Glucocorticoid Receptor Density and Binding Affinity in Horses with Systemic Inflammatory Response Syndrome

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ABSTRACT

There were three objectives of this study. The first was to determine if commercially available fluorochromes could be used to determine the glucocorticoid receptor (GR) density and binding affinity (BA) in equine peripheral blood mononuclear cells. The second was to determine if there was a correlation between elevated plasma cortisol and GR density or binding affinity in healthy adult horses. The third objective was to evaluate the HPA axis in adult horses presenting with systemic inflammatory response syndrome (SIRS), and to determine where any alterations in HPA axis function occur in these patients compared to healthy adults. For the first part of the study, peripheral venous blood was collected from 3 healthy research horses on 3 days. Peripheral blood mononuclear cells were isolated using Ficoll gradient centrifugation. Phycoerythrin (PE)-CD44 was then used to extracellularly label leukocytes, and then an intracellular GR antibody was used to determine a baseline measurement of GR density and fluorescein isothiocyanate (FITC)-dexamethasone was used to determine binding affinity via flow cytometric analysis. Comparison of control samples to those for CD44, GR density, and GR binding affinity showed a statistically significant difference for all samples ($P<0.0001$, $P<0.0001$, and $P<0.0001$ respectively). This showed that the CD44, GR antibody, and FITC-dexamethasone could successfully be used to analyze equine peripheral blood mononuclear cells for GR activity.

For the second part of the study, an ACTH stimulation test was performed on 8 healthy horses in order to induce an increase in endogenous cortisol production. Plasma cortisol levels, GR density, and GR binding affinity were measured at baseline, 4, 8, and 24 hours after treatment. Median basal cortisol concentration was 4.9, range 3.2-6.1 µg/dl. This initially increased following ACTH stimulation to 5.6, range 4.8-7.4 µg/dl, then showed a significant decrease by 8 hours post ACTH administration to 1.4, range 1.1-2.7 µg/dl ($P=0.0221$). No correlation was observed between plasma cortisol concentration in healthy horses and GR density or binding affinity ($r=-0.145$, $P=0.428$ and $r=0.046$, $P=0.802$, respectively).

For the third phase of the study, horses (N=10) with systemic inflammatory response syndrome (SIRS) were compared to healthy, age and sex matched controls (N=10) presenting for

lameness evaluation or ophthalmologic examination. Blood was collected from SIRS cases and controls on presentation to the Equine Medical Center. A CBC, serum biochemistry, and serum ACTH and cortisol measurements were performed. GR density and binding affinity were also determined. Nonsurvivors had a significantly decreased GR binding affinity (P=0.008) and demonstrated a trend towards an increase in the ACTH:cortisol ratio. ROC analysis was performed for serum ACTH and cortisol concentrations, the ACTH:cortisol ratio, GR density and GR binding affinity, and triglycerides to determine cut-off values associated with nonsurvival. These were then used to analyze this population using Fischer's exact test to determine the odds ratio (OR) associated with nonsurvival for each variable. This revealed that a serum triglyceride concentration greater than 28.5 mg/dl was associated with nonsurvival (OR=117, 95% CI, 1.94-7060). The other variables were not found to be significantly associated with nonsurvival, although a Delta BA% of less than 35.79% was found to be closely associated with nonsurvival (OR=30.33, 95% CI, 0.96-960.5). Additionally, a significant negative correlation was detected between the plasma ACTH concentration and Delta BA% (r=-0.685, P=0.029) and the ACTH:cortisol ratio and the Delta BA% (r=-0.697, P=0.025).

This study showed that nonsurviving horses with SIRS had a significantly decreased GR binding affinity compared to survivors, and a tendency toward an increase in their ACTH:cortisol ratios. This confirms that HPA axis dysfunction occurs in adult horses with SIRS as tissue resistance to glucocorticoids, and potentially relative adrenal insufficiency as well. These results suggest that there are horses with SIRS that might benefit from "physiologic" doses of synthetic glucocorticoids to complement their relative adrenal insufficiency in addition to their poor tissue sensitivity. Further research should focus on methods to more rapidly determine which horses might benefit from treatment with glucocorticoids on presentation, as well as to more accurately determine prognosis for survival.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophin hormone
Ala	alanine
ALP	alkaline phosphatase
ANOVA	analysis of variance
AP-1	activator protein 1
APC	allophycocyanin
APP	acute phase protein
AST	aspartate aminotransferase
AVP	arginine vasopressin
BA	binding affinity
bands	band neutrophils
BUN	blood urea nitrogen
Ca	calcium
CBC	complete blood count
CBG	cortisol binding globulin
CD	cluster of differentiation
CI	confidence interval
CIRCI	critical illness related corticosteroid insufficiency
Cl	chloride
COX-2	cyclo-oxygenase 2
Creat	creatinine
CRH	corticotropin releasing hormone
CK	creatine kinase
DAMP	damage associated molecular pattern
D Bili	direct bilirubin
Delta BA%	percentage difference between the FITC-dexamethasone binding and control
Delta GR%	percentage difference between the FITC-GR binding and control
dl	deciliter
eos	eosinophils
fib	fibrinogen
FITC	fluorescein isothyocyanate
FL1	channel on flow cytometer specific for detecting FITC
FL2	channel on flow cytometer specific for detecting PE
FL3	channel on flow cytometer specific for detecting PI
FSC	forward scatter
g	standard acceleration due to gravity
GGT	gamma-glutamyl transpeptidase
GR	glucocorticoid receptor
GRα	glucocorticoid receptor α
GRβ	glucocorticoid receptor β
^{3}H	tritium
hct	hematocrit
HPA	Hypothalamic-Pituitary-Adrenal
IgG	immunoglobulin G
Igs	immunoglobulins

IL	interleukin
IKK	inhibitor of κB kinase
iNOS	inducible nitric oxide synthase
K	potassium
kg	kilogram
l	liter
LBP	LPS binding protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
lymphs	lymphocytes
M	molar
MAPK	mitogen activated protein kinase
MC2R	melanocortin 2 Receptor
Mg	magnesium
µg	microgram
µl	microliter
ml	milliliter
MODS	multiple organ dysfunction syndrome
monos	monocytes
mRNA	messenger ribonucleic acid
Na	sodium
neuts	neutrophils
NLR	nucleotide binding domain leucine-rich repeat containing proteins
NF-κB	nuclear factor κB
ng	nanogram
nmol	nanomole
OR	odds ratio
p450scc	cholesterol side chain cleavage enzyme
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
pg	pictogram
Phos	phosphorus
PI	propidium iodide
plt	platelets
PRR	pattern recognition receptor
RA	rheumatoid arthritis
ROC	receiver operator characteristic
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
RR	relative risk
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SSC	side scatter

T Bili	total bilirubin
TF	tissue factor
TG	triglycerides
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
TNFR	TNF receptors
TP	total protein
VCAM-1	vascular cell adhesion molecule 1
WBC	white blood cell

Chapter 1. Introduction and Review of the Literature

The Systemic Inflammatory Response Syndrome and Endotoxemia

Sepsis, which is defined as the systemic inflammatory response to infection, represents a major cause of death in critically ill human patients, and has been reported to have a case fatality rate approaching 50% in a variety of veterinary species [1, 2]. However, the systemic inflammatory response can be the result of non-infectious causes as well. The term Systemic Inflammatory Response Syndrome (SIRS) has been coined to describe this clinical syndrome, irrespective of its cause [1, 3]. In the equine patient, SIRS plays a major role in severe pathologic conditions, including neonatal septicemia, colitis, various causes of colic, and pleuropneumonia and is characterized by widespread uncontrolled inflammation leading to systemic disease, shock, and death [4, 5]. Regardless of the initiating cause, SIRS in the equine patient is defined when two or more of the following criteria are met: (1) leukocytosis or leukopenia (white blood cell count greater than 12,000 cells/µl or less than 5,000 cells/µl), or the presence of 10% or greater band neutrophils, (2) hyperthermia (rectal temperature of greater than 101.5^{o}F for adults and greater than 102.5^{o}F in foals), or hypothermia in foals (rectal temperature less than 98.9^{o}F) (3) tachycardia (heart rate greater than 60 beats per minute in an adult or greater than 120 beats per minute in a foal), and/or (4) tachypnea (respiratory rate greater than 30 breaths per minute) [5-7].

SIRS is most often associated with sepsis and endotoxemia in the equine patient. It is initiated when pattern recognition receptors (PRR) on various effector cells of the immune system – including macrophages, neutrophils, dendritic cells, and lymphocytes – bind to pathogen-associated molecular patterns (PAMP) found on a potential pathogen [2]. These PAMP's are a key component in the ability of the innate immune system to discern "self" from "non-self". They are only produced by microbial pathogens and are generally fixed molecules that are shared by entire classes of microorganisms, such as lipopolysaccharide (LPS) on the outer cell membrane of gram-negative bacteria or lipoteichoic acid (LTA) in the cell membrane of gram-positive bacteria. This allows the evolutionary preservation of a relatively small number of PRR's that are able to recognize and respond to a large number of potential pathogens. These PRR's include the toll-like receptors (TLR), the nucleotide binding domain, and leucine-rich repeat containing proteins (NLR) [2]. A large number of PRR's have been identified in animal models as well, demonstrating the importance of this interaction across species [8-15].

Additionally, PRR's can react to both pathogen- and host-derived ligands (PAMP's and damage associated molecular proteins (DAMP's), respectively), leading to propagation of the inflammatory response in a non-pathogen induced inflammatory state [16].

TLR's, which were first recognized as a transmembrane glycoprotein in *Drosophila melanogaster,* play a significant role in the mammalian innate immune response [17-19]. When a PAMP binds to the PRR, this leads to activation of a chain of kinases through the cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain of the TLR [2]. This signaling cascade leads to the activation of inhibitor of κB kinase (IKK) pathway or the mitogen-activated protein kinase (MAPK) pathway [2]. The end result of these pathways is the production of nuclear factor κB (NF-κB) and activator protein-1 (AP-1), which can then enter the nucleus and activate transcription sites for a variety of genes, including: acute phase proteins, inducible nitric oxide synthase (iNOS), coagulation factors, eicosanoids, pro-inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interleukin (IL) – 1β and 6, and anti-inflammatory cytokines, including IL-10 [2, 20, 21].

Endotoxin, which is one of the most significant causes of SIRS in horses, is the LPS portion of the outer cell wall of gram-negative bacteria [22, 23]. It is comprised of three structural components, including the lipid A portion, the O-specific chain, and the core region [24, 25]. The lipid A portion is highly conserved amongst bacterial species, and is the portion responsible for most of the toxic effects seen following LPS accumulation [25]. The normal equine gastrointestinal tract contains large numbers of gram-negative bacteria, which release endotoxin when they die or multiply rapidly [22, 23]. This endotoxin is confined to the intestinal lumen by the intestinal mucosal barrier consisting of tight junctions between mucosal epithelial cells, their secretions, and other normal bacterial flora. However, this barrier is not 100% effective, and small amounts of endotoxin normally escape into the hepatic portal circulation [22]. These molecules are typically rapidly removed by hepatic macrophages, or Kupfer cells, or by anti-endotoxin antibodies within the systemic circulation, preventing the development of a systemic inflammatory response [22]. However, under certain disease conditions including inflammation, ischemia or trauma to the gastrointestinal system, the intestinal mucosal barrier can become disrupted [22, 25-27]. This leads to a massive absorption of endotoxin by the portal circulation, exceeding the clearance capacity of the hepatic mononuclear phagocytic system, and allowing endotoxin to gain access to the systemic circulation [26]. Alternatively, LPS

originating from other sites in the body, including the peritoneal cavity, pleural cavity, or uterus, can bypass the portal circulation and enter the systemic circulation directly [24].

When LPS gains access to the systemic circulation, it can interact with plasma proteins and blood cells [22]. This process is initiated when LPS binds to lipopolysaccharide binding protein (LBP) at the lipid A moiety [22, 27]. LBP is an acute phase protein, synthesized by hepatocytes in response to inflammation, and it has a strong affinity for the lipid A portion of LPS as well as the cluster of differentiation 14 (CD14) receptor located in the cell membrane of various phagocytes of the innate immune system, including: peripheral blood mononuclear cells, neutrophils and tissue macrophages [2, 27, 28]. Thus, LBP acts as a shuttle to facilitate delivery of LPS to these cells. The LBP/LPS/CD14 complex increases the cell's sensitivity to endotoxin, however, the interaction of endotoxin with TLR-4 and myeloid differentiation factor 2 (MD-2) on the cellular surface is necessary to stimulate intracellular signal transduction pathways as described previously [22]. MD-2 is a protein that is associated with TLR-4 on the surface of the cell, and serves increase the responsiveness of TLR-4 to LPS binding [28, 29]. NFκB and AP-1 can then enter the cell nucleus, where they bind to promoter regions of genes responsible for the synthesis of various pro- and anti-inflammatory mediators [2, 29-31].

TNF-α is the first mediator released in response to endotoxin and is produced predominately by activated macrophages and T-cells [32]. TNF-α concentrations have been shown to be increased in 20% of horses presenting to an equine hospital for colic, and higher concentrations are associated with increased mortality [33]. It exerts its effects by interaction with one of 2 receptors, TNF receptors 1 and 2 (TNFR1 and TNFR2) [2]. Activation of the first receptor type mediates the pro-inflammatory and apoptotic pathways associated with inflammation, while activation of the second receptor type is involved in angiogenesis and tissue repair [34]. At the endothelium, TNF-α induces increased production of iNOS and cyclooxygenase 2 (COX-2), leading to vasodilation and decreased local blood flow as well as stimulation of endothelial adhesion molecules and vascular cell adhesion molecule 1 (VCAM-1) [35]. Activation of these molecules causes leukocytes to adhere to the endothelial wall, which is followed by transmigration into the interstitium and the disruption of vascular homeostasis, ultimately leading to fluid extravasation and tissue edema [36]. TNF-α also induces the expression of pro-coagulant proteins, such as tissue factor (TF), and causes the down-regulation of anti-coagulant factors, leading to activation of the coagulation cascade [37]. Additionally,

there is some interaction between the 2 receptor types, particularly at low TNF-α concentrations, leading to further NF-κB and AP-1 production and further propagation and/or amplification of the inflammatory response [38].

A number of pro-inflammatory interleukins, including IL-1 and IL-6, are also released with the activation of NF-κB. IL-1 acts synergistically with TNF-α to induce the synthesis of adhesion molecules and cytokines by endothelial cells and subsequent endothelial tethering and disrupted endothelial homeostasis. This ultimately leads to neutrophil migration into injured tissues, causing inflammation, tissue destruction and loss of function [39]. Additionally, IL-1 causes further stimulation of neutrophils to produce inflammatory phospholipid derivatives (prostanoids, thromboxane) and reactive oxygen species (superoxide anion, hydroxyl radicals, and nitric oxide) [4]. IL-1 and TNF-α also act in concert to directly affect the myocardium leading to impaired myocardial contractility [40]. Other effects of IL-1 include its action as a major endogenous pyrogen and increases in the release of endogenous glucocorticoid via hypothalamic effects [41, 42]. Similarly, IL-6 also has stimulatory effects on leukocyte activation as well as myeloid progenitor cell proliferation, in addition to serving as a potent pyrogen [43]. In both human and equine studies, IL-6 concentrations appear to be a more useful marker of disease severity and mortality compared to TNF-α and IL-1 [39, 44].

Serum concentrations of anti-inflammatory cytokines, most notably IL-10, also increase in sepsis [45]. IL-10 acts to directly inhibit the release of the pro-inflammatory cytokines from monocytes and macrophages. Additionally, it induces the production of IL-1 receptor antagonist protein (IRAP) and soluble TNF receptor to reduce the concentrations of these cytokines in circulation [46]. Cytokine activation also leads to the release of large amounts of acute phase proteins (APP) from hepatocytes [2]. These proteins have a variety of functions designed to re-establish homeostasis via facilitation of the local inflammatory response and elimination of pathogens. APP's that aid in the elimination of pathogens include complement proteins C3 and C4, which are important in the innate immune responses to bacteria, and granulocyte colony-stimulating factor, which provides a protective role against bacterial infection via its effect on neutrophils, among others [4, 47]. However, the APP's plasminogen and tissue plasminogen activator (TPA) have anticoagulant activities that further complicate tissue perfusion by contributing to the development of coagulopathies [4]. Additionally, APP's, including serum

amyloid A, C-reactive protein, and haptoglobin, can serve as serum markers of acute inflammation and as prognostic indicators during treatment of sepsis [48, 49].

The summation of the events produced with SIRS is the production of hypovolemia caused by increased vascular permeability and peripheral vasodilation, culminating in cardiovascular dysfunction due to vasodilatory shock [50]. Decreased tissue blood flow results in cellular oxygen and energy deficits [50]. Blood flow is re-directed to vital tissues to increase oxygen delivery, and anaerobic glycolysis occurs in other tissues to meet energy and oxygen demands [50]. When these compensatory mechanisms fail, this results in multiple organ dysfunction syndrome (MODS) and eventually death [4, 50].

Hypothalamic Pituitary Adrenal Axis

The body's response to inflammation is kept in check via action of the Hypothalamic-Pituitary-Adrenal (HPA) Axis, which helps to coordinate a response to maintain homeostasis by modulating and responding to signals from the endocrine, neural, and the immune systems [51]. Circulating pro-inflammatory cytokines, including IL-1 and IL-6 are detected by vagal afferent fibers, which then leads to stimulation of the parvocellular neurons of the paraventricular nuclei of the hypothalamus, causing secretion of corticotropin-releasing hormone (CRH) [52, 53]. CRH then circulates to the pars distalis of the pituitary gland via blood vessels that make up the hypothalamic-hypophyseal portal system. In the pars distalis, CRH activates type 1 CRH receptors on pituitary corticotrophs to induce the release of adrenocorticotropic hormone (ACTH), which is then released into the systemic circulation [54, 55]. Numerous other factors also contribute towards up-regulating ACTH synthesis at the same time, including noradrenergic stimulation, vasopressin, serotonin, angiotensin, vasoactive intestinal peptide, and increased circulating IL-6 [52, 53, 56, 57]. ACTH then binds to the melanocortin 2 receptor (MC2R) on adrenocortical cells where it stimulates the synthesis and secretion of cortisol by the zona fasciculata of the adrenal cortex [51, 54].

Cortisol is lipophilic, like all other steroid hormones, and thus circulates in the plasma predominately bound to plasma proteins such as cortisol binding globulin (CBG) and albumin. Cortisol bound to CBG is protected from hepatic clearance, with a circulating half-life of about 70-90 minutes, and typically 90-95% of the circulating cortisol is bound to CBG at any given time [58]. At inflammatory sites, elastase, which is produced by neutrophils, liberates cortisol

from cortisol binding protein, providing localized delivery of cortisol [59]. The unbound, or free cortisol, is the physiologically active form that is available to enter cells via diffusion across the plasma membrane to bind to intracellular glucocorticoid receptors (GR) [60]. In the absence of ligand, the GR exists as a monomer bound to several different heat shock proteins. When cortisol binds to the GR, the heat shock proteins dissociate, and the cortisol-GR complex then dimerizes and translocates to the nucleus where it binds to specific glucocorticoid response elements in the DNA to modulate gene expression. Hormone activated GR can also bind to NF-κB and AP-1 to prevent the production of inflammatory cytokines [61].

Many cell types are sensitive to cortisol, which allows it to exert diverse actions during the stress response. One essential effect of cortisol is the provision of energy to tissues via increasing the rate of hepatic gluconeogenesis, inhibiting adipose tissue glucose uptake, and stimulating free fatty-acid release from adipose tissue and amino acid release from body proteins. They also contribute to the maintenance of cardiac contractility, vascular tone and blood pressure via increased synthesis of catecholamines and modulation of vascular permeability. Additionally, they possess anti-inflammatory and immunosuppressive effects via modulating the production or activity of various cytokines. The summation of these effects serves to neutralize the stressors that initially activated the HPA axis and then return HPA axis activity to baseline once this has occurred. This last step occurs because cortisol itself acts via negative feedback mechanisms on the HPA axis to inhibit its own secretion and down-regulate HPA axis activity [60].

Critical Illness Related Corticosteroid Insufficiency

Definition

In both human and veterinary medicine, adrenal insufficiency can either be transient or permanent [62-65]. The most commonly encountered form of permanent HPA axis impairment is Addison's disease, however, genetic defects in adrenocortical enzymes have also been shown to lead to permanent cortisol deficiency in people [60, 66]. Acute illness may also lead to dramatic changes in endocrine function [67, 68]. During severe illness, circulating pro-inflammatory cytokines, including IL-1 and IL-6, normally stimulate the production of CRH and ACTH. This leads to an increase in circulating ACTH and cortisol, both of which are essential for maintenance of homeostasis and adaptation during this time [69]. However, studies in

humans and animals have shown a marked heterogeneity in this response in critically ill patients [64, 65, 70-75]. The expected response is an elevation in plasma cortisol and ACTH concentrations; however, a loss of correlation between the ACTH concentrations and cortisol concentrations can develop with critical illness, as evidenced by a low plasma cortisol level and a poor response to ACTH stimulation [64, 76]. This lack of adrenal responsiveness has been shown to be associated with an increase in mortality [64, 65, 70].

The syndrome of critical illness-related corticosteroid insufficiency (CIRCI), which was previously referred to as relative adrenal insufficiency, is characterized by an inappropriately low basal cortisol or inadequate production of cortisol in response to the increased demands during periods of severe physiologic stress, especially with critical illnesses such as sepsis or septic shock [62]. In patients with CIRCI, cortisol concentrations are inadequate to maintain homeostasis, leading to insufficient corticosteroid mediated down regulation of inflammatory transcription factors [76]. Transient HPA axis dysfunction is commonly encountered in critically ill patients with a variety of conditions, including: sepsis/septic shock, trauma, respiratory distress syndrome, and surgery [70, 77]. Severe sepsis has been shown to produce an altered HPA axis response in humans and animals, resulting in profound changes in the circulating concentrations of ACTH, cortisol, and arginine vasopressin (AVP) [62-65, 76, 78].

Hypofunction of the HPA axis can occur due to decreased cortisol synthesis or from decreased glucocorticoid activity at the target tissue or cells [76]. Cortisol is synthesized from cholesterol in the adrenal cortex. However, the amount of glucocorticoid produced at any one point in time is not sufficient to maintain normal rates of secretion for more than a few minutes in the absence of continued biosynthesis. Thus, any factor leading to a disruption of glucocorticoid synthesis will result in glucocorticoid insufficiency [53]. CRH synthesis can be suppressed with sepsis due to neuronal apoptosis triggered by an increase in substance P or iNOS in the hypothalamus [79, 80]. Elevated concentrations of TNF-α have been shown to inhibit corticotropin releasing factor stimulated ACTH production in cultures of rat anterior pituitary cells, and thus have been proposed to decrease ACTH production and contribute to mortality in human cases of severe septic shock [81]. Similarly, elevated concentrations of TNF-α have also been shown to block ACTH-induced cortisol production when incubated with cultured fetal human adrenal cells [82]. Corticostatins, such as α-defensin, are produced by immune cells in response to infection, and have been shown to impair cortisol production by competing with

ACTH at the MC2R [62]. They have been shown to increase 20-fold in plasma, 10-fold in adrenal tissue, and 5-fold in the hypothalamus of rabbits during bacterial infection, and human patients with sepsis have been shown to have elevations in their plasma defensin concentration [83, 84]. Critical illness can also lead to decreased production of CRH, ACTH, and cortisol via damage to the hypothalamus, pituitary gland or adrenals [63, 80]. Necrosis and hemorrhage of the hypothalamus and pituitary gland have been reported in humans with sepsis from prolonged hypotension or the development of a severe coagulopathy [80]. Additionally, animal models of septic shock have shown that this can lead to extensive pathology of the adrenal glands, including: hemorrhagic necrosis, massive hematomas, microthrombi, and platelet aggregation [85]. Extensive adrenal hemorrhage (Waterhouse-Friderichsen Syndrome) has been documented in up to 30% of critically ill humans who did not survive septic shock [71]. This occurs due vascular derangements and ischemia associated with the primary disease, and has also been reported as a common finding in horses with endotoxic shock [54, 86, 87].

Reduced access of glucocorticoids to their target tissues and cells can also occur via several mechanisms. CBG, which is essential for transporting cortisol to the tissues and cells, has been shown to decrease by up to 50% in human patients with sepsis/septic shock because of direct inhibition of CBG gene transcription related to an increase in the circulating IL-6 concentrations [59, 76, 88]. Therefore, cortisol delivery to the tissues can be hindered with systemic inflammation. Cortisol concentrations in the tissue are also regulated by enzymatic conversion of cortisol to its inactive form, cortisone, by 11β-hydroxysteroid dehydrogenase type 2. Increased cytokine concentrations, including IL-2, IL-4, and IL-13, have been shown to increase 11β-hydroxysteroid dehydrogenase type 2 activity in the tissues, leading to increased deactivation of cortisol at this level [89]. Additionally, multiple studies have suggested a cytokine-mediated response leading to a decrease in the number and activity of glucocorticoid receptors in various cell types [90-97]. One mechanism proposed to explain this finding is the inhibition of glucocorticoid receptor translocation from the cytoplasm to the nucleus leading to a reduction in glucocorticoid receptor-mediated gene transcription, decreasing the ability of cells to respond to cortisol [90]. These mechanisms for decreasing glucocorticoid access to its target tissues and cells could account for the development of CIRCI, even in the face of normal cortisol concentrations.

Foals

Function of the fetal and neonatal foal HPA axis has been examined in a number of studies, and has been shown to differ from that of adult horses in a number of important ways, which can have an effect on the capacity of the neonatal foal to respond to various stressors. Maturation of the HPA axis occurs in the few days leading up to parturition in the equine fetus and continues through the first 12 weeks of life, which is much later than what has been reported in other species studied [98-102]. The mean basal cortisol for healthy 12-24 hour neonatal foals (2.0-3.6 μg/dl) is reported to be lower than that of healthy adults (3.0-7.0 μg/dl), despite having similar or greater concentrations of ACTH [103-105]. This decrease in cortisol production in response to endogenous ACTH in foals can be related to either an impaired adrenocortical responsiveness to ACTH, decreased synthetic capacity for cortisol, or both when compared to adult horses. The key steroidogenic enzymes required for cortisol synthesis (p450scc and 3-beta-hydroxysteroid dehydrogenase) in the equine fetus have been shown to be absent or present in small amounts until just prior to parturition, which may impact the ability of the neonatal adrenal gland to synthesize cortisol [106, 107]. Additionally, several studies have demonstrated a decreased cortisol response to exogenous ACTH administered to healthy foals up to 12 weeks of age [102, 105]. While the specific cause of this impaired response in neonatal foals is currently unknown, this evidence of HPA axis immaturity may contribute to an impaired response to disease in the foal.

Dysregulation of the HPA axis, evidenced by increased concentrations of circulating ACTH and a relative decreased concentration of cortisol, has also been reported in both septic and sick non-septic hospitalized foals [64, 65, 73, 108-115]. In these studies, nonsurvival of septic foals was associated with both decreased and increased cortisol concentrations, as well as an increased ACTH:cortisol ratio, suggesting that nonsurvival in these cases was associated with an impaired HPA axis response to disease [64, 65, 108, 110, 112-114]. Because both ACTH and cortisol values should increase proportionately with HPA axis stimulation, abnormalities of the ratio of these two concentrations suggests an abnormality in their production or their response at the tissue level. Studies examining the response of septic, sick non-septic, and healthy foals to exogenous ACTH showed that an inadequate increase in cortisol concentration following ACTH administration was associated with a decreased survival rate [64, 73, 102].

Arginine vasopressin (AVP) is also released from the hypothalamus in response to HPA axis stimulation. AVP is released when increased serum osmolality is detected, and functions to conserve water via its action in the renal collecting ducts. Additionally, AVP has been shown to increase release of ACTH in horses via its action on the V3 receptor in the pars distalis [116, 117]. As with ACTH and cortisol, increases in AVP are indicative of HPA axis stimulation, and AVP depletion has been observed in both adults and human children in association with HPA axis dysfunction and is negatively correlated with survival [118-121]. In foals, AVP concentrations and AVP:ACTH ratios were shown to be significantly higher in sick foals compared to sick non-septic and healthy foals, and elevations in AVP concentrations were associated with nonsurvival in these studies [65, 108].

Adult Horses

Basal cortisol concentrations have been reported for healthy adult horses, ranging from 1.09-7.86 µg/dl, and this is released in a circadian rhythm with the peak secretion in the morning and the nadir in the evening [74, 75, 103, 122-128]. However, this circadian rhythm can be disrupted by factors even as minimal as changes in their routine; thus disease states can disrupt this as well [124].

The response of the HPA axis to pathophysiologic stress has been evaluated in several studies of adult equine patients. Hinchcliff et al examined the relationship between serum cortisol concentrations and clinical signs, routinely measured clinicopathologic variables, and outcome in a population of horses presenting for colic [74]. This study demonstrated that elevated serum cortisol concentrations were significantly associated with an increased risk of nonsurvival, most likely as a reflection of the severity of the disease process [74]. Similarly, Ayala et al examined concentrations of cortisol and ACTH in horses presenting for laminitis, acute abdominal syndrome, castration surgery, acute diseases, chronic diseases and healthy controls, to assess the response of the HPA axis to various stressors [75]. The most significant elevations in cortisol and ACTH were observed in horses with strangulating obstruction of the small and large intestine [75]. In another similar study by Mair and Sherlock, cortisol was measured within 36 hours of hospitalization for colic, and then daily after that until dismissal or death [127]. In these horses with colic, they found a mean cortisol at admission of 17.98 µg/dl, which was much higher than the controls reported in this same study (0.91-5.62 µg/dl) [103, 122,

123, 127]. Additionally, higher cortisol concentrations were detected in horses with more severe disease processes, including strangulating lesions, and nonsurvivors had persistently elevated cortisol that did not decrease during hospitalization [127]. Lastly, in a study by Hart et al, ACTH and cortisol were evaluated serially over a 12-hour period in 7 sick horses that met SIRS criteria [109]. This study group consisted of horses that had a celiotomy for a strangulating intestinal lesion as well as horses with colitis or enteritis [109]. They found that the cortisol and ACTH concentrations varied over a large range, but the sickest horses had an ACTH that stayed greater than 100 pg/ml during the 12-hour monitoring period while the least sick had concentrations that tended to stay below 50 pg/ml. Additionally, cortisol concentrations tended to follow the same pattern, with persistently high concentrations in the sickest horses [109]. In all of these studies, elevations of ACTH and cortisol were attributed to increased HPA axis tone associated with pain, hypovolemia, and SIRS. However, it is unknown whether these animals were able to respond to the increased ACTH and cortisol appropriately at the tissue level.

Additionally, CIRCI has been demonstrated in at least one study of critically adult equine patients, where plasma ACTH and serum cortisol concentrations, as well as cortisol concentrations 30 minutes after the administration of exogenous ACTH (0.1 μg/kg) were measured in horses presenting with colic or systemic illness on admission, and 2, 4, and 6 days after presentation [129]. Of the 58 horses included in this study, 11 were classified as having mild disease, 30 with moderate disease, and 17 with severe disease. They found that the admission ACTH and cortisol were highest in horses with severe disease (93 ± 198 pg/ml and 13.1 ± 4.97 μg/dl, respectively), compared to those with moderate and mild disease. Additionally, admission cortisol concentrations were overall higher in severely ill horses, however, there were 4/17 horses in this group with low cortisol measurements. In this group as well, 11/13 horses had a lower than expected delta cortisol after the administration of exogenous ACTH, which was found to be associated with marked adrenal hemorrhage on post-mortem in nonsurvivors [129].

Table 1. Cortisol and ACTH measurements in adult horses with acute abdominal disease

Study	Cortisol Measured (µg/dl)	*Reference Range/Normal Cortisol(µg/dl)*	ACTH Measured (pg/ml)	*Normal ACTH (pg/ml)*
Hinchcliff et al [74]	15.4 (9.1-25.7)	*0.5-2*	-	-
Ayala et al [75]	6.8 ± 1.9	*1.3 ± 0.2*	54 ± 5.4	*22.9 ± 2.7*
Mair and Sherlock [127]	17.9 ± 11.2	*0.9-5.6*	-	-
Hart et al [109, 125]	3.7-17.8	*2.6 ± 0.6*	12-830	-
Stewart et al [128, 129]	13.1 ± 4.9	*6.2 ± 1.6*	93 ± 198	*19.2 ± 5.6*

Diagnosing Critical Illness Related Corticosteroid Insufficiency

CIRCI should be suspected in critically ill patients presenting with sepsis/septic shock (often termed endotoxemia in equine patients) and trauma. Presently, there is no consensus in the veterinary literature concerning the identification of CIRCI. However, there are 2 general categories of testing methods that can be used to determine if adrenocortical insufficiency is present including: assessment of endogenous basal adrenocortical activity by measuring circulating hormone concentrations and dynamic assessment of adrenocortical responsiveness by measuring hormone production after administration of exogenous regulatory hormones [54].

Measurement of basal hormone concentrations offers a rapid and safe means of assessing adrenocortical function. There are several commercially available radioluminescent or chemiluminescent immunoassays for the measurement of equine cortisol [54]. Several other commercial immunoassays are also available for most other adrenal corticosteroids, with the measurement of aldosterone and adrenal androgens being described in horses [65, 122, 130, 131]. Although these tests are simple to perform, cortisol and other adrenal steroids are typically secreted in a pulsatile fashion, and plasma concentrations can vary widely over a 24-hour period in sick and healthy animals, depending on the time of day, season, emotional state, and other changes in physiologic stressors [54, 125, 132]. Hart et al reported a mean 24 hour ± SD cortisol concentration of 2 ± 0.4 µg/dl for foals and 2.6 ± 0.6 µg/dl for adult horses [125]. Ayala et al collected a single cortisol sample from healthy control horses, and reported a mean ± SD of

1.26 ± 0.21 μg/dl [75]. Similarly, Stewart et al collected a total of 40 baseline cortisol concentrations prior to performing ACTH stimulation tests, and found a mean ± SD of 6.24 ± 1.62 μg/dl [128]. This shows that there can be wide variations between cortisol measurements, and documentation of a single random low basal concentration of cortisol should be interpreted with caution.

Evaluation of cortisol concentrations in conjunction with its regulatory hormone concentrations (ACTH, CRH) can provide a more thorough analysis of HPA axis function. CRH is predominantly secreted into the hypothalamic-hypophyseal portal vessels, rather than systemic circulation, so measurement of CRH concentrations requires collection of pituitary venous blood or other such complicated sampling methods, making measuring concentrations of this hormone less practical [133]. There are commercial immunoassays available for the measurement of ACTH concentrations. The study by Stewart et al examined the cortisol response to ACTH stimulation reported a mean ± SD baseline ACTH concentration of 19.2 ± 5.6 pg/ml [128]. Similarly, the study by Ayala et al reported a mean ± SD of 22.9 ± 2.7 pg/ml [75]. While these values in adults appear more consistent, reports of normal ACTH values in foals are much more varied, ranging from 14.4-60.98 pg/ml [102, 110, 115]. In healthy individuals, ACTH and cortisol concentrations are fairly closely correlated. However, several studies in foals have supported ACTH-cortisol dissociation during illness, with an elevated ACTH:cortisol ratio indicating HPA axis dysfunction [64, 65, 73, 108, 110, 112, 114]. Studies examining ACTH and cortisol concentrations in adult horses for the most part have not demonstrated such disparity between ACTH and cortisol values in critically ill patients, however, one study by Stewart et al showed significantly lower admission cortisol in 24% of severely ill horses relative to their admission ACTH [75, 109, 129]. Use of the ACTH:cortisol ratio to detect relative adrenal insufficiency has not been evaluated in adult horses to date.

Dynamic tests have been designed to circumvent some of the limitations posed by the assessment of endogenous basal HPA axis activity. The most commonly utilized dynamic test to diagnose CIRCI is the ACTH stimulation test, which measures the cortisol response to exogenous synthetic ACTH (cosyntropin, α1-24 corticotropin) [54]. For the high-dose ACTH stimulation test, cortisol concentration is measured at baseline, and then 30 and 90 minutes after intravenous or intramuscular administration of a supraphysiologic dose of ACTH (1.0-2.0 μg/kg) [134]. By using this supraphysiologic dose of ACTH, a maximal adrenal response is elicited.

Therefore, an inadequate increase in cortisol concentration with this test is used to diagnose adrenal insufficiency of any cause [70, 72, 76]. However, in some patients with CIRCI, the adrenal glands may respond adequately to this high-dose of ACTH but fail to respond to a lower dose [64, 72]. In these cases, a low-dose of ACTH (0.01-2.0 μg/kg) may help to more accurately diagnose CIRCI [64, 72, 135].

However, the ACTH stimulation tests only evaluate the adrenal component of the HPA axis. For patients where the cortisol insufficiency stems from malfunction of the HPA axis at the hypothalamic and/or pituitary levels, an ACTH stimulation test may lead to an appropriate cortisol response, resulting in a false negative test result for CIRCI. A CRH stimulation test would help to assess function at these higher levels of the HPA axis, and would allow for exact localization of the malfunction if both ACTH and cortisol concentrations are measured prior to CRH stimulation [54]. The effect of varying doses of ovine CRH on plasma and saliva cortisol concentrations in horses has been evaluated [136]. However, due to its expense and variable results reported in humans, this test is not commonly used to evaluate the critically ill equine patient [137].

In humans, the metyrapone test has been used to assess the integrity of the entire HPA axis [138]. Metyrapone is a specific inhibitor of the adrenocortical steroidogenic enzyme 11β-hydroxylase, which catalyzes the conversion of 11-deoxycortisol to cortisol in the last step of adrenocortical cortisol synthesis [138]. Administration of metyrapone results in a decrease in the circulating cortisol concentration, leading to feedback to the HPA axis to increase secretion of ACTH and CRH in an individual with an intact HPA axis. Additionally, since metyrapone inhibits cortisol synthesis, concentrations of cortisol's precursor, 11-deoxycortisol, increase. Therefore, decreased concentrations of ACTH and 11-deoxycortisol after metyrapone administration indicates dysfunction of the HPA axis at the hypothalamic or pituitary level, whereas increased concentrations of ACTH and low concentrations of 11-deoxycortisol indicate adrenocortical dysfunction [138]. Measurement of 11-deoxycortisol has been described in one equine study, however, a commercial assay is not readily available [139]. Additionally, there has been only one study describing the use of metyrapone in healthy horses, which was administered intravenously over a 3 hour period, while cortisol, ACTH, AVP, and CRH concentrations were measured from pituitary venous blood samples starting 30 seconds before this infusion and every 30 seconds during the infusion [124]. In this study, they found that the plasma cortisol

concentration decreased linearly during treatment to a mean nadir of 15% of the pre-treatment concentrations, while CRH, AVP and ACTH concentrations increased, indicating an intact response of the HPA axis to decreased circulating cortisol [124].

The insulin tolerance test is another dynamic test used to diagnose HPA axis dysfunction and has been described in both foals and adult horses [140-142]. For this test, hypoglycemia is induced through the administration of exogenous insulin, and the cortisol response to the physiologic stress of hypoglycemia is measured. A decreased or absent cortisol response implies HPA axis dysfunction. ACTH concentrations can also be measured to differentiate between HPA axis dysfunction stemming from the adrenal glands or more centrally [142]. However, due to the risks associated with severe hypoglycemia, in addition to the potential for pre-existing derangements in glucose concentrations or peripheral insulin sensitivity in the critically ill patient, its clinical utility for the diagnosis of CIRCI is limited [140].

As discussed previously, CIRCI can arise due to both decreased circulating cortisol as well as resistance to cortisol in the peripheral tissues [52, 76, 93, 143]. In these cases, the above-mentioned tests may suggest intact HPA axis function, despite clinical evidence to the contrary. Methods for assessing cortisol activity at the level of the GR are currently limited [91, 92, 144-150]. There have been no studies to date to examine the equine GR in health or disease.

Glucocorticoid Receptor

The GR is a member of the steroid receptor family of nuclear receptors, which act primarily as transcription factors controlling gene expression [151]. It is the product of a single gene containing of 9 exons, of which 8 are translated to form a protein containing 777 amino acids [151]. The 9th exon undergoes alternative splicing to create two isoforms of GR – GRα and GRβ [151]. Through its interaction with glucocorticoids, GRα can respond to internal or external stress stimuli in order to maintain homeostasis at the cellular and organismal levels [152]. In the absence of stimulation, GRα is typically located in the cytoplasm in complex with various heat shock proteins, which modulate both expression level and activity of the receptor [61]. In the presence of glucocorticoids, GRα transforms into a chaperone-free form, dimerizes and then moves to the nucleus where it regulates gene expression via binding to regulatory DNA sequences in the promoter of the target genes. In its monomer form, it also inhibits or promotes various other transcription factors from binding to or that are already bound to this promoter

region, respectively [153]. The metabolic effects of glucocorticoids are mediated by GRα binding to specific regulatory DNA sequences, while their anti-inflammatory and immunosuppressive effects are mediated by the interaction of GRα with transcription factors including AP-1 and NFκB [61]. The GRβ form is usually present in much lower quantities compared to GRα, and is believed to act as a primary inhibitor of GRα [144, 154]. Increased GRβ expression has been demonstrated in a multitude of conditions characterized by tissue-specific insensitivity to glucocorticoids, including: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), glucocorticoid-resistant asthma, and chronic lymphocytic leukemia [155-158]. In a study by Webster et al, incubation of various cell lines with both TNFα and IL-1 stimulated an increase in both GRα and GRβ production, with GRβ production favored. This led to the hypothesis that an increase in GRβ production is stimulated by inflammatory cytokines, and due to its inhibitory effects on GRα, may contribute to cortisol resistance at the tissue level [159].

The GR is ubiquitously present in almost all nucleated cells, and their cellular level is controlled through a negative feedback mechanism in relation to the circulating concentration of its ligand [160]. GR concentration, of both the α and β isomers, in the cells has been shown to be altered by various acute and chronic disease or inflammatory states in humans and animals, including: endotoxemia, sepsis, trauma, parturition, post-traumatic stress disorder, multiple sclerosis, systemic lupus erythematosus, neoplasia, and following organ transplantation [91, 147-151, 160-168]. Additionally, several studies have demonstrated alterations in GR binding affinity produced by these various inflammatory states [91, 149, 150, 161, 162, 165, 167, 168]. Techniques used in these studies to measure receptor concentration and binding affinity include real-time reverse-transcriptase polymerase chain reaction for GR mRNA, a radio-ligand-binding assay using [^{3}H]dexamethasone as the ligand, and flow cytometry [91, 147-151, 160-168].

Flow cytometric analysis of GR

Voutsas et al compared the radio-ligand-binding assay to flow cytometry for the measurement of GR concentration in various human cell lines [151]. They found that these methods produced similar accuracy in their measurements, however, flow cytometric analysis provided higher precision compared to the radio-ligand-binding assay [151]. Subsequent studies have used flow cytometry to measure GR concentration and GR binding affinity in patients with

systemic lupus erythematosus, in periparturient cows, and in patients with post-traumatic stress disorder [160, 165, 167].

Flow cytometry is a powerful tool that can be used to distinguish individual cells suspended in a fluid stream by their size, cytoplasmic granularity and positive or negative expression of different receptors using fluorochromes conjugated to antibodies that recognize a particular protein of interest [169]. There are five main components of a basic flow cytometer: a light source (laser), a flow cell, optical components to focus different wavelengths of light on to their respective detectors, electronics to amplify and process the signals produced, and a computer to analyze and report these signals. Additionally, gating techniques can be used to limit analysis to only cells of a particular interest. Benefits of using flow cytometry to analyze the GR compared to other methods include its rapid production of results, its ability to detect heterogeneity in individual cells, it requires fewer cells, and does not require the use of radioactive materials [151].

The multicolor direct-immunofluorescence staining technique allows the use of commercially available antibodies to be employed for the surface detection of multiple cell surface markers simultaneously [170]. Additionally, staining for intracellular proteins can be performed, with the extra steps of cell permeabilization and fixation. The cells are fixed to maintain their structural integrity and made permeable to allow antibodies to reach their intended intracellular targets [170]. Both cell surface staining and intracellular staining can be used to increase the sensitivity of detection of a particular type of protein within a particular type of cell. To do this, the cell surface antigen is stained first, the cell is then permeabilized, and then the intracellular staining is performed. Fluorochromes with different spectra are typically used for this type of analysis (i.e. fluorescein isothiocyanate (FITC) and phycoerythrin (PE)). When these samples are analyzed, gating techniques can then performed to identify the cells that are positive for both fluorochromes, and thus containing both the cell surface marker and intracellular protein of interest [170].

In the studies by Du et al, Gotovac et al, and Voutsas et al, the GR monoclonal antibody used was a mouse monoclonal to human GR, containing amino acids 150-175 and 304-428 [151, 160, 165]. In the study by Du et al, blood samples were obtained from patients with systemic lupus erythematosis as well as healthy volunteers. These samples were incubated with antibodies for CD3 and CD14, which were labeled with phycoerythrin (PE) and allophycocyanin

(APC) respectively, to identify lymphocytes and monocytes in the sample. The erythrocytes were then lysed with a lysing solution, and then the cells were washed with PBS and fixed in a buffered paraformaldehyde solution. The cells were then permeabilized, and then were incubated with an anti glucocorticoid monoclonal antibody, and an anti-mouse IgG-FITC secondary antibody was used to detect GR. Samples were then analyzed using flow cytometry, with simultaneous detection of the PE- or APC-labeled antibodies and FITC-GR to identify the concentration of GR in lymphocytes and monocytes. In this study they also analyzed the binding affinity of the GR. They followed the same protocol for cell surface staining and erythrocyte lysis, but then incubated cells with FITC-dexamethasone. For a control, they prepared another tube by adding a 500-fold excess amount of unlabeled dexamethasone prior to adding the FITC-dexamethasone probe [165]. The study by Gotovac et al used a similar protocol, except PE conjugated anti-CD3, anti-CD16, and anti-CD56 as well as peridinin chlorophyll protein (PerCP) conjugated anti-CD20 monoclonal antibodies were used for surface staining. The monoclonal GR antibody used in this study was the same as in the study by Du et al [160, 165]. Lastly, in the study by Voutsas et al, they started with specific cell lines that had been cultured. To detect GR, they developed their own monoclonal antibody with human GR peptide Ala304-Ala428. Because these cells were from pure cultures of specific cell lines, no surface staining was used in their protocol for analysis [151].

Analysis of the equine and human nucleic acid sequences for the glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1) shows 91% homology between these species, with 93% homology for amino acids 176-289 [171]. This suggests that the human GR monoclonal antibody could be used to examine GR concentration and binding in equine cell lines.

Corticosteroids and Critical Illness Related Corticosteroid Insufficiency

Humans

Human studies have shown conflicting results when it comes to the treatment of septic patients with CIRCI with glucocorticoid supplementation. When glucocorticoid supplementation of humans with septic shock was first evaluated, the use of supraphysiologic doses, or doses exceeding that which the body normally produces, was common [172, 173]. In these older studies, short-term (1-2 days) administration of high doses of glucocorticoids (up to 40g of

hydrocortisone equivalent per day) in early septic shock was not associated an improved outcome, or was even considered harmful most likely due to the profound immunosuppression created with these high doses leading to an increased incidence of infection [172, 173].

However, several more recent studies, including a meta-analysis, have shown an improvement in outcome when septic patients with CIRCI were treated with glucocorticoid supplementation in the form of low-dose hydrocortisone therapy [71, 174-179]. In the meta-analysis by Annane et al, 12 trials investigating the effects of long term low-dose corticosteroid treatment showed an improvement in 28-day mortality rates when humans with septic shock were treated with corticosteroids versus those that were not treated (37.5% mortality vs. 44%). They reported a relative risk (RR) of 0.84 (95% CI, 0.72-0.97) for mortality in treated patients. Additionally, treatment with low-dose corticosteroids showed an increased 28-day shock reversal in 6 trials (69.9% vs. 58.6%, RR, 1.12, 95% CI, 1.02-1.23), and a reduced intensive care unit length of stay by 4.49 days in 8 trials (95% CI, -7.04- -1.94) [71]. The study by Keh et al reported an increase of mean arterial pressure, systemic vascular resistance, and a decline of heart rate, cardiac index, and norepinephrine requirement in shock patients treated with hydrocortisone compared to placebo controls [175]. Similarly, Oppert et al showed a significantly shorter time to the cessation of vasopressor therapy in patients treated with a low-dose of hydrocortisone compared to controls (53 hours vs. 102 hours, $P<0.02$) as well as decreased circulating concentrations of IL-6 in the treated group [176]. Conversely, there continues to be some debate as to the usefulness of even low-dose glucocorticoid therapy in the treatment of these patients. For example, in a multi-center, randomized, double blind, placebo-controlled trial by Sprung et al, patients treated with low-dose hydrocortisone supplementation did not show an improvement in survival or reversal of septic shock compared to controls [177].

The rationale for the use of glucocorticoids in these patients has been their potent anti-inflammatory properties including inhibition of cytokine production and prevention of the migration of circulating inflammatory cells into the tissues [51]. Other beneficial effects of glucocorticoids in patients with CIRCI include their ability to enhance vasoactive tone and catecholamine responsiveness [51]. Thus, treatment of septic patients with glucocorticoids is postulated to prevent desensitization of the β-adrenergic receptor and to up-regulate receptors that have been down regulated in response to systemic inflammation [85, 180]. However, due to their potential negative effects on immune function there is concern that corticosteroid treatment

can lead to increased episodes of "superinfection" including new episodes of sepsis or septic shock [177]. This has led to the current recommendation for hydrocortisone replacement regimens for septic adults of a 50 mg intravenous bolus every 6 hours (approximately 2.5-3 mg/kg/day) or a 100 mg loading dose followed by a continuous rate infusion of 10 mg per hour (total dose of 4-6 mg/kg/day) [72, 175]. These dosing strategies were derived from the maximal cortisol secretory rate in response to exogenous HPA axis stimulation with cosyntropin or by multiplying the daily endogenous cortisol production rate in healthy, unstressed individuals by various illness factors to account for the increases in cortisol production associated with stress and illness [69, 181].

Equine

There have been no studies to date examining the effect of glucocorticoid therapy on outcome in equine patients. Hart et al examined the effects of low-dose hydrocortisone therapy on the ex vivo immune function of neonatal foals, and showed that foals treated with hydrocortisone had significantly decreased endotoxin-induced expression of TNF-α, IL-6, IL-8 and IL-1β [182]. Additionally, neutrophil phagocytosis and reactive oxygen species production were unchanged, suggesting that low-dose hydrocortisone therapy did not impair the innate immune response of the equine neonate [182]. In another study by Hart et al, the daily endogenous cortisol production rate and the pharmacokinetics of an intravenous bolus of hydrocortisone were compared between foals and adult horses [125]. For adult horses, a daily endogenous cortisol production rate of 2,140 ng/kg/day was reported, which is similar to what has been reported for adult humans at approximately 1,630 ng/kg/day [125, 183]. This suggests that adult horses may respond to similar doses of glucocorticoid replacement therapy as used in humans during septic shock or endotoxemia (2.5 to 3 mg/kg/day of hydrocortisone) [72]. However, this study reported a much higher endogenous daily cortisol production rate of 6,710 ng/kg/day in healthy neonatal foals, which was attributed to both an increased systemic clearance rate and an increased volume of hydrocortisone distribution in foals compared to adult horses [125]. Based on this finding, an appropriate replacement hydrocortisone dose for the use in septic foals with CIRCI is thought to differ from what has been reported in septic infants, although this remains unknown at this time.

Summary of Literature and Hypothesis of Present Study

CIRCI, due to disruptions in the regulation of the HPA axis, has been identified in both foals and adult horses with systemic inflammation from a variety of pathologic conditions including colitis, pleuropneumonia, neonatal sepsis, and colic. In these patients, an inadequate or inappropriate cortisol response to illness is correlated with increases in morbidity and mortality. No studies to date have examined the role of peripheral resistance to cortisol mediated by the GR in the equine patient. However, studies in other species have demonstrated that with a pro-inflammatory state there is a decrease in both the density of GR's as well as their binding affinity. These findings suggest that tissue resistance to cortisol plays a pivotal role in the development of CIRCI. In humans, this has lead to the use of a "physiologic" or low-dose of steroids in septic patients with CIRCI, resulting in improved shock reversal and survival. We hypothesize that a similar mechanism occurs in the equine patient.

To test this hypothesis, a study involving three parts was performed. The first part of the study examined whether a human monoclonal GR antibody could be used to detect and quantify the GR in equine peripheral blood mononuclear cells, and if fluorescently labeled dexamethasone could be used to determine GR binding affinity. The second phase of the study examined the effects of exogenously produced increases in the circulating cortisol on the GR density and binding affinity in a healthy population of horses. Finally, the third part of the study examined the response of the GR in horses with SIRS compared to age and sex matched healthy controls. We predicted that horses with systemic inflammation would have increased cortisol and ACTH compared to controls, and a lower GR density and binding affinity. If peripheral resistance to endogenously produced glucocorticoids occurs in horses with SIRS, then this might support further investigation into the use of "physiologic" doses of synthetic glucocorticoids for the management of endotoxemia or sepsis in the adult equine patient.

Chapter 2. Glucocorticoid Receptor Density and Binding Affinity in Equine Peripheral Blood Mononuclear Cells in Horses with Systemic Inflammatory Response Syndrome.

Introduction

Critical illness-related corticosteroid insufficiency (CIRCI), due to disruptions in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, has been identified in both foals and adult horses with systemic inflammation from a variety of pathologic conditions including colitis, pleuropneumonia, neonatal sepsis, and colic [64, 74, 112, 115, 129]. This HPA axis dysregulation has been demonstrated in equine patients via measuring circulating concentrations of cortisol, adrenocorticotropic hormone (ACTH), as well as performing ACTH stimulation tests to measure the ability of the adrenal glands to respond to an increased demand for circulating cortisol [64, 74, 112, 115, 129]. In these patients, a low basal cortisol concentration, inadequate cortisol response to ACTH-stimulation, increased or decreased endogenous ACTH concentration, and an increased ACTH:cortisol ratio have all been associated with increases in morbidity and mortality. In these studies, dysregulation of the HPA axis was localized to either the adrenal glands or the central nervous system. However, at present, there are no studies in horses examining the role of peripheral resistance at the level of the glucocorticoid receptor (GR) in the development of CIRCI.

Several human and animal studies have investigated the contribution of tissue resistance to endogenous cortisol in the development of CIRCI via flow cytometry and biochemical binding assays of the GR [91, 148, 149, 161, 162, 165]. These have shown that with severe acute inflammation, there is a decrease in the number of GR as well as their binding affinity, suggesting that tissue resistance to cortisol plays a pivotal role in the development of CIRCI. In humans, these findings have led to the use of a low-dose of hydrocortisone in septic patients with CIRCI, which has led to improved shock reversal and survival [71, 72, 76, 175, 184, 185]. This "physiologic" dose of glucocorticoid supplementation was derived from studies examining the maximal cortisol secretory rate in response to exogenous HPA axis stimulation with cosyntropin or by multiplying the daily endogenous cortisol production rate in healthy, unstressed individuals by various illness factors to account for the increases in cortisol production associated with stress and illness [69, 181]. This lower dose was developed to avoid the negative effects on immune function with corticosteroid treatment encountered in earlier studies of treating septic shock with glucocorticoids, where such treatment was associated with increased episodes of

"superinfection" including new episodes of sepsis or septic shock [177].

There were three objectives of this study. The first was to determine if commercially available human and mouse fluorochromes could be used to determine the glucocorticoid receptor (GR) density and binding affinity in equine peripheral blood mononuclear cells. The second was to determine if there was a correlation between elevated plasma cortisol and the GR density or binding affinity in healthy adult horses. The third objective was to evaluate the HPA axis in adult horses presenting with systemic inflammatory response syndrome (SIRS), and to determine where any alterations in HPA axis function occur in these patients compared to healthy adults. The hypothesis was that horses presenting with SIRS/CIRCI would have inappropriate plasma concentrations of ACTH and cortisol, and would have a decreased GR density and binding affinity. Determining the sites of HPA axis dysfunction in the equine patient with SIRS will help to guide treatment in these cases, particularly in regards to the use of a "physiologic" dose of glucocorticoids.

Materials and Methods

Part I - Flow cytometry analysis of glucocorticoid receptor density and binding in the healthy equine patient.

Animals

Three healthy adult research horses residing at the Marion DuPont Scott Equine Medical Center were used after the Virginia Tech University Institutional Animal Care and Use Committee approved the study protocol. The horses were determined to be healthy based on physical examination findings.

Peripheral Blood Mononuclear Cell (PBMC) Collection and Isolation

Peripheral venous blood samples (50 ml in 5, 10 ml BD Vacutainer® potassium EDTA tubes) were collected via jugular venipuncture at 8 a.m. on three separate days. Following blood collection, PBMC's were collected by density gradient centrifugation. Briefly, samples from these horses were mixed 1:1 with Phosphate Buffered Saline (PBS) without calcium or magnesium, then 8 ml aliquots of this solution were slowly layered over 5 ml of Ficoll-Hypaque[a] in 15 ml conical tubes (BD Falcon®). These tubes were then centrifuged (400g for 40 minutes at 4°C) to separate cells on the gradient. Cells at the plasma-Ficoll-Hypaque interface were then aspirated using a 1000 µl micropipette and transferred to a 5 ml round bottom tube containing 4

ml of PBS for washing. Care was taken to avoid removing excess Ficoll-Hypaque or plasma to limit inclusion of granulocytes or platelets in the sample. The PBMC's in PBS were then centrifuged (400 g for 10 minutes at 4°C). The resulting supernatant was discarded, the PBMC's were re-suspended in another 4 ml of PBS, and centrifugation followed by supernatant removal was repeated an additional two times. Once washing in PBS was complete, isolated PBMC's were then re-suspended in 5 ml PBS.

A small amount (100 µl) of the PBMC suspension was then removed and a manual cell count performed using a hemocytometer; cell numbers were adjusted to $1x10^6$ cells/ml for subsequent flow cytometry analysis.

Flow Cytometry

Cells were separated into nine aliquots containing $1x10^6$ cells per sample. Preliminary trials were performed with various volumes of primary antibody (results not shown), and the following protocol was developed from these results. The first sample served as a control for autofluorescence. Two aliquots were used to determine CD44 expression on PBMC's. One of these was stained with PE-Mouse IgG[b] as an isotype control, by incubating 10 µl of antibody in the dark at 4°C for 10 minutes, while the other was stained with PE-anti-mouse CD44[c] in a similar manner. Following this 10-minute incubation, cells were washed with 2 ml of PBS, and the sample was centrifuged (400 g for 10 minutes at 4°C). The resulting supernatant was discarded, and the tubes were gently vortexed to re-suspend the cells. The washing process was repeated an additional two times, with 2 ml of PBS on each occasion. An additional 1 ml of PBS was added to each sample following the third wash.

Three additional aliquots were used to determine the concentration of GR within the cytosol of PBMC's. PBMC's were surface stained with PE CD44 as described above. Following cell surface staining, PBMC's were permeabilized by the addition of 0.25 ml of CytoFix/CytoPerm solution[d] per tube and allowed incubated in the dark at 4°C for 15 minutes. The cells were then washed with 2 ml CytoFix/CytoPerm wash buffer[d] followed by centrifugation (400 g for 10 minutes at 4°C). The resulting supernatant was discarded, and the tubes were gently vortexed to re-suspend the cells. An additional 1 ml of PBS was added to each sample. Ten microliters of purified mouse anti-glucocorticoid receptor[e] was then added to one of these samples. An equal volume of purified mouse IgG[f] was added to the other two aliquots as an isotype control for the primary antibody and a control for the secondary antibody, which was

added next. These samples were allowed to incubate in the dark at 4°C for 10 minutes. Following this, 10 µl of a FITC goat anti-mouse secondary antibody[g] was added to the sample containing the anti-mouse GR antibody as well as one of the aliquots stained with mouse isotype control. Again these samples were allowed to incubate in the dark at 4°C for 10 minutes. The samples were then washed 3 times as described above using 2 ml of PBS. An additional 1 ml of PBS was added to each sample following the third wash step.

Two aliquots were also prepared to assess GR binding affinity. PBMC's were surface stained with PE CD44 and permeabilized as described above. One sample was then incubated with 5 µg of dexamethasone[h] for 10 minutes at 37°C, and the other sample without dexamethasone prior to staining with 1.5 x 10^{-7} M fluorescein labeled dexamethasone[i]. These tubes were then incubated in the dark at room temperature for 1 hour, gently mixing the sample every 10 minutes. The cells were then washed with PBS as described above. Finally, one aliquot was used to determine cell viability. Cells were surface stained for CD44 as described above then 2 µl of propidium iodide solution[j] was added to the sample. This was allowed to incubate in the dark at room temperature for 5 minutes, prior to analysis of this sample using flow cytometry.

For analysis by flow cytometry[k], a dot plot of forward scatter (FSC) and side scatter (SSC) enabled visualization of voltage adjustments in order to move PBMC's into a gated region. Dot plots were then created with FL1 on the y-axis and FSC on the x-axis, FL2 on the y-axis and FSC on the x-axis, and FL2 on the y-axis and FL1 on the x-axis. The unstained cells, cells stained with only PE-CD44, and cells stained with PE-CD44 and the secondary FITC goat anti-mouse Ig were used to set the voltages for FL1 and FL2. A fifth dot plot was created with FL3 on the y-axis and FL2 on the x-axis to analyze cells positive for PE-CD44 and PI stain. The results from the FL1 vs. FL2 dot plot were used to determine the percentage of PBMC's positive for both PE-CD44 and the GR as well as PE-CD44 and the dexamethasone binding. Twenty thousand gated events were analyzed for each sample. Cell Quest software was used to analyze the results.

Table 2. Aliquots for Flow Cytometry Analysis

Tube number	Fluorochrome I (FITC)	Fluorochrome II (PE)	Comments
1	None	None	Autofluorescence Control/Gating PBMC's
2	None	PE-Mouse IgG	Isotype control for CD44
3	None	PE-CD44	CD44 expressed on PBMC's
4	Mouse IgG	PE-CD44	Control for GR antibody
5	Mouse IgG + FITC-goat anti-mouse Ig	PE-CD44	Control for FITC-goat anti-mouse Ig
6	Purified anti-mouse GR + FITC-goat anti-mouse Ig	PE-CD44	Cells expressing GR
7	5 µg Dexamethasone, then Fluorescein-Dexamethasone	PE-CD44	Control for GR binding affinity
8	Fluorescein-Dexamethasone	PE-CD44	GR binding affinity
9	Propidium Iodine	PE-CD44	PBMC Viability

Statistical Analysis

Statistical analysis was performed using commercial statistical software (GraphPad Prism 6[l]). Data sets were tested for normality by the Shapiro-Wilk statistic. The control samples for the GR antibody and those incubated with the GR + FITC goat-anti-mouse Ig were compared using a student t-test. Because the results for CD44 antibody binding and dexamethasone antibody binding were non-normally distributed, the CD44 controls were compared to the samples stained with the PE-CD44 antibody using the Mann-Whitney test, as were the controls for GR binding affinity and the samples evaluating GR binding affinity. The overall GR density (Delta GR%) was defined as the difference in the percentage of PBMC's positive for binding of a FITC labeled antibody between the control samples for the GR antibody and those incubated with the GR + FITC goat-anti-mouse Ig. The overall GR binding affinity (Delta BA%) was defined as the difference in the percentage of PBMC's positive for binding of a FITC labeled antibody between the samples incubated with dexamethasone and then FITC-dexamethasone and those incubated with just FITC-dexamethasone. Repeated measures analysis of variance

(ANOVA), with multiple comparisons conducted using Tukey's test was used to compare the cell viability, CD44 binding and Delta GR% between the 3 horses over the course of the 3 days. For the non-parametric variables, the Friedman test was used to compare these values, with Dunn's multiple comparison test when significant differences were found. Hypothesis tests were 2 tailed, and statistical significance was set at $P < 0.05$ for all analyses. Data are expressed as mean ± standard deviation, or median and range where appropriate.

Part II - GR density and binding affinity in horses with increased circulating cortisol.

Animals

Four healthy adult horses residing at the Marion duPont Scott Equine Medical Center and four healthy client-owned horses were used after the study design was approved by the Virginia Tech University Institutional Animal Care and Use Committee. These horses were determined to be healthy based on physical examination findings.

Adrenocorticotrophin Stimulation Test

Horses were placed in a stall the evening prior to performing the ACTH stimulation test. The horses were each weighed, and a thorough physical examination was performed. An intravenous jugular catheter[m] was placed aseptically in the right or left jugular vein of each horse 12 hours prior to intervention. The catheter was flushed every six hours with heparinized saline to prevent clotting while they were in place.

The following morning, an ACTH stimulation test was performed using 0.5 μg/kg dose of cosyntropin[n] administered intravenously to each horse. This was acquired as a lyophilized powder containing 250 μg of α1-24 corticotropin/vial, which was reconstituted according to the manufacturer's recommendations immediately prior to use. The cosyntropin dose used for ACTH stimulation testing (0.5 μg/kg) was selected based on the dose that created the most profound effect on cortisol in a previous study of healthy adult horses [128]. Blood was collected from the intravenous catheter at baseline, and then times 4, 8, and 24 hours post-ACTH injection for cortisol measurement and GR density and binding as described above.

Sampling

A total of 10 ml of venous blood was obtained at each time point for measurement of cortisol concentration. This was collected into a sterile glass tube without additives, allowed to clot at room temperature for 30-60 minutes, and then stored at -30°C until shipment for analysis

at the Cornell University College of Veterinary Medicine Animal Health Diagnostic Center. A total of 30 ml of venous blood was obtained for GR density and binding affinity determination at each time point, which was stored at 4°C for a maximum of 1 hour prior to PBMC isolation.

Cortisol Assay

Cortisol concentrations were measured using an automated chemiluminescent enzyme immunoassay[o] previously validated for horses [186].

GR Density and Binding Affinity

PBMC's were isolated and counted as described in Part I. However, after the last wash phase, cells were re-suspended in Roswell Park Memorial Institute (RPMI) 1640 cell culture media with L-glutamine and glucose[p] and stored for a maximum of 24 hours at 4°C. Antibody staining of the samples was performed as described in Part I. Prior to analyzing samples via flow cytometry, a color compensation step was performed. To do this, a sample of cells labeled with only PE-CD44 and a sample labeled only with the GR monoclonal antibody and FITC-secondary antibody was obtained. Compensation adjustments were made while examining the PE and FITC samples on dual fluorescence dot plots until there was no longer any overlap between the FITC and PE samples. Once this was performed, the samples were mixed into 1 and analyzed again to ensure there was no overlap when PE and FITC markers were in the same sample. Following this, GR density and binding affinity were determined as described in Part I.

Statistical Analysis

Statistical analysis was performed using commercial statistical software (GraphPad Prism 6 and SAS[q]). Data sets were tested for normality by the Shapiro-Wilk statistic. Differences in the PBMC count and cell viability over the course of the study were evaluated using a repeated measures ANOVA, with Tukey's test for multiple comparisons. Differences in cortisol concentrations, Delta GR% and Delta BA% over the 24-hour study period were analyzed by a Friedman test, with multiple comparisons conducted using Dunn's test. Additionally, Spearman's rank correlation test was used to evaluate for an effect of cortisol on Delta GR% and Delta BA%, as well as a relationship between Delta GR% and Delta BA%. Statistical significance was set at $P < 0.05$ for all analyses. Data are expressed as mean ± standard deviation, or median and range where appropriate.

Part III - GR density and binding affinity in clinical cases of horses with SIRS.

Animals

Ten adult horses presenting to the Marion DuPont Scott Equine Medical Center that met the criteria for SIRS, and ten healthy adult horses presenting for orthopedic or ophthalmic evaluation were used after the Virginia Tech University Institutional Animal Care and Use Committee approved the study design.

Data Collection

Horses were included in the SIRS group if they met two or more of the following criteria on presentation: (1) leukocytosis or leukopenia (white blood cell count greater than 12,000 cells/µl or less than 5,000 cells/µl), or the presence of 10% or greater band neutrophils, (2) hyperthermia (rectal temperature of greater than 101.5^{o}F), (3) tachycardia (heart rate greater than 60 beats per minute), and/or (4) tachypnea (respiratory rate greater than 30 breaths per minute) [5-7]. Horses presenting to the EMC for orthopedic or ophthalmic evaluation were used as age and sex matched controls. Data collected on presentation included a history, primary complaint and baseline physical examination findings. Blood variables assessed included a CBC, serum biochemistry, blood fibrinogen concentration, blood hormone concentrations (ACTH and cortisol), and GR density and binding affinity.

Sampling

Blood samples were collected by jugular venipuncture using plastic tubes containing 3.6 mg potassium EDTA for the measurement of plasma endogenous ACTH and cortisol concentrations, CBC, fibrinogen and measurement of the GR density and binding affinity. Blood was also collected into a plastic tube containing sodium heparin for serum biochemistry analysis. CBC, serum biochemistry, and fibrinogen measurements were performed immediately after blood collection. The CBC was performed using an automatic blood cell counter[r] and manual differential cell count. Plasma fibrinogen concentration was determined using refractometry and the heat precipitation method. Serum biochemistry analysis was performed using an automated spectrophotometry chemistry analyzer[s]. A total of 10 ml of venous blood was obtained for hormone assays, and centrifuged within 20 minutes of collection at 5°C, 2000 rpm for 15 minutes. Plasma was then aliquoted and stored at -30°C until shipment for hormone concentration analysis. A total of 20 ml of venous blood was obtained for GR density and binding affinity determination, which was stored at 4°C for a maximum of 1 hour prior to PBMC

isolation.

Hormone Assays

Plasma ACTH and cortisol concentrations were determined using an automated chemiluminescent enzyme immunoassay at the Cornell University College of Veterinary Medicine Animal Health Diagnostic Center[o] that was previously validated for horses [186, 187].

GR Density and Binding Affinity

PBMC's were isolated and counted as described in Part I. However, after the last wash phase, cells were re-suspended in Roswell Park Memorial Institute (RPMI) 1640 cell culture media with L-glutamine and glucose[p] and stored for a maximum of 24 hours at 4°C. Following this, GR density and binding affinity were determined as described in Part II.

Statistical Analysis

Statistical analysis was performed using commercial statistical software[a]. Data sets were tested for normality by the Shapiro-Wilk statistic. Data were expressed as ± standard deviation, or median and range where appropriate. Age, white blood cell count, neutrophil count, lymphocyte count, hematocrit, platelets, total protein, serum calcium, sodium, potassium, chloride, lactate dehydrogenase (LDH), magnesium, and phosphate, PBMC count, cell viability after PBMC isolation, and Delta BA% were compared between SIRS cases and controls as well as survivors and nonsurvivors using student t-tests. The monocyte count, eosinophil count, fibrinogen, albumin, alkaline phosphatase (ALP), amylase, aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatine kinase (CK), creatinine, direct bilirubin, gamma-glutamyl transpeptidase (GGT), glucose, total bilirubin, triglycerides, plasma ACTH, plasma cortisol, ACTH:cortisol ratio, and Delta GR% were compared between SIRS cases and controls as well as survivors and nonsurvivors using the Mann-Whitney test because these values were not distributed normally. Spearman's rank correlation test was used to determine any correlations between serum hormone values, Delta GR% and Delta BA%, and serum biochemistry and CBC variables.

In order to determine a cut-point above which nonsurvival could be most reliably predicted by plasma concentration of ACTH or cortisol, ACTH:cortisol, Delta GR%, Delta BA%, and serum triglyceride concentration, receiver-operator curve (ROC) analysis and sensitivity specificity analyses were also performed. The cut-points for these values determined by the sensitivity and specificity combination with the highest likelihood ratio, were then used to

assess these values in this population using Fisher's exact test and to determine an odds ratio for nonsurvival. Hypothesis tests were 2 tailed, and statistical significance was set at $P < 0.05$ for all analyses.

Results

Part I

Mean age of the horses included in this part of the study was 13.3 ± 7.5 years. All three horses were female, and consisted of a Quarter Horse, a Paint Horse, and a Thoroughbred. The median PBMC count recovered from these horses on all 3 days was 1.7×10^6 cells/ml. There was no significant variation between horses or day of collection on PBMC recovery. The mean PBMC viability was 95.16 ± 1.65%. Again, there was no significant effect of horse or day of collection on PBMC viability.

In order to evaluate the density and binding affinity of GR by equine PBMC's, it was important to first identify mononuclear cells from each sample. Cells were first analyzed without any added antibodies, and dot diagrams were used to visualize cells separated by side scatter and forward scatter. Cells with characteristic forward and side scatter were identified as lymphocytes and monocytes, and these cells were gated for further sampling, as seen in Figure 1. CD44 is a glycoprotein expressed on all leukocytes, endothelial cells, hepatocytes and mesenchymal cells, thus it was used in this study to identify both lymphocytes and monocytes. Binding of an isotype control for the CD44 antibody was used to control for the CD44 antibody to account for any background effect of the flow cytometer and analysis software. This was then compared to samples incubated with the CD44 antibody as seen in Figure 2. The median percentage of PBMC's that bound the isotype control was 0.70% (range 0.51-3.92%), and the median percentage of PBMC's that bound the CD44 antibody was 44.20% (range 23.10-64.68%). These values were significantly different ($P<0.0001$), and there was no significant effect of horse on these measurements, but there was a significant effect on the day these samples were collected ($P=0.0145$).

Figure 1. Example of a forward scatter and side scatter flow cytometric dot plot used to set voltages and gate lymphocytes and monocytes.

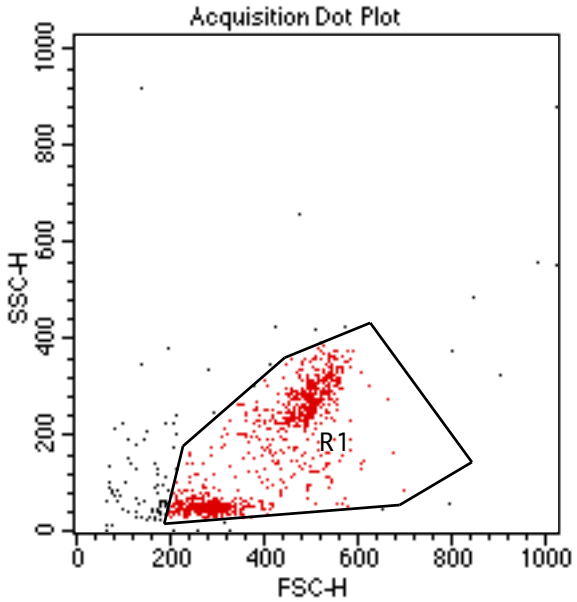
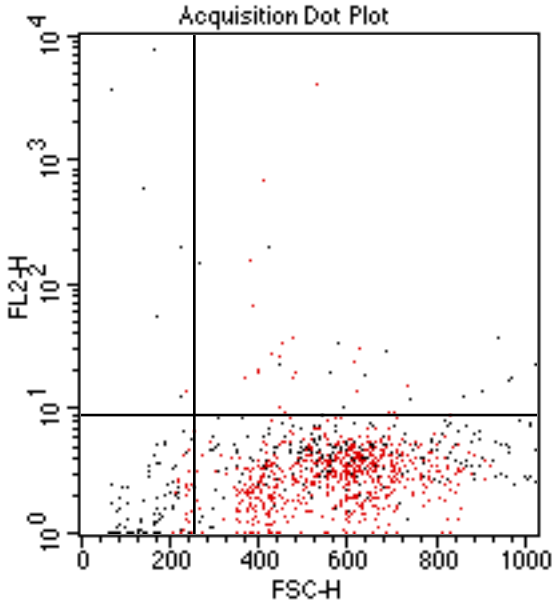
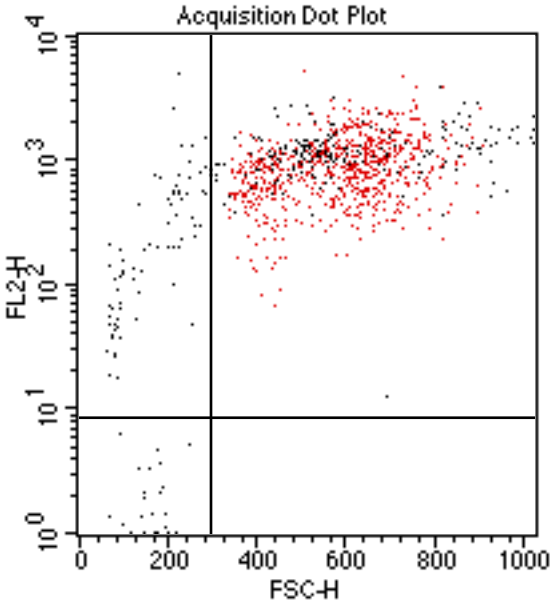


Figure 2. CD44 Controls vs. CD44+ Cells. CD44+ equine peripheral blood mononuclear cells were identified by comparing cells incubated with an isotype control to those incubated with a monoclonal CD44+ antibody.

Isotype	CD44+
	

Following identification of the CD44+ population of cells, the density of GR in this cell population was determined. Dot diagrams were used to visualize expression of two receptors simultaneously. Figure 3 shows expression of GR in CD44+ PBMC's. After correcting for the FITC-goat anti-mouse Ig control, the mean percentage of PBMC's that bound both the GR and CD44 antibodies, was 8.87 ± 4.61%. There was a significant difference between the FITC-control cells and the cells expressing GR ($P<0.0001$), and there was no significant effect of horse or time on these measurements. Using this same population of CD44+ cells, the binding affinity of the GR in the PBMC's was determined. Again, dot diagrams were used to visualize expression of the two receptors simultaneously. Figure 4 shows GR binding FITC-dexamethasone in CD44+ PBMC's. There was a significant difference between the control group, where the receptors were blocked by dexamethasone, and those incubated with the FITC-dexamethasone ($P<0.0001$). Again, there was no significant effect of horse or time on these measurements. After correcting for the control samples, a median percentage of PBMC's that bound both the FITC-dexamethasone and CD44 antibodies, was 2.15%, range 0.97-11.29%. Figure 5 summarizes the results of the control fluorochrome binding to PBMC's vs. the binding of the fluorochromes used to characterize the GR in this study.

Figure 3. Glucocorticoid Receptor (GR) Density in Equine Peripheral Blood Mononuclear Cells. Expression of glucocorticoid receptors by CD44+ equine peripheral blood mononuclear cells using flow cytometry. Dot plots show GR density in CD44+ cells. Isotype control for GR (FITC-goat anti-mouse Ig) is shown for comparison. Cross hatch lines were determined by comparing with isotype control antibodies.

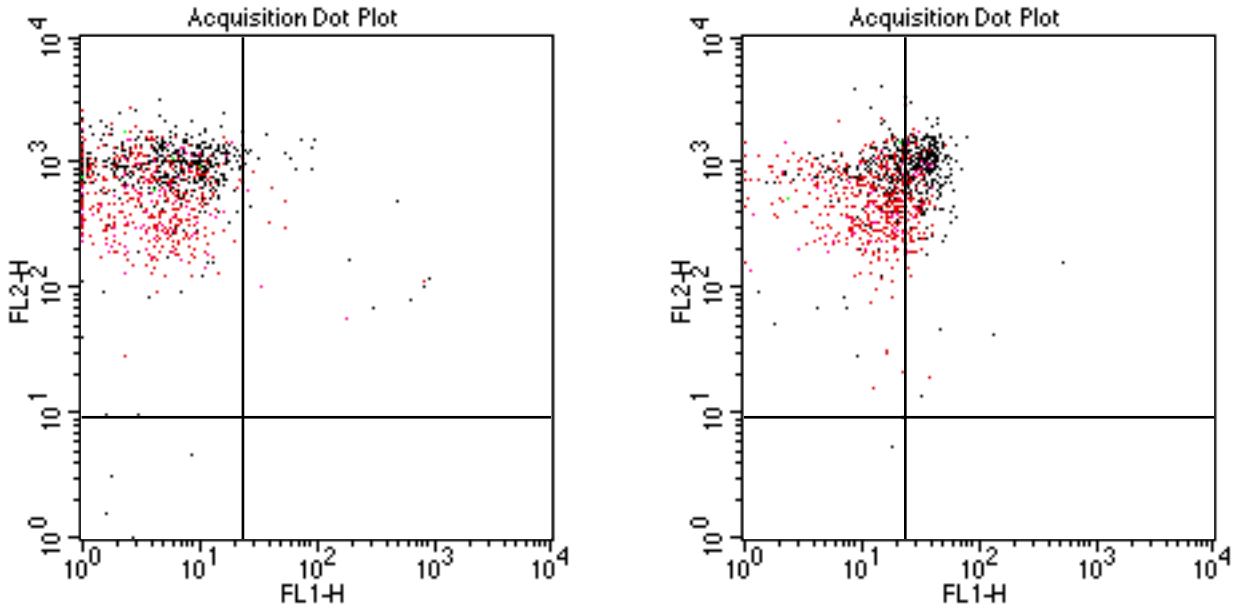


Figure 4. Glucocorticoid Receptor (GR) Binding Affinity in Equine Peripheral Blood Mononuclear Cells. Binding affinity of glucocorticoid receptors in CD44+ equine peripheral blood mononuclear cells. Dot plots show GR binding affinity in CD44+ cells. The control for GR binding affinity (dexamethasone prior to FITC-dexamethasone) is shown for comparison. Cross hatch lines were determined by comparing with control sample (dexamethasone).

Dexamethasone Control	GR Binding Affinity

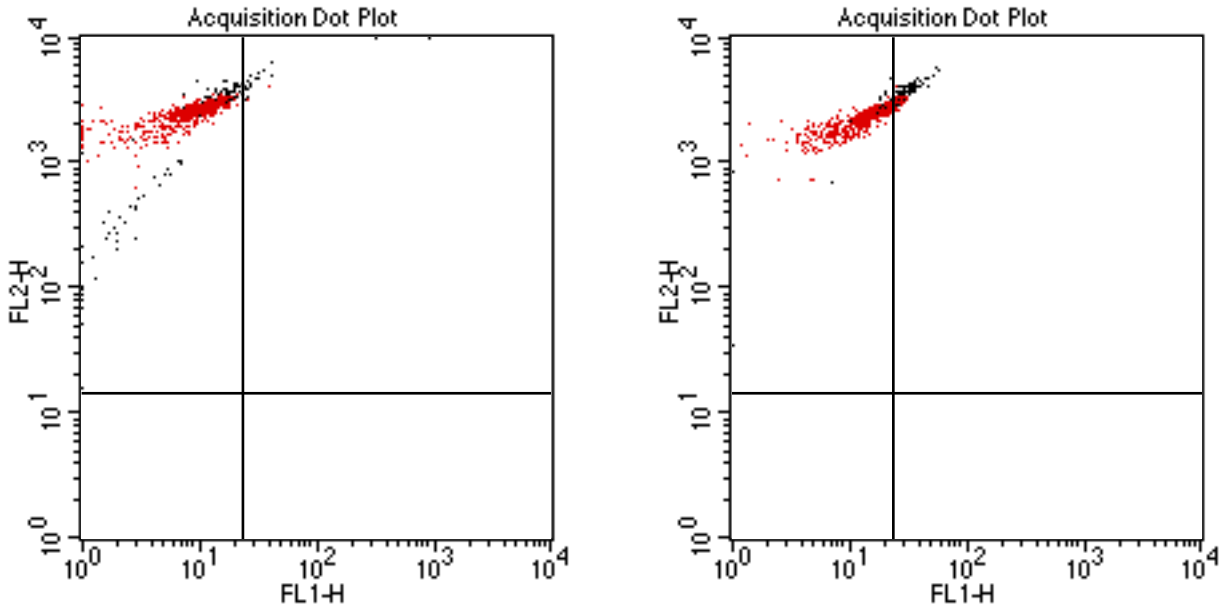
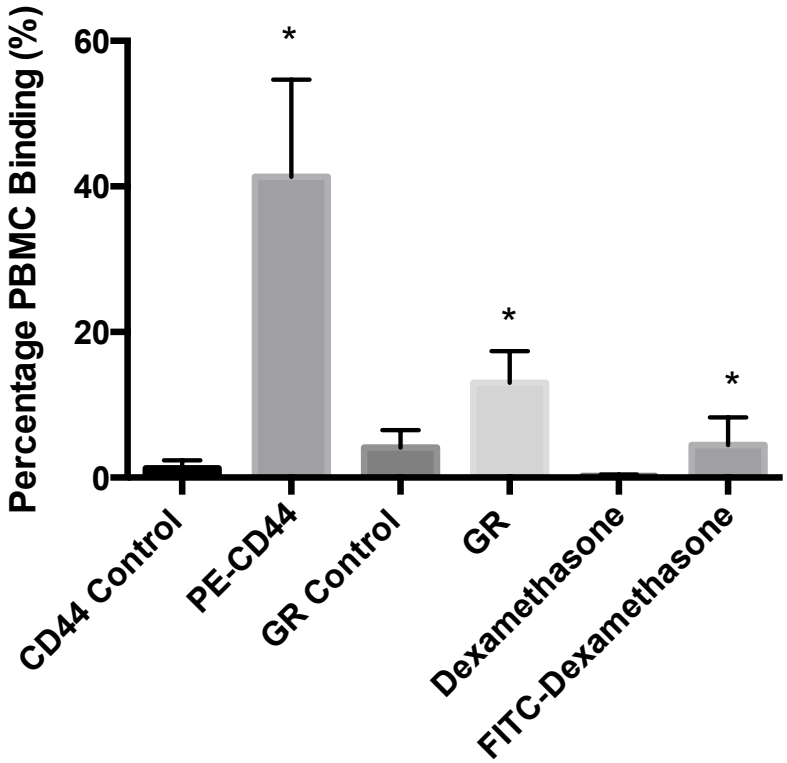


Figure 5. Percentage PBMC binding for CD44 control, PE-CD44, GR control, FITC-GR, dexamethasone control and FITC-dexamethasone. Asterisk (*) indicates a significant difference between the control and fluorescent antibody.



Part II

Mean age of horses used in this part of the study was 12.0 ± 6.7 years, and this group consisted of 4 mares and 4 geldings. There were 3 Paint Horses, 2 Quarter Horses, 2 Thoroughbreds, and 1 Warmblood cross. All 8 horses were normal on physical examination. The mean PBMC count recovered from these horses at all 4 time points was $2.4 \times 10^6 \pm 1.0 \times 10^6$ cells/ml. There was no significant variation between horses, however, significantly more cells were collected at the 8 hour collection point compared to all other times (P=0.0266, P=0.0039, and P=0.0011 respectively). The mean PBMC viability was 92.46 ± 3.33%, and there was no significant variation between horses or time of PBMC collection.

The median serum cortisol at baseline was 4.9, range 3.2-6.1 µg/dl. This was significantly greater than the median serum cortisol at 8 hours after the ACTH stimulation test of 1.4, range 1.1-2.7 µg/dl (P=0.0221). Additionally, the 4-hour cortisol concentrations were also significantly greater than the 8-hour concentrations, with a median of 5.6, range 4.8-7.4 µg/dl (P=0.0006). Figure 6 shows the median concentrations of cortisol over the 24-hour period. The median Delta GR% over the course of the 24-hour period was 16.28, range 1.21-53.63%, and

there was a significant difference in these values between horses (*P*=0.0359) and between the 8 hour post-ACTH administration and 24 hour samples (*P*=0.0402). Figure 7 shows the median Delta GR% during the 24-hour period. The median Delta BA% over the course of the 24-hour period was 45.00, range 7.01-90.98%. There were no significant differences in this value between horses or over the 24 hours of analysis. Figure 8 shows the median Delta BA% during this time period.

There was no correlation between cortisol concentration and Delta GR% (r=-0.145, *P*=0.428) or Delta BA% (r=0.046, *P*=0.802). However, there was a correlation between Delta GR% and Delta BA% (r=-0.483, *P*=0.005).

Figure 6. Median cortisol concentration over time following the administration of exogenous ACTH. Asterisk (*) indicates a significant difference in the cortisol concentration at a specific time point.

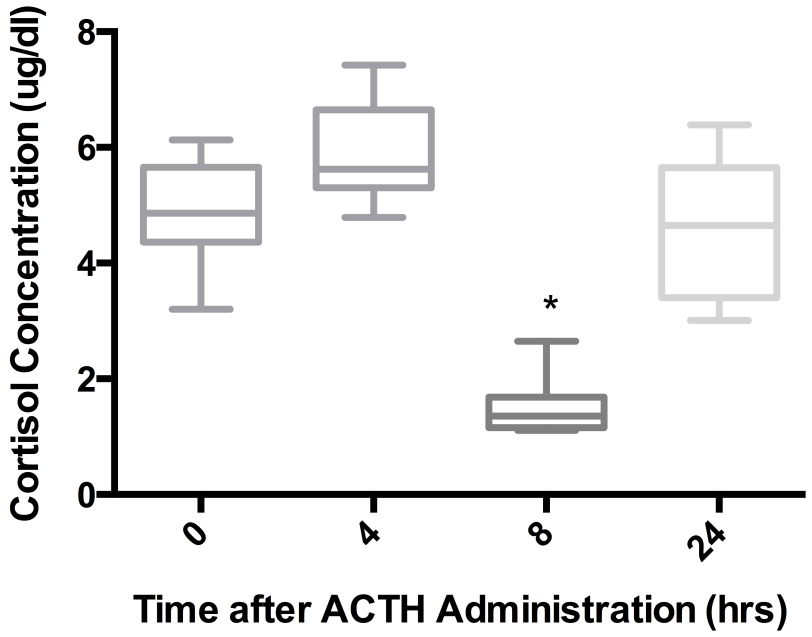


Figure 7. Median Delta GR% over time following the administration of exogenous ACTH.

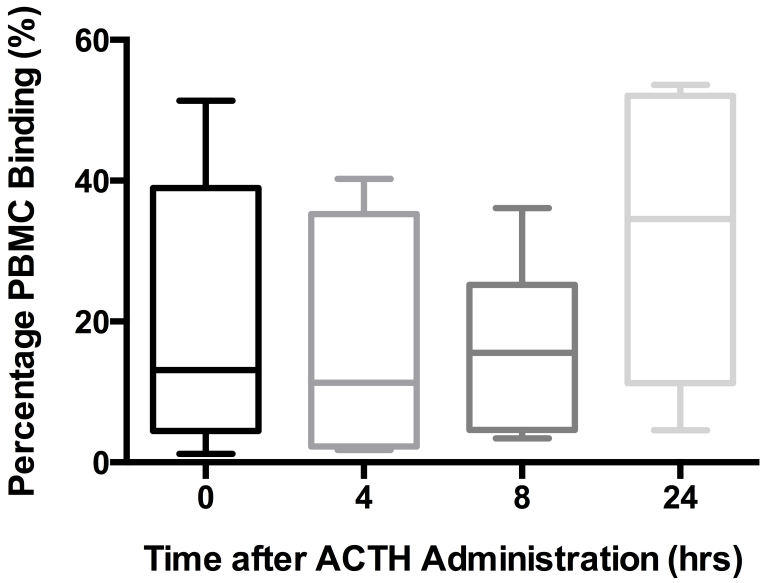
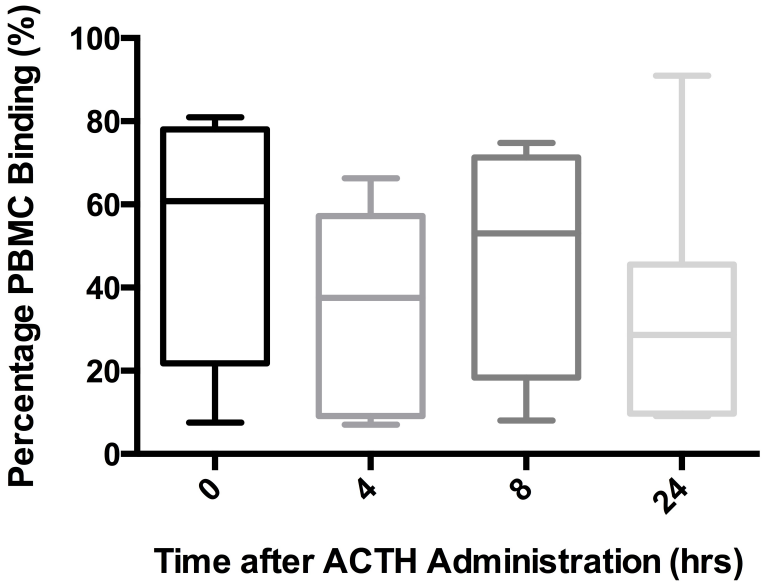


Figure 8. Median Delta BA% over time following the administration of exogenous ACTH.



Part III

There were 20 horses total included in this part of the study, 10 controls and 10 SIRS cases. See Table 3 for the signalment and diagnosis for each horse. The mean age was 12.7 ± 4.1 years, and there was no significant difference in age between SIRS cases or controls or survivors and nonsurvivors. There were 8 mares and 12 geldings. Breeds included, 6 Warmbloods, 5 Quarter Horse/Quarter Horse crosses, 4 Thoroughbreds, 3 Standardbreds, 1 Paint Horse, and 1 Tennessee Walker. The mean PBMC count was $1.6 \times 10^6 \pm 0.6 \times 10^6$ cells/ml, with no significant difference between SIRS cases and controls. The mean PBMC viability was 95.28 ± 2.58%, with no significant difference between SIRS cases and controls.

Table 3. Summary of Horses Included in Part III – Signalment and Diagnosis. (TB=Thoroughbred, WB=Warmblood, STB=Standardbred, QH=Quarter Horse, QHX=Quarter Horse Cross, Perch=Percheron, TW=Tennessee Walker)

Group	Age (years)	Sex	Breed	Diagnosis
Healthy Controls (n=10)	9	4 mares	TB	Hock Arthritis
	12	6 geldings	Paint	Navicular Syndrome
	12		WB	Suspensory Ligament Desmitis
	17		STB	Uveitis
	17		WB	Suspensory Ligament Desmitis
	12		WB	Lameness of Unknown Origin
	13		WB	Lameness of Unknown Origin
	6		QH	Navicular Syndrome
	10		QHX	Desmitis of Lateral Collateral Ligament of Coffin Joint
	20		QH	Laceration
SIRS Cases (n=10)	9	4 mares	TB	Hemoabdomen
	10	6 geldings	Perch	Enteritis
	15		TB	Small Colon Impaction
	15		STB	Colitis
	8		STB	Epiploic Foramen Entrapment
	16		TB	Ruptured Stomach
	15		WB	Hemoabdomen
	12		QH	Red Maple Leaf Toxicity
	6		WB	Aortic Aneurysm
	19		TW	Lymphosarcoma

A summary of the CBC and serum biochemistry values can be seen in Tables 4, 5, 6, and 7, with Tables 4 and 6 comparing values for SIRS cases and controls and Tables 5 and 7 comparing values for survivors and nonsurvivors. When the CBC's of the survivors and nonsurvivors were compared, significant differences were observed between SIRS cases and controls in their WBC (P=0.0191), percentage band neutrophils (P=0.0031), percentage lymphocytes (P=0.0045), percentage eosinophils (P=0.0007), and percentage monocytes (P=0.0080). When the serum biochemistry of SIRS cases and controls were compared, significant differences were found in the AST concentration (P=0.0049), BUN concentration (P=0.0002), CK concentration (P=0.0173), creatinine concentration (P=0.0489), chloride concentration (P=0.0050) and LDH concentration (P=0.0231). When the serum biochemistry of survivors and nonsurvivors were compared, a significant difference in the serum triglyceride concentrations was detected (P=0.0095).

Table 4. Complete Blood Cell Count values in healthy controls and horses presenting with SIRS, including: White Blood Cell Count (WBC), Neutrophils (Neuts), Band Neutrophils (Bands), Lymphocytes (Lymphs), Eosinophils (Eos), Monocytes (Monos), Toxic Morphology, Hematocrit (Hct), Platelet Count (Plt), Total Protein (TP), and Fibrinogen (Fib). Values with an asterisk (*) indicate a significant difference between groups.

Group	WBC (cells/µl)	Neuts (%)	Bands (%)	Lymphs (%)	Eos (%)	Monos (%)	Toxic (#/total)	Hct (%)	Plt (x10^3 cells/µl)	TP (g/dl)	Fib (mg/dl)
Healthy Controls (n=10)	7,300 ± 1,712	67.90 ± 10.84	0	29.00 ± 10.33	0	4.0 (1.0-18.0)	0/10	38.50 ± 6.654	206.1 ± 43.14	6.98 ± 0.649	250 (100-700)
SIRS Cases (n=10)	11,940 ± 5,437*	76.00 ±16.76	4.50 (0-11.0)*	14.00 ±10.32*	2.0 (0-3.0)*	1.0 (0-4.0)*	3/10	43.40 ± 13.91	185.7 ± 68.43	7.31 ± 1.76	200 (100-700)

Table 5. Complete Blood Cell Count values in survivors and nonsurvivors, including: White Blood Cell Count (WBC), Neutrophils (Neuts), Band Neutrophils (Bands), Lymphocytes (Lymphs), Eosinophils (Eos), Monocytes (Monos), Toxic Morphology, Hematocrit (Hct), Platelet Count (Plt), Total Protein (TP), and Fibrinogen (Fib). Values with an asterisk (*) indicate a significant difference between groups.

Group	WBC (cells/µl)	Neuts (%)	Bands (%)	Lymphs (%)	Eos (%)	Monos (%)	Toxic (#/total)	Hct (%)	Plt ($x10^3$ cells/µl)	TP (g/dl)	Fib (mg/dl)
Survivors (n=4)	13,300 ± 5,932	80.5 (59.0-83.0)	4.0 ± 3.2	14.5 ± 6.6	0	5.0 (1.0-12.0)	0/4	39.8 ± 10.4	177.3 ± 85.1	7.2 ± 2.2	150 (100-200)
Nonsurvivors (n=6)	11,033 ± 5,436	85.0 (36.0-91.0)	4.7 ± 4.9	13.7 ±12.9	0	3.0 (2.0-18.0)	3/6	45.8 ± 16.3	191.3 ± 63.2	7.4 ± 1.6	300 (100-700)

Table 6. Serum Biochemistry results in healthy controls and SIRS cases, including: Albumin, Alkaline Phosphatase (ALP), Amylase, Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN), Calcium (Ca), Creatine Kinase (CK), Creatinine (Creat), Direct Bilirubin (D Bili), Gamma Glutamyl Transferase (GGT), Glucose, Sodium (Na), Potassium (K), Chloride (Cl), Lactate Dehydrogenase (LDH), Magnesium (Mg), Phosphorus (Phos), Total Bilirubin (T Bili), and Triglycerides (TG). Values with an asterisk (*) indicate a significant difference between groups.

Group	Albumin (g/dl)	ALP (U/L)	Amylase (U/L)	AST (U/L)	BUN (mg/dl)	Ca (mg/dl)	CK (U/L)	Creat (mg/dL)	D Bili (U/L)	GGT (U/L)	Glucose (mg/dl)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)	LDH (U/L)	Mg (mg/dl)	Phos (mg/dl)	T Bili (U/L)	TG (mg/dl)
Healthy Controls (n=10)	2.9 (2.6-3.4)	127.5 (79.0-298.0)	3.0 (0-6.0)	256 (163-336)	14 (11-21)	10.81 ± 0.735	205.5 (106-951)	1.2 (0.8-2.4)	0.1 (0-0.3)	21.5 (13-34)	100.5 (84-232)	134.7 ± 4.76	3.30 ± 0.43	100.6 ± 3.20	248 ± 113.2	1.97 ± 0.47	2.58 ± 0.57	1.35 (0.7-5.5)	21 (6-41)
SIRS Cases (n=10)	2.95 (1.8-3.4)	170.5 (55.0-342.0)	6.5 (1.0-517.0)	391 (189-762)*	24.5 (15-67)*	10.49 ± 1.92	530 (150-3060)*	1.6 (0.8-2.8)*	0.1 (0-0.1)	21.5 (10-229)	159.5 (77-282)	131.4 ± 4.27	3.15 ± 0.66	96.0 ± 3.23	418.5 ± 185.1*	1.62 ± 0.68	2.47 ± 1.05	2.7 (1.0-9.4)	35.5 (10-612)

Table 7. Serum Biochemistry results in survivors and nonsurvivors, including: Albumin, Alkaline Phosphatase (ALP), Amylase, Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN), Calcium (Ca), Creatine Kinase (CK), Creatinine (Creat), Direct Bilirubin (D Bili), Gamma Glutamyl Transferase (GGT), Glucose, Sodium (Na), Potassium (K), Chloride (Cl), Lactate Dehydrogenase (LDH), Magnesium (Mg), Phosphorus (Phos), Total Bilirubin (T Bili), and Triglycerides (TG). Values with an asterisk (*) indicate a significant difference between groups.

Group	Albumin (g/dl)	ALP (U/L)	Amylase (U/L)	AST (U/L)	BUN (mg/dl)	Ca (mg/dl)	CK (U/L)	Creat (mg/dL)	D Bili (U/L)	GGT (U/L)	Glucose (mg/dl)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)	LDH (U/L)	Mg (mg/dl)	Phos (mg/dl)	T Bili (U/L)	TG (mg/dl)
Survivors (n=4)	2.9 (1.8-3.0)	175.0 ± 96.9	38.5 (1.0-517.0)	364.5 ± 32.2	27.0 (22.0-67.0)	10.2 ± 1.9	1100 (401-1670)	1.5 (0.8-2.4)	0.1 (0 – 0.1)	21.5 (10.0-26.0)	171.5 ± 84.6	128.8 ± 4.8	3.1 ± 0.5	96.8 ± 2.9	395.0 ± 44.3	1.5 ± 0.5	2.7 ± 1.5	2.0 (1.0-2.7)	16.0 (10.0-28.0)
Non-survivors (n=6)	3.1 (1.9-3.4)	182.2 ± 98.2	3.0 (2.0-12.0)	430.0 ± 201.3	24.5 (15.0-37.0)	10.7 ± 2.1	424 (150-3060)	1.8 (1.2-2.8)	0.1	21.5 (10.0-229.0)	164.7 ± 57.6	133.2 ± 3.1	3.2 ± 0.8	95.5 ± 3.6	434.2 ± 244.5	1.7 ± 0.8	2.3 ± 0.7	4.2 (1.7-9.4)	58.0 (29.0-612.0)*

Plasma ACTH, cortisol, and ACTH:cortisol ratios were compared between SIRS cases and controls as well as survivors and nonsurvivors (see Tables 8 and 9). The median baseline ACTH of healthy controls was 28.75 pg/ml (range 9.07-202.0), which was significantly different from the cases with SIRS with a median of 72.50 pg/ml (range 39.90-689.0) (P=0.0002). The median baseline cortisol of healthy controls was 4.46 µg/dl (range 2.29-14.00), which was also significantly different from the cases with SIRS, with a median of 9.46 µg/dl (range 0.81-41.20) (P=0.0232). The ACTH:cortisol ratios were not significantly different between SIRS cases and controls or survivors and nonsurvivors, although the ACTH:cortisol ratio tended to be greater in the nonsurvivors when compared to survivors. The Delta GR% and Delta BA% were compared between SIRS cases and controls as well as survivors and nonsurvivors (Tables 10 and 11). The only significant difference in these values was found in the Delta BA% when compared between survivors and nonsurvivors (P=0.008).

Table 8. Median basal endogenous plasma ACTH concentration, total plasma cortisol concentration, and basal ACTH: cortisol ratio for healthy controls and horses presenting with SIRS. Values with an asterisk (*) indicate a significant difference between groups.

Group	ACTH Concentration (pg/ml)	Cortisol Concentration (µg/dl)	ACTH:Cortisol
Healthy Controls (n=10)	28.75 (9.07-202.0)	4.46 (2.29-14.00)	4.85 (2.13-27.82)
SIRS Cases (n=10)	72.50 * (39.90-689.0)	9.46 * (0.81-41.20)	8.09 (4.49-97.14)

Table 9. Median basal endogenous plasma ACTH concentration, total plasma cortisol concentration, and basal ACTH: cortisol ratio for survivors and nonsurvivors. Values with an asterisk (*) indicate a significant difference between groups.

Group	ACTH Concentration (pg/mL)	Cortisol Concentration (µg/dL)	ACTH:Cortisol
Survivors (n=4)	54.54 (39.90-202.0)	9.33 (5.20-14.0)	6.43 (4.50-20.63)
Nonsurvivors (n=6)	150.1 (60.00-689.0)	10.97 (0.81-41.20)	20.83 (4.69-97.14)

Table 10. Median and mean Delta GR and Delta BA for SIRS cases and controls. Values with an asterisk (*) indicate a significant difference between groups.

Group	Delta GR (%)	Delta BA (%)
Healthy Controls (n=10)	33.06 (20.64-73.56)	31.06 ± 25.29
SIRS Cases (n=10)	56.13 (40.67-68.43)	28.57 ± 25.36

Table 11. Median and mean Delta GR and Delta BA for survivors and nonsurvivors. Values with an asterisk (*) indicate a significant difference between groups.

Group	Delta GR (%)	Delta BA (%)
Survivors (n=4)	55.35 ± 12.64	51.51 ± 24.53
Nonsurvivors (n=6)	52.51 ± 19.98	13.28 ± 9.77 *

Receiver operator characteristic (ROC) analysis was then performed for the results of plasma ACTH concentration, plasma cortisol concentration, ACTH:cortisol, Delta GR% and Delta BA%. Figure 9 shows the ROC curves for plasma ACTH, plasma cortisol, ACTH:cortisol, and the Delta BA%. ROC analysis and the area under the curves (AUCs) were used to determine the predictive value of these variables for predicting nonsurvival. Table 12 summarizes the AUC for each of these variables. For values with an AUC > 0.50, the cut-off value with the highest likelihood ratio was then used to perform contingency analysis of the results of the survivors and nonsurvivors in this study to determine the odds ratio for nonsurvival. Table 13 summarizes these results.

Figure 9. ROC Analysis of plasma ACTH concentration, plasma cortisol concentration, ACTH:cortisol ratio and Delta BA%.

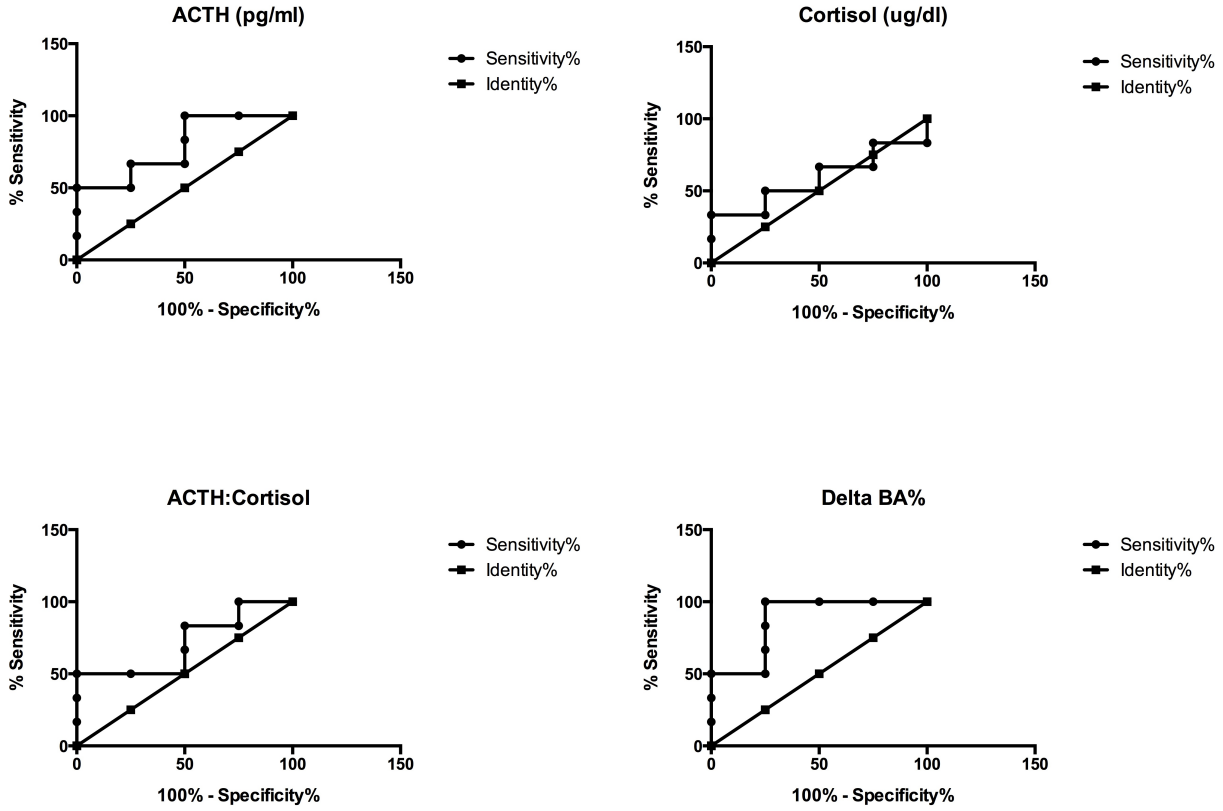


Table 12. Mean (±SE) areas under the curve (AUCs) for receiver operator characteristic curves for predictive value of plasma ACTH concentration, plasma cortisol concentration, ACTH:cortisol ratio, Delta GR% and Delta BA% for predicting nonsurvival in horses presenting with SIRS.

Variable of Interest	Mean (±SE) AUC
Plasma ACTH concentration (pg/ml)	0.79 ± 0.15
Plasma cortisol concentration (µg/d)	0.58 ± 0.19
ACTH:cortisol	0.71 ± 0.17
Delta GR (%)	0.50 ± 0.19
Delta BA (%)	0.88 ± 0.13

Table 13. Cut-off values determined by Likelihood Ratios from ROC Analysis, the sensitivity, specificity and odds ratio for nonsurvival for plasma ACTH concentration, plasma cortisol concentration, ACTH:cortisol ratio, and Delta BA%.

Variable of Interest	Cut-off Value	Sensitivity (%)	Specificity (%)	Odds Ratio	95% Confidence Interval
Plasma ACTH concentration (pg/mL)	> 72.50	66.67	75.0	6.0	0.35-101.6
Plasma cortisol concentration (µg/dL)	> 11.30	50.0	75.0	3.0	0.19-48.0
ACTH:cortisol	> 14.36	50.0	75.0	3.0	0.19-48.0
Delta BA (%)	< 35.79	100	75.0	30.33	0.96-960.5

Spearman correlation analysis was then performed between the serum triglycerides and HPA axis hormones in horses that presented with SIRS. This showed that there were no correlations observed between the serum triglyceride concentration and the plasma ACTH or cortisol concentration, the ACTH:cortisol ratio, or the Delta GR% and Delta BA%, although the relationship between the serum triglyceride concentration and Delta BA% was almost significant (r=-0.600, *P*=0.067). In this group, there was a significant negative correlation observed

between the plasma ACTH and ACTH:cortisol and Delta BA% (r=-0.685, *P*=0.029 and r=-0.697, *P*=0.025 respectively). Additionally, no correlation between Delta GR% and Delta BA% was observed (r=0.176, *P*=0.627).

Lastly, ROC analysis with a calculated AUC was performed using the serum triglyceride concentrations of the horses presenting with SIRS. The cut-off value with the highest likelihood ratio was then again used to perform contingency analysis of the survivors and nonsurvivors in this study to determine the odds ratio for nonsurvival. Table 14 summarizes these results.

Table 14. Analysis of serum triglycerides for predicting HPA axis dysfunction and nonsurvival. The cut-off value was determined by ROC analysis.

Variable of Interest	Mean AUC (±SE)	Cut-off Value	Sensitivity (%)	Specificity (%)	Odds Ratio	95% Confidence Interval
Triglycerides (mg/dl)	1.0	> 28.5	100.0	100.0	117	1.94-7067

Discussion

Measurement of the density and binding affinity of the equine GR in PBMC's was successfully performed in this study, and was technically simple to perform using flow cytometry. Although we found no correlation between cortisol concentrations and the GR density or binding affinity, a decrease in the Delta BA% and an increase in the serum triglycerides were found to be significantly associated with increased odds of nonsurvival in these cases. These horses also tended to have an increase in their ACTH:cortisol ratio. These findings suggest that HPA axis dysfunction may occur at multiple levels in adult horses presenting with SIRS, and further research is needed to further elucidate methods for rapidly detecting which horses are at risk of nonsurvival and those that may benefit from supplementation with glucocorticoids.

In this study, CD44 was chosen as a cell surface marker to identify mononuclear cells. This is a cell-surface glycoprotein expressed on all leukocytes, endothelial cells, hepatocytes and mesenchymal cells. This specific antibody was chosen to allow both lymphocytes and monocytes to be simultaneously highlighted. In *Part I* of this study, the PE-CD44 antibody was shown to be effective at highlighting both equine lymphocytes and monocytes. There was no

significant difference in the percentage of CD44+ cells between horses on any given day, however, a significantly greater percentage of CD44+ cells were identified in all 3 horses on day 3 compared to day 2. There was no significant difference in the cell viability detected between days, so this could not have contributed to the difference in CD44+ cells observed between days.

It has been demonstrated in humans that as T cells become activated, CD44 expression on the cell surface increases [188]. T cell activation can occur due to antigen presentation via MHC protein or through co-stimulatory proteins such as CD80 and CD86 binding to CD28 on the T-cell during an innate response to infection [189]. More simply put, T cell activation occurs in response to an inflammatory or infectious stimulus. In this case, all 3 horses showed a similar elevation in CD44 binding on the third day of the study. None of the horses showed signs of systemic illness on this day or any others during the study. Additionally, in *Part II* and *Part III* of the study, there was no difference observed in CD44 binding between both healthy and sick horses, making T cell activation a less likely explanation for this elevation in *Part I*. Another possibility explaining increased CD44 expression is sample preparation. In a human study by Schmielau and Finn, they demonstrated that storing samples at room temperature for prolonged periods can lead to granulocyte activation, which affects their buoyancy profile, resulting in less efficient separation by density gradient procedures [190]. In a study by McKenna et al, they showed that delayed processing of blood by 8 hours increased granulocyte contamination by 2.3 fold compared to samples processed in less than 3 hours [191]. While these samples were not left at room temperature for that long, sample processing was delayed for 2-3 hours on the third day of the study due to the weather, and this may have led to both increased granulocyte activation and granulocyte contamination of the PBMC's gated on flow cytometry analysis. Following this, samples were either processed immediately, or refrigerated for up to 1 hour prior to PBMC isolation, and this may have prevented any further discrepancy of results due to delayed sample processing.

Cell viability of PBMC's was determined in this study using flow cytometry based on the uptake of propidium iodide by cells that were simultaneously labeled with CD44. This is a DNA-binding dye, which is known to only pass through the cell membrane of dead or dying cells [192]. The mean PBMC viability obtained for all 3 parts of this study was, 93.78 ± 3.187%. There is no standard value for the viability level below which a specimen yields data that is unacceptable for flow cytometry analysis, however, samples with decreased cell viability

can have an effect on cell staining and can induce cell clumping [170]. Because all of the samples in this study had similar cell viability, we do not believe this contributed to any artifact in our results.

A mouse monoclonal GR antibody was shown to be effective to detect and quantify the GR in equine PBMC's using flow cytometry analysis. This is the first study to date using the purified mouse anti-glucocorticoid receptor antibody to analyze the equine GR, although, a similar antibody was successfully used in both rat and human studies previously [146, 147, 151, 160, 164-166, 193]. However, there was substantial variation in antibody binding between horses, ranging from 20.64%-73.56% in healthy horses, making it difficult to detect differences between groups. In the study by Du et al, the percentage of GR identified in human PBMC samples differed when monocytes or lymphocytes were examined individually [165]. In that study, when CD3+ cells (T-lymphocytes) were examined in healthy individuals, a mean GR density of 72.7% was identified, while a mean of 43.6% was found in CD14+ cells (monocytes) [165]. In the study by Gotovac et al, there was no significant difference in the GR density in T (CD3) and B (CD20) lymphocytes (45.3% and 45.6%, respectively) in healthy individuals, however, patients with post-traumatic stress disorder showed variation in the GR density in various cell types [160]. A significant difference in the lymphocyte and monocyte concentrations were detected between SIRS cases and controls in *Part III* of this study. Thus, using separate cell surface markers to identify lymphocytes and monocytes might have helped decrease the variability in the GR density determined, and potentially highlighted differences in the GR density between SIRS cases and controls as well as survivors and nonsurvivors.

Similarly, fluorescently labeled dexamethasone was successfully used in this study to determine the binding affinity of the GR in equine PBMC's. There was also substantial variation in the binding affinity, ranging from 5.47-56.64% in healthy horses in *Part III* of this study, and from 3.51-23.05% in nonsurvivors with SIRS. However, unlike the GR density, a significant decrease in the binding affinity was detected between survivors and nonsurvivors in this study. This is similar to the findings in the study by Du et al, where steroid resistant human patients with systemic lupus erythematosus had a significantly decreased GR binding affinity when compared to steroid sensitive patients and normal controls [165]. This same technique to determine the binding affinity of the GR in PBMC's was used in a study of periparturient Holstein cows as well [167]. In this study by Preisler et al, the GR binding affinity was

decreased in lymphocytes and monocytes of cows immediately post-calving compared to the 28 days prior to calving, and this decrease persisted up to 14 days post-calving [167]. Thus the decrease in GR binding affinity detected with systemic inflammation in this study coincides with what has been detected in inflammatory states of other species.

The median Delta GR% and Delta BA% obtained in *Part I* of this study were much lower than the measurements obtained in *Parts II and III*. In *Part I*, color compensation was not performed, which decreased the percentage of FITC signal detection. Because of the broad emission spectra of fluorochromes, the light collected by an optical filter of a specific wavelength which reaches a photomultiplier tube detector, consists of signals not only from the fluorochrome of interest, but also signals from other fluorescent dyes. In the case of the FITC and PE combination, as was used in this study, the overlap in their emission spectra causes a higher percentage of FITC signals to be detected by the PE detector. In order to correct this "spillover", a fraction of the FITC fluorescence must be subtracted from the total fluorescence measured by the PE detector, a step referred to as compensation [170]. In order to correct for this in the second and third part of the study, compensation adjustments were performed as described in the materials and methods section.

In the study by Preisler et al, a negative correlation between serum cortisol concentration and the GR binding affinity was detected in post-parturient dairy cows [167]. They hypothesized that the dramatic increase in the plasma cortisol concentration, which occurred during parturition, led to altered gene transcription in the lymphocytes and monocytes, and degradation of the GR during this time period [167]. In *Phase II* of our study, we did not detect a correlation between serum cortisol concentration and GR density or binding affinity. This was similar to what was reported in studies by Molijn et al and Siebig et al, where no correlation between plasma cortisol concentrations and GR density or binding affinity were detected [148, 194]. Additionally, in *Phase III* of the present study, the GR density was not significantly different between groups, but showed a trend towards being increased in the SIRS cases, despite the fact that cases with SIRS had a significantly greater plasma cortisol concentration. Therefore, the degradation of the GR did not appear to be a cause of decreased binding affinity in the cases with SIRS. These studies support the hypothesis that the relationship between plasma cortisol concentration and GR behavior is complex, and most likely influenced by other local factors. In the study by Molijn et al, they demonstrated that incubating PBMC's with IL-2 led to decreased

GR binding affinity [194]. Similarly, a study by Kam et al human PBMC's incubated with IL-2 and IL-4 also demonstrated a decreased GR binding affinity, which was reversed when the cells were then incubated in medium without cytokines present [195]. Thus there appears to be a local effect of inflammatory cytokines on GR binding affinity acting separately from the effects of circulating cortisol. Additionally, studies examining the different GR isoforms have shown an increase in both the GRα and GRβ in inflammatory states and a concomitant decrease in the GRα:GRβ ratio [154, 157-159]. In the study by Webster et al, cells that were incubated with TNFα and IL-1 showed an increase in the GR density of both isoforms, with the β form increasing more than the α [159]. The GRβ isoform has been shown to have a dominant-negative effect on GRα-induced transcriptional activity, thus in the presence of local inflammatory cytokines, an increase in GRβ could explain the decreased binding affinity seen in this study [154].

In *Part II* of this study, exogenous ACTH was administered to healthy horses to produce an increase in their serum cortisol concentration to determine the effects of an increase in circulating endogenous cortisol on GR density and binding affinity. The median basal serum cortisol measured in healthy horses was 4.9, range 3.2-6.1 μg/dl. This sample was collected at 8 am, prior to initiation of the ACTH stimulation test. This was significantly greater than the median serum cortisol at 8 hours after the ACTH stimulation test of 1.4, range 1.1-2.7 μg/dl (P=0.0221), which was collected at 4 pm. Additionally, the 4-hour cortisol concentrations, which were collected at 12 pm, were also significantly greater than the 8-hour concentrations, with a median of 5.6, range 4.8-7.4 μg/dl (P=0.0006). Previous studies of the normal cortisol concentration for adult horses have demonstrated diurnal variations in concentrations of plasma cortisol, with a significantly higher mean concentration observed early in the morning and a gradual decrease to a nadir in the evening [125, 126]. In a study by Hart et al examining the normal basal cortisol production in healthy adult horses, the mean cortisol production observed around 8 am was between 4.5 and 5.5 μg/dl, which decreased to approximately 1.0-2.0 μg/dl by 4 pm, as was observed in this study [125]. Additionally, in a study by Stewart et al examining the effect of various doses of exogenous ACTH on cortisol concentrations, a significant effect on cortisol concentration was observed up to the 4 hour time point after the administration of a 0.5 μg/kg dose of ACTH, as was used in this study [128]. Thus, the decrease in cortisol concentration between the 8 am and 4 pm samples was not surprising. Although the cortisol

concentration of the samples collected 4 hours after exogenous ACTH was administered were not significantly higher than the baseline samples, the median concentration was greater, which was consistent with what was found in the Stewart study following ACTH stimulation of cortisol production. The Delta GR% also showed a delayed trend to increase after the cortisol concentration decreased, with a significantly greater Delta GR% observed at the 24-hour post-ACTH administration sample compared to the 8-hour sample. There have been no previous studies examining the effect of exogenous ACTH on HPA axis dynamics for greater than 4 hours in adult horses. However, in the absence of inflammation, this increase in Delta GR% most likely reflected a delayed feedback response of cortisol and the GR, where increased cortisol leads to degradation of intracellular GR, and a decreased concentration of cortisol might have the opposite effect [167, 195].

ROC analysis was used to determine cut-off points for the HPA axis variables, the serum TG concentration, the Delta GR% and the Delta BA% that were most likely to predict nonsurvival. An ROC curve is a graphical representation of the relationship between the true-positive rate, or sensitivity, and the false-positive rate, or 1 minus the specificity. This curve describes the overall accuracy of detection of a single test. Additionally, the area under this curve integrates the essential measures of sensitivity, specificity, and decision threshold, and can be used to compare the diagnostic utility of numerous tests [196]. As the area under the curve approaches 1, the sensitivity and specificity of that test improve. If the area under the curve is less than 0.5, then the test has no utility in predicting the presence or absence of disease. Therefore, in this study the variables with the greatest area under the curve, and thus were the most robust for predicting nonsurvival, included the serum triglyceride concentration, the Delta BA%, and the plasma ACTH concentration. The area under the curve for the Delta GR% was equal to 0.5, and thus this variable was excluded from further analysis.

Horses with SIRS that did not survive had a significantly decreased Delta BA% compared to survivors. Additionally, nonsurvivors had a trend towards an increase in their ACTH:cortisol ratio compared to survivors. In previous studies in foals, the ACTH:cortisol ratio has been used as a measure of decreased adrenal responsiveness to ACTH, as cortisol concentrations should increase in proportion to ACTH concentrations when the adrenal glands are functioning appropriately. These findings suggest that there is HPA axis dysfunction in horses with SIRS both at the level of the adrenal gland and the GR. The elevated concentration

of ACTH is consistent with an appropriate response to critical illness. The elevated cortisol concentration in horses with SIRS compared to controls is also consistent with an expected response to critical illness. However, a decreased cortisol release in relation to the elevated ACTH concentrations in nonsurvivors suggested inadequate cortisol production and release in the adrenal glands. Extensive adrenal hemorrhage (Waterhouse-Friderichsen Syndrome) has been documented in up to 30% of critically ill humans who did not survive septic shock [71]. This occurs due vascular derangements and ischemia associated with the primary disease, and has also been reported as a common finding on post-mortem examination in horses with endotoxic shock [54, 86, 87]. A decreased GR binding affinity in nonsurvivors with no change in the GR density suggests insensitivity to the cortisol that is present at the level of the GR as well. As described above, this may have been due to a local effect of inflammatory cytokines causing a disruption in the GRα:GRβ ratio.

In *Part III*, it was seen that an elevation in serum triglyceride concentration was associated with nonsurvival, and there was also a trend towards a negative association between the serum triglyceride concentration and the Delta BA%. In several human and animal models, increased concentrations of circulating endotoxin have been found to produce an increase in serum triglyceride concentrations [197-200]. Mechanisms that have been proposed to explain this include an increase in lipoprotein production by the liver in response to an increase in inflammatory cytokine concentration, including TNF-α and IL-1, as well as a decrease in lipoprotein clearance due to a decrease in lipoprotein lipase activity [197-200]. Additionally, this increase in circulating lipoproteins is thought to be at least partially protective, and represents an innate immune response intended to bind and neutralize LPS [201]. Complicating this increase in serum triglycerides due to an inflammatory state is the fact that fasting alone, without taking into account systemic inflammation, can also lead to mobilization of fat stores and increases in the triglyceride concentration as well [202]. Additionally, HPA axis activation can also lead to elevated concentrations of cortisol, which will further propagate energy substrate mobilization, leading to further increases in the triglyceride concentration [203]. All of these factors make it difficult to determine if the elevation in serum TG seen in these patients is directly due to systemic inflammation, HPA axis activation, decreased appetite, or if it is the combination of all of these factors that is required for producing these changes.

Hypertriglyceridemia has been previously documented in horses with SIRS, but has not been evaluated as a biomarker for determining prognosis or disease state in this species [204]. In a human study examining the utility of serum biomarkers in predicting in-hospital mortality, it was found that patients with a higher serum level of triglycerides (>300 mg/dL) had a higher mortality rate compared to patients with low serum levels (37% versus 7%) [205]. Similarly, we found that a TG concentration of greater than 28.5 mg/dl was highly associated with nonsurvival. Although the negative correlation between TG concentration and the Delta BA% was not significant, this may have been related to the small population size examined in this study. Thus, elevated triglycerides are commonly observed in horses with SIRS, and may have a role as a biomarker of HPA axis activation and potentially a poor prognosis for survival.

One limitation of this study was that the health of horses in Part I and Part II of this study was determined from physical examination alone, without any screening CBC, serum biochemistry, or plasma hormone concentrations to determine if there was any evidence of underlying infectious or metabolic disease. However, these horses consisted of horses either from the research herd at the Equine Medical Center, or client owned horses. All had a well documented history of being healthy in the weeks leading up to and after the study, none of the horses had been receiving medication prior to/during the study, and all of the horses were up to date on their vaccination status. Therefore, although subtle underlying illness or metabolic derangements may have been present, which may have altered the results for cortisol concentration and GR density and binding affinity in the "normal horses", these were not significant enough to require medical intervention. Additionally, these horses were not included in the comparison with SIRS cases, so these did not alter our results comparing healthy horses to sick horses, or survivors to nonsurvivors.

For the controls in this study, we used horses presenting to the EMC for lameness evaluation or ophthalmic evaluation. Although these horses were presumed to be clinically healthy, some had evidence of chronic inflammation from their injury or ophthalmic disease process. This may have caused some amount of HPA axis activation in our control cases, which may have affected our analysis. We elected to use these cases for our controls because we wanted to account for the effect of shipping and the stress associated with being in a new environment on our HPA axis analysis, as shipping horses for even short periods has been shown to produce changes in their cortisol concentration [123]. In hind sight, using horses transported

to the EMC for routine farrier work with the on site farrier, and no apparent lameness issues or systemic disease, may have been more appropriate for these control cases.

Another limitation of this study was the sample size. With only 10 cases of SIRS, 6 of which were nonsurvivors, it became more difficult to separate those horses with appropriate HPA axis stimulation from those with relative adrenal insufficiency and or those with peripheral GR resistance. Separating these horses into three distinct groups may have revealed some subtle differences that would be beneficial for determining both prognosis and appropriate treatment, i.e. when it is appropriate to intervene with glucocorticoid therapy. However, with such small numbers, this was not possible in this study. Performing a power analysis to determine how many horses would be required for each group to determine if there are significant differences in these variables would have been beneficial.

Although CIRCI has been more clearly documented in foals prior to this study, we elected to examine this response in adult horses partially due to our expectation that these cases would be more readily available and would help to increase the power of this study. However, not only did fewer horses than we expected meet our selection criteria, but these cases also represented a vast array of pathologies, which introduced a substantial amount of variability into this population that we were unable to control. Therefore, it would have been ideal to examine a more uniform population (i.e. only cases with colitis) to eliminate any variability inherent to the disease process from our results. While the SIRS cases were classified as survivors and nonsurvivors for the analysis of the various HPA axis hormones, GR density and binding affinity, and TG's as diagnostic tools, this classification could have been effected due to perceptions of a poor prognosis by the attending clinician. In such a small group of horses, this may have altered the results for the diagnostic utility of these variables. Therefore, this should be examined in a larger group of horses to determine if the associations found in this study can are applicable. Additionally, it would have been beneficial to perform a post-mortem examination on all of the nonsurvivors to determine if adrenal pathology was present. This would have helped to make a stronger argument for the presence or absence of adrenal dysfunction in these cases.

Conclusions and Future Directions

The commercially available PE-CD44 fluorochrome, mouse anti-glucocorticoid receptor with a secondary FITC-goat anti-mouse immunoglobulin, and FITC-dexamethasone can be used

to evaluate the equine GR on PBMC's. The technique was easy to perform, and took approximately 8 hours per sample. There was a large amount of variability in both the GR density and binding affinity, which may be related to the grouping of lymphocytes and monocytes, and the concentrations of these leukocytes in various states of inflammation. Therefore, in future studies, it may be beneficial to label these individually with CD markers specific for each.

In healthy horses, cortisol concentration was not correlated with either GR density or binding affinity, although there may have been a delayed response of GR density to circulating cortisol. This supports previous work suggesting a complex interaction between cortisol and cellular GR, involving local factors as well, mainly inflammatory cytokines. In further studies, it would be beneficial to attempt to characterize these cytokines to determine if there are ways in which we could pharmacologically manipulate these to increase GR binding affinity in our critically ill patients. As was seen in *Part III* of this study, decreased GR binding affinity was associated with mortality in equine patients with SIRS, so reversing this process could facilitate treatment of these horses. Additionally, it would be beneficial to examine GR activity in foals as well to determine if peripheral resistance to cortisol also occurs in neonates. Current research supports HPA axis dysfunction at the level of the adrenal gland, and has led to the suggestion of supplementing these patients with "physiologic" concentrations of glucocorticoids (i.e. hydrocortisone). However, we do not know if decreased GR binding affinity is present in these foals as well, and how this would affect their response to treatment with hydrocortisone.

Additionally, it would be interesting to further characterize the density of the two GR isoforms produced during the inflammatory response, to determine if the GRβ isoform preferentially increases in the presence of inflammation as has been shown in humans, as well as the way in which this effects the ability of the GRα to bind cortisol and translocate to the nucleus. In human studies, this has been performed through various methods including immunofluorescence staining, immunostaining, western blot, and real-time polymerase chain reaction, which could easily be applied to equine PBMC's.

As was seen in *Part III* of this study, an elevation in the serum TG concentration was associated with nonsurvival, and potentially negatively associated with Delta BA%. Thus, the serum TG concentration may serve as a proxy for evaluating HPA axis dysfunction in addition to predicting a poor prognosis for survival. In human medicine, there are multitudes of studies

looking at biomarkers for diagnosing sepsis and SIRS, and predicting outcome [206-208]. Biomarkers evaluated in these studies are vast and include C-reactive protein, procalcitonin, IL-6, IL-18, metalloproteinases, sE-selectin, and many others. Therefore, further work in larger populations of horses is needed to determine the effectiveness of easily measured variables, such as the serum TG concentration, as proxies for evaluating the HPA axis. Additionally, if the specific cytokines involved in peripheral cortisol resistance in horses are identified, assay of these in affected patients may also aid in diagnosing HPA axis dysfunction as well as predicting survival in these cases.

Footnotes

a. Histopaque®-1077, Sigma-Aldrich, St. Louis, MO.
b. PE-Cy™5 Mouse IgG1 κ Isotype Control, BD Pharmingen™, San Jose, CA.
c. PE anti-mouse/human CD44, BioLegend, San Diego, CA.
d. BD CytoFix/Cytoperm™ Fixation/Permeabilization Solution Kit, BD Biosciences, San Jose, CA.
e. Purified Mouse Anti-Glucocorticoid Receptor, BD Transduction Laboratories™, San Jose, CA.
f. Purified Mouse IgG1 κ Isotype Control, BD Pharmingen™, San Jose, CA.
g. FITC Goat Anti-Mouse Ig, BD Pharmingen™, San Jose, CA.
h. Dexamethasone, Sigma-Aldrich, St. Louis, MO.
i. Dexamethasone Fluorescein, Life Technologies Corporation, Carlsbad, CA.
j. Propidium Iodide Staining Solution, BD Pharmingen™, San Jose, CA.
k. FACSCaliber, BD Biosciences, San Jose, CA.
l. GraphPad Prism Statistical Software, version 6, GraphPad Software Inc, San Diego, CA.
m. Abbocath, 14-gauge 5.5 inch PEP polymer, Medline Industries Inc, Mundelein, IL.
n. Cortrosyn®, Amphstar Pharmaceuticals, Rancho Cucamonga, CA.
o. Immulite, Diagnostic Products Corporation, Los Angeles, CA.
p. Cellgro®, RPMI-1640, Corning™, Manassas, VA.
q. SAS, version , SAS Institute Inc, Cary, NC.
r. AC-T diff™ Analyzer, Beckman Coulter®, Brea, CA.
s. Synermed© IR-500 Chemistry Analyzer, Synermed, Westfield, IN.

Resources

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