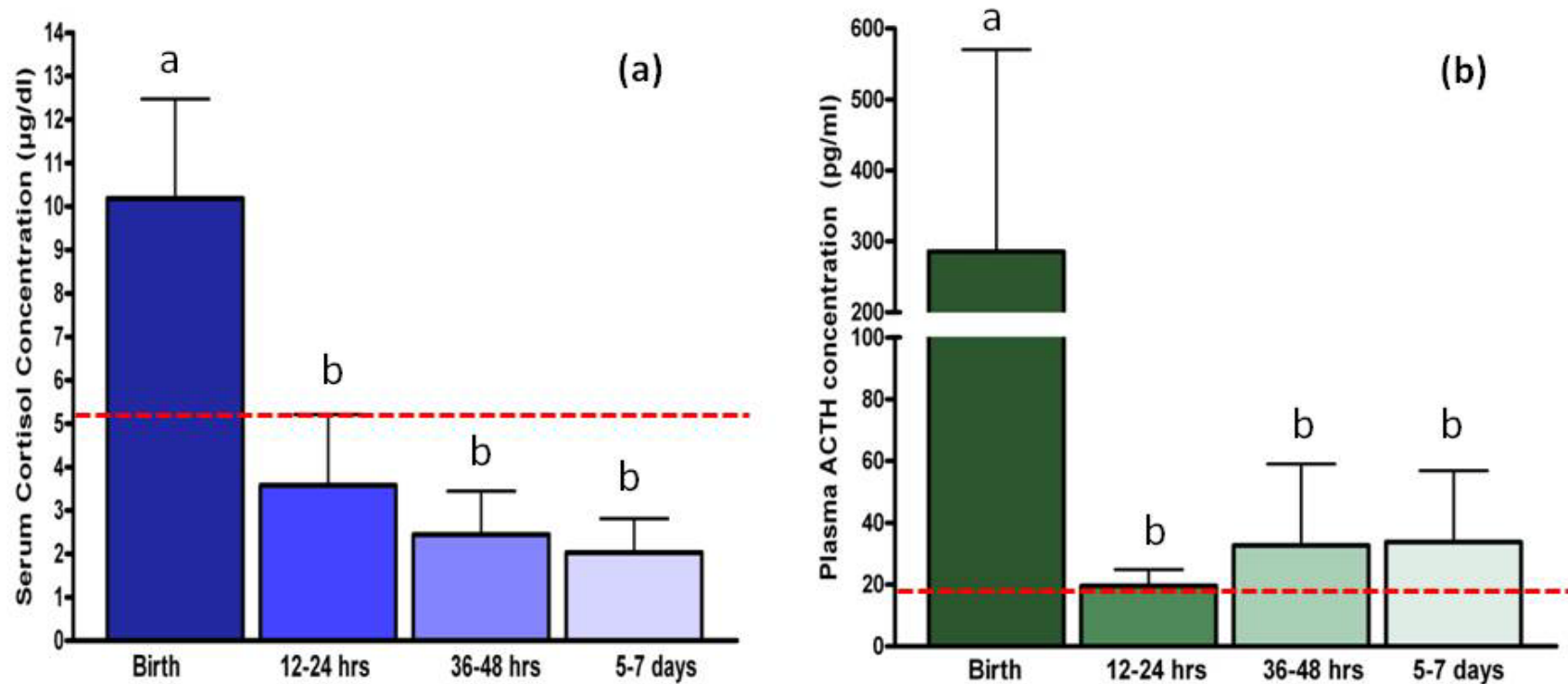


Figure 9.1. Basal serum cortisol concentrations (a) and plasma ACTH concentrations (b) in 11 healthy neonatal foals at 4 ages during the first week of life. For comparison with adult HPA axis parameters, mean basal cortisol and ACTH concentrations in healthy unstressed adult horses described in previously studies⁴⁴⁻⁴⁶ are represented by the dashed red line on each graph.