

**Peripheral Blood Mononuclear Cell Cytokine Expression in Horses
Treated with Dexamethasone**

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Masters of Science

In

Biomedical and Veterinary Sciences

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August 04, 2005

Blacksburg, VA

Keywords: Cytokines, Dexamethasone, Horses

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(Abstract)

Glucocorticoids are widely used in horses for a variety of autoimmune and inflammatory conditions. Its potent antiinflammatory properties have been associated with the suppression of a number of different inflammatory cytokines. The purpose of the study was to evaluate the effect of dexamethasone treatment in horses on mRNA cytokine expression, including interleukin-1 β , interferon-gamma, interleukin-4 and interleukin-6, during a five day treatment period and a five day post treatment period.

A randomized complete block design was performed on 16 healthy horses. Group I (8 horses) received 0.1 mg/kg of dexamethasone sodium phosphate by intravenous injection once daily for 5 days. Group II (8 horses) received an equivalent volume of sterile saline by intravenous injection daily for 5 days. A sample of 5x10 milliliters of blood in acid citrate dextrose was obtained prior to initial treatment. Thirty minutes after each treatment injection (placebo or dexamethasone) a sample of blood was obtained during the 5 day treatment period and 24, 48, 72, 96 and 120 hours after the last treatment injection was administered. Peripheral-blood mononuclear cells were isolated from the blood samples and stimulated with concavalin A. RNA was isolated using the QIAGEN RNeasy kit. cDNA first strand synthesis was achieved using QIAGEN's OMMIScript RT KIT. cDNA was also constructed for the house keeping gene β actin. Primer pairs specific for each cytokine were designed using equine cytokine sequences available on

Genbank. cDNA for each cytokine and β -actin was amplified using Real Time PCR technique.

Interleukin-4, interleukin-6 and interferon-gamma mRNA expression was statistically significant suppressed in horses treated with dexamethasone when compared to control horses. Interleukin-1 β was only significantly suppressed on day 5. Interleukin-4, interleukin-6 and interferon-gamma mRNA expression suppression was initially observed on day 2 and lasted 24 hours after the last dose of dexamethasone was administered. Interleukin-6 mRNA expression was significantly higher when compared to control group on day 10.

Our results suggest that dexamethasone treatment of healthy horses suppresses mRNA expression of several cytokines, including interleukin-4, interleukin-6 and interferon-gamma. This effect could explain part of corticosteroid's mechanism of action for controlling inflammation in a variety of disease conditions. The time-course effect of dexamethasone showed that the effect on mRNA cytokine expression suppression is only observed on day 2 of treatment and mRNA suppression is maintained for 24 hours after discontinuation of treatment.

ACKNOWLEDGEMENTS

I would like to thank the members of my graduate committee, Dr. Virginia Buechner-Maxwell, for your patience, dedication and support, Dr. Sharon Witonsky for your encouragement and support, Dr. Willian Huckle, for assistance in the laboratory and guidance.

Thanks to Dan Ward for helping me understand the statistical analysis of my data.

Thanks to Barbara Dryman for your patience, dedication and enjoyable time spent at the laboratory.

I would also like to thank my family in Brazil for the constant support and encouragement during these 3 years in the United States.

DEDICATION

Dedicated with love to my mom, Ilsa Regina, for her friendship and understanding despite the distance she was always supporting me emotionally and encouraging me to never give up. I also would like to dedicate my thesis to my dad Paulo Roberto, for his love and dedication to his daughters, to my sisters, Fatima Beatriz and Fernanda Maria, for their friendship and encouragement, to my fiancé, Benjamin Lepene, for his patience and friendship.

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LIST OF ABBREVIATIONS:

Adreno-corticotropic hormone: ACTH

Bronchoalveolar lavage: BAL

Equine Protozoal Myeloencephalitis: EPM

Glucocorticoid: GC

Glucocorticoid receptor: GC-R

Granulocyte/Monocyte-colony stimulating factor: GM-CSF

Granulocyte stimulating factor: GSF

Immunoglobulin E: IgE

Inhibitory Kappa B: IκB

Intercellular adhesion molecule 1: ICAM-1

Interleukin 1 beta: IL-1β

Interleukin 2: IL-2

Interleukin 4: IL-4

Interleukin 5: IL-5

Interleukin 6: IL-6

Interleukin 8: IL-8

Interleukin 10: IL-10

Interleukin 12: IL-12

Interleukin 13: IL-13

Interferon gamma: INF-gamma

Monocyte Chemoattractant protein 1: MCP-1

Monocyte stimulating factor: MSF

Nuclear factor kappa B: NFkB

Recurrent Airway obstruction: RAO

Summer Pasture Associated Obstructive Pulmonary Disease: SPAOPD

T helper 1: Th1

T helper 2: Th2

Tumor necrosis factor a: TNF-a

Vascular cell adhesion molecule: VCAM

Chapter I: Introduction

1.1) Inflammation: overview

Inflammation is initiated as a consequence of a complex cascade of reactions that result from tissue damage caused by mechanical, thermal or chemical trauma. It is characterized by increased permeability and dilation of the capillaries; increased formation of fibrinogen and protein extravasation; migration of granulocytes and mononuclear cells into the tissues; and swelling of the tissue cells.[1]

Inflammatory cytokines are found to participate in some of the changes observed during the inflammatory process. For example, interleukin-8 (IL-8) is known to be an important chemoattractant for neutrophils. During acute disease, horses with recurrent airway obstruction show an increased amount of IL-8 in bronchoalveolar lavage cells. The accumulation of neutrophils in the airways aggravates tissue injury in the lungs.[2] Interleukin-4 (IL-4) activates B cell differentiation and proliferation consequently increasing the production of immunoglobulins (especially IgE) in inflammatory response to allergens. IL-4 in the presence of IL-6 also promotes Th2 differentiation.[3, 4] Interleukin-6 (IL-6) activates B and T lymphocytes increasing the degree of inflammatory reaction and also stimulating production of acute-phase proteins in the liver.[5]

Adhesion molecule expression is up-regulated by cytokines, such as IL-4 in the microvasculature near an inflammatory site. Leukocytes adhere to endothelial cells by selectin mediated emigration and migrate to the tissue where the injury has occurred. In

humans, eosinophils are known to migrate into the lung via adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 expressed on the endothelium.[6, 7]

Interleukin-1 β (IL-1 β) is released by macrophages at the beginning of the inflammatory process and is primarily responsible for activating the production of more inflammatory cytokines and stimulating immune cells. This cytokine specifically activates cells in the connective tissue, such as fibroblasts and endothelial cells of blood vessels in the periphery, resulting in production of additional cytokines.[8]

The production of granulocytes and mononuclear cells by the bone marrow is stimulated by tumor necrosis factor - α (TNF- α), IL-1, granulocytes-monocyte stimulating factor (GM-CSF), granulocyte stimulating factor (G-CSF) and monocyte stimulating factor (M-CSF) that are produced by activated macrophages and T-cells in the inflamed tissue. Interferon-gamma (INF-gamma) is also released in the presence of intracellular organisms and it is responsible for activation of cellular immunity.[1]

The role of cytokines in the inflammatory cascade is essential since cytokines provide a means of communication between immune cells including activation of their defense mechanisms and protection of the body against foreign antigens and tissue injury.

1.2) Cytokines

Cytokines are low molecular weight proteins that play an important role in the communication between immune cells involved in inflammatory reactions. They influence the cell by binding to receptors located on the surface of the cell membrane. Their effect on specific cell types is dependent on the presence and amount of these receptors.[8] The body's immune function is highly influenced by these proteins due to

their effect on activation, proliferation, and differentiation of different types of immune cells. Their activity affects a great number of tissue cells locally where the primary injury occurs and they are absorbed into the circulation, causing systemic clinical signs.[9]

The term cytokine represents many families of growth and differentiation factors that participate in immune interaction between cells during inflammation. These factors are generally called lymphokines, interleukins, colony-stimulating factors, tumor necrosis factor (TNF), and interferons. Communication between white blood cells is mediated by cytokines called interleukins. The growth and expansion of cell populations, such as progenitor cells is initiated by colony-stimulating factors. Tumor necrosis factor was previously found to be able to destroy specific types of tumors and that was considered its main function. Later on it was discovered that its role in inflammation involves the chemotaxis of leukocytes, activation of neutrophils and expression of adhesion molecules on the surface of endothelial cells. Viral replication in tissue cells was previously found to be effected by specific cytokines called interferons. They are now known to be one of the main cytokines involved in cellular immunity and protection against intracellular organisms.[8]

1.3) T-helper 1 and T-helper 2 responses in horses and their cytokine pattern:

The immune system is structured to protect the body against invading organisms and tissue injury. To effectively protect the body, the immune system is composed of a great variety of effector immune cells with specific functions and characteristics.

Antibodies are produced by B lymphocytes that neutralize specific antigens. T-cells are

more complex since they are composed of separate categories of cells depending on the specific marker present at their surface membrane. For example T helper cells express a CD4+ molecule while cytotoxic cells express CD8+ molecule on their surface.[10]

Cell mediated immune responses are generated by T helper 1 (Th1) cells that produce a specific subset of cytokines. The Th1 response is important in cases of intracellular parasites, viral infections and neoplastic cells. Cytokines released by these cell types will stimulate the activity of cytotoxic T cells and natural killer cells that are responsible for identification and destruction of cells infected by microorganisms. In contrast, humoral responses are mediated by T helper 2 (Th2) cells that stimulate the production of antibodies in response to a specific subset of cytokines. One example is the immediate type hypersensitivity reaction observed in cases of allergy. Welsh et al observed the polarization of the T helper (Th) response from Th1 to Th2 in cases of bovine tuberculosis that deteriorated clinically. It was suggested that Th1 response may be important in the defense against pathogenic intracellular organisms such as *Mycobacterium bovis*. [11, 12]

Interferon-gamma, TNF-a and interleukin-2 (IL-2) are examples of cytokines produced by Th1 cells that participate in cellular immune responses. Interleukin-4, interleukin-5 (IL-5), interleukin-13 (IL-13), interleukin-10 (IL-10), interleukin-12 (IL-12), and IL-6 are produced by Th2 cells that participate in humoral responses by stimulating the production of antibodies. [13, 10] Substantial evidence exists suggesting the importance of interferons in the stimulation of Th1 responses. T lymphocytes and natural killer cells release interferons, which activate macrophages, neutrophils, T lymphocytes and also determine the class of antibody produced by B cells.[8]

Once Th cells specialize into Th1 or Th2, the cytokines that these subsets produce also serve to perpetuate and maintain this differentiation. It appears that individuals that grow up in clean environments without being exposed to microorganisms that would induce a Th1 response have higher tendency to develop atopic asthma. The development of a Th1 response would potentially diminish the Th2 cell population essential for the pathophysiology of allergic diseases.[14] Pregnancy is maintained in a Th2 type environment to prevent maternal rejection of the fetus. Thus newborns have a tendency toward Th2 responses unless provided a strong stimulus, like bacterial infection. In cases where the newborn is not exposed to antigens in the environment the Th2 response is maintained leading to predisposition to atopic diseases. This theory is currently called the hygiene hypothesis.[15]

A great number of *in-vivo* and *in-vitro* studies have been performed to evaluate the expression of cytokines. It has been suggested that data from *in-vitro* studies may not be reliable because the pattern of cytokine expression is altered by cell manipulation. Therefore, *in-vivo* studies appear to be more reliable since these conditions provide better representation of the interactions between immune cells, tissue cells and cytokines that occur in the body during an inflammatory reaction.[16, 17]

1.4) The role of cytokines in disease process in horses:

Cytokines are involved in the pathogenesis of many diseases affecting humans and animals. Many studies have demonstrated the different pattern of cytokine expression in different disease processes.

1.4.1) Interferon-gamma

Influenza virus infection in humans promotes a Th1 response. There is some indication that cytokines that appear early in the inflammatory process are responsible for the clinical signs associated with disease. These cytokines, such as INF-gamma, TNF-a and IL-1a and β are released at the inflammatory site secondary to infection causing local and systemic changes.[17]

Gene-knock out mice studies have made an important contribution in understanding how specific cytokines play a role in diseases like influenza. Mice with “knock out” gene for IL-1 β and IL-6 infected with influenza virus did not show a remarkable increase in body temperature when compared to mice capable of producing both cytokines.[18, 19] Horses infected with influenza virus also demonstrated a Th1 type response by expressing INF-gamma.[20]

Equine recurrent uveitis has been suggested to be an immune-mediated disease. Recent evidence indicates that this disease is likely characterized by a Th1 response since high levels of IL-2 and INF-gamma mRNA expression were identified in samples collected from horses affected with the disease.[21]

Boyd et al evaluated the changes in cytokine expression in neonatal foals from birth until they were 28 days old. Interferon-gamma was found to increase significantly overtime. It was suggested that at birth Th1 response was not well developed but develops as the immune system matures.[22] This finding would be in agreement with the hypothesis that the immune system in a newborn shifts from a Th2 response to a Th1 response and that this change is mediated by exposure to antigens in the environment.[23]

Equine protozoal myeloencephalitis (EPM) is one of the most common neurologic diseases of the horse. It is caused by the organism, *Sarcocystis neurona* and results in injury to the nervous system. Approximately 60 to 70 % of horses have positive serum titers for the disease suggesting that degree of exposure to the organism is high. However, only a small percentage (approximately 1%) of animals develops neurologic signs. The reason why horses are predisposed to EPM is not well understood, but there is some suggestion that immune compromised animals are more susceptible to the disease and that the protozoa itself may also induce immune suppression. The profile of cytokine expression in horses affected with EPM indicates that INF-gamma expression is suppressed in peripheral blood lymphocytes obtained from horses positively infected with the protozoa when compared to negative controls. The decreased expression of INF-gamma could indicate an immune compromised animal. However, it was also hypothesized that *Sarcocystis neurona* may be able to induce a cellular immunosuppression, thus facilitating its proliferation and infection in the body.[24]

Rhodococcus equi infection commonly causes pneumonia in foals between 3 to 6 months of age. Experimental studies in mice have demonstrated that clearance of *Rhodococcus equi* infection in lungs is mediated by a Th1 profile of cytokines. Treatment of mice with anti-INF-gamma antibodies resulted in persistence of lung infection and development of pulmonary granulomas. In contrast, treatment of mice with anti-IL-4 antibodies resulted in resolution of infection by 21 days. These mice also expressed increased levels of INF-gamma in bronchial lymphocytes. This study demonstrates the importance of a Th1 driven response in resolution of infection in cases of pneumonia caused by *Rhodococcus equi*. [25]

1.4.2) Interleukin-4

Interleukin-4 was previously known as B-cell-stimulating factor 1, T-cell-growth factor II, and mast cell growth factor II. Interleukin-4 is an essential cytokine for the activation, proliferation, and differentiation of B cells. It is also responsible for the increase in the expression of class II major histocompatibility complex on the surface of B cells, and stimulates the proliferation of Th2 cells and mast cells. [26]

Surface receptors for IL-4 are present on B and T cells, macrophages, mast cells, myeloid cells, granulocytes, erythroid progenitors, natural killer cells, megakaryocytes, endothelial cells and fibroblasts. Interleukin-4 increases the formation and expression of adhesion molecules on the surface of endothelial cells, which will consequently trigger the migration of immune cells to the site of inflammation. It also appears to be involved in the development of atherosclerosis in humans by the up-regulation of VCAM-1, E-selectin, monocyte chemoattractant protein-1(MCP-1) and IL-6. [26]

Current studies suggest that Th2 response is responsible for the pathogenesis of allergic and atopic diseases. Increased production of IgE antibodies in patients with allergic asthma, rhinitis and conjunctivitis support this theory.[27] Another indication that Th2 cytokines are important for the development of airway inflammation in asthma is that activated peripheral blood T lymphocytes obtained from humans with the disease release high amounts of IL-4 in-vitro.[28]

Recurrent airway obstruction (RAO) commonly affects aged horses and it is proposed to have an allergic etiology. Studies have demonstrated high levels of IL-4 and IL-5 expressed in bronco-alveolar lavage cells from clinically ill horses. RAO is also associated with decreased INF-gamma expression.[29, 30] An elevated level of IL-4 was also

correlated with high serum and bronchoalveolar lavage concentrations of immunoglobulin IgE.[31] These studies support the idea that RAO is mediated by a Th2 response based on the pattern of cytokines expressed during exacerbation of the disease.[32] One theory is that the pathophysiology of the disease involves the degranulation of basophils secondary to increased levels of immunoglobulin IgE in the airways.[33] Ainsworth et al questioned the common belief that RAO is a disease with a Th2 predominant response and showed that high levels of INF-gamma was present in bronchoalveolar lavage cells obtained from chronically affected horses.[2]

Another disease with similar pathogenesis is summer pasture associated obstructive pulmonary disease (SPAOPD). In contrast, Beadle et al found that SPAOPD was associated with a Th2 response based on findings of elevated levels of IL-4 in bronco-alveolar lavage and blood cells from affected horses.[34]

The nuclear factor-kappa B (NF-kB) has been shown to participate in the activation of DNA inflammatory genes. It has also been found to be increased in bronchial cells and bronchial epithelial cells in horses with RAO. This finding may suggest the role of NF-kB in initiating airway inflammation.[35, 36]

1.4.3) Interleukin-6

Interleukin-6 stimulates the development and antibody production by B cells. It is also called B cell differentiation factor. IL-6 promotes T cell growth, IL-2 synthesis, and production of acute-phase protein by hepatocytes. Macrophages, T cells, stromal cells, fibroblasts, and a variety of other cell lines are responsible for the production of IL-6. Clinically, one of its main effects is the development of fever since it acts as an

endogenous pyrogen by stimulation of the hypothalamus. Interleukin-6 is considered an important cytokine in inflammatory reactions because it mediates innate immunity.[37]

Interleukin-6 has been found to be a cytokine that strongly stimulates megakaryocytopoiesis. Hauser et al demonstrated elevated concentrations of interleukin-6 in bone marrow cultures obtained from mice. Another interesting finding was that adding anti-IL-6 antibody to the cultures produced a significant suppression of myeloid cell production.[37]

Horses are frequently affected by gastrointestinal disorders, including a wide variety of clinical conditions. Barton and Collatos found that horses with inflammatory or strangulating obstructions involving the gastrointestinal tract have elevated levels of IL-6 in the blood and peritoneal fluid. Those horses were also found to have increased levels of endotoxin in the peripheral blood when compared to controls.[38] Significant evidence exists which shows that endotoxin influences the production of IL-6. In-vivo and in-vitro studies have identified an increased production of IL-6 in the blood when horses are exposed to endotoxin and enhanced activity of the cytokine when peritoneal macrophages are exposed to endotoxin, respectively.[39, 40, 41, 42]

Additionally, equine infectious anemia in another disease condition of horses that appears to be associated with increased IL-6 production. This response is thought to stimulate the inflammatory response observed during the acute stage of the disease. Sellon et al showed that ponies acutely infected with equine infectious anemia released a significant amount of IL-6 in the serum.[43]

1.4.4) Interleukin-1 β

Interleukin-1 β is essentially produced by activated macrophages, and its main function is to control immune responses and initiate inflammatory reactions. When acting locally at low concentrations it stimulates T cell and B cell proliferation and differentiation. In the periphery of the inflammatory site it stimulates its own production and IL-6 production while increasing the adhesion between leukocytes and endothelial cells. Interleukin-1 gains access to the systemic circulation when present in higher concentrations, causing fever, stimulating the liver to release acute-phase proteins and inducing cachexia. IL-1 α and IL-1 β produced by separate genes in the cell. These cytokines are commonly released in response to several stimuli, including endotoxin, immune complexes, toxins, tissue physical damage, and other types of inflammatory processes. There is some evidence that injury to the central nervous system results in increased expression of IL-1 β and that this cytokine may be responsible for neurodegeneration seen in ischemic or inflammatory insults to the brain.[44]

Horses are athletic animals; consequently they are frequently affected by arthritic conditions. Synoviocytes and chondrocytes from horses with arthritis have been found to produce high levels of IL-1 β . This cytokine is believed to play a role in arthritic diseases by loss of cartilage matrix.[45, 46,47] It also appears to be important in the mechanisms that lead to laminitis in horses. Fontaine et al showed that IL-1 β was highly expressed by cells that surround the vessels in laminar tissues of horses during the initial stages of experimentally induced laminitis. This finding indicates that local production of cytokines, like IL-1 β may participate in the development of the disease.[48]

Equine ehrlichiosis is a disease condition caused by the organism *Anaplasma phagocytophila* and it is transmitted by ticks. The pathogenesis of the disease is suspected to involve the presence of inflammatory cytokines, especially because of the low number of *Anaplasma phagocytophila* in the blood of equine patients in the acute stage of the disease. Expression of IL-1 β and TNF are believed to contribute to the fever, neutropenia and thrombocytopenia commonly observed in horses clinically affected.[49]

Follicular maturation and ovulation in horses may be regulated by IL-1 β . Martoriati et al showed that granulosa cells obtained from healthy mares increased the expression of IL-1 β during the development of the follicle leading to ovulation.[50]

Expression of IL-1 β is also elevated in the airways of patients with asthma resulting in exacerbation of the inflammatory response initiated by the presence of allergens.[51]

The pathogenesis of septic shock has been extensively studied and recently there have been increased indications that the cytokine release plays an important role in the cascade of events. Lipopolysaccharide originates from gram negative bacteria and is the molecule mainly responsible for alterations observed during septic shock. It binds a plasma protein called lipopolysaccharide binding protein. Subsequently, this complex formed between these two proteins then binds to a receptor on the surface of mononuclear cells called CD14. This receptor initiates the expression of specific cytokines, such as TNF and IL-1 β that contribute to the inflammatory process as previously described.[52]

1.4.5) Applications

Evidence suggests that inflammatory cytokines contribute to allergic, inflammatory and infectious equine and human diseases. Potential development of new therapeutic strategies that manipulate these responses would be an interesting approach to reduce symptoms and progression of disease. The possibility of using recombinant cytokines or anticytokine antibodies to manipulate the immune system could potentially provide clinical improvement without the adverse effects of corticosteroids. There is evidence that IL-4 antagonists produced clinical improvement in human patients with asthma.[53, 54, 51] Another example is the expression of IL-1 receptor antagonist in the joints of horses that were treated with gene therapy. When compared to controls, these horses showed significant clinical improvement with reduced signs of arthritic pain and there was also decreased damage to articular cartilage and reduced inflammation in the synovial membrane.[55] This study suggests that manipulation of the expression of inflammatory cytokines could benefit clinical patients.

1.5) Adrenal gland and endogenous cortisol: its function in the organism

The adrenal gland is divided into adrenal medulla and adrenal cortex. Corticosteroids such as cortisol and mineralocorticoids are released by the adrenal cortex, both originating from cholesterol. Seventy-five percent of the adrenal cortex constitutes the zona fasciculata that is responsible for producing cortisol and corticosterone. The adreno-corticotrophic hormone (ACTH) secreted by the pituitary controls the production and secretion of steroids by the gland. Release of ACTH is also controlled by the release of adrenocorticotrophic releasing hormone from the hypothalamus.[56]

The majority of cortisol released in the plasma conjugates with a plasma protein called transcortin. A small amount of the cortisol binds with albumin. Cortisol has long half life because it is highly conjugated with plasma proteins. The catabolism of corticosteroids is mediated by the liver and it is excreted in the bile in the form of glucuronic acid. Part of it is excreted in the feces and part is reabsorbed and excreted by the kidneys.[57]

Pathways affected by cortisol in the body include gluconeogenesis in the liver, mobilization of amino acids from other tissues like muscle and decrease use of glucose by cells via increase insulin resistance. These alterations ultimately result in increased glucose concentration in the blood. There is a significant mobilization of fat from the adipose tissue. Hyperglycemia caused by excessive cortisol release such as in cases of equine Cushing's disease may be partially responsible for vascular changes that predispose those animals to laminitis. [59]

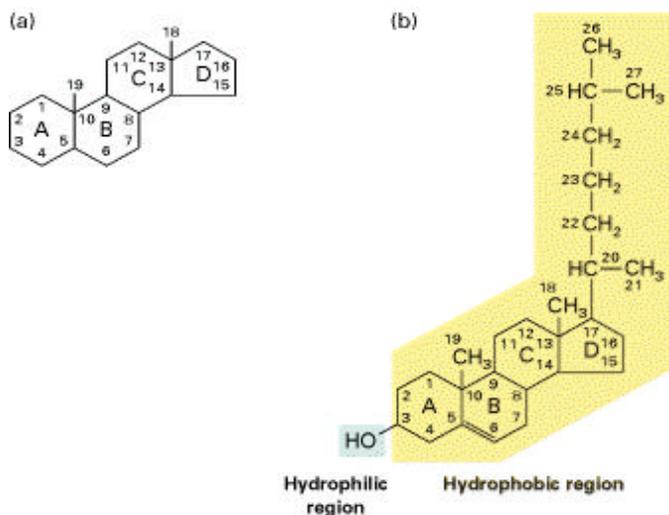
The Adrenocorticotrophic hormone (ACTH) is responsible for the release of cortisol from the adrenal gland in the blood stream. However, ACTH is also influenced by the hypothalamus via neuroendocrine control. The hypothalamus secretes corticotrophic-releasing hormone that stimulates secretion of ACTH from the pituitary gland. The increase in serum levels of cortisol provide a direct negative feedback mechanism to inhibit the release of corticotrophic-releasing hormone from the hypothalamus.[1]

1.6) Glucocorticoids: mechanism of action, effects and cytokine suppression

The first glucocorticoids (GC) were found in the adrenal cortex. Glucocorticoids, mineralocorticoids, such as aldosterone, and the adrenal sex hormones form the three corticoid groups produced by the adrenal gland.[58]

Glucocorticoids that are normally produced by the body (cortisol and cortisone) exhibit some antiinflammatory properties, but also have some mineralocorticoid effects such as sodium and water retention. The basic GC molecule structure has intentionally been altered to produce analogs with higher antiinflammatory activity and less mineralocorticoid activity. The basic structure that composes the steroid molecule is a four ring structure.[58]

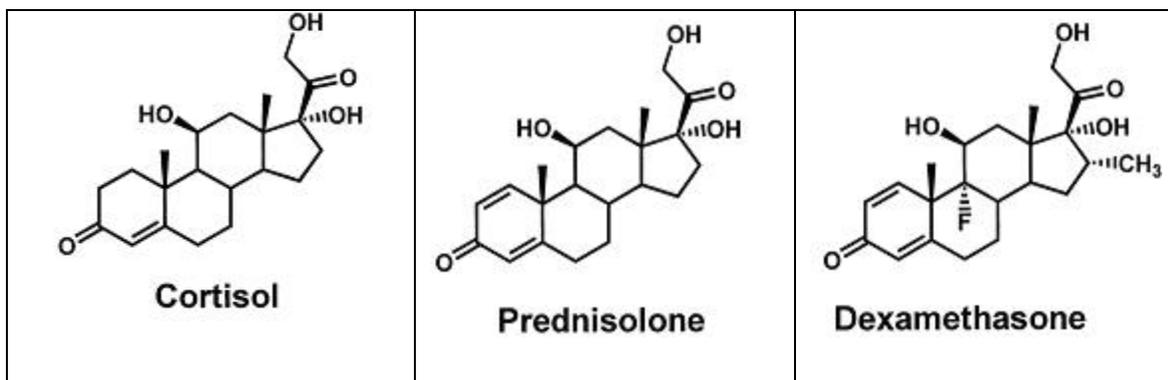
Figure 1: (a) Steroid structure. Hydrocarbon rings labeled A, B, C, and D. (b) Cholesterol - hydrophobic (yellow) and hydrophilic (green).



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Figure 2: Examples of different types of steroid structures



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Examples of GCs are dexamethasone, prednisone, prednisolone and methyl-prednisolone. The natural GCs are cortisol and hydrocortisone with mild anti-inflammatory properties. The active form of CGs is characterized by the presence of a hydroxyl group at position 11. The addition of a fluoro group increases the antiinflammatory activity of dexamethasone and the addition of a methyl group minimizes its mineralocorticoid activity. Once absorbed GCs bind to plasma proteins (transcortin or albumin) and the remaining drug is free to activate its receptor.[57] Fluticasone is an inhaled GC used in equine patients and appears to produce significant clinical improvement in horses affected with RAO. [60]

The duration of action of GCs depends on its formulation, for example, water-soluble drugs produce fast absorption via intravenous or intramuscular injection. Long

acting forms include acetate, diacetate, acetonide, valerate, and isonicotinate which are water insoluble ester formulations.[61]

Glucocorticoids are commonly used in the treatment of allergic and autoimmune disorders. Their antiinflammatory effects are produced secondary to activation or repressing of target genes.[62] Their effects include stabilization of lysosomal membranes, decreased vascular permeability, inhibition of leukocyte migration, decreased leukocyte adhesion to endothelial cells, reduction of leukocyte superoxide production and inhibition of platelet aggregation.[58]

Glucocorticoids are lipophilic molecules and are able to enter the cell by means of passive diffusion. The glucocorticoid receptor (GC-R) enters the nucleus after binding with a GC molecule. It is usually present in the cytoplasm of the cell and it is classified as: type I or type II. Type I is a mineralocorticoid receptor and is mostly expressed at the kidney, colon, salivary and sweat glands, and hippocampus. Type II is a glucocorticoid receptor and is expressed in almost all tissues and cells.[63]

The activation of gene transcription by the glucocorticoid-receptor complex appears to be mediated by a group of coactivator proteins. Examples of nuclear coactivator proteins are the CREB-binding protein and p300.[64, 65] Another example of a coactivator protein is the steroid receptor coactivator 1. They are able to enhance transcription by permitting a connection between the corticosteroid-receptor complex with transcriptions factors in the DNA. In addition, the coactivator proteins produce conformational alterations on the nucleosomes via histone acetylation to permit binding of transcription factors in specific regions of the DNA.[66]

Chromosomal DNA is compacted into chromatin by histones molecules, which inhibits gene expression. Corticosteroid-receptor complex requires enzymes that are capable of acetylation of histones and unfolding the chromatin to permit access of transcriptions factors to the gene promoter. Once the acetylation of histones occurs the DNA becomes accessible to the glucocorticoid-receptor complex.[62, 67]

In contrast, another group of proteins with opposite activity of the histone acetylation enzymes has been identified. They are called corepressor proteins with histone deacetylase activity.[67, 68, 69] These proteins are capable of condensing the chromatin by inhibition of histone acetylation and consequently decrease binding of transcription factors. This deacetylase activity has been suggested to be involved in suppression of inflammatory genes by glucocorticoids.[70]

It is not known which specific genes are responsible for the antiinflammatory activity of glucocorticoids. Their antiinflammatory effects are mediated by stimulation of transcription of genes with antiinflammatory activity and inhibition of transcription of genes with inflammatory activity. Synthesis of lipocortin-1 was initially suggested to be the mechanism of action of corticosteroids.[71] Lipocortins inhibit the production of inflammatory mediators such as prostaglandins, leukotrienes, and platelet-activating factor by blocking the activity of the enzyme phospholipase A₂. The suggested mechanism of action of glucocorticoids has been recently questioned.[62]

A more current theory is that glucocorticoids inhibit the expression of several inflammatory cytokines, including IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, IL16, granulocyte/monocyte-colony stimulating factor (GM-CSF), tumor necrosis factor- α , matrix metalloproteinase 9, and the chemokines IL-8, RANTES, eotaxin, macrophage

inflammatory protein 1a, and monocyte chemoattractant protein 1 (table 1).[63, 72, 73]

They are also capable of inhibiting the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and VCAM-1, decreasing the migration of immune cells into tissues.[74,75]

The table below shows a wide variety of molecules that are inhibited or stimulated by the effect of glucocorticoids in gene expression. It is clear that the effect of corticosteroids is complex and involves many pathways.

Table 1: Glucocorticoid-sensitive genes

Decreased transcription	Increased transcription
<u>Chemokines:</u> IL-8 RANTES Macrophage inflammatory protein-1a Monocyte chemoattractant protein (MCP)-1 MCP-3, MCP-4 Eotaxin	<u>Lipocortin-1/ Annexin-1</u> Phospholipase A ₂ inhibitor
<u>Cytokines:</u> Interleukins- 1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18 Tumor necrosis factor- α Granulocyte macrophage colony-stimulating factor Stem cell factor	<u>Clara cell protein</u> CC10 Phospholipase A ₂ inhibitor
<u>Inducible enzymes:</u> Inducible nitric oxide synthase Cyclooxygenase-2 Cytoplasmic phospholipase A ₂	<u>Others:</u> β_2 -adrenoceptor Secretory leukocyte inhibitory protein (SLPI) IL-1 receptor antagonist IL-1R ₂ (decoy receptor) IkBa (inhibitor of NF- κ B) CD163 (scavenger receptor) MAP Kinase phosphatase 1 (MKP-1)
<u>Endothelin-1 receptors</u> Neurokinin NK ₁ -receptors NK ₂ -receptors	
<u>Adhesion molecules:</u> Intercellular cell adhesion molecule-1 E-selectin	

Table obtained from the *European Journal of Pharmacology* 500 (2004) 51-62. Effects

of glucocorticoids on gene transcription.

Cytokine production may be differentially altered by high circulating levels of endogenous or exogenous GCs. Studies have shown that physiologic cortisol levels decrease the expression of IL-12, but the level of IL-10 remains unchanged. During periods of stress the body increases the release of corticosteroids which may result in the activation of a Th2 response. One example is the decreased cellular immunity that occurs during pregnancy. Alternatively, treatment with exogenous corticosteroids has been shown to suppress IL-10.[76] It has been demonstrated that *in-vitro* treatment of rat T cells with dexamethasone induced IL-4 production.[77] However, the opposite effect has been observed in humans T cells treated with corticosteroids *in-vitro*.[78]

Nuclear-factor kappa B (NF- κ B) and activator protein-1 (AP-1) stimulate gene expression of many different inflammatory cytokines. Consequently, it seems likely that the mechanism of action of corticosteroids involves interfering with their activity.[79] Nuclear-factor kappa B affects a wide range of different cytokines, as well as chemokines and adhesion molecules. Only a limited number of cytokines is controlled by AP-1 protein. Nuclear factor-kappa B is located in the cytoplasm and it is normally inactivated by a protein called inhibitory-kappa B (I- κ B) which prevents its translocation into the nucleus.[79] The expression of IL-1 β and TNF is activated by NF- κ B. Increased levels of IL-1 β and TNF initiates lysis of the I- κ B protein allowing the translocation of NF- κ B into the nucleus and activating the inflammatory process.[80] One study showed that the production of inflammatory cytokines in the lung of foals infected with a virulent strain of *Rhodococcus equi* was mediated by activation of NF- κ B. It was also suggested that pathogenic bacteria may be directly responsible for this activation.[81]

Studies have demonstrated that GCs suppress the effects of NF- κ B by directly binding with it at the gene promoter region. Activity of RNA polymerase II is blocked resulting in decreased transcription.[82]

Several different types of stimulus are capable of activating NF- κ B such as, TNF, IL- 1β , viruses, and antigens. Authors have recently shown that phosphorylation of membrane receptors result in phosphorylation of I- κ B protein which forces it to be removed from the NF- κ B. It then moves freely into the nucleus of the cell to alter the expression of specific genes.[83] A second way in which GC attenuate cytokine gene expression is by transcriptional activation of the I- κ B protein gene. This results in an increased expression of I- κ B protein in the cytoplasm and decreased translocation of NF- κ B into the nucleus (figure 4).[84]

Another mechanism by which GCs attenuate inflammation is by inducing apoptosis in lymphoid cells, especially CD4⁺ and CD8⁺ thymocytes. However, circulatory T lymphocytes are reasonably unaffected. Osteoclasts, osteocytes, dendritic cells and some neuronal cells have also been shown to suffer apoptosis induced by corticosteroids. It is still unclear which mechanism leads to cell apoptosis.[85]

A small number of asthmatic human patients are nonresponsive to corticosteroids, needing increased doses to produce clinical improvement. Nonresponsive individuals may result from a defect in acetylation of histones in the DNA molecule impeding access of corticosteroids to target genes.[86]

1.7) Glucocorticoids: their use in equine diseases

Glucocorticoids are successfully used in the treatment of RAO. The general clinical impression is that different horses respond differently to the medication, some requiring higher doses to obtain significant clinical improvement. Systemic GCs commonly used in horses are dexamethasone and prednisolone. Dexamethasone is considered more potent and its clinical effect remains for a longer period of time. Cornelisse et al showed that oral administration of dexamethasone intravenous formulation provided even longer clinical effect in horses with RAO when compared to intravenous administration, especially if the horses were fasted prior to treatment. Peak activity for oral formulations was 24 hours. [87]

The dose of 0.1 mg/kg of dexamethasone intravenously once a day produce improvement in horses with RAO with the maximal result obtained after 3 days of therapy. Treatment was maintained daily for 10 days. Clinical improvement remained for one to two days after discontinuation of treatment. The number of neutrophils is significantly reduced in BAL samples of treated animals when compared to controls. [88, 89] Cornelisse et al also showed better pulmonary function values within four to six hours after initiation of intravenous dexamethasone treatment. Both intravenous and oral routes of drug administration provided a significant effect for up to 30 hours.[87]

Recently new formulations have permitted the use of corticosteroids by inhalation. Improvement of pulmonary function was observed in RAO affected horses treated with beclomethasone dipropionate by inhalation (3750 micrograms) twice a day for 2 weeks.[90] Inhaled fluticasone (2000 micrograms of fluticasone propionate every 12 hours) was effective and changed airway cell cytokine profile from Th2 to Th1 type.[60]

Glucocorticoids are often administered with bronchodilators.[36] This association is beneficial due to the fact that dexamethasone reverses the suppression of β_2 -adrenoceptors induced by bronchial inflammation and/or long term use of β_2 -agonists.[91]

Athletic horses frequently develop joint inflammatory conditions. The treatment with intra-articular corticosteroids is a successful approach to control pain associated with this condition.[58] Triamcinolone is a commonly used intra-articular steroid in performance horses to reduce or block the inflammatory cascade. Insulin-like growth factor is an anabolic drug that has been shown to increase cartilage healing. The effect of triamcinolone and insulin growth factor on equine cartilage treated with IL-1, which is known to induce or accelerate cartilage damage, has been evaluated in some studies. Beneficial effects of this combination were observed in studies using both *in-vivo* and *in-vitro* models. [92, 93]

Many skin allergic and inflammatory diseases are commonly treated with corticosteroids, such as dexamethasone, prednisolone and prednisone. The effectiveness of prednisone has been questioned due to poor gastrointestinal absorption of the medication.[94] Multisystemic eosinophilic epitheliotropic disease is an uncommon disease of horses and it is usually accompanied by a poor outcome. One recent report described clinical improvement in a case treated with dexamethasone.[95] Purpura haemorrhagica which is a disease caused by an immune mediated vasculitis is also commonly treated with corticosteroids.[96]

1.8) Glucocorticoids: side effects in the equine patient

The beneficial therapeutic effects of GCs are largely accepted. However, adverse effects are also frequently observed especially after prolonged therapies or use of high doses.

1.8.1) Adrenal suppression:

Adrenal suppression occurs when exogenous administration of corticosteroids causes negative feedback in the production of endogenous cortisol. This effect has been reported in the horse in many occasions.[97, 100, 101]

Horses affected by adrenal insufficiency show clinical signs of lethargy, low blood glucose levels, decreased appetite, poor hair coat and muscle weakness. Administration of high doses, potency of the drug and route of administration all influence the adrenal suppression response. Dexamethasone induced suppression of cortisol for five to seven days in horses, especially in those animals treated with higher doses (0.088 mg/kg, IM q 24 hours).[102] Interestingly, adrenal suppression was maintained for longer periods of time when the drug was administered via intramuscular versus intravenous injection.[101]

Because of the potential for adrenal suppression, corticosteroids are often delivered in tapering doses. This approach is also used to determine the lowest effective dose when long term treatment is required.[61]

1.8.2) Hepatic injury:

Liver injury has been reported in horses secondary to the treatment with corticosteroids. Evidence of liver damage is demonstrated by altered serum chemistry values such as, increased gamma glutamyltransferase, aspartate aminotransferase and bile acids.[97, 98] This adverse effect is often called steroid hepatopathy and it has been previously described in humans and dogs.[98]

1.8.3) Laminitis:

Laminitis has been reported as an adverse effect to the use of steroids on several occasions. Local digital vasoconstriction is believed to be activated by systemic corticosteroids leading to poor blood supply to the lamina. Cases of laminitis induced by steroids are frequently associated with the use of the drug triamcinolone. [103, 98]

1.8.4) Arthropathy:

Administration of corticosteroids in joints with some evidence of intra-articular pathology can result in progression or worsening of cartilage degradation.[104] Due to this potential side effect horses are usually rested after intra-articular injections and joints with significant pathology are avoided. There is also a high risk for joint infection, since defense mechanisms will be suppressed by the medication.[105]

Occasionally horses that receive an intra-articular steroid injection develop worsening of the inflammatory reaction instead of improvement of clinical signs. This is often observed 24 hours after drug administration. It likely results from a reaction to the delivery vehicle used in steroid drugs. Occasionally, there is calcification of tissues close

to the joint were the previous site of injection was located. This effect is common with the use of long acting corticosteroids. Iatrogenic calcification is called osseous metaplasia.[58]

1.8.5) Immunosuppression

Immunosuppression is a common sequela to the use of steroids, allowing secondary infections to take place or reactivating latent viral infections. Two factors that permit the occurrence of immunosuppression are: a) the decreased gene expression of inflammatory cytokines and, b) the retention of lymphocytes in the reticuloendothelial system.[106] When inflammatory cytokines are suppressed the communication between immune cells becomes compromised permitting pathogenic organisms to establish infection. Without the expression of adhesion molecules inflammatory cells are not capable of migrating to the site of injury.[107]

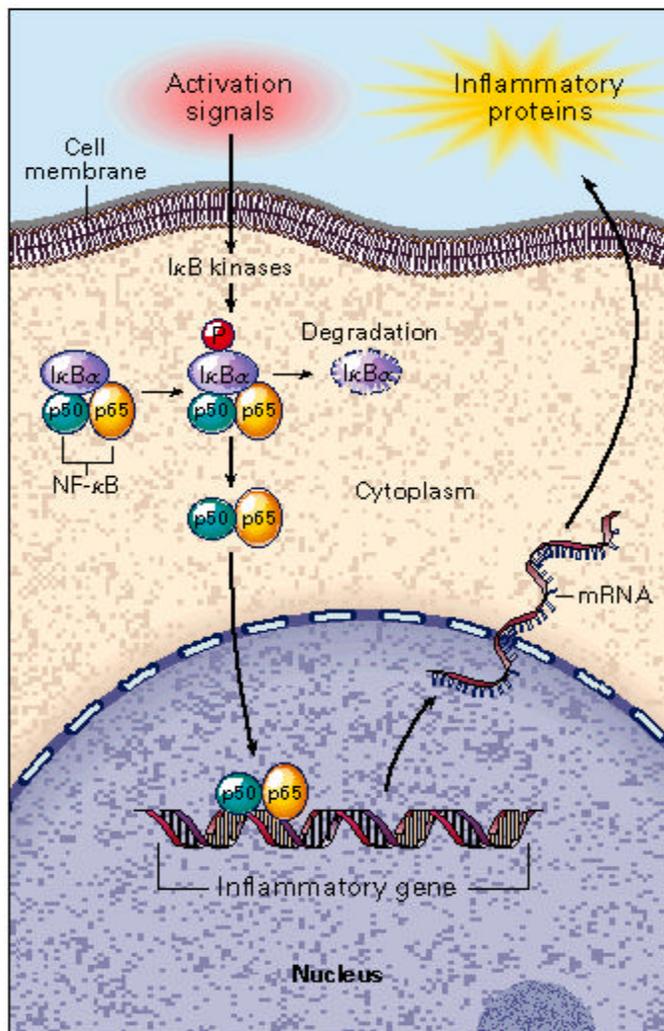
1.9) Future considerations:

Understanding of the mechanism of action of GCs will allow the development of similar antiinflammatory drugs without the undesirable side effects associated with many steroids. Nuclear factor-kappa B appears to be a key point in the control of inflammatory reactions. Inhibitory kinase-2 is an enzyme responsible for activating NF-kB. A drug that blocks the action of inhibitory kinase-2 has been developed but its clinical efficacy is still unknown.[83]

The knowledge obtained from studying cytokine expression in a variety of disease conditions provides the possibility of different therapeutic approaches. Polarization of T helper response may be manipulated in favor of resolution of the pathological condition. For example, in psoriasis, T helper 1 cells participate in the pathophysiology of the disease. Recent evidence suggests that manipulating the immune system by administering IL-4 provides improvement of the disease in humans.[53]

Diseases such as RAO, endotoxemia, parasitic conditions and others would potentially benefit from understanding how glucocorticoids affect cytokine expression. It would also be interesting to be able to correlate cytokine suppression with clinical improvement in RAO horses treated with GCs. Understanding the profile of cytokines responsible for clinical condition would allow us to select specific therapeutic strategies modulating the expression of those cytokines. Barnes suggested that antibody neutralization of IL-4 may improve humans patients affected with chronic airway obstruction (COPD) due to the fact that IL-4 promotes IgE production. Inhibition of IL-1 β was also suggested to block the inflammatory cascade initiated by this cytokine during the course of the disease. [51]

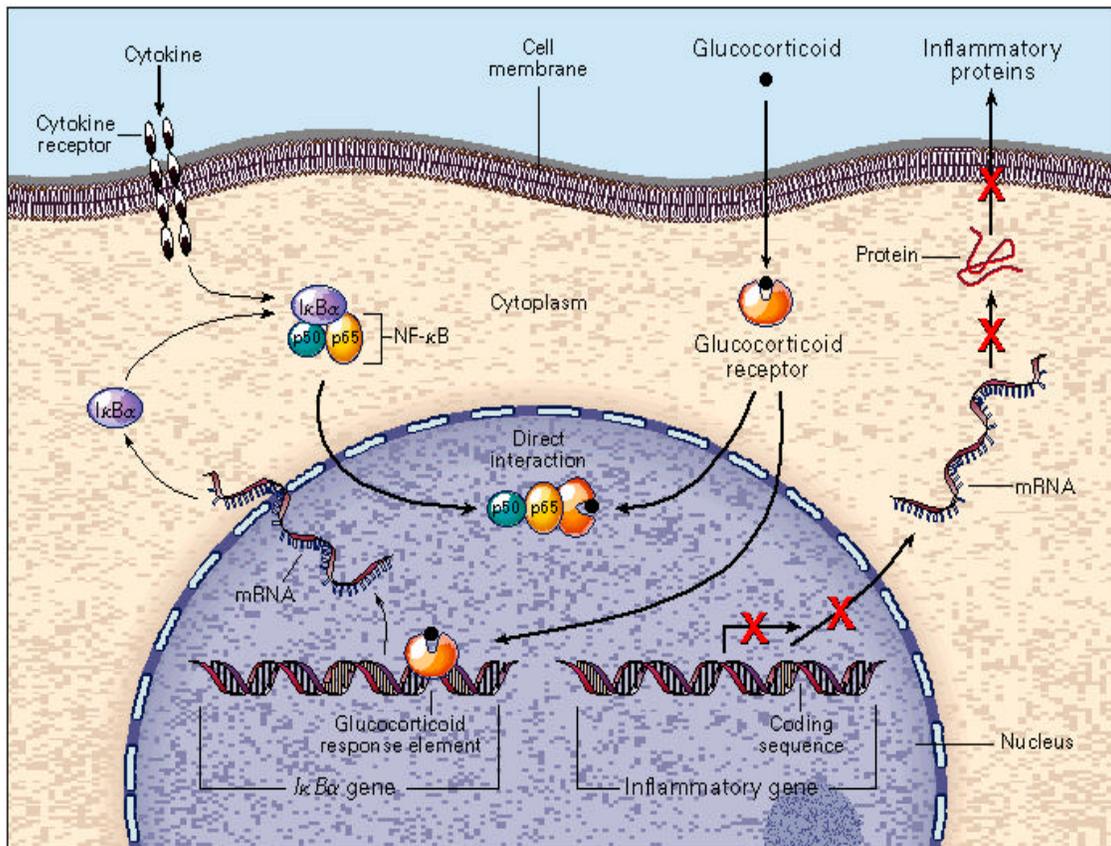
We hypothesize that dexamethasone treatment in horses will suppress the expression of IL-4, IL-6, IL-1 β and INF-gamma in our study due to the fact that corticosteroids are known to suppress these cytokines in humans and other animals. We also believe that the clinical improvement produced by corticosteroids is mediated by interference with these cytokines due to the important role they play in inflammation.



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Figure 3: Nuclear factor-kB activation.

Nuclear factor-kB is located in the cytoplasm and moves into the nucleus when the IκB protein is removed by specific enzymes. In the nucleus, NF-kB (composed of a p50 and p65 heterodimer) will affect the expression of genes in the DNA.



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Figure 4: Effects of glucocorticoids on nuclear factor-κB activation.

Glucocorticoid-receptor complexes have two mechanisms of action: 1) It blocks the NF-κB directly (binds to p65 heterodimer) once it has reached the nucleus; 2) It increases the expression of I-κB protein that moves back to the cytoplasm and inactivates NF-κB.

1.10) Hypothesis:

We hypothesize that dexamethasone treatment of healthy horses at a dose of 0.1 mg/kg intravenous once a day for 5 days will attenuate expression of IL-4, IL-6, IL-1 β and INF-gamma mRNA expression in a cytokine specific way and will be influenced by the duration of treatment. We also hypothesize that recovery of gene expression after treatment will occur at a rate that is cytokine specific.

1.11) Objectives

The objectives of this study were to evaluate the effect of dexamethasone treatment on cytokine expression in healthy horses, including IL-4, IL-6, IL-1 β and interferon-gamma compared to control horses treated with saline solution during a 5 day treatment and 5 day post-treatment period.

Specifically, the goals of this study were to:

- 1) Measure relative cytokine expression in activated peripheral blood mononuclear cells (PBMC) from normal horses treated with once a day intravenous dexamethasone, administered at a dose of 0.1 mg/kg. Gene expression was evaluated daily during 5 days of treatment and 5 days post treatment.
- 2) Measure relative cytokine expression in PBMC retrieved from horses receiving once a day intravenous saline (placebo) for 5 days and 5 post treatment days.
- 3) Compare relative cytokine expression in both treatment and placebo groups to determine if changes in gene expression were associated with treatment.

CHAPTER 2:

2.1) MATERIALS AND METHODS

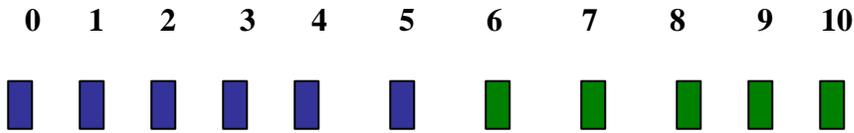
2.1.1) Horses

Sixteen clinically healthy horses from the University Research and Teaching herd, of various light riding breeds, and ranging from 6 to 22 years old were used in the study. Only normal horses, based on history, physical examination, and complete blood counts, were included. Horses were randomly assigned to treatment or placebo groups and randomly assigned to 2 distinct blocks: 1) the first block consisted of 8 horses that were turned out in pasture during the treatment period and sample collection; 2) the second block consisted of 8 horses that were housed in 12 x 12 foot stalls, with 12 x 14 foot attached outdoors pens for the duration of the study.

The data was analyzed as a generalized randomized complete block design, considering that there was more than one horse in each block, that they were randomly assigned and that each block had horses from each treatment group.

Diagram 1: treatment days

Days



Legend:

Day 0: prior to treatment/placebo

Days 1-5: Treatment/Placebo

Days 6-10: Post-treatment/Placebo

Table 1: Treatment and control horses, age, gender and breed

Horses	treatment	age	gender	breed
Horse 1	1	6 y	F	Morgan cross
Horse 2	0	19 y	F	Thoroughbred cross
Horse 3	1	12 y	F	Warmblood cross
Horse 4	0	26 y	F	Standardbred
Horse 5	0	6 y	F	Thoroughbred/Warmblood
Horse 6	1	22 y	F	Appaloosa cross
Horse 7	1	20 y	F	American Quarter-Horse
Horse 8	0	22 y	F	American Quarter-Horse
Horse 9	1	17 y	F	Morgan
Horse 10	1	12 y	F	Appaloosa cross
Horse 11	-	10 y	F	Thoroughbred
Horse 12	0	12 y	F	Thoroughbred
Horse 13	0	15 y	F	American Quarter-Horse
Horse 14	-	17 y	F	Thoroughbred
Horse 15	1	16 y	F	American Quarter-Horse
Horse 16	0	13 y	F	Arabian

Treatment 1= dexamethasone group; treatment 0= saline group; - horses removed from study; y= years

2.1.2) Treatments

Horses on block 1 were housed on pasture and treated at 8:00 AM. The horses on block 2 were placed in the research barn for a minimum of 24 hours before the study was initiated. In each block, 4 horses were randomly assigned to the treatment group and 4 were randomly assigned to the placebo group. On the first treatment day (Day 1), 5 x 10 mls of blood in acid citrate dextrose was taken by jugular venopuncture prior to the administration of treatment (Day 0). Horses in the treatment group then received 0.1 mg/kg of dexamethasone sodium phosphate by intravenous injection. Horses in the placebo group received an equivalent volume of sterile normal saline by intravenous route. Horses received their assigned treatments for 5 consecutive days.

2.1.3) Samples

On the first day, 50 mls of blood were removed from each horse prior to initiating treatment (day 0). Thereafter, 50 mls of blood in acid citrate dextrose was taken by jugular venopuncture 1 hour after administration of the medication or saline. On day 6, 50 mls of blood in acid citrate dextrose was taken by jugular venopuncture exactly 24 hours after the last dose was administered. Subsequently, blood was taken at 48, 72, 96 and 120 hours (5 days) after the last dose of dexamethasone or placebo was administered.

Blood samples were centrifuged at 500 RCF (relative centrifugal force) for 15 minutes, 27°C. The buffycoat from each tube was harvested and combined with an equal

volume of calcium and magnesium free Hanks buffer (CMFH)¹. This was layered over a 59 % Percoll gradient at a ratio of 5 mls of buffycoat-CMFH to 5 mls 59 % Percoll gradient. The loaded gradient was centrifuged at 940 RCF for 40 minutes, 27°C. After centrifugation, mononuclear cells were removed from the top of the gradient and washed twice with 30 ml of CMF DPBS (calcium, magnesium free Dulbecco's phosphate-buffered saline) and then the cells were re-suspended in RPMI² medium without fetal calf serum. Cell concentration was determined by manual count with a hemocytometer, and volumes were adjusted to produce a final cell concentration of 2×10^6 cells per ml of complete RPMI with 10 % heat activated fetal calf serum.

To evaluate the capacity of mononuclear cells to produce inflammatory cytokines, cells were stimulated with a mitogen. Peripheral blood mononuclear cells (PBMS) at a concentration of 2×10^6 cells per ml were placed in culture with Concanavalin A (ConA) at concentrations of 5 mcg/ml. Cells remained in culture for 4 hours, at 37°C, with 5 % CO₂.

2.1.4) Cell harvesting after incubation:

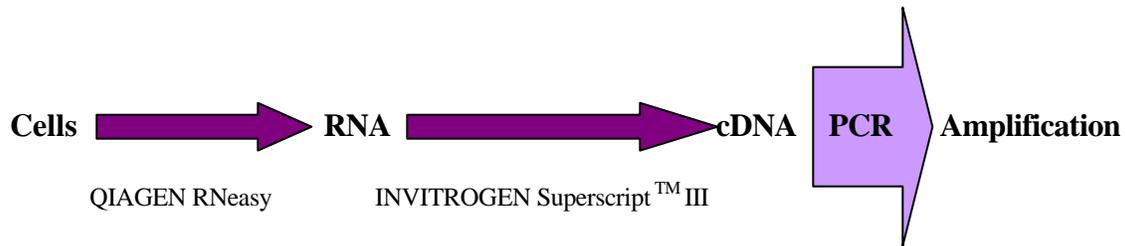
Approximately 10×10^6 cells were harvested per sample, washed with 8 to 10 ml of cold CMF DPBS and centrifuged at 4°C, 500 RCF for 15 minutes. The supernatant was discarded and another 10 mls of cold CMF DPBS was added. The sample was centrifuged a second time at 4°C, 500 RCF for 15 minutes. The supernatant was discarded and 1 ml of cold CMF DPBS was added and the sample vortexed. After the

¹ Hanks buffer – Grand Island, NY, 14072.

² RPMI medium without fetal calf serum – Grand Island, NY, 14072.

previous washing steps, 50 μ l was removed from the sample to determine the cell count. Cells were then transferred to a 1.5 ml (RNase and DNase free) microcentrifuge tube at a concentration of about 5×10^6 cells/ml. The sample was centrifuged in microcentrifuge (Eppendorf®) at 16,000 relative centrifugal force (RCF) for 2 minutes. The supernatant was aspirated and 1 ml of RNAlater® was added to the sample, vortexed and frozen at -80°C for future evaluation.

Figure 1:



2.1.5) RNA isolation:

RNA was isolated from the cell pellet using the QIAGEN RNeasy kit³. Residual genomic DNA was removed from the sample using the QIAGEN RNase-free DNase, which was added to the RNA isolation column during the RNA isolation process. RNA was quantified using an Eppendorf Biophotometer⁴.

³ QIAGEN RNeasy kit – cat. # 74104, Valencia, CA.

⁴ Eppendorf Biophotometer – Brinkman Instruments, Inc., Westbury, NY.

ConA stimulated samples for IL-1 β , INF-gamma, IL-4 and IL-6 saved in RNA laterTM were thawed and washed in approximately 1 ml of cold CMF DPBS, and centrifuged (Eppendorf centrifuge) at 16,000 RCF for 2-3 minutes at - 20°C.

The supernatant was aspirated and 1 ml of buffer RLT (RNeasy Lysis Buffer) was mixed with 10 μ ls of β -mercaptoethanol. Six hundred μ ls of the RLT with β -mercaptoethanol was added to the sample if the cell count was between 5×10^6 - 10×10^6 cells, and 350 μ ls were added if the cell count was less than 5×10^6 cells.

The sample was pipeted several times until it was homogeneously mixed. Then an equal volume of 70% ethanol was added to the homogenized lysate and mixed well by pipeting. Seven hundred μ ls of the sample was transferred into a RNeasy® mini column that was placed in a 2 ml collection tube and centrifuged for 15 seconds at 8,160 RCF. The flow-through was discarded. Then 350 μ ls of buffer RW1 was added into the RNeasy® mini column and centrifuged for 15 seconds at 8,160 RCF. The flow-through was again discarded.

DNase I stock solution (12 μ ls) was mixed with 70 μ ls of buffer RDD mix to prepare a DNase incubation mix. The DNase incubation mix (82 μ ls) was pipeted directly into the RNeasy® silica-gel membrane and rested for 30 minutes. Then 350 μ ls of buffer RW1 was added into the RNeasy® mini column and centrifuged for 15 seconds at 8,160 RCF. The flow-through was discarded. This step was performed to remove any residual genomic DNA.

The RNeasy® mini column was transferred into a new 2 ml collection tube. Then 500 μ ls of buffer RPE was added onto the RNeasy® mini column and centrifuged for 15 seconds at 8,160 RCF to wash the column. The flow-through was discarded. Another 500

µls of buffer RPE was added onto the RNeasy® mini column and centrifuged for 2 minutes at 8,160 RCF to dry the silica-gel membrane.

Finally, to isolate the RNA the RNeasy® column was removed and transferred to a new 1.5ml collection tube. Fifty µls of RNase free water was pipeted directly into the silica-gel membrane and the tube was centrifuged for 1 minute at 8,160 RCF. The flow-through was removed and added back into the silica-gel membrane for another centrifugation step of 1 minute at 8,160 RCF. The filter was discarded and the RNA left in the tube was frozen at - 80°C.

2.1.6) cDNA isolation:

The cDNA first strand synthesis was achieved using INVITROGEN Superscript™ III⁵, and following the manufacturer's protocol, making a RT⁺ (reverse transcriptase positive sample) and a RT⁻ (negative reverse transcriptase - sample that did not contain reverse transcriptase enzyme). The RT⁻ reaction was performed to confirm mRNA dependence of target detection.

The protocol for cDNA synthesis using INVITROGENS Superscript™ III was designed to convert from 1 pcg to 5 mcg of total RNA into first strand cDNA. The protocol consisted of mixing 5 µg of total RNA with RNase/DNase Free water to equal a total volume of 8 µls, and adding that mixture to 1 µl of 50µM oligo (dT)₂₀ and 1 µl of 10µM dNTP mix. The sample was incubated at 65°C for 5 minutes and then the sample was placed on ice for 1 minute to stop the reaction.

⁵ INVITROGEN Superscript™ III – Carlsbad, CA.

cDNA Synthesis master mix (10 µls), which consisted of 2 µls of 10 x RT buffer, 4 µls of 25 mM MgCl₂, 2 µls of 0.1 M DTT, 1 µl of RNaseOUT (40U/mcl) and 1 µl of SuperScript III RT (200 U/mcl), was added to each RNA/primer mixture. The negative RT samples did not contain the SuperScript III RT (reverse transcriptase enzyme) in the cDNA Synthesis master mix. The negative RT samples should not amplify a product in the PCR reaction due to the fact that it does not contain reverse transcriptase to form cDNA. If a band is amplified from the RT⁻ samples, it indicates the presence of contaminant DNA. Samples were then incubated in 50°C water bath for 50 minutes. The reaction was terminated by heating on heat block at 85°C for 5 minutes and chilling it on ice for 2 minutes. Rnase H (1 µl) was added to each tube, homogenized by pipetting and incubated at 37°C for 20 minutes. The samples were finally frozen at - 80°C.

2.1.7) Primer design:

Primer pairs specific for each of the cytokines were designed using published equine cytokine sequences available on GenBank (table 2). The primers were designed using the BECON designer 2.0 primer designer software⁶.

Primers were designed with a GC content of 50-60% and the annealing temperature was maintained between 50 and 60°C. Primer dimer formation was avoided by checking the sequence of forward and reverse primers, amplicon length was kept around 75-150 base pairs and each primer was between 18-24 base pairs to maximize PCR efficiency.

⁶ BECON designer 2.0 - Biosoft International, Palo Alto, CA

2.1.8) Standards: purification of plasmid DNA

The standards for each cytokine were obtained from cloned genes (Dr. Crisman's laboratory, Maryland-Virginia Regional College of Veterinary Medicine, Blacksburg VA) for IL-1 β , INF-gamma, IL-4 and IL-6.

E. coli cells containing each cloned gene were added to 10 mls of LB broth. Cultures were incubated at 37°C for 18 hours. After incubation 1.5 mls of each culture was added to a 2 ml microcentrifuge tube and centrifuged at 16,000 RCF for 5 minutes. The supernatant was discarded and another 1.5 mls of culture was added. The sample was centrifuged again at 16,000 RCF for 5 minutes. The pellet was purified using QIA prep⁷ miniprep kit.

The pellet was resuspended in 250 μ ls of buffer P1 (with RNase A) and transferred to a microcentrifuge tube. Buffer P2 (250 μ ls) was added and mixed by inverting the tube 4 to 6 times. Then 350 μ ls of buffer N3 was added and also mixed by inverting the tube gently 4 to 6 times. The solution was centrifuged at 13,800 RCF forming a compact white pellet. The supernatant was applied to the QIAprep Spin column by pipetting and then centrifuging again for 30 to 60 seconds. The flow-through was discarded.

The QIAprep Spin column was washed by adding 0.5 mls of buffer PB and centrifuged for 30-60 seconds. The flow through was discarded. This step was necessary to remove any trace of nuclease activity. The QIAprep Spin column was washed again by adding 0.75 μ ls of buffer PE and centrifuging for 30-60 seconds. The flow-through was

⁷ QIAGEN, Valencia, CA.

discarded and the column was centrifuged for an additional 1 minute to remove any residual wash buffer.

The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute the purified plasmid 50 μ ls of distilled water (RNase/Dnase Free) was added to the center of the column, allowed to stand for 1 minute, then centrifuged at 16,000 RCF for 1 minute.

Restriction enzymes were prepared to extract the cloned sequences from the purified plasmids. They remove the cloned gene sequence out of the plasmid by cutting the DNA on specific points called recognition sequence. Finally, the cloned sequences removed from the plasmid were run in a 1% agarose gel at 60 volts (25 μ ls of cloned sequences with 6 μ ls of loading dye-bromophenol xylene cyanol) for 3 hours to verify the correct size (specific base pairs number) of each cytokine cloned sequences. All cytokines demonstrated the expected fragment sizes consistent with the plasmids containing the cytokines of interest.

2.1.9) Standard: quantification and qualification

The purified plasmids obtained from the previous step were diluted (1:100) in distilled water (5 μ ls added to 495 μ ls of distilled water-RNase/DNase Free) to be used in PCR reactions.

Each PCR reaction contained 2 x QIAGEN mix 50 μ ls with 4 μ ls of template (diluted 1:100), 2 μ ls of primer sense and 2 μ ls of primer antisense for each specific cytokine.

PCR samples were run with the following protocol: 15 minutes at 95°C, 32 cycles of (30 seconds at 94°C, then 1 minute and 30 seconds at 60°C and 1 minute and 30 seconds at 72°C), and then 10 minutes at 72°C. All samples were quantified and qualified by a Bioanalyser⁸.

2.1.10) Real time PCR reaction:

IQTM SYBR[®] Green super mix⁹ was used for real-time PCR applications. The syber green dye will bind to double stranded molecule of DNA during the PCR amplification. As the cDNA amplification occurs during the PCR reaction the fluorescence increases secondary to increase amount of syber green bound to DNA. The mRNA quantity is estimated based on internal standards curves for each cytokine.

Samples were loaded (25 µl) into a 96 well plate for the real-time PCR reactions. Real-time amplification was performed using a BioRad (iCycler)¹⁰. Specific annealing temperatures were set for each cytokine based on the optimal annealing temperature determined by the temperature gradient analysis protocol.

The plates were set up with all the standards for one cytokine contained in each plate. The standards were run as triplicates at five different concentrations with a 10 fold dilution starting at 5 pcgs. Each plate contained samples from 0 to 10 days for each horse (2 horses per plate) and duplicates of the negative RT (reverse transcriptase) samples for

⁸ Bioanalyser - Agilent Technologies 2100 Bioanalyzer, Bio sizing, Version A.02.12 SI292

⁹ IQTM SYBR[®] Green super mix – Hercules, CA, 94547.

¹⁰ BioRad (iCycler) – Hercules, CA, 94547.

days 0 and 5 for each horse. Duplicates of negative controls were also run in each plate. These contained H₂O as a template (substituting cDNA).

The objective of running negative controls in each plate was to ascertain if there was DNA contamination in any of the reagents used in the PCR reaction. Also the negative RT samples were another form of control to verify the presence of genomic DNA possibly contaminating the sample. With the absence of the enzyme reverse transcriptase cDNA was not synthesized. Consequently, no amplification should occur during the PCR reaction unless the sample was contaminated with genomic DNA. The negative RT samples verify the purity of the RNA template and the negative control with H₂O as a template verifies the purity of the reagents.

The samples were arranged in each PCR plate (figure 2) with all the standards dilutions for one cytokine, samples for two horses including all 10 days for each horse and negative controls for both horses in each plate. This arrangement permitted that samples from each horse for all days of the experiment was compared with its own standards in the same plate, minimizing variation between plates. The arrangement of samples for each day for each horse in the same plate also minimized variation within the same horse.

B-actin was used as “house keeping gene”. It’s expression should not change over time; particularly, it should not change due to the influence of corticosteroids. All samples of cytokine expression were calculated as a percentage of β-actin. β-actin was an internal control for constant gene expression to compare the expression of different cytokines over time. Our data showed that β-actin expression was suppressed in the treatment group compared with the control group. Suppression of β-actin was statistically

significant for the treatment effect group. However, when the ratio (cytokine/ β -actin) for each cytokine was obtained the ratios were mildly increased. That effect would not be a concern considering that the data obtained for treated horses shows the opposite effect of suppression of cytokines.

Each PCR reaction consisted of a 3 step melt protocol as follows: 95 °C for 3 minutes; 40 cycles of 10 seconds at 95 °C followed by 15 seconds at 55 °C (annealing temperature) followed by 20 seconds at 72 °C (extension step); then 1 minute at 95 °C, followed by 1 minute at 55 °C; then 80 cycles were run at 55 °C for 10 seconds each to determine the melt curve.

The amount of fluorescence was measured for each sample during the PCR reaction. The amount of mRNA expression for each cytokine was estimated from the different standard dilutions.

Figure 2: Real-Time PCR plate setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	SD 5pg	SD 0.5pg	SD 0.05 pg	SD 0.005 pg	SD .0005p g	Horse 1 Day 0	→	→	Horse 1 Day 1	→	→	X
B	↓	↓	↓	↓	↓	Horse 1 Day 2	→	→	Horse 1 Day 3	→	→	X
C	↓	↓	↓	↓	↓	Horse 1 Day 4	→	→	Horse 1 Day 5	→	→	X
D	Horse 1 Day 6	→	→	Horse 1 Day 7	→	→	Horse 1 Day 8	→	→	Horse 1 Day 9	→	→
E	Horse 1 Day 10	→	→	Horse 2 Day 0	→	→	Horse 2 Day 1	→	→	Horse 2 Day 2	→	→
F	Horse 2 Day 3	→	→	Horse 2 Day 4	→	→	Horse 2 Day 5	→	→	Horse 2 Day 6	→	→
G	Horse 2 Day 7	→	→	Horse 2 Day 8	→	→	Horse 2 Day 9	→	→	Horse 2 Day 10	→	→
H	Horse 1 -RT Day 0	→	Horse 1 -RT Day 5	→	Horse 2 -RT Day 0	→	Horse 2 -RT Day 5	→	Neg Contr	→	Neg Contr	→

Legend:

- * SD indicates standard samples
- * - RT indicates negative reverse transcriptase samples
- * Neg Contr. indicates negative control samples
- * pg indicates picograms
- * X indicates wells not used.
- * → indicates repeats of the sample

Table 2: Sequences for equine cytokine-specific primer pairs

Cytokine	Primer sequence	Accession number (Genbank)
IL-1 β	Sense: CAGCAGGCGGGTGGAG Anti-sense: TGAGTAGCAGAGGTGAGAGG	U92481
IL-4	Sense: AATGCCTGAGCGGACTG Anti-sense: TGCTCTTCTTGCTTCATTC	L06010
IL-6	Sense: TCACTCCAGTTGCCTTCTC Anti-sense: AGGTGGTTTCATCTTCTCCC	U64794
INF-gamma	Sense: TGAAGGTCCAGCGCAAAGC Anti-sense: CTGACTCCTCTTCCGCTTCC	D28520
B - actin	Sense: TGGACTTCGAGCAGGAGATG Anti-sense: CGTTGCCGATGGTGATGAC	AF035774

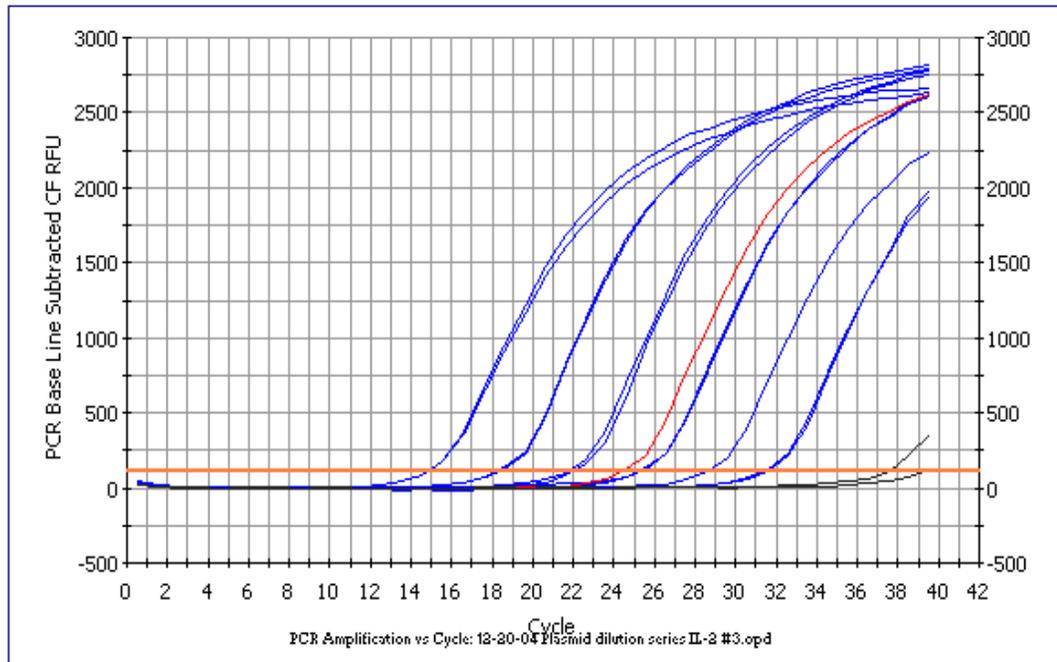
2.2) Statistical analysis

The variance in mRNA expression for both treatment and control horses was evaluated between groups of horses and within groups. A mixed-model repeated measures ANOVA (analysis of variance) was used to estimate the variance among the 14 horses.

The model tested for effects of treatment, time and treatment by time interaction. The model also corrected for different blocks of horses, starting quantity of mRNA, different plates and baseline values for each horse by including them as co-variantes. The data was corrected for baseline values (Day 0) to compensate for individual horse variation. The means were compared between treatments within each time. The mixed procedure of the SAS system was used for calculating the ANOVA. All comparisons were considered significant at $p < 0.05$.

First a standard curve was estimated using the mean CT of the 3 repeats for each ten fold concentration of the standards, and calculating that value against the known concentration of that sample. An estimate of starting mRNA quantity was made by determining the mean CT value of the three repeats and calculating relative mRNA quantity from the standard curve. Relative starting quantity was calculated by dividing estimated starting mRNA quantity by the estimated starting mRNA quantity of β -actin (presumed constitutively expressed gene). These results were then log transformed to stabilize variation. Note on diagram 2 that the sample (red curve) falls into the range of standard curves.

Diagram 2: PCR amplification of standards shown by blue color curves (10 fold dilution of each standard separated by 3.5 cycles). The red curve represents PCR amplification of one sample. Y axis denotes amount of fluorescence emitted and X axis denotes number of cycles during the PCR reaction.



CHAPTER 3: RESULTS

The complete blood counts and serum biochemistry profiles did not reveal any significant abnormalities in any of the horses prior to initiation of the study. Daily physical exams were within normal limits for all horses, except for horse number 14. Horse 14 developed cellulitis on the right hind limb and had to be removed from the study. Horse 11 was also removed from the study due to erroneous drug administration. No significant reaction was noted at the site of intravenous injection in all horses.

3.1) Interleukin-4:

When compared to controls, IL-4 mRNA expression (samples with Con A) in horses treated with dexamethasone showed significantly lower values at days 2, 3, 4 and 6 (table 1 and figure 1). On day 7 mRNA expression for IL-4 for horses treated with dexamethasone returned to values similar to controls and on days 8, 9 and 10 mRNA expression was higher when compared to controls but not statistically significant (figure 1).

Table 1: F values and P values for interleukin-4

IL-4 Treatment day	F values	P values (significant if P<0.05)
Day 1	0.82	0.3686
Day 2	7.84	0.0061 *
Day 3	13.56	0.0004 *
Day 4	7.8	0.0062 *
Day 5	2.50	0.1170
Day 6	6.67	0.0111 *
Day 7	0.06	0.8056
Day 8	3.28	0.0727
Day 9	1.17	0.2810
Day 10	3.32	0.0710

* denotes significant values

Interleukin 4

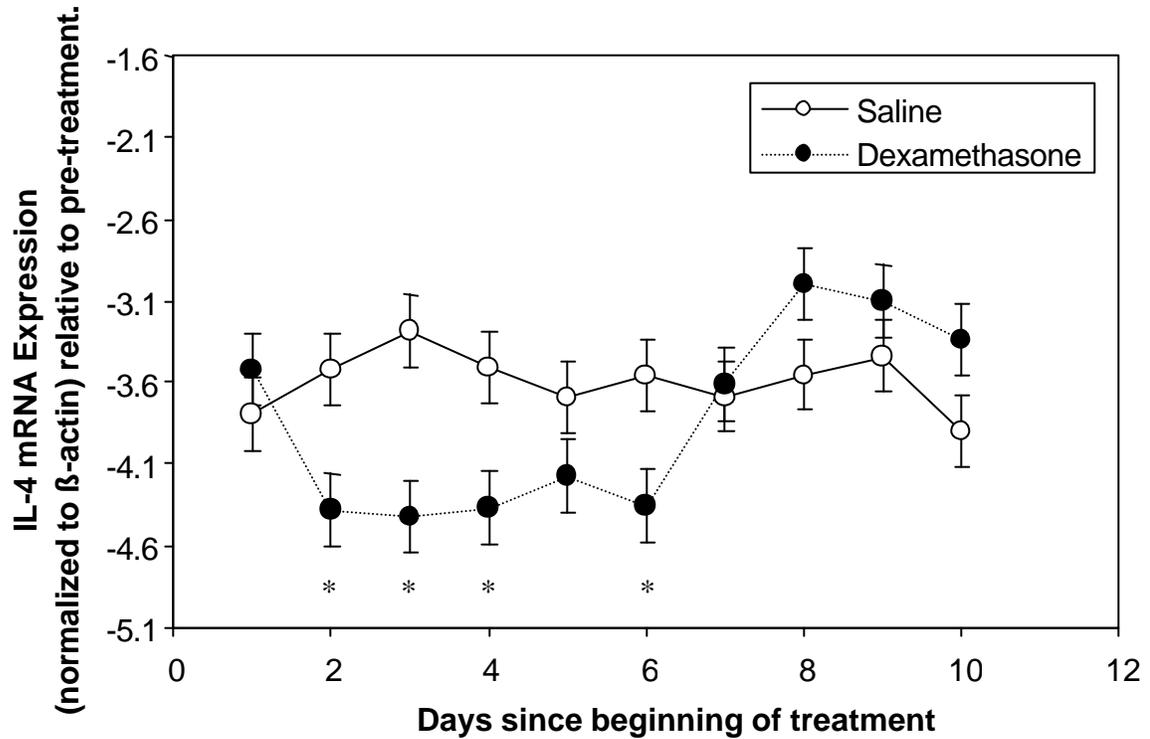


Figure 1: Linear model of interleukin-4 mRNA expression in horses treated with dexamethasone (0.1 mg/kg, intravenously daily for 5 days) compared to control (saline treated horses).

3.2) Interleukin-6:

Interleukin-6 mRNA expression (samples with Con A) in horses treated with dexamethasone showed significantly lower values at days 2, 3, 4, 5 and 6 (table 2 and figure 2), compared to control group. On day 7 mRNA expression for IL-6 for horses treated with dexamethasone returned to values similar to controls and on days 8 and 9 mRNA expression was higher when compared to controls but not statistically significant (figure 2). On day 10 however, mRNA expression for IL-6 was significantly higher when compared to the control group.

Table 2: F values and P values for interleukin-6

IL-6 Treatment day	F values	P values (significant if P<0.05)
Day 1	0.89	0.3473
Day 2	10.98	0.0013 *
Day 3	15.68	0.0001 *
Day 4	12.17	0.0007 *
Day 5	10.08	0.0020 *
Day 6	3.94	0.0497 *
Day 7	0.34	0.5627
Day 8	2.33	0.1294
Day 9	2.42	0.1225
Day 10	6.78	0.0105 *

* denotes significant values

Interleukin-6

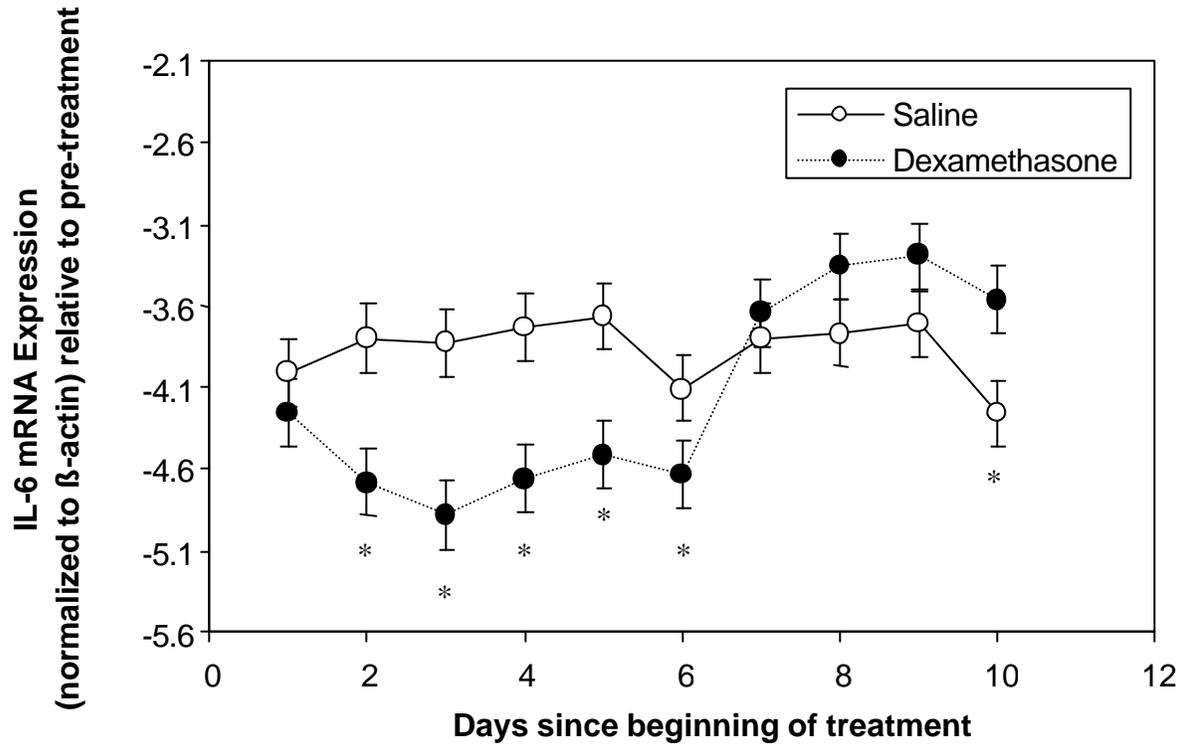


Figure 2: Linear model of interleukin-6 mRNA expression in horses treated with dexamethasone (0.1 mg/kg, intravenously daily for 5 days) compared to control (saline treated horses).

3.3) Interferon- gamma:

Interferon-gamma mRNA expression (samples with Con A) in horses treated with dexamethasone showed significantly lower values at days 2, 3, 4, 5 and 6 (table 3 and figure 3) compared to control group receiving saline . On day 7 mRNA expression for INF-gamma for horses treated with dexamethasone was still lower when compared to the control group but higher than values obtained on day 6. On days 8 and 9 mRNA expression was almost equal to the control group (figure 3). On day 10 INF-gamma mRNA expression in the treatment group was higher than control group but not statistically significant.

Table 3: F values and P values for interferon-gamma

Interferon-gamma Treatment day	F values	P values (significant if P<0.05)
Day 1	2.14	0.1468
Day 2	6.14	0.0148 *
Day 3	26.10	< 0.001 *
Day 4	17.98	< 0.001 *
Day 5	21.26	< 0.001 *
Day 6	14.57	0.0002 *
Day 7	1.35	0.2484
Day 8	0.00	0.9624
Day 9	0.01	0.9389
Day 10	1.01	0.3164

* denotes significant values

Interferon-gamma:

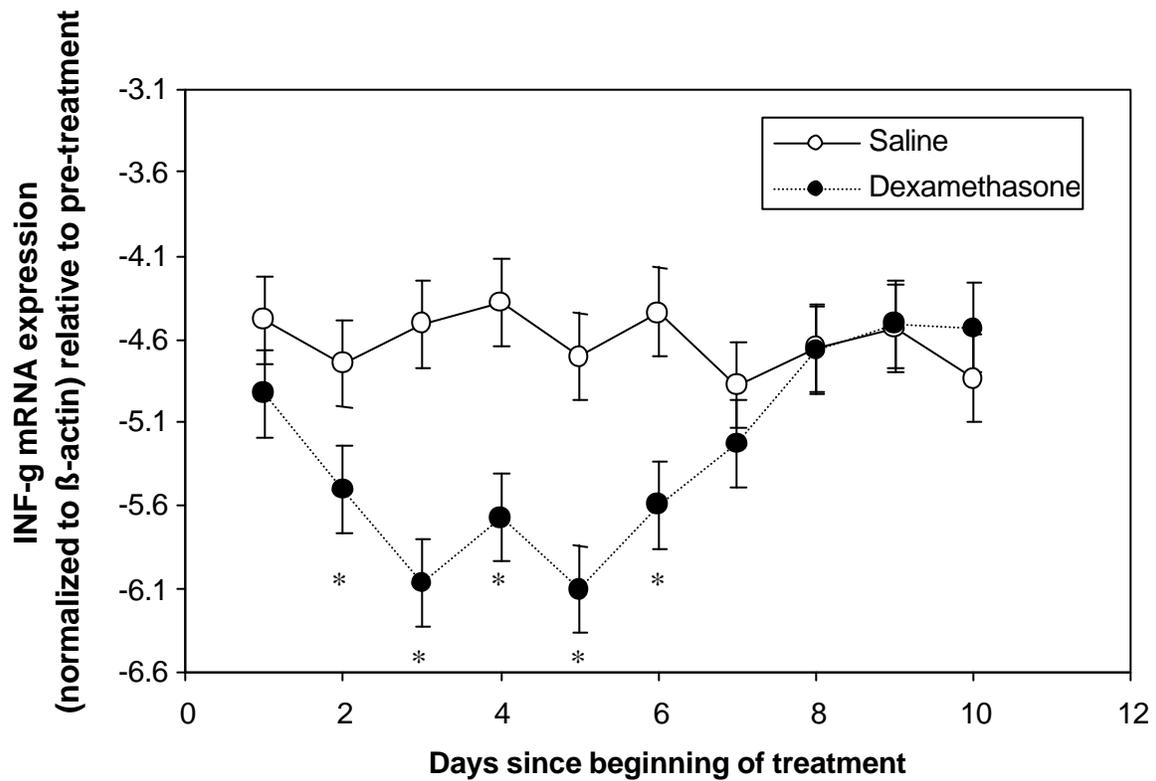


Figure 3: Linear model of interferon-gamma mRNA expression in horses treated with dexamethasone (0.1 mg/kg, intravenously daily for 5 days) compared to control (saline treated horses).

3.4) Interleukin-1 β :

Interleukin-1 β mRNA expression (samples with Con A) in horses treated with dexamethasone showed significantly lower values only at day 5 (table 4 and figure 4) compared to control group receiving saline. On day 6, mRNA expression for IL-1 β in the treatment group was close to values obtained in the control group.

Table 4: F values and P values for interleukin-1 β

Interleukin-1β Treatment day	F values	P values (significant if P<0.05)
Day 1	0.06	0.8037
Day 2	0.45	0.5026
Day 3	1.85	0.1772
Day 4	3.34	0.0703
Day 5	4.79	0.0308 *
Day 6	0.08	0.7800
Day 7	0.11	0.7455
Day 8	0.03	0.8599
Day 9	0.55	0.4618
Day 10	0.93	0.3383

* denotes significant values

Interleukin-1 β :

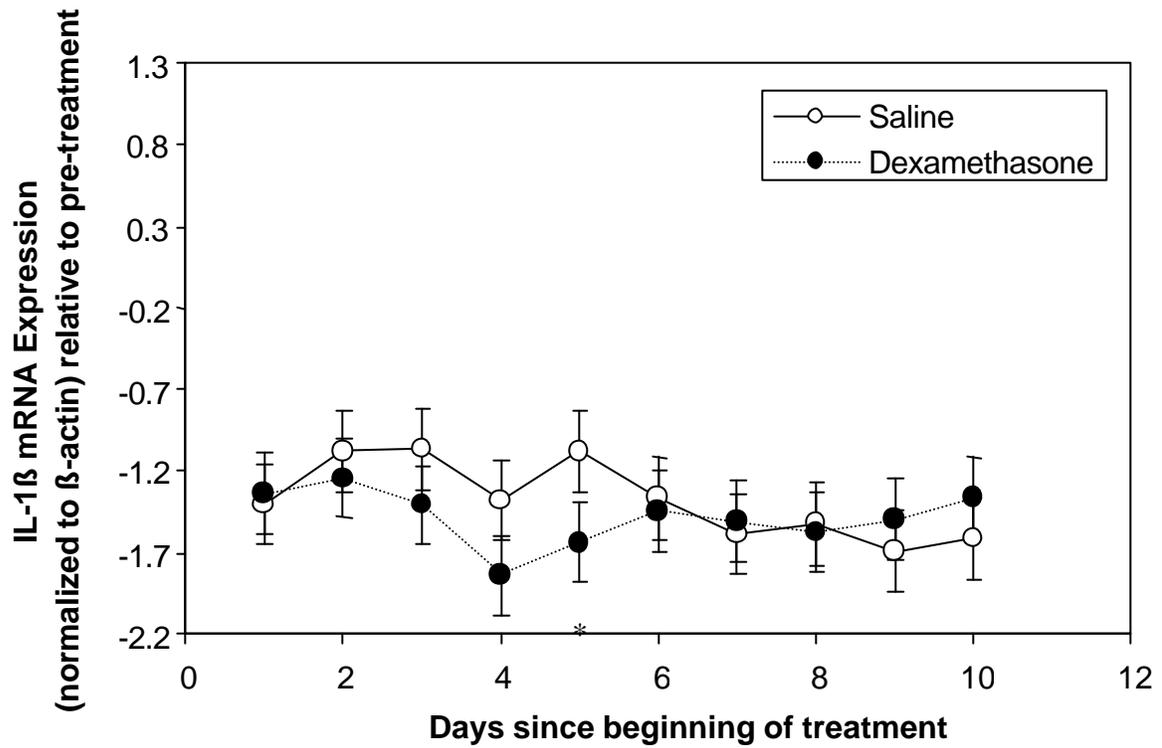


Figure 4: Linear model of interleukin-1 β mRNA expression in horses treated with dexamethasone (0.1 mg/kg, intravenously daily for 5 days) compared to control (saline treated horses).

CHAPTER 4: DISCUSSION

The effect of dexamethasone treatment on cytokine expression has not been completely studied in horses. It is one of the most extensively used steroids for treatment of a variety of inflammatory, allergic and auto immune diseases in equine patients. Understanding of its effect on cytokine expression in horses could provide important information regarding dose and duration of treatment on specific disease processes.

4.1) Interleukin-4:

Interleukin-4 is an important cytokine in the pathogenesis of allergic diseases because it enhances the T helper 2 response, which is responsible for activating the production of immunoglobulins. Immunoglobulin IgE primary function is to degranulate mast cells and initiate an inflammatory reaction characteristic of allergies. Down regulation of IL-4 may provide a key interruption in this inflammatory process. This pathway may explain why GCs are successfully used in the treatment of allergic conditions. In humans there is some evidence that GCs inhibit the production of IL-4 and IL-5 in mast cells.[108] Glucocorticoids have also been shown to suppress the expression of IL-4 receptor on the surface of immune cells in treated human patients.[109]

All of these studies in humans agree with the findings of our study that IL-4 expression is suppressed in peripheral blood mononuclear cells obtained from healthy horses treated with dexamethasone. IL-4, especially in the presence of IL-6 drives the

immune response towards a Th2 phenotype. Suppression of both of these cytokines should result in attenuation of T cells developing a Th2 phenotype.

Interleukin-4 did not show significant suppression of day 5. This effect could have been caused by an outlier or technical error especially because the suppression was then observed on day 6.

4.2) Interleukin-6:

Another cytokine that may contribute to the pathophysiology of allergic conditions such as human asthma or RAO in horses is IL-6. It was found that bradykinin activates the release of interleukin-6 from the airway smooth muscle cells via β_2 receptor. Treatment with dexamethasone blocked the release of interleukin-6 mediated by bradykinin.[110]

In our study IL-6 was suppressed in cells from horses treated with dexamethasone compared to cells from horses treated with placebo. The down regulation of IL-6 may interrupt the activation of a T helper 2 response resulting in a decreased production of immunoglobulins. In cases of allergic diseases IgE would be the main targeted immunoglobulin. Other disease processes that involve the excessive production of immunoglobulins such as auto immune diseases may also benefit from suppression of IL-6. Interleukin-6 is known to be an important inflammatory cytokine in the pathogenesis of equine infectious anemia, and in the development of endotoxemia. It also appears to be an important stimulus for hematopoietic cell production in the bone marrow.[37, 39, 40, 41, 43]

4.3) Interleukin-1 β :

Interleukin-1 β is one of the first cytokines released by macrophages during the beginning of the inflammatory cascade. It participates in the activation of many immune cells and also increases production of other cytokines and adhesion molecules.

Understanding the effect of corticosteroids on IL-1 β expression is interesting to us due to its role in the initiation of an inflammatory reaction and innate immunity.

In humans it appears that IL-1 gene expression is suppressed by the use of corticosteroids. Authors have shown a reduction in the expression of IL-1 α , IL-1 β and IL-6 by the use of dexamethasone in human mononuclear cells in a dose dependent manner. It was also found that the addition of steroid receptor antagonist RU486 to the cells was able to reverse the effect.[111]

In our study IL-1 β was not significantly suppressed by treatment with dexamethasone. It may be that IL-1 β needs 5 days of treatment to show suppression and that it returns to normal values soon after discontinuation of the medication. However, it seems questionable that the suppression produced on day 5 would be clinically significant. It is also possible that dexamethasone does not play a significant role in suppressing innate immunity. Horses with RAO respond well to treatment with dexamethasone so it may be that IL-1 β suppression is not detrimental for the control of the disease. Also our samples were composed of mostly lymphocytes which are not the main cells responsible for the production of IL-1 β .

4.4) Interferon-gamma:

Interferon-gamma is a key cytokine in the activation of a cell mediated immune response, such as viral infections. In our study, treatment of horses with dexamethasone produced suppression of this cytokine. This effect may contribute to the immunosuppression frequently observed in cases where steroids are used for a prolonged period of time. It may predispose horses to opportunistic viral and bacterial infections due to the suppression of cellular immunity.

Some authors have suggested that the T helper 1 response is inhibited and that the T helper 2 response is enhanced by the use of corticosteroids. This opinion is based on the fact that corticosteroids are known to block the production of INF-gamma which is a cytokine that suppresses T helper 2 cell proliferation. However, this suggestion is questionable because the expression of IL-4 (a cytokine release by T helper 2 cells) is also usually inhibited by the drug.[112, 113] One study proposed that the mechanism responsible for the change from T helper 1 to T helper 2 response during pregnancy may be induced by progesterone and glucocorticoids.[112]

4.5) Time course effect of dexamethasone on cytokine expression:

In our study, we demonstrated that dexamethasone treatment in horses (at the dose of 0.1 mg/kg, given intravenously every 24 hours) significantly suppressed IL-4, IL-6 and INF-gamma mRNA expression in peripheral blood lymphocytes. In all of these cytokines the suppression was first observed on day two of treatment and it lasted for

approximately three to four days during treatment. This finding suggests that treatment of healthy horses with corticosteroids reduces inflammatory cytokines. This may partially explain the mechanism of action controlling inflammation in equine patients.

Suppression of cytokine expression (IL-4, IL-6 and INF-gamma) by dexamethasone was apparent 24 hours after administering treatment, which usually correlates with the time maximal improvement of clinical signs is observed in horses affected by RAO. It also appeared that different cytokines showed a different pattern of suppression overtime.

After the last day of dexamethasone treatment (day 5) IL-4, IL-6 and INF-gamma still showed significant suppression for 24 hours. The prolonged effect of cytokine suppression observed in horses treated with dexamethasone suggests that it is reasonable to treat horses on an every other day regimen and still observe clinical improvement or no progression of clinical signs.

Clinically, this effect is specially seen in horses treated with dexamethasone for RAO, although these horses need to be treated for consecutive days initially in order to allow treatment regimen to be changed to every other day interval. Maybe consecutive daily treatment is needed to produce significant cytokine suppression to permit alternate treatment. It is also known that IL-4 is over expressed in horses affected by RAO, which suggests that the suppression of this cytokine is a mechanism by which corticosteroids interfere with inflammation in RAO affected horses.

4.6) Th1 and Th2 phenotypes affected by dexamethasone:

The response produced by T helper cells is divided into T helper 1 and T helper 2. Interferon-gamma is produced by T helper 1 cells which participate in cellular immune

reactions. Interleukin 4 and 6 are produced by T helper 2 cells and are involved in humoral responses.[13, 10] Our study showed that dexamethasone could suppress antibody production in disease processes, due to the suppression of IL-4 and IL-6, which are Th2 induced cytokines. It is possible that dexamethasone affects T cell maturation into a Th1 or Th2 type phenotypes since both Th1 and Th2 responses are suppressed with dexamethasone treatment.

Interleukin-1 β is primarily secreted by macrophages during the inflammatory process. In this study interleukin-1 β was not significantly suppressed by dexamethasone treatment, suggesting that glucocorticoids may have a more profound effect on antibody production or cell mediated immunity rather than affecting innate immunity. Also, our cell population contained mainly lymphocytes rather than macrophages, which are the cells mostly responsible for the production of IL-1 β . It is difficult to make such conclusions without evaluating the effect of dexamethasone in the expression of all of the other cytokines involved in the inflammatory process. Also mRNA expression is an indirect measurement of cytokine expression. The actual amount of cytokine synthesized by the cell's mRNA will determine its activity in the body.

The fact that the expression of INF-gamma is inhibited in horses treated with corticosteroids partially clarifies the mechanism that leads to immunosuppression in treated individuals. Interferon-gamma is an essential cytokine for the development of cellular defenses of the body; therefore it would be expected to observe immunosuppression when its production is compromised such as in cases of steroid treatment. Interferon-gamma stimulates macrophages to destroy pathogenic organisms and stimulates the activity of

antigen presenting cells. Their suppression of T helper 2 cells may also contribute to the mechanisms that result in immunosuppression.

4.7) Suppression of cytokines and application on equine diseases:

To the author's knowledge, this is the first study that closely evaluates the effect of dexamethasone treatment in horses on peripheral lymphocyte cytokine expression over time. One study showed that inhaled fluticasone considerably suppressed the expression of IL-4 in samples obtained from bronchoalveolar lavage in horses with recurrent airway obstruction.[60] Our study showed that IL-4 mRNA expression on peripheral blood lymphocytes was suppressed in horses treated with dexamethasone compared to control animals. However, there may be a difference in cytokine expression in cells obtained from the peripheral blood when compared to cells obtained from BAL in horses treated with corticosteroids.

Several other studies have evaluated the pattern of cytokine expression in different disease processes in horses. One example is the increased IL-4 and IL-5 mRNA expression in bronchoalveolar cells obtained from horses affected by RAO.[29] Interferon-gamma was found to be suppressed in those horses.[29] Based on the results of our study, the effect of dexamethasone would likely be beneficial in horses with RAO by decreasing the expression of IL-4.

Ponies infected with influenza virus have shown significantly increased levels of INF-gamma when compared to control animals. It is possible that the high levels of INF-gamma represent a protective response to infection or that INF-gamma participates in the pathophysiology process. The same effect is observed in horses with recurrent uveitis.[13]

If INF-gamma is protective for the disease, then corticosteroid treatment would be contraindicated.

Authors have shown that IL-1 β is an important cytokine that participates in the development of equine arthritis.[45, 46] Considering the findings of our study corticosteroids would not be expected to provide improvement in cases of equine arthritis due to the finding that this cytokine remains unaffected by it. However, steroids are known to reduce the degree of inflammation in arthritic conditions of horses. It could be speculated that clinical improvement is due to reduced expression of other inflammatory cytokines in the synovial fluid other than IL-1 β . If IL-1 persists in these joints then cartilage degradation would continue to progress as typically observed in these cases. [45, 46, 47] It is also possible that steroids do inhibit IL-1 when steroids are administered intra-articularly. Future studies should help to elucidate these questions.

4.8) Rebound effect and adverse effects:

Interestingly, all cytokines (IL-1 β , INF-gamma, IL-4 and IL-6) evaluated in this study demonstrated increased mRNA expression on day 10 when compared to values obtained from control horses. However, this difference was only statistically significant on interleukin-6 mRNA expression. This rebound effect of interleukin-6 mRNA expression it has not been described in other studies. We speculate that there may be a mild rebound effect on cytokine production after abrupt discontinuation of dexamethasone treatment.

Some authors have observed worsening of clinical signs secondary to interruption of long term steroid treatment in cases of human renal transplants.[114] This effect was evaluated by studying the expression of cytokines after the acute interruption of steroid

treatment in humans. A rebound effect was also observed with a transient increase in the expression of inflammatory cytokines such as interleukin-2 (IL-2), interleukin-7 (IL-7), interleukin-12 (IL-12) and INF-gamma. The slow withdraw of the drug was found more appropriate to avoid this side effect.[72, 115] Another possible explanation for the mechanism that lead to the rebound effect produced by use of corticosteroids is the over expression of cytokine receptors in immune cells such as IL-1 receptor, IL-2 receptor and IL-6 receptor. [116]

No systemic adverse effects were observed in almost all horses treated with dexamethasone in our study during the 5 day treatment period and the 5 day post treatment period. However, horse-11 did develop cellulitis on the right hind limb and had to be removed from the study due to the need for antimicrobial therapy. It is possible that treatment with dexamethasone predisposed this animal to a bacterial infection leading to cellulitis. Immunosuppression is a well known side effect of using corticosteroid treatment and it may have played a role in this case.

The 5 day treatment period was chosen based on results from previous studies that showed that blastogenic activity of peripheral blood mononuclear cells (PBMC) was maximally reduced 5 days after initiation of dexamethasone therapy. The 5 day post-treatment period was also chosen based on the observation that PBMCs blastogenic activity was restored within three to five days post-treatment.[117] We observed similar effects by demonstrating suppression of cytokine expression in horses treated with dexamethasone suppressed by day five of treatment, and mRNA expression was restored by day five post-treatment.

4.9) Future studies and clinical applications:

Knowing the pattern of cytokine suppression by dexamethasone treatment can provide useful information about which equine disease processes should be more appropriately treated by GCs. Interleukin-4 is well known to play a role in equine RAO and our study confirms that the use of dexamethasone in horses suppresses mRNA expression for this cytokine. The suppression of IL-4 may be the mechanism in which dexamethasone provides clinical improvement in RAO horses.

Barnes et al showed that treatment of humans with anti-IL-4 via nebulization did improve clinical signs of asthma especially in severe cases. INF-gamma nebulization of asthmatic human patients did not produce significant clinical improvement even though INF-gamma is known to suppress Th2 response. [51]

The time course effect of dexamethasone on cytokine expression is another important point addressed in our study. Interleukin-4, IL-6 and INF-gamma were significantly suppressed for 24 hours after the last dose of dexamethasone was administered. These results may provide guidelines for strategic treatments regimes for an efficient every other day treatment protocol if the targeted cytokine is suppressed for a prolonged period of time after the last dose of dexamethasone. To the authors knowledge, no other study has evaluated the post treatment period of cytokine expression in horses treated with dexamethasone.

Based on the results of our study, it would be interesting to develop future studies to evaluate a greater number of different cytokines affected by dexamethasone in horses.

It would also be interesting to evaluate this effect in diseased animals such as horses with RAO. It is not known if dexamethasone produces the same effect of suppression of inflammatory cytokines in diseased animals or if that effect is even more pronounced by the overexpression of cytokines during inflammatory conditions. Studies evaluating the effect of different doses of dexamethasone on cytokine expression may also be beneficial by providing alternative treatment plans based on the degree of suppression of specific cytokines. For example if a lower dose of dexamethasone suppresses IL-4 within 24 of initiation of therapy and the suppression persists for 24 hours after discontinuation of the medication, as found in our study, than a lower dose may be indicated due to reduced risks of side effects.

CHAPTER 5: SUMMARY

Dexamethasone suppresses cytokines involved in inflammatory responses, such as IL-4, IL-6 and INF-gamma. Its potent antiinflammatory effects in the treatment of a variety of equine diseases may be due to suppression of cytokine expression. Every other day treatment schedule could be justified due to the finding in our study that cytokine suppression persists for 24 hours after discontinuation of treatment.

CHAPTER 6: CONCLUSION

Our study supports our hypothesis that treatment of healthy horses with dexamethasone suppresses the expression of IL-6, IL-4, IL-1 β and INF-gamma. Most of the cytokines show a similar pattern of suppression and recovery of expression after discontinuation of treatment, except IL-1 β . We speculate that dexamethasone may affect the differentiation of Th cells into Th1 or Th2 phenotypes, consequently affecting cytokines produced by these 2 cells subsets (IL-6, IL-4, INF-gamma).

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