LEUKOCYTE PRODUCTION OF REACTIVE OXYGEN SPECIES, AND CIRCULATING CONCENTRATIONS OF HYDROGEN PEROXIDE AND GLUTATHIONE IN HEALTHY HORSES AND HORSES WITH GASTROINTESTINAL DISEASE

By

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(Under the Direction of Stephen J. Lewis, Ph.D.)

ABSTRACT

The first goal of this project was to characterize the inflammatory state of healthy horses. The average endogenous and stimulated production of reactive oxygen species for 80 horses was 3.03 ± 0.26 and 259.20 ± 8.87 average fluorescent units (AFU), respectively. Average plasma levels of hydrogen peroxide for 54 healthy horses were 0.41 ± 0.03 OD, and reduced (GSH) and oxidized (GSSG) glutathione for 41 horses averaged 641.3 ± 53.5 and 13.5 ± 2.5 μM, respectively. The mean GSH/GSSG ratio was 147.1 ± 30.0. An overall stress index was calculated as the product of endogenous reactive oxygen species production and total leukocyte count.

The same parameters were measured in 62 horses with gastrointestinal diseases, and were compared with method of treatment (medical or surgical) and outcome (survived or died). The results obtained suggest that GSH/GSSG ratio, plasma level of hydrogen peroxide and the overall stress index may help distinguish horses requiring medical or surgical treatment, and provide information regarding prognosis.

Key Words: Reactive oxygen species, neutrophils, horse, oxidant, antioxidant, gastrointestinal disease

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DEDICATION

I would like to dedicate this thesis to my family. I am indebted to my mother and brother for their unwavering support and encouragement. I would like to thank my parents for showing me that hard work pays off and that perseverance is underrated. I am truly grateful to my family for always being there to give me advice and to be there when I just needed a break. I couldn't have done this without you, thank you.

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TABLE OF CONTENTS

Page

CHAPTER 1

LITERATURE REVIEW

Evidence of the Impact of Inflammatory State on the Development of Laminitis after Administration of Black Walnut Heartwood Extract

Laminitis is an incapacitating disease in horses that occurs secondary to diseases affecting the gastrointestinal tract or musculoskeletal system.[1-4] In laminitis the integrity of the digital laminae is lost, leading to disruption of the normal interdigitation between the distal phalanx and hoof wall.[3-5] Although the causes of laminitis are unclear, some of the pathophysiological changes in the laminar microvasculature have been identified.[3-7] There are currently two models used for the induction of laminitis in horses. The first is via carbohydrate overload of the gastrointestinal tract, and the second is by intragastric administration of black walnut heartwood extract. Induction of laminitis by carbohydrate overload is less consistent than by administration of black walnut heartwood extract.[7,8] Moreover, the changes seen in the laminar microvasculature during naturally occurring laminitis are also seen in black walnut heartwood extract induced laminitis.[5,7,8] The mechanism by which black walnut heartwood extract causes laminitis is unknown, as is the case for the naturally occurring disease.

In recent studies at the University of Georgia, the striking observation was made that horses that were induced with black walnut extract were statistically more likely to develop signs of Obel grade 1 laminitis if they had elevated endogenous and inducible

production of reactive oxygen species (ROS)[2]. It was also, determined that the number of white blood cells recovered from whole blood samples from horses administered black walnut heartwood extract who developed signs of laminitis were significantly lower than the number of white blood cells recovered from either control horses or from horses that did not develop laminitis over the first six hours after administration of the black walnut heartwood extract.[2] Furthermore, leukocyte production of ROS before administration of the black walnut extract was significantly greater in horses that developed laminitis when compared to horses that did not develop signs of laminitis.[2] Finally, leukocytes from the horses that developed signs of laminitis had a decreased capacity to produce ROS, starting 2 hours after black walnut administration, when compared to leukocytes from horses that did not develop signs of laminitis. Due to these findings we decided to measure ROS as an indication of the inflammatory state of horses, establish the normal inflammatory range of healthy horses and attempt to use the inflammatory state as a predictor of the severity of clinical disease in horses.

Preliminary experiments were then performed to determine whether it would be possible to rapidly and accurately obtain leukocytes for this purpose. In these initial experiments, two methods were compared to the well established method involving the collection of buffy coat cells. The collection of a leukocyte rich plasma as a source of leukocytes was proven to be superior and yielded results that were indistinguishable from the values obtained with buffy coat cells.

Inflammation and Reactive Oxygen Species

Inflammation is a complex reaction of the innate immune system that is designed to control infections and promote tissue repair[9]. The first phase of inflammation is characterized by vasodilation, which promotes leukocyte recruitment and leads to the second phase of inflammation, which is characterized by the infiltration of neutrophils.[10] Neutrophils routinely make up 60% of the circulating leukocytes in the horse, and can often represent up to 85% of the circulating leukocytes in this species. At the site of infection, cell injury, exposure to toxins or foreign bodies, neutrophils adhere to the endothelial cells and become activated.[9,11] The activated leukocytes release a variety of defensive molecules, such as ROS, that are designed to kill invaders. Because activated neutrophils release ROS at the site of inflammation, measurement of ROS can be used as an indication of the inflammatory state of the animal. Results obtained in other studies have also suggested that production of ROS by activated neutrophils can be used as a measure of an individual's inflammatory state.[12,13]

ROS are oxygen derivatives that are unstable due to the presence of one or more unpaired electrons. ROS include superoxide anion, hydrogen peroxide (H_2O_2), the hydroxyl radical, and peroxyl radicals to name a few.[14] The major sources of ROS are from the mitochondria of activated neutrophils at the site of inflammation.[15-17] Formation of the superoxide anion is mediated enzymatically by NADPH oxidase and xanthine oxidase, and non-enzymatically by components in the mitochondrial electron transport chain.[18] The other ROS are formed from the superoxide anion. In one such reaction,

superoxide dismutase transforms superoxide into H_2O_2. In platelets, superoxide formation occurs via the xanthine oxidase system.[19] Some non-enzymatic reactions resulting in the generation of reactive oxygen species include electron leakage, exposure to pollutants and activation of oxygen by irradiation.[16,20,21]

The NADPH oxidase system, which consists of cytochrome b558 subunits, cytosolic proteins, a quinone, and a GTP-binding protein, is an electron transport chain that is present in endocytic vacuole walls of neutrophils.[22] In resting neutrophils the NADPH oxidase system is dissociated and inactive, with some of its components being membrane bound and others stored in the cytosol.[23] When the neutrophils become activated, protein kinase C phosphorylates one of the cytosolic components, $p47^{PHOX}$, which causes translocation of the cytosolic components of the NADPH oxidase system. These cytosolic components assemble with the membrane-bound components, activate the enzyme system and initiate a respiratory burst characterized by an influx of oxygen.[24,25] This electron transport system then uses the NADPH reducing equivalents it generates to reduce the oxygen generated by the respiratory burst to form superoxide anions, which are then converted to other reactive oxygen species such as H_2O_2 by the activity of superoxide dismutase.[26-29]

The phorbol ester, phorbol myristate acetate (PMA), bypasses cellular receptor systems and directly binds protein kinase C, resulting in the phosphorylation of $p47^{PHOX}$ and production of ROS by neutrophils.[28,30,31] Therefore, stimulation of ROS production by PMA provides information about the capacity of an animal's neutrophils to produce ROS, or mount an inflammatory response when needed.

Inflammation in equine gastrointestinal diseases

Colic is a general term meaning abdominal pain, which is caused by many gastrointestinal conditions in horses, and can affect horses of all ages, genders, and breeds. In 1998 the incidence of colic was reported to be 4.2 events per 100 horses per year.[32] Colic is a major problem in terms of determination of medical or surgical treatment, loss of use, cost of treatment and morbidity and mortality rates.[33-36] Predisposing factors to colic have been attributed to many diverse situations from deworming to changes in diet, and even to changes in the weather.[37] In this study, we looked at gastrointestinal diseases that were categorized into 1 of 4 categories. These disease categories included non-strangulating large intestinal conditions, non-strangulating small intestinal conditions, strangulating small intestinal conditions, and inflammatory gastrointestinal conditions.[38]

Many gastrointestinal diseases in horses have been found to be characterized by transmural movement of bacterial endotoxins from the intestinal lumen into the bloodstream, and initiation of a systemic inflammatory response.[38-40] The end results of this systemic response include complications such as thrombophlebitis, intra-abdominal adhesions, and acute laminitis.[41-44] The results of recent studies suggest that activation of peripheral blood leukocytes plays an important role in development of these complications. Activation of neutrophils have been observed in large and small strangulating lesions[45,46] and in inflammatory gastrointestinal conditions.[40]

Measurement of reactive oxygen species

Production of ROS can be measured using two dyes, namely 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and dihydrorhodamine 123 (DHR-123). Both dyes are oxidized by hydroxyl radicals, peroxynitrites, and H_2O_2,[47,48] and DHR-123 is also oxidized by superoxide anions.[49] These dyes donate an electron to the ROS, which in turn causes the dyes to be converted to their fluorescent state. Fluorescence of the dyes is quantified, thereby providing a measurement of the amount of ROS produced. DCF-DA is a negatively charged dye and is oxidized and accumulates in the cytosol, whereas DHR-123 is a neutral dye and can accumulate either in the cytosol or in the mitochondria. However, the fluorescent product of DHR-123, rhodamine-123 (RHO-123) is positively charged and usually accumulates in the mitochondrial compartments.[49] DHR-123 is more efficiently trapped intracellularly than is DCF-DA,[48] and often is the dye of choice in studies of reactive oxygen species production by isolated cells.

Measurement of H_2O_2 in plasma

The mitochondria of activated neutrophils are the primary producers of ROS in cells and tissues.[15-17] Of the ROS produced, H_2O_2 is not only the most stable, but also the most abundant.[15,50] H_2O_2 is produced as the result of the enzymatic action of superoxide dismutase on superoxide anions arising from the respiratory burst and the action of the NADPH oxidase enzyme system.[20,51-54] The production of ROS is usually measured with a fluorescent dye that reacts with H_2O_2.[55]

Due to the aforementioned reasons, the endogenous level of H_2O_2 was measured in horses included in this study. The levels of H_2O_2 were first measured by the

fluorescence of scopoletin, which is still used today.[15] This method has been criticized for its sensitivity and interference by NADPH fluorescence. Another method commonly used to measure hydrogen peroxide is that of DCFDA. Problems, as mentioned earlier, with this measurement include leakage and interference with cytochrome C and other proteins.[15] In this study the level of H_2O_2 was determined by monitoring the change in color produced from the reaction of H_2O_2 and 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS), which was catalyzed by the horseradish peroxidase enzyme. This reaction is widely exploited in the design of ELISA assays.

Measurement of GSH and GSSG in blood

As with virtually every physiologic system, the oxidants are balanced by an antioxidant system. These antioxidants ensure that ROS serve their purpose of defense, but are then neutralized before they can cause significant tissue damage and destruction. Antioxidants neutralize ROS by either accepting or donating an electron, thereby rendering the ROS more stable.

Glutathione is one of the most important non-enzymatic antioxidants.[56,57] Glutathione's reduced form (GSH) neutralizes H_2O_2 and hydroxyl radicals, and its oxidized form (GSSG) neutralizes other unstable glutathione molecules. GSH is a tripeptide of glutamate, cysteine, and glycine, and is a primary reducing agent of ROS.[58,59] Whether GSH directly neutralizes ROS or catalytically reduces ROS through

glutathione peroxidase, the end result is the conversion of GSH to GSSG.[59] The cysteine residues of two GSH molecules form a disulfide bond during the course of being oxidized, thereby forming GSSG. GSSG is catalytically recycled back to GSH by NADPH through glutathione reductase.[59] Measurements of circulating concentrations of GSH and GSSG, and calculation of the ratio of GSH to GSSG have been used to provide information about the antioxidant status of people. In most studies, clinically diseased patients have increased circulating concentrations of GSSG, decreased concentrations of GSH, and reduced GSH/GSSG ratios.[25,60-68]

Blood concentrations of GSH and GSSG can be determined utilizing an assay based on oxidation of GSH by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduction of GSSG by NADPH catalyzed by glutathione reductase as described by Tietze.[69,70] This assay provides determination of the reduced and oxidized forms of glutathione, and provides information about concentrations of these antioxidants in blood rather than plasma; a much greater percentage of glutathione is present in whole blood than in plasma samples.[18,59] This assay also utilizes the thiol scavenger, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate, which does not underestimate or overestimate GSSG as has occurred with the use of other thiol scavenging agents.[69]

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CHAPTER 2

CIRCULATING CONCENTRATIONS OF HYDROGEN PEROXIDE AND GLUTATHIONE, AND PRODUCTION OF OXYGEN RADICALS BY BLOOD LEUKOCYTES FROM HEALTHY HORSES[1]

[1] Goan et al. To be submitted to Journal of Equine Veterinary Sciences

ABSTRACT

Measurements of inflammatory activation (plasma hydrogen peroxide [H_2O_2] concentration, endogenous and phorbol ester inducible production of reactive oxygen species [ROS] by peripheral blood leukocytes) and antioxidant status (measured as reduced and oxidized glutathione, and their ratio) were conducted on blood samples collected from 96 clinically normal horses. H_2O_2 was measured using a colormetric assay containing horseradish peroxidase enzyme and 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS). The average plasma level of H_2O_2 measured for a subpopulation of 54 horses was 0.41 ± 0.03 OD (All values represented as mean ± SEM). Endogenous ROS was measured by incubating leukocytes with dihydrorhodamine 123 (DHR-123) in culture for 1 hour, and inducible ROS measured by adding 10^{-7}M phorbol myristate acetate (PMA) to leukocytes in separate wells. The level of ROS was determined by measuring the conversion of DHR-123 to its fluorescent form, rhodamine-123. The average endogenous and inducible production of ROS by leukocytes from the subpopulation of 80 horses were 3.0 ± 0.3 and 259 ± 9 arbitrary fluorescent units (AFU), respectively. Both the H_2O_2 and ROS measurements clustered tightly around the mean. Glutathione was measured using a micro-volume modification of a commercial kit with separate measurements of the reduced (GSH) and oxidized (GSSG) forms. The mean blood concentrations of GSH and GSSG for a subset of 41 horses were 641 ± 54 and 14 ± 3 μM, respectively. The mean GSH/GSSG ratio for these horses was 147 ± 30. The glutathione values were broadly distributed with no clustering around the mean. In addition, seasonal variations in inducible ROS, plasma H_2O_2, GSH, GSSG, and the GSH/GSSG ratio were observed, with samples collected in the spring having the greatest

level of inflammatory activation and summer having the greatest level of antioxidant activity. This study appears to provide an additional set of tools and a set of normal baseline data from a large set of horses that will allow for further dissection of the role of inflammation in disease processes.

INTRODUCTION

Inflammatory responses in horses are dominated by neutrophils, as these cells comprise 60 - 85% of circulating leukocytes in this species. As neutrophils are recruited to sites of inflammation, they become activated, and release a variety of defensive molecules, such as reactive oxygen species (ROS), designed to kill invaders. ROS, which are unstable oxygen-derived radicals having one or more unpaired electrons, include hydrogen peroxide (H_2O_2), superoxide anion, hydroxyl radical, and peroxyl radicals.[1,2] These ROS are neutralized by endogenous antioxidants, thereby minimizing tissue damage. Antioxidants neutralize ROS by accepting or donating an electron, depending on the particular ROS.

Glutathione is an antioxidant whose reduced form (GSH) neutralizes H_2O_2 and hydroxyl radicals, and whose oxidized form (GSSG) neutralizes other unstable glutathione molecules. GSH is a tripeptide of glutamate, cysteine, and glycine. The cysteine residues of two GSH molecules form a disulfide bond during the course of being oxidized, thereby forming GSSG. GSSG is recycled back to GSH by NADPH; this reaction is catalyzed by glutathione reductase.[3]

This study had three primary objectives; namely, to assess the endogenous and inducible capacity of neutrophils from healthy horses to produce ROS, to estimate circulating concentrations of H_2O_2 as measures of current, future potential, and immediate past oxygen radical production, respectively. In addition, we measured the concentrations of GSH and GSSG in whole blood, and calculated a simple ratio of GSH to GSSG as an index of the horse's antioxidant status. The overall goal of this study was to establish the normal ranges for these parameters in healthy horses as indicators of the

variability of the inflammatory state among horses that are defined as healthy on the basis of clinical signs. A clear understanding of the typical inflammatory state of healthy horses will allow for a better understanding of measurements of the magnitude and duration of inflammation over the course of disease. Often measurements of the inflammatory state of a horse are not made until the horse has developed clear signs and symptoms of disease, so it is difficult to directly assess changes from the animal's healthy state. Thus, a definition of the typical state and the range of inflammatory activity seen in healthy horses are important first-steps in the development of new diagnostic assays to detect inflammatory conditions in horses or to monitor the effects of nutritional and management methods designed to reduce inflammation in the horse.

MATERIALS AND METHODS

Horses in the Study

Ninety-six horses determined to be healthy based on their recent histories were used in this study; blood samples from 27 of these horses were obtained on more than one occasion (detailed below). The population of horses included horses on the University of Georgia Equestrian Team, horses from the Department of Animal and Dairy Science's teaching herd, horses used as standing blood donors in the Veterinary Medical Teaching Hospital, horses owned by personnel working in the laboratory, and one utilized in another research project. There were 19 Thoroughbreds, 47 Quarter Horses and Paints, 23 Warmbloods, and 7 Arabians. Seventy of the horses sampled were geldings, 23 were mares, and 3 were stallions. The ages of the horses utilized ranged from 1 to 26 years, with an average age of approximately 10 years.

Preparation of Leukocytes

In an effort to generate baseline data that would be useful in the development of new methods to assess the role of inflammation in diseases, we were interested in finding a simple and easy way to isolate leukocytes for our assessments. Thus, our first task was to determine whether we could use leukocyte-rich plasma (LRP) as a source of plasma and cells, or if it was necessary to use a buffy coat preparation as the source of cells and plasma. We conducted a preliminary experiment comparing these two preparatory methods using blood from 10 horses owned by the University of Georgia.

To prepare LRP, a modification of the method reported recently by Okano et al was used.[4] 14mL of whole blood was collected into a vacuum-evacuated tube containing EDTA. The tube was mixed by inverting at the time of collection. The blood was transported to the laboratory and the tube again mixed by inverting several times. A 12 mL portion of the blood was transferred to a 12mL syringe. The syringe was placed in a standing position on the plunger in a biocontainment hood, and the erythrocytes were allowed to settle by gravity for 30 minutes. The LRP was transferred to a 15mL centrifuge tube by gently expressing the plasma and cells above the erythrocyte boundary from the syringe through a needle bent to 135 degrees. The LRP was then centrifuged at approximately 1300 x g for 10 min, after which the plasma was collected. The leukocytes in the pellet were washed with 10mL of Dulbecco's phosphate buffered saline without Ca^{+2} or Mg^{+2} (PBS, Roche, Indianapolis, USA), and centrifuged at 1300 x g for 10 minutes. The supernatant was decanted and the cells suspended in 5mL of PBS; leukocytes in an aliquot were counted using a hemocytometer, the number of viable cells recovered was calculated, and the cells were diluted to 5.0×10^6/ mL in PBS.

To process buffy coat preparations, 60mL of blood was collected into 60mL syringes containing 1.5mL of ethylenediamine tetraacetic acid (EDTA, Sigma, St. Louis, USA). The blood was then centrifuged at 1400 x g for 20 minutes, after which the plasma was transferred to a sterile tube. The buffy coat layer containing relatively few red blood cells was collected and transferred to a 50mL centrifuge tube, diluted with PBS, and centrifuged at 800 x g for 10 minutes. The supernatant was removed, 10mL of sterile double-distilled water added to the pellet, the sample was vortexed for 30 seconds, and then tonicity restored by the addition of 10mL of sterile 1.8% saline, with vortexing for 10 seconds. PBS was added to bring the total volume to 50mL, and the tubes were centrifuged at 800 x g for 10 minutes. The supernatant was removed, and the cells washed with PBS a second time. The cells were then suspended in 50mL of PBS and centrifuged at 800 x g for 5 minutes and the supernatant was removed.[5] The cells were counted using a hemocytometer, the number of viable cells recovered was calculated, and the cells were diluted to 5.0 x 10^6/ mL in Dulbecco's phosphate buffered saline containing 5mM glucose (Sigma, St. Louis, USA) and 0.5% sterile tissue culture grade bovine serum albumin (PBG, Sigma)

ROS Production by Leukocytes

Leukocytes obtained using these two methods were plated in 96-well flat bottom plates, with 3 replicates for each treatment. Production of ROS was measured by the conversion of colorless dihydrorhodamine-123 (DHR-123, Molecular Probes, Eugene, USA) to fluorescent rhodamine-123 (RHO-123). DHR-123 enters the cell as a freely permeable dye and is oxidized to RHO-123 upon accepting an electron. RHO-123 is

excitable at 488 nm and emits at 515nm.[6] Phorbol myristate acetate (PMA, Sigma) was used at concentrations from 10^{-9}M to 10^{-6}M to activate the enzymes that produce ROS in the cells.[5]

Each plate included the following samples: medium only (background), medium plus DHR-123 (dye control), cells plus DHR-123 (endogenous ROS production), and cells plus PMA and DHR-123 (capacity to produce ROS). An incubation of 1 h at 37˚C was allowed for accumulation of the fluorescent signal. The plates were read using a fluorescent plate reader with excitation and emission filters of 485nm and 538nm, respectively (Fluoroskan Ascent FL, ThermoLabsystems, Helsinki, Finland). The ROS data were collected in arbitrary fluorescent units (AFU) using Ascent software, version 2.6. Data obtained using the two methods were indistinguishable. Consequently, the more rapid LRP method was used to collect leukocytes for the rest of the study, and the same controls and treatments were used. To determine the variability of endogenous ROS production within individual horses, 27 horses were sampled at least twice.

Plasma Concentration of H_2O_2

For the measurement of H_2O_2 in circulation, the plasma recovered after centrifugation of the LRP was immediately transferred to another 15mL centrifuge tube and aliquoted into a 96-well flat bottom plate in sets of 3 replicates for each treatment. The concentration of H_2O_2 in the sample was assessed by measuring the change in color of the substrate, 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS, Sigma). The reaction between the H_2O_2 and ABTS was catalyzed by the horseradish peroxidase

enzyme (HRP, Sigma), which was added in excess to the samples, and optical density OD) was measured.

The concentrations of H_2O_2 in the 100ul samples of plasma from each horse were estimated by adding an equivalent amount of ABTS used in the ELISA and a quantity of HRP equivalent to the total amount of labeled antibody used in the ELISA. This allowed detection of the quantity of H_2O_2 in the plasma at the time of collection. To obtain an estimate of the relative quantity of H_2O_2 present, 0.003%, 0.03% and 0,3% H_2O_2 (Fisher, Pittsburg, USA) were added to separate samples of the plasma to generate representative OD values. All samples were set up in triplicate in 96 well flat bottom plates. The plates were incubated at 37˚C and read using an optical density plate reader with a 405nm filter (Multiskan Ascent, TheromLabsystems). The H_2O_2 data were collected in OD units using Ascent software, version 2.6. To determine the variability of plasma concentrations of H_2O_2 within individual horses, 13 horses were sampled at least twice. The addition of H_2O_2 to the samples was used to test the capacity of the assay to measure the amount of H_2O_2 in the plasma sample. If the OD values for plasma, HRP and ABTS and those with added H_2O_2 were equivalent, then the assay conditions either were saturated or the reagents were not working properly. Only samples having concentration dependent increases in ABTS conversion with the addition of exogenous H_2O_2 were included in the analysis of the data.

Blood Concentrations of GSH and GSSG

Blood concentrations of GSH and GSSG were determined utilizing a commercial kit (Oxis International Bioxytech GSH/GSSG-412 kit) based on 5,5'-dithiobis-(2-

nitrobenzoic acid) oxidation of GSH and reduction of GSSG by NADPH catalyzed by glutathione reductase, an enzymatic recycling assay first introduced by Tietze.[7] This procedure was slightly modified to result in colorimetric determination of reduced and oxidized glutathione and also allowed for use in microtiter plates in our laboratory, and the conditions were optimized using the standards provided with the kit

To determine concentrations of GSH, 50µL of whole blood was aliquoted into 500µL centrifuge tubes and frozen at -80˚C to lyse the erythrocytes. To determine concentrations of GSSG, 100µL of whole blood and 10µL of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) were aliquoted into 500µL centrifuge tubes and frozen at -80˚C to lyse the erythrocytes and maximize the concentration of GSSG.

Samples used to determine GSH concentrations were thawed, vortexed, and 350µL of cold 5% metaphosphoric acid (MPA) was added to the microcentrifuge tubes. The tubes were then vortexed for 15-20 seconds, centrifuged at approximately 1000 x g for 10 minutes, and 50µL of the MPA extract was added to 3mL of cold assay buffer. All samples were maintained on ice until assayed.

Samples used to determine GSSG concentrations were thawed, vortexed, and incubated at room temperature. After approximately 5 minutes, 290µL of cold 5% MPA was added to the tubes and the samples vortexed for 15-20 seconds. The samples were then centrifuged at approximately 1000 x g for 10 minutes, after which 50µL of the MPA extract was added to 700µL of cold GSSG assay buffer on ice. The blank for the GSSG assay was prepared by adding 50µL MPA to 700µL GSSG assay buffer. All samples were maintained on ice until assayed.

Six GSH standards (0 – 0.5μM) and the GSSG blank were used to construct calibration curves for the GSH and GSSG assays. 40μL of standards, blank, or samples were aliquoted into 96-well flat bottom plates. After addition of an equal volume of the chromogenic substrate and enzyme, and a 5-minute incubation at room temperature, 40μL of NADPH was added and the optical density read at 405nm for 3 minutes using an optical density plate reader (Multiskan Ascent, Labsystems) and Ascent software, version 2.6. To determine the variability of blood concentrations of GSH and GSSG within individual horses, 6 horses were sampled at least twice.

A simple ratio of the concentration of GSSG to GSH was calculated based on the measurement of each at each sampling: [GSH]/[GSSG] = ratio. This ratio was used as an indicator of the relative concentrations of glutathione at each redox state. This calculated ratio differed from that recommended by the manufacturers of the kit, but it provided us with a consistent indicator of the relationship between GSH and GSSG in each horse.

Data Analysis

All data were transferred to an Excel spreadsheet, where the means and standard error of the mean were calculated for each parameter. Any ROS experiments for which spontaneous conversion of DHR-123 to RHO-123 exceeded 20 AFU were excluded from the data as being outside our quality range. Mean values for number of white blood cells recovered, endogenous and induced production of ROS, plasma concentration of H_2O_2, and blood concentrations of GSH, GSSG, and the GSH/GSSG ratio were compiled by parameter and separately tested for normality. Subsequent analyses included ANOVA, multiple means comparisons, or T-tests, where appropriate. All analyses were performed

using Prism (Version 4, GraphPad Software, Inc San Diego, CA) with significance defined as $P < 0.05$.

RESULTS

Recovery of White Blood Cells

The average number of white blood cells (WBC) recovered per milliliter of whole blood for all horses in this study was 1.30×10^7 cells/mL $\pm$ 0.03×10^7 cells/mL. There were no significant differences in the number of WBC recovered based on breed, age group, gender, or the season of the year in which the samples were collected.

Endogenous ROS Production

The average production of ROS by WBC from the 80 horses in the dataset was 3.03 ± 0.26 AFU. There were no significant differences in endogenous ROS production based on breed, age group, gender, or the season in which the samples were collected.

Stimulated ROS Production

The average value for PMA-stimulated production of ROS by WBC from the 80 horses in the dataset was 259.20 ± 8.87 AFU at a PMA concentration of 10^{-7}M; the other concentrations of PMA tested caused less production of ROS in all horses. There were no significant differences in the level of ROS production stimulated by PMA among the animals based on breed, age group, or gender. However, there was a significant effect of the season of the year in which the samples were collected on ROS production. These differences are summarized in Table 1.

Plasma Level of H_2O_2

The average plasma level of H_2O_2 measured for the 54 horses in the dataset was 0.41 ± 0.03 OD. Seasonal differences are summarized in Table 1. There were no significant differences measured based on gender or age group.

Blood Concentrations of GSH and GSSG, and the Ratio of GSH to GSSG

The mean blood concentration of GSH of the 41 horses included in the dataset was 641.3 ± 53.5 μM. The range of these measurements was very broad (180.8 to 1517.1), however the data were normally distributed. Significant seasonal differences in blood concentrations of GSH are summarized in Table 1. There were no significant differences identified among the age groups, breeds, or genders.

The mean blood concentration of GSSG of the 41 horses in the dataset was 13.5 ± 2.5 μM. Significant differences in blood GSSG concentrations due to season of sample collection are summarized in Table 1.

The mean GSH/GSSG ratio for the 41 horses in the dataset was 147.1 ± 30.0. The range of these measurements was very broad (4.4 – 813.1), however, only 2 of the 41 values exceeded 500. The data were not normally distributed and there were no significant differences between values for different breeds, age groups, or genders. Significant differences in the GSH/GSSG ratio due to season of sample collection are summarized in Table 1.

DISCUSSION

The first objective of this study was to measure endogenous production of ROS by leukocytes from healthy adult horses. Our primary goal was to obtain a 'snapshot' of each horse's inflammatory state and to determine a range of values for each assessment we made within this population of 80 horses. While there are other well-established markers of inflammation in the horse, including fibrinogen, C-reactive protein, and serum amyloid-A,[8] changes in concentrations of these markers occur over days and require special reagents not available in most clinical pathology laboratories. In contrast, measurement of endogenous ROS production requires only a fluorescent microtiter plate reader and an inexpensive fluorescent substrate. In order to provide the basis for development of an assay that could be used in most clinical laboratories, one of our first aims was to make the sample processing as simple and rapid as possible. Therefore, we compared a simple procedure for obtaining leukocytes from LRP to a more laborious method using buffy coat preparations. When we determined that values for ROS production were indistinguishable between cells prepared using the two methods, all subsequent assays were performed using LRP as the source of leukocytes. Although many factors can affect an individual's inflammatory state, we were impressed with the finding that endogenous production of ROS by leukocytes from healthy horses falls within a fairly tight range and that there was no effect of the season in which the samples were collected. This finding suggests that production of ROS by leukocytes is a well-controlled process under resting conditions.

Another objective of the study was to determine the capacity of leukocytes from healthy horses to produce ROS. We utilized stimulation with PMA, a strong activator of

PKC activity, to drive production of ROS. Maximal production of ROS by equine leukocytes is generally observed at a concentration of 10^{-7}M in horses.[5] In these experiments, incubation of the leukocytes with that concentration of PMA increased ROS production by 50-fold or greater over endogenous levels of production. Again, we observed the ability of leukocytes from healthy horses to produce ROS after stimulation was similar for the 80 healthy horses included in this study. These findings suggest that there was minimal stimulation of the cells during the isolation procedure, thereby facilitating detection of the effect of enzyme stimulation.

An intriguing finding in this study was the large increase in the capacity of leukocytes collected in the spring to produce ROS (Table 1). While the underlying cause for this change remains to be determined, it is interesting to speculate that changes in exposure to environmental factors, such as inhaled pollens, as well as dietary differences, changes in day length, and different exercise and management protocols between horses may be responsible. Although the number of leukocytes in each well was constant throughout the present study, the number of neutrophils was not determined. Thus, it is possible that the number of neutrophils included in each well may have been higher in the spring months. The capacity for production of ROS is controlled at the level of the NADPH oxidase system. Thus, it may be that neutrophils released into circulation in the spring have a higher content of the enzymes responsible for ROS production. This hypothesis could be tested by monitoring cellular expression of these enzymes over the year to determine if there is a relationship with the pronounced increase in the capacity to produce ROS identified in the present study.

The H_2O_2 assay is a simple and rapid assay based on colorimetric determination of the level of H_2O_2 in a plasma sample. This assay utilizes commercially available components that are common to many ELISAs and can be performed using standard laboratory equipment. We chose to utilize an excess of HRP enzyme and ABTS substrate in the assay and allow H_2O_2 to be the limiting reagent. This assay has the potential to be developed as a stall side diagnostic assay, although there will need to be some improvements made in sensitivity. For example, we have observed some inconsistencies when 0.3% and 0.03% H_2O_2 were added to plasma samples from a subpopulation of the horses tested in this study. The inconsistencies observed in the 0.3% and 0.03% H_2O_2 controls could reflect the endogenous level of H_2O_2. We recognize that this assay is still relatively untested, however, we believe that it shows the basis for a useful clinical assessment.

While the results of the endogenous and stimulated ROS assays provided snapshots of the horse's current and likely future inflammatory states, respectively, and the H_2O_2 assay revealed the immediate past history of the horse's inflammatory activity, we wanted to be able to generate a broader picture of the horse's inflammatory response. To do so, we felt it necessary to assess the horse's antioxidant status. Therefore, the third objective of this study was to measure blood concentrations of GSH and GSSG. The measurement of GSH shows the animal's level of antioxidant available to reduce ROS, whereas the amount of GSSG present is an indication of the amount of ROS that have been reduced. Therefore, the ratio shows the animal's current antioxidant status. Using the same assay system, de Moffarts *et al* (2004) reported GSH levels of 958 ± 32μM and GSSG levels of 23.2 ± 5.8 μM for the 6 horses measured in their study.[9] Differences in

GSH and GSSG levels between those reported by de Moffarts *et al* (2004) and this study may be due to the effects of the defined exercise protocol used in their study and the lack of a defined level of exercise for the horses in our study, the common and defined diet in their study but the use of many different diets used in our study, and the modifications we made to the commercial assay system for use in microtiter plates.

At the outset of this study, we strived to develop rapid and accurate laboratory assays that could be used to assess the oxidant and antioxidant status of individual horses. In completing this work, we have established normal ranges for the assays assessing the oxidant status of individual horses, measured as endogenous and induced cellular production of ROS and plasma H_2O_2 values obtained from healthy horses were tightly clustered around the mean. In contrast, the ranges of values obtained for GSH and GSSG, and for the calculated GSH/GSSG ratio were broadly distributed. These findings suggest that the oxidant producing systems in healthy horses are more tightly controlled than the antioxidant system, and as such may provide more accessible information regarding regulation of inflammation in horses. Thus, these assessments may provide the tools needed to develop new clinical assays to help understand and predict the role of inflammation in equine disease.

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Table 1. Effects of season of sample collection on measured parameters.

Parameter	Winter	Spring	Summer	Fall
End ROS (AFU)	2.9 ± 0.7^{a}	2.2 ± 0.6^{a}	3.0 ± 0.4^{a}	4.2 ± 0.5^{a}
Stim ROS* (AFU)	$223 \pm 17^{a,c,d}$	324 ± 16^{b}	$263 \pm 12^{a,c}$	$207 \pm 15^{a,d}$
H_2O_2 (OD)	$0.3 \pm 0.2^{a,b}$	$0.1 \pm 0.0^{a,b}$	0.5 ± 0.0^{a}	0.3 ± 0.1^{b}
GSH (μM)	582 ± 82^{a}	ND	497.2 ± 52^{a}	1025.0 ± 110^{b}
GSSG (μM)	7 ± 1^{a}	ND	23 ± 5^{b}	7.7 ± 2.9^{a}
GSH/GSSG	$156 \pm 54^{a,b}$	ND	47 ± 15^{a}	230 ± 59^{b}

The data are mean ± SEM. *End* = endogenous. *Stim* = stimulated. ND = Not done, * PMA at 10^{-7}M used to stimulate the response. All values within each row (for the respective parameter) with a different letter indicate significant differences for the corresponding season of sample collection

CHAPTER 3

BLOOD CONCENTRATIONS OF HYDROGEN PEROXIDE AND GLUTATHIONE, AND PRODUCTION OF OXYGEN RADICALS BY BLOOD LEUKOCYTES FROM HORSES WITH GASTROINTESTINAL DISEASES[2]

[2] Goan et al. To be submitted to American Journal of Veterinary Research

ABSTRACT

Blood samples from 62 horses admitted to The University of Georgia Large Animal Veterinary Teaching Hospital with signs of gastrointestinal disease were assessed for indicators of oxidant (endogenous and PMA-stimulated production of reactive oxygen species [ROS] and plasma hydrogen peroxide [H_2O_2] concentration) and antioxidant status (reduced [GSH] and oxidized [GSSG] glutathione, and their ratio [GSH/GSSG]). Other assessments included quantification of total circulating leukocytes, segmented and band neutrophils, and calculation of an overall stress index based on the product of endogenous ROS production and circulating leukocyte count. Based on data in medical records, the horses were assigned to one of four gastrointestinal disease categories, non-strangulating large intestinal conditions, non-strangulating small intestinal conditions, strangulating small intestinal conditions, or inflammatory intestinal conditions, and by method of treatment (medical or surgical) and outcome (survived or died) were recorded. The results obtained suggest that the values obtained for GSH/GSSG ratios, plasma levels of hydrogen peroxide and the overall stress index may be valuable adjuncts to current diagnostic methods used to distinguish between horses requiring medical or surgical treatment, and provide additional information regarding prognosis for survival.

INTRODUCTION

Colic is a general term for the presence of abdominal pain in horses with gastrointestinal diseases. Many of these diseases are characterized by transmural movement of bacterial endotoxins from the intestinal lumen into the bloodstream, and initiation of a systemic inflammatory response. The end results of this systemic response include complications such as thrombophlebitis, intra-abdominal adhesions, and acute laminitis. The results of recent studies suggest that activation of peripheral blood leukocytes plays an important role in development of these complications. Furthermore, results obtained in other species suggest that production of reactive oxygen species (ROS) by activated neutrophils can be used as a measure of an individual's inflammatory state.[1,2] When neutrophils are activated in vivo, one of the first ROS produced by the NADPH oxidase system is superoxide. Superoxide is rapidly converted to hydrogen peroxide by superoxide dismutase, and increased circulating concentrations of hydrogen peroxide reflect in vivo activation of leukocytes.[3] The body's defense against damage induced by ROS are naturally occurring antioxidants, the most abundant of which is glutathione.[1,4] Consequently, diagnostic techniques that assess leukocyte activation and alterations in antioxidants may provide important data that can be used to assess the level of inflammation in horses with gastrointestinal diseases.

Normal ranges of ROS production by leukocytes, and circulating concentrations of hydrogen peroxide and glutathione have recently been established for healthy horses.[5] With that information as a background, the purposes of the present study were to quantify

these parameters of inflammatory status in horses with naturally occurring gastrointestinal diseases, to determine whether these parameters are altered in horses with diseases having either ischemia or inflammation as a component of their pathogenesis, and to determine whether a relationship existed between inflammatory status and patient outcome. We hypothesized that horses having a higher level of inflammation at admission have a poorer prognosis than horses admitted with a lower level of inflammation.

MATERIALS AND METHODS

Horses in the Study

Sixty-two horses admitted to the University of Georgia Large Animal Veterinary Teaching Hospital with gastrointestinal diseases between July 19, 2005 and January 26, 2006 were used in this study, which was approved by the University of Georgia Animal Care and Use Committee, and all samples were obtained with the client's consent. After review of the medical records, each case was assigned to one of the following categories[6]: non-strangulating large intestinal conditions (NSLI), non-strangulating small intestinal conditions (NSSI), strangulating small intestinal conditions (SSI), and inflammatory intestinal conditions (IC). Four horses originally assigned to the SSI category were euthanized due to financial considerations. These horses were excluded from this study, leaving a total of 58 horses with gastrointestinal diseases. Although a variety of breeds were included, the majority were Quarter Horses and Paints (n=23), and Thoroughbreds (n=9). There were 24 mares, 27 geldings, and 7 stallions included in the study, with 9 horses being less than or equal to three years of age, 31 horses between 4

and 10 years of age, 8 horses between 11 and 17 years of age, and 10 horses were 18 years of age or older (mean age 10 years, range 10 months to 25 years).

Preparation of leukocytes

To prepare leukocyte rich plasma (LRP), a modification of the method reported recently by Okano et al was used.[7] 14mL of whole blood was collected into a vacuum-evacuated tube containing EDTA (Sigma, St Louis, USA). The tube was mixed by inverting at the time of collection. The blood was transported to the laboratory and the tube again mixed by inverting several times. A 12 mL portion of the blood was transferred to a 12mL syringe. The syringe was placed in a standing position on the plunger in a biocontainment hood, and the erythrocytes were allowed to settle by gravity for 30 minutes. The LRP was transferred to a 15mL centrifuge tube by gently expressing the plasma and cells above the erythrocyte boundary from the syringe through a needle bent to 135 degrees. The LRP was then centrifuged at approximately 1300 x g for 10 min, after which the plasma was collected. The leukocytes in the pellet were washed with 10mL of Dulbecco's phosphate buffered saline without Ca^{+2} or Mg^{+2} (PBS, Roche, Indianapolis, USA), and centrifuged at 1300 x g for 10 minutes. The supernatant was decanted and the cells suspended in 5mL of PBS; leukocytes in an aliquot were counted using a hemocytometer, the number of viable cells recovered was calculated, and the cells were diluted to 5.0 x 10^6/ mL in PBS.

ROS Production by Leukocytes

Leukocytes were transferred to a 96-well flat bottom plate, with 3 replicates for each treatment. Production of ROS was measured by the conversion of colorless dihydrorhodamine-123 (DHR-123, Molecular Probes, Eugene, USA) to fluorescent rhodamine-123 (RHO-123). DHR-123 enters the cell as a freely permeable dye and is oxidized to RHO-123 upon accepting an electron. RHO-123 is excitable at 488 nm and emits at 515nm.[8] Phorbol myristate acetate (PMA, Sigma, St. Louis, USA) was used at concentrations from 10^{-9}M to 10^{-6}M to activate the enzymes that produce ROS.[9]

Each plate included the following samples: medium only (background control), medium plus DHR-123 (dye control), cells plus DHR-123 (endogenous ROS production), and cells plus PMA and DHR-123 (capacity to produce ROS). An incubation of 1 h at 37˚C was allowed for accumulation of the fluorescent signal. The plates were read using a fluorescent plate reader with excitation and emission filters of 485nm and 538nm, respectively (FluoroskanAscent FL, ThermoLabsystems, Albertville, USA). The ROS data were collected in arbitrary fluorescent units (AFU) using Ascent software, version 2.6. Data obtained using the two methods were indistinguishable. Consequently, the more rapid LRP method was used for the collection of leukocytes for the remainder of the study, and the same controls and treatments were used. To determine the variability of endogenous ROS production within individual horses, 27 horses were sampled at least twice.

Plasma Concentration of H_2O_2

For the measurement of H_2O_2 in circulation, the plasma recovered after centrifugation of the LRP was immediately transferred to another 15mL centrifuge tube and aliquoted into a 96-well flat bottom plate in sets of 3 replicates for each treatment. The concentration of H_2O_2 in the sample was assessed by measuring the change in color of the substrate, 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS, Sigma). The reaction between the H_2O_2 and ABTS was catalyzed by the horseradish peroxidase enzyme (HRP, Sigma), which was added in excess to the samples, and optical density (OD) was measured.

The concentrations of H_2O_2 in the 100ul samples of plasma from each horse were estimated by adding an equivalent amount of ABTS used in the ELISA and a quantity of HRP equivalent to the total amount of labeled antibody used in the ELISA. This allowed detection of the quantity of H_2O_2 in the plasma at the time of collection. To obtain an estimate of the relative quantity of H_2O_2 present, 0.003%, 0.03% and 0,3% H_2O_2 were added to separate samples of the plasma to generate representative OD values. All samples were set up in triplicate in 96 well flat bottom plates. The plates were incubated at 37˚C and read using an optical density plate reader with a 405nm filter (Multiskan Ascent, ThermoLabsystems). The H_2O_2 data were collected in OD units using Ascent software, version 2.6. To determine the variability of plasma concentrations of H_2O_2 within individual horses, 13 horses were sampled at least twice. The addition of H_2O_2 to the samples was used to test the capacity of the assay to measure the amount of H_2O_2 in the plasma sample. If the OD values for plasma, HRP and ABTS and those with added H_2O_2 were equivalent, then the assay conditions either were saturated or the reagents

were not working properly. Only samples having concentration dependent increases in ABTS conversion with the addition of exogenous H_2O_2 were included in the analysis of the data.

Blood Concentrations of GSH and GSSG

Blood concentrations of GSH and GSSG were determined utilizing a modified version of the assay for use in microtiter plates of a commercial kit (Oxis International Bioxytech GSH/GSSG-412 kit, Portland, USA) based on 5,5'-dithiobis-(2-nitrobenzoic acid) oxidation of GSH and reduction of GSSG by NADPH catalyzed by glutathione reductase, an enzymatic recycling assay first introduced by Tietze.[10] This procedure was optimized using the standards provided with the kit

To determine concentrations of GSH, 50µL of whole blood was aliquoted into 500µL microcentrifuge tubes and frozen at -80˚C to lyse the erythrocytes. To determine concentrations of GSSG, 100µL of whole blood and 10µL of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) were aliquoted into 500µL microcentrifuge tubes and frozen at -80˚C to lyse the erythrocytes and maximize the concentration of GSSG in the sample.

Samples used to determine GSH concentrations were thawed, vortexed, and 350µL of cold 5% metaphosphoric acid (MPA) was added to the microcentrifuge tubes. The tubes were then vortexed for 15-20 seconds, centrifuged at approximately 1000 x g for 10 minutes, and 50µL of the MPA extract was added to 3mL of cold assay buffer. All samples were maintained on ice until assayed.

Samples used to determine GSSG concentrations were thawed, vortexed, and incubated at room temperature. After approximately 5 minutes, 290µL of cold 5% MPA was added to the tubes and the samples vortexed for 15-20 seconds. The samples were then centrifuged at approximately 1000 x g for 10 minutes, after which 50µL of the MPA extract was added to 700µL of cold GSSG assay buffer on ice. The blank for the GSSG assay was prepared by adding 50µL MPA to 700µL GSSG assay buffer. All samples were maintained on ice until assayed.

Six GSH standards (0 – 0.5µM) and the GSSG blank were used to construct calibration curves for the GSH and GSSG assays. 40µL of standards, blank, or samples were aliquoted into 96-well flat bottom plates. After addition of an equal volume of the chromogenic substrate and enzyme, and a 5-minute incubation at room temperature, 40µL of NADPH was added and the optical density read at 405nm for 3 minutes using an optical density plate reader (Multiskan Ascent, Labsystems) and Ascent software, version 2.6. To determine the variability of blood concentrations of GSH and GSSG within individual horses, 6 horses were sampled at least twice.

A simple ratio of the concentration of GSH to GSSG was calculated based on the measurement of each at each sampling: [GSH]/[GSSG] = ratio.

This ratio was used as an indicator of the relative concentrations of glutathione at each redox state in each horse. This calculated ratio differed from that recommended by the manufacturers of the kit, but it provided us with a consistent indicator of the relationship between GSH and GSSG in individual horses.

Circulating Leukocyte Counts

As horses were admitted to the Teaching Hospital, peripheral blood was obtained via venipuncture and transferred to 2 vacuum evacuated tubes, 1 containing EDTA and 1 containing a serum separator. The anti-coagulated blood was processed by the Clinical Pathology Laboratory for complete blood count (CBC) and white blood cell differential determinations. The serum was evaluated for serum chemistry profiles.

Overall Stress Index

An overall stress index for each horse was calculated by multiplying the number of circulating WBC by the value obtained for endogenous ROS production. The purpose of calculating this index was to provide an indicator of the total circulating ROS production at the time of admission for each horse.

Data Analysis

All data were transferred to an Excel spreadsheet, where the means and standard error of the mean were calculated. Any ROS experiments for which spontaneous conversion of DHR-123 to RHO-123 exceeded 20 AFU, indicating a problem with the dye, were excluded from the data. Mean values for number of white blood cells (WBC) recovered, number of circulating WBC, number of circulating segmented neutrophils, number of circulating band neutrophils, overall stress index, endogenous and induced production of ROS, plasma concentration of H_2O_2, and blood concentrations of GSH, GSSG, and the GSH/GSSG ratio were compiled by parameter and separately tested for normality. Subsequent analyses included ANOVA, multiple means comparisons, or T-

tests, where appropriate. All analyses were performed using Prism (Version 4, GraphPad Software, Inc , San Diego, USA) with significance defined as $P < 0.05$, and a tendency was ascribed to $P \leq 0.15$ and > 0.05.

RESULTS

The overall survival rate of the 58 horses in this study was 81%. Thirty-three of the 58 horses were treated medically, and 26 survived. Twenty-five of the 58 horses were surgically treated, and 21 survived.

Survival rates for each of the disease categories were as follows: NSLI 22/25 (88%); NSSI 14/15 (93%); SSI 3/6 (50%); IC 8/12 (67%). The number of horses in each of the disease categories treated medically were as follows: NSLI 12/25; NSSI 10/15; SSI 0/6; IC 11/12. Nine of the 12 horses in the NSLI category treated medically survived, 9 of the 10 horses in the NSSI category treated medically survived, and 3 of the 11 horses in the IC category survived. The number of horses that underwent surgery in each of the disease categories were as follows: NSLI 13/25; NSSI 5/15; SSI 6/6; IC 1/12. Thirteen of the 13 horses in the NSLI category treated surgically survived, 5 of the 5 horses in the NSSI category treated surgically survived, 3 of the 6 horses in the SSI category treated surgically survived, and the 1 horse in the IC category treated surgically did not survive.

When values for endogenous and stimulated production of ROS, overall stress index, reduced and oxidized glutathione, and GSH/GSSG ratios were compared irrespective of the method of treatment (medical or surgical), there were no significant differences between horses that survived and horses that died (Table 1). There were,

however, significant differences in plasma concentrations of hydrogen peroxide between horses that survived and those that died (Table 1).

The only parameter that was significantly different between the horses that survived or died in this study was the plasma level of H_2O_2 (Table 1), which reflects immediate past activation of the horse's inflammatory cells. Horses that died also had higher levels of endogenous ROS production and GSSG, and higher values for OSI and the GSH/GSSG ratio than horses that survived, but none of these differences were significant.

Values for endogenous production of ROS by leukocytes from horses compared by method of treatment (medical or surgical) and outcome (survived or died) for the respective treatment method are presented in Table 2. The endogenous level of ROS production on admission tended (P=0.01) to be lower in the horses that received medical treatment after admission than those that required surgical treatment. Further, within the group of animals that received medical treatment and survived, endogenous production of ROS on admission tended (P=0.01) to be lower than the medically treated animals that died. The medically treated animals that died had a nearly identical level of endogenous ROS production on admission as the animals that required surgical treatment

The OSI values compared by treatment method and outcome of the respective treatment method are presented in Table 3. The overall stress index was calculated based on the product of the total leukocyte count and the level of endogenous ROS production. Thus, it is not surprising that the OSI tended (P=0.15) to be less for medically treated horses than for surgically treated horses and significantly less for medically treated horses that survived than for those that died, since these two comparisons (medical versus

surgical, and medically treated horses that survived versus medically treated horses that died) tended to be different with respect to production of endogenous ROS on admission as well (Table 2). The total circulating WBC also tended to be less for horses treated medically that survived and for those that died, $p = 0.1$ (data not presented). It is interesting to note that the OSI had a different relationship for the horses that were treated surgically and died than either all horses undergoing surgery or those that survived after surgical treatment compared to the values obtained for the endogenous ROS for these same comparisons. The OSI was lower in the surgically treated horses that died, but the endogenous production of ROS was higher for the surgically treated horses that died, suggesting that OSI is not a good indicator of treatment method, but seems to be of outcome of medically treated horses. The small number of animals that were surgically treated and died made any significance of these differences difficult to establish.

GSH/GSSG ratio for horses based on method of treatment and outcome of the respective method of treatment are presented in Table 4. In addition to evidence of lower endogenous leukocyte activation among the horses that were treated medically compared with those requiring surgical treatment, we observed a significantly higher antioxidant capacity in the horses that were medically treated to those that were surgically treated. This suggests the measurement of the GSH/GSSG ratio may be of value in aiding current diagnostic methods in determination of treatment method. However, there is an overlap in the values on the lower end of the medically treated horses and the upper end of the surgically treated horses, which reduces the usefulness of this measurement in the clinical setting.

The total number of circulating WBC, segmented and band neutrophils for horses presented with gastrointestinal diseases are summarized in Table 5. Leukocyte production of ROS, overall stress index and plasma concentrations of H_2O_2 for the different disease categories are summarized in Table 6. To determine whether endogenous production of ROS by WBC was influenced by the number of WBC in circulation, and, potentially either the level of activation or the stage of maturation, total WBC count and endogenous ROS production were analyzed for 58 horses by linear regression; circulating WBC count did not affect endogenous ROS production (r^2 = 0.00001). Blood concentrations of GSH and GSSG, and the calculated ratio of GSH/GSSG for the different disease categories are summarized in Table 7.

DISCUSSION

The results of this study suggest that the GSH/GSSG ratio serves as an adjunct to current diagnostic methods in determining whether horses with gastrointestinal diseases should be treated medically or surgically Further, the results of this study suggest that determining the plasma concentrations of hydrogen peroxide and calculation of an overall stress index (the product of endogenous ROS production and total leukocyte count) may provide additional information concerning the outcome of such horses.

Although blood concentrations of glutathione have been measured in horses with naturally occurring respiratory diseases,[11,12] we are unaware of any studies in which glutathione has been measured in horses with gastrointestinal diseases. In the present study, all horses treated medically and the horses treated medically that survived had significantly higher GSH/GSSG ratios than values obtained for healthy horses in our

laboratory. Furthermore, the horses treated medically that died had a strong trend (p-value = 0.06) towards higher GSH/GSSG ratios than for the healthy horses. Although the GSH/GSSG ratios for horses treated medically were significantly higher than those for horses treated surgically, the clinical usefulness of this ratio is reduced by the overlap in values on the lower end of the medically treated horses and upper end of horses treated surgically. We are at a loss to explain the increased GSH/GSSG ratios in the horses in the present study.

Plasma levels of hydrogen peroxide were significantly different between the horses that survived and those that died. The level of hydrogen peroxide in plasma is an indicator of inflammatory cell activity immediately before the sample was collected, and thus is a good indicator of the current inflammatory state of the horse. Thus, monitoring plasma levels of hydrogen peroxide may provide important information regarding the inflammatory state of horses with gastrointestinal diseases as well as an indication of prognosis. As was the case for the GSH/GSSG ratio, the horses that died had significantly higher plasma levels of hydrogen peroxide than values obtained previously in our laboratory for healthy horses. Additionally, horses in the IC disease category had a strong trend towards increased plasma levels of hydrogen peroxide than values obtained for healthy horses (p-value = 0.06). Larger studies of more horses with various diseases need to be performed to determine whether or not results of hydrogen peroxide testing would be clinically useful on an individual horse basis.

To our knowledge, the overall stress index calculated in this study has not been used previously in horses. As this calculation combines the endogenous production of reactive oxygen species by leukocytes with the total number of circulating leukocytes, it

may reflect the on-going production of reactive oxygen species in circulation. As such, this measurement could prove helpful in determining the aggressiveness of medial therapy required (horses treated medically that survived had significantly lower overall stress indices than horses that did not survive) or in differentiating horses that should be medically treated from those that require surgical intervention. Indeed, there was a trend towards this distinction in the present study (p-value = 0.15). Larger clinical studies are needed to determine the usefulness of this calculation on an individual horse basis.

There were no significant differences found in clinical horses compared to healthy horses for the measurement of the endogenous production of ROS in horses treated medically or surgically or between survivors and non-survivors, but did yield some trends worthy of mention. When broke down by disease category there are some significant differences between healthy horses and clinical horses. The endogenous ROS production from healthy horses is significantly less than the endogenous ROS production from horses in the SSI disease category (p-value = 0.0497), horses in the SSI disease category that survived (p-value = 0.007), and significantly lower than the endogenous ROS production from horses in the IC disease category that did not survive (p-value = 0.0268). However, this measurement did not prove to be of value in aiding current diagnostic methods in the prediction of the best treatment method, disease severity, or prognosis for recovery in clinical horses admitted with gastrointestinal disease.

Clinical case studies such as this inherently are difficult to control. For example, in the current study there likely were wide discrepancies in the duration of the disease before admission even within the same disease category. Unfortunately, there is no way to control for this factor, and the impact of the findings obtained depend on the number of

animals involved. Furthermore, one of our aims was to determine the production of reactive oxygen species by circulating leukocytes. There is no way to know whether the results obtained reflect leukocytes recruited to sites of inflammation or to the peritoneal cavity. Another complicating factor in clinical case studies such as this is that sometimes it is difficult to determine from information in medical records whether or not individual horses were euthanized for financial constraints. Although we eliminated 4 horses with SSI lesions that fit this condition, it is possible that other horses that did not survive were euthanized later in their clinical course due to financial constraints. It is also important to mention that the healthy horse values obtained in a previous study completed in our laboratory, which were cited in this paper were not transported prior to sampling.

Our hypothesis at the outset of this study was that horses with a higher level of inflammation at admission would have a poorer prognosis and require more aggressive treatment than horses with a lower state of inflammation. Our results suggest that the GSH/GSSG ratios, plasma levels of hydrogen peroxide and the overall stress index would support this hypothesis. Additional studies involving more horses will be needed to more fully test the hypothesis. Additionally, the parameters measured in this clinical study may prove valuable when measured in horses with inflammatory conditions other than the gastrointestinal diseases included in the current study.

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Table 1. Values for endogenous and stimulated production of ROS, overall stress index, plasma concentration of hydrogen peroxide, blood concentrations of reduced and oxidized glutathione, and GSH/GSSG ratios associated with outcome (survived or died).

Parameter	**Survived**	**Died**
Endogenous production of ROS	3.5 ± 0.4; n=45	4.5 ± 0.8; n=10
Stimulated production of ROS	214 ± 14; n=45	198 ± 19; n=10
Overall stress index	34 ± 4; n=44	47 ± 10; n=10
Endogenous level of H_20_2	0.4 ± 0.0; n=41*	0.6 ± 0.2; n=10
GSH	849 ± 90.1; 26	936 ± 163; n=6
GSSG	14 ± 5; n=26	21 ± 17; n=6
GSH/GSSG ratio	227 ± 46; n=26	257 ± 93; n=6

The data are mean ± SEM.

*$P < 0.05$, significantly different from horses that did not survive.

Table 2. Endogenous production of ROS by leukocytes compared by method of treatment (medical or surgical) and outcome (survived or died).

Medically Treated Horses		**Surgically Treated Horses**	
[a]3.2 ± 0.3; n=32		4.3 ± 0.6; n=23	
Survived	**Died**	**Survived**	**Died**
[b]3.0 ± 0.4; n=26	4.4 ± 0.9; n=6	4.2 ± 0.7; n=19	4.8 ± 1.7; n=4

The data are mean ± SEM.

[a]$P = 0.10$, comparison with surgically treated horses.

[b]$P = 0.10$, comparison with medically treated horses that died.

Table 3. Overall stress index values compared by method of treatment (medical or surgical) and outcome (survived or died).

Medically Treated Horses		**Surgically Treated Horses**	
31.3 ± 4.3; n=31*		42.9 ± 7.3; n=23	
Survived	**Died**	**Survived**	**Died**
25.7 ± 3.3†; n=25	54.3 ± 15.4; n=6	44.2 ± 8.8; n=19	37.0 ± 5.7; n=4

The data are mean ± SEM.

*$P = 0.15$, comparison with surgically treated horses

$^{\dagger}P < 0.05$, significantly less than medically treated horses that died

Table 4. GSH/GSSG ratio values compared by method of treatment (medical or surgical) and outcome (survived or died).

Medically Treated Horses		**Surgically Treated Horses**	
289 ± 60; n = 20*		129 ± 28; n = 14	
Survived	**Died**	**Survived**	**Died**
275 ± 70; n = 16	344 ± 118; n = 4	137 ± 32; n = 12	83 ± 2; n = 2

The data are mean ± SEM.

*$P < 0.05$, significantly greater than surgically treated horses

Table 5. Total circulating WBC, segmented neutrophils, and band neutrophils for each category of gastrointestinal disease.

Category	Total WBC (10^3/µL)	Segmented Neutrophils (10^3/µL)	Band Neutrophils (10^3/µL)
NSLI	9.1 ± 0.7; n=23	6.9 ± 0.6	0.1 ± 0.0
Survived	9.1 ± 0.8; n=20	6.9 ± 0.7	0.2 ± 0.0
Died	8.8 ± 2.0; n=3	6.6 ± 2.4	0.0 ± 0.0
NSSI	10.8 ± 0.6; n=15	8.3 ± 0.6	0.2 ± 0.1
Survived	10.5 ± 0.5; n=14	8.1 ± 0.6	0.1 ± 0.0
Died	15.2 ± 0.0; n=1	11.6 ± 0.0	2.0 ± 0.0
SSI	11.0 ± 0.7; n=6	8.6 ± 0.5	0.7 ±0.3
Survived	9.7 ± 0.2; n=3*	8.2 ± 0.5	0.2 ± 0.2[a]
Died	12.2 ± 0.7; n=3	9.0 ±0.9	1.2 ± 0.5
IC	8.5 ± 1.3; n=12	6.4 ± 1.2	0.4 ± 0.2
Survived	7.9 ± 0.9; n=8	5.5 ± 1.0	0.5 ± 0.2
Died	9.7 ± 3.7; n=4	8.0 ± 3.0	0.2 ± 0.1

Data are presented as mean ± SEM.

Values are presented as total for each disease category, and for horses that survived or died within each category. NSLI = non-strangulating large colon conditions, NSSI = non-strangulating small intestinal conditions, SSI = strangulating small intestinal conditions, IC = inflammatory conditions, n = number of horses.

*$P < 0.05$, Significantly less than SSI horses that died

[a]$P = 0.12$, Comparison with SSI horses that died,

Table 6. Endogenous ROS production, stimulated ROS production, overall stress index, and plasma concentration of H_2O_2.

Disease	Endogenous ROS (AFU)	Stimulated ROS (AFU)	Overall Stress Index	H_2O_2 (OD)
NSLI	3.4 ± 0.4; n=23	233 ± 20; n=22	30 ± 5; n=20	0.4 ± 0.0; n=19
Survived	3.2 ± 0.4; n=21	239 ± 22; n=20	29 ± 6; n=11	0.4 ± 0.5; n=17
Died	5.0 ± 0.3; n=2	172 ± 36; n=2	33 ± 8; n=9	0.4 ± 0.1; n=2
NSSI	3.6 ± 0.7; n=14	188 ± 20	40 ± 10	0.4 ± 0.1
Survived	3.7 ± 0.8; n=13	189 ± 21	41 ± 11	0.3 ± 0.1
Died	2.3; n=1	167	35	0.6
SSI	5.1 ±1.4; n=6	219 ±27	52 ± 12	0.5 ± 0.2
Survived	6.9 ± 2.3; n=3	234 ±5.6	66 ± 22	0.4 ± 0.1
Died	3.2 ± 0.9; n=3	204 ± 15	38 ± 8	0.6 ±0.3
IC	3.7 ± 0.8; n=12	212 ± 22	84 ± 31[b]	0.6 ± 0.1
Survived	27 ± 0.7; n=8 [a]	211 ± 27	18 ± 4	0.5 ± 0.1
Died	5.8 ± 1.7; n=4	215 ± 45	215 ± 45*	0.7 ± 0.4

Values are mean ± SEM.

Values are presented as total for each disease category, and for horses that survived or died within each category. NSLI = non-strangulating large colon conditions. NSSI = non-strangulating small intestinal conditions. SSI = strangulating small intestinal conditions. IC = inflammatory conditions. n = number of horses.

*$P < 0.05$, Significantly greater than IC horses that survived and all IC horses

[a]$P = 0.07$, Comparison with IC horses that died.

[b]$P = 0.11$, Comparison with IC horses that survived.

Table 7. Mean ± SEM values for reduced (GSH) and oxidized (GSSG) glutathione in whole blood, and the calculated GSH/GSSG ratio.

Disease	GSH	GSSG	GSH/GSSG
NSLI	1021 ± 105; n=21	15 ± 7; n=16	263 ± 70; n=15
Survived	1029 ± 116; n=19	9 ± 3; n=14*	272 ± 81; n=13
Died	939 ± 120; n=2	54.6 ±50.3	204.6 ± 15.0
NSSI	1167 ± 169; n=12	9 ± 3; n=8	268 ± 93; n=8
Survived	1143 ± 184; n=11	10 ± 6; n=7	207 ± 82; n=7
Died	1437.0	2.1 ± 0.0	694.1 ± 0
SSI	772 ± 158; n=4	28.4 ± 19.0	89.2 ± 25.6
Survived	902 ± 127; n=3	36.4 ± 24.4	92.0 ± 36.0
Died	384; n=1	4.6	80.8
IC	825 ± 97; n=10	28 ± 17; n=8	504 ± 344; n=8
Survived	764 ± 114; n=7	35 ± 23; n=6	612 ± 459; n=6
Died	968 ± 187; n=3	6 ±1; n=2	180 ± 94; n=2

The data are mean ± SEM. Values for GSH and GSSG are in OD units. Values are presented as total for each disease category, and for horses that survived or died within each category. NSLI = non-strangulating large colon conditions, NSSI = non-strangulating small intestinal conditions, SSI = strangulating small intestinal conditions, IC = inflammatory conditions, n = number of horses (for clarity values consistent within a row are only given under the GSH column).

*$P < 0.05$, Significantly less than NSLI horses that died

CHAPTER 4

GENERAL CONCLUSIONS AND DISCUSSIONS

The goals of this project were three-fold: 1) to establish simple and rapid laboratory methods to assess the inflammatory state of horses, 2) to evaluate the inflammatory state of a large number of clinically healthy horses, and 3) to determine if the level of inflammatory state of horses admitted with gastrointestinal disease correlates with the method of treatment and/or outcome. In order to pursue these goals, we had to address several objectives, including finding a simple and rapid method to use to collect leukocytes and plasma, a straightforward protocol for making the measurements that would provide reproducible results, and a simple medium that would support repeatable results.

An important foundation for this project was establishing the leukocyte-rich plasma method for collecting leukocytes and plasma. This method was simpler and less time consuming than the method used to isolate buffy coat cells, and method involving repeated lysis of erythrocytes with distilled water. The ease and reliability of the method involving the use leukocyte-rich plasma will facilitate transition of these assays to the clinical setting, as will the finding that PBS can be used as the medium for the cellular assays.

In addition, the development of a simple enzymatic method to measure plasma hydrogen peroxide provided us with another measure that could potentially be taken to

the stall for immediate feedback. The method is simple and direct and would lead itself to a development of a potential dipstick kit in the future.

Assays for cellular production of ROS and plasma levels of hydrogen peroxide were used to establish the range of values reflecting the basal inflammatory state of healthy horses. Once we had established a clear range for these values, we used these tests to assess the status of horses admitted for gastrointestinal disease. Although we also measured the oxidized and reduced forms of glutathione in the blood of healthy horses, the values were widely distributed without any clear clustering of values around the mean. In contrast, blood glutathione concentrations in the horses admitted with gastrointestinal diseases correlated with the diagnosis, the method of treatment (medical or surgery) and/or outcome (death or survival). This was an exciting finding as it appears that glutathione pools change more over time than the levels of individual oxidant products, and may reflect the pathogenesis of disease. Thus, a combination of oxidant measures that narrowly define the healthy state combined with the relationship between oxidized and reduced glutathione may provide insight into the role of inflammation in the pathogenesis of disease and indicate the best course of treatment.

In the clinical case study, there were no significant differences in the production of ROS by leukocytes from horses with gastrointestinal diseases that were treated medically versus those treated surgically or between survivors and non-survivors. However, we did determine that leukocytes from healthy horses produced significantly less reactive oxygen species than cells from horses with strangulating small intestinal conditions as a whole, as well as from horses with strangulating small intestinal conditions that survived. The leukocytes from some horses with strangulating small

intestinal conditions had normal or below normal amounts of reactive oxygen species production. This suggests that many of the activated neutrophils were being sequestered in the strangulated tissue or in the peritoneal cavity.

Horses with inflammatory gastrointestinal conditions that died had a strong tendency ($P = 0.07$) to produce more reactive oxygen species than horses with inflammatory conditions that survived, and significantly more than healthy horses. This finding supports our hypothesis that horses with a greater level of inflammation at admission would have a poorer prognosis than horses admitted at a lower inflammatory state. Based on these results, it would appear to be beneficial to evaluate horses admitted with inflammatory conditions not involving the gastrointestinal system.

One potential complication in this study is the common use of flunxin meglumine and the occasional use of dimethyl sulfoxide in horses before they are referred to the Large Animal Teaching Hospital. While dimethyl sulfoxide is administered as an ROS scavenger,[1] the effects of flunxin meglumine on the leukocyte production of ROS has not been determined. Although used less commonly for treatment of abdominal pain in horses, phenylbutazone has been shown in an in vitro study to decrease the number of neutrophils phagocytosing bacteria .[2] The direct effects of these drugs on the assays used in this study should be further investigated.

Although not studied in this project, it is important to note that serious injury to the affected intestine can occur after correction of the strangulation as a result of reoxygenation of the previous ischemic tissue. As a result of this process, the intestinal mucosa is damaged due to the production of reactive oxygen species via the xanthine oxidase system..[1,3-6] One possible remedy for the insult caused by reoxygenation could be

the use of drugs such as tempol, dimethyl sulfoxide, and allopurinol. Tempol and allopurinol are drugs with superoxide dismutase activity that inhibit xanthine oxidase resulting in a decrease in the production of reactive oxygen species.[7,8] During ischemia the increase in vascular permeability has been shown to be minimized by dimethyl sulfoxide, a known scavenger of reactive oxygen species, and by allopurinol.[5,9] These treatments could prove helpful in minimizing or eliminating the oxidative damage that occurs during reoxygenation of strangulated tissue.

In summation, these studies have provided both the background and tools necessary to address development of a family of laboratory assessments to study the role of systemic inflammation and conversion of the oxidized and reduced forms of glutathione in the pathogenesis of equine diseases. The measures of ROS and circulating H_2O_2 provide a tight definition of the normal inflammatory state in the healthy horse. A combination of measurements, including those for oxidant and antioxidant activity, provides correlations that discriminate across treatment options and relate to survival outcomes. More studies may allow the development of a predictive model based on these measurements that can be used to optimize treatment choices and outcomes.

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