This is to certify that the
dissertation entitled

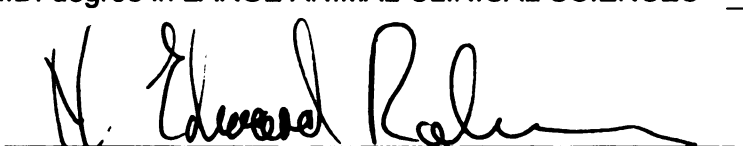
DEXAMETHASONE: THE TREATMENT OF HEAVES AND THE RISK OF LAMINITIS

presented by

CORNELIS JAN CORNELISSE

has been accepted towards fulfillment
of the requirements for the

__ Ph.D. degree in LARGE ANIMAL CLINICAL SCIENCES __



Major Professor's Signature

Feb. 18th 2005

Date

DEXAMETHASONE: THE TREATMENT OF HEAVES AND THE RISK OF LAMINITIS

By

Cornelis Jan Cornelisse

A DISSERTATION

Submitted
to Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Large Animal Clinical Sciences

2005

ABSTRACT

DEXAMETHASONE: TREATMENT OF HEAVES AND THE RISK OF LAMINITIS

By

Cornelis Jan Cornelisse

Glucocorticoids are potent anti-inflammatory drugs that are used with great success in the treatment of recurrent airway obstruction (RAO, also known as heaves) in the horse. However, up to date clinical studies into an optimal dose have not been performed, and the speed of onset of action is unknown. Additionally, data regarding the clinical efficacy of oral application of glucocorticoids to horses during an RAO-crisis are not available either. Oral application is easy, and would facilitate the treatment of RAO-exacerbations by the horse owner. Unfortunately glucocorticoid treatment in the horse has been associated with reports of laminitis, a debilitating disease of the equine hoof. A vascular etiology has been hypothesized. Up to date *in vivo* studies on horses regarding the modulation of vascular tone by glucocorticoids have not been performed.

The objectives of my Ph.D. studies were: 1) to study the efficacy of various oral doses of dexamethasone on RAO in horses, and to compare this to the effect of a standard intravenous dose of 0.1 mg/kg dexamethasone or an equivalent volume of saline; 2) to compare the clinical onset of improvement in lung function in RAO-affected horses after 0.1 mg/kg intravenous dexamethasone treatment to intravenous saline and 3) to study the *in vivo* modulation of dexamethasone on vascular responses after intradermal injections of vasoconstricting or vasodilating substances. The changes in dermal perfusion from the intradermal injections were studied as changes in dermal temperature with a

thermographic imager and compared between dexamethasone and saline treated horses.

The results showed that all oral applications of dexamethasone were as efficacious as intravenous dexamethasone in the treatment of RAO in the horse. Dexamethasone at a dose of 0.164 mg/kg given orally prior to food and intravenous dexamethasone both resulted in clinically relevant improvement of lung function (as judged by the ΔP_{PLMAX}) from 6 to 24 hours. Intravenous dexamethasone resulted in a statistical improvement of lung function after 2 hours and a clinical improvement after 4 hours. Intravenous dexamethasone treatment resulted in a significant decrease of baseline skin temperature, indicating an increase in vasomotor tone. The vascular responsiveness to intradermal injections with α_1-agonist phenylephrine was greater (i.e. increased efficacy) and potentiated (i.e. left shift of the EC_{50}). The effects of intradermal injections with vasoconstrictor endothelin-1 were superimposed on the lower skin baseline temperature, resulting in a greater decrease in skin temperature compared to the saline treatment. The statistical analysis of the data on nitric oxide controlled vasodilation from intradermal injections of methacholine was difficult to interpret. The reasons for this were a rise in skin temperature over time due to anxiety of the horses, likely in combination with other confounding experimental factors.

In conclusion, oral dexamethasone is an easy applicable and efficacious treatment for heaves with an onset, magnitude and duration of effect that is comparable to 0.1 mg/kg intravenous dexamethasone. Dexamethasone treatment in horses results in an altered vasomotor tone with a greater and potentiated response to catecholamines. These studies indicate that oral and intravenous dexamethasone is of great benefit for the treatment of RAO, a disease not associated with circulatory risk factors.

Dedication

ACKNOWLEDGEMENTS

This thesis would have been impossible without the help and support of many people. First of all my warm and special thanks to my advisor Dr. N. Edward Robinson for his guidance and stewardship of my PhD program, and his incredible enthusiasm for my studies. Dear Ed I hope when I am your age I still can be that much involved in projects as you are. Also many many thanks to Dr. Fred Derksen, Dr. Greg Fink, Dr. Ray Nachreiner and Dr. Hal Schott who as members of my graduate committee believed in my projects, and gave me the support and confidence to see it all through. I am in great great debt to Cathy Berney and Sue Eberberhardt. They were instrumental to the good ending of all my experiments, some which were done under bizarre weather conditions. Sorry for the artic exposures and thank you so much for your friendship, support and putting up with me. Many thanks to Dr. Jeff Bunn, practitioner in Howell, who was so gracious to lend me his thermograph imager for pilot experiments on intradermal injections of vasoactive substances in horses. Similarly so many thanks to Christina Kobe and Dan Boruta for that I always could count on them during the collection of the lung function data on the horses, even if that sometimes ended up being in weekend hours. Of course all data collected needed statistical analysis and I owe many thanks to Dr. Joe Hauptman and Dr. Wilfried Karmaus for their interest in, and help with statistics on my projects. It appeared that the art of editing papers is not one of my strongest points. Luckily I could count on the expertise of Vicki Hoelzer-Maddox who did do such a great job to get everything seemingly easy up to the required standards. Vicki thank you so much for that. I also like to thank Heather de Feiter-Rupp, Annerose Berndt and Lisa Bartner for their upbeat lab spirit and helping me out on several occasions.

Funding and contributions from many sources were essential to the completion of my Ph.D program. I like to acknowledge the Matilda Wilson Equine Respiratory Endowment and the Office of the Associate Dean for Research and Graduate Studies of the College of Veterinary Medicine for their annual stipend for the duration of the program. Additionally I like to thank the Office of the Dean of the Graduate School of Michigan State University for a writing grant in support of my thesis. My warm thanks go to Dr. Hans Swaan, Surgeon and friend of my father, and his wife Wil for financing a laptop computer which was a great asset to me in analyzing my data and writing this thesis in and outside the office.

Many thanks to the horses Bright, Casey, Cindy, Dancer, Lacey, Missy, Rusty, Sioux, Sugar, Sunny, Wendelina and White Fang for their ongoing contributions to veterinary science.

So many thanks to my run-bike-swim and ski buddies Amanda, Amy, Bob, James, Jean, Justin, John, Kent, Lauri, Lyndsay, Mat, Mary, Sarah, Seth, Sue and Tom for their friendship and the awesome time we spent on great outdoors activities that helped to keep some sanity in me. I will miss you guys. And finally I like to thank my mother Alice and brothers Dick and Rogier for their ongoing support during all these years of continuing education.

TABLE OF CONTENTS

LIST OF TABLES and FIGURES

CHAPTER 1

TABLES

FIGURES

CHAPTER 2

TABLES

CHAPTER 3

TABLES

FIGURES

CHAPTER 6

TABLES

FIGURES

CHAPTER 7

TABLES

FIGURES

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ARG	Argine
AT-1	Angiotensin type-1 receptor
BALF	Bronchoalveolar lavage fluid
BCL	Beclomethasone
BDP	Beclomethasone dipropionate
Cdyn	Dynamic compliance
CONC	Concentration
DEX	dexamethasone
DMSO	Dimethyl sulfoxide
EC_{50}	50% effective dose
ECE	endothelin converting enzyme
ET-1	Endothelin-1
ET_A	Endothelin Type A receptor
ET_B	Endothelin Type B receptor
FP	Fluticasone dipropionate
GC	Glucocorticoid
GR	Glucocorticoid receptor
HDF	High dose, food
HDNF	High dose, no food
IA	Intra-articular
ID	Intradermally
IL	interleukin
INF	Interferon
IV	Intravenously
LDNF	Low dose, no food
L-NAME	N^G-nitro-L-arginine methyl ester
M	Molar concentration
M_2	Muscarine receptor, type 2
M_3	Muscarine receptor, type 3
MCH	Methacholine
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MPA	Methyl prednisolone acetate
NF	Nuclear factor
NO	Nitric oxide

NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PEF	Peak expiratory flow
PHE	Phenylephrine
PIF	Peak inspiratory Flow
PO	Per os (oral)
PREDNL	Prednisolone
RAO	Recurrent airway obstruction
R_L	Airway resistance
RR	Respiratory rate
RRA	Relative receptor affinity
RX	various injection solutions
SAL	Saline solution
SNAP	S-nitroso-N-acetylpenicillamine
TAA	Triamcinolone acetonide
T_{AVG}	Average temperature
TELF	Tracheal epithelial lining fluid
TM	Time
TMC	triamcinolone acetonide
TNF	Tumor necrosis factor
T_V	Tidal volume
TX	Treatment
V_{min}	Minute volume
VSMC	Vascular smooth muscle cell
α_1	alpha adrenergic receptor, type1
β_2	B2-adrenergic receptor, type 2
ΔPpl_{max}	Maximal intrapleural pressure change (cm H_2O)

INTRODUCTION

Recurrent airway obstruction (RAO; also known as heaves) is an allergic and inflammatory condition of the lower airway of the horse that can be induced by exposure to organic dusts that are associated with feeding roughage and stabling on straw bedding. The disease has many similarities to human asthma, including bronchospasm, mucus production and inflammatory cell infiltrates into the lower airway.

Glucocorticoids are potent anti-inflammatory drugs that successfully are used for the treatment of exacerbations of RAO in horses. Literature indicates that the effects by glucocorticoids are due to genomic and non-genomic actions. Genomic effects by glucocorticoids involve the modulation of the transcription of cellular proteins. This can result in transactivation or transrepression of these products. Non-genomic effects involve cytosolic post translational modifications of proteins in cellular pathways. These effects are faster and hypothesized to be more prominent with higher doses of a glucocorticoid. In the light of this new literature it is no surprise that glucocorticoid therapy can result in desired as well adverse effects.

Dexamethasone is a glucocorticoid that is used with great success for the treatment of exacerbations of RAO in the horse. However up to date no clinical dose-response studies have been conducted regarding the systemic or oral application of this drug. Owners and veterinarians would be very interested to know what a minimal efficacious dose of an easy way to administer glucocorticoid would be for the treatment of RAO.

Glucocorticoid therapy in the horse has unfortunately been overshadowed by reports of induction of laminitis, a debilitating disease of the equine foot. The reports are

few and the exact nature of how glucocorticoid treatment can result in this complication in horses is unknown. The broad actions by glucocorticoids suggest that it is likely that glucocorticoid induced laminitis follows the same pathways that have been hypothesized to be important in the etiology of clinical laminitis from severe disease. This includes a vascular model. The *in vivo* vascular effects of glucocorticoids in horses have not been studied.

From the above it is clear that there exist many questions regarding the efficacy and safety of glucocorticoid therapy in horses. This Ph.D. thesis reports on 3 studies with dexamethasone in horses in order to help to answer some of these questions. Study 1 (chapter 3) reports on a study with different doses of oral dexamethasone in the treatment of heaves, and compares this to the efficacy with a standard intravenous dose. Speed of onset is additionally studied as well. In regard to the possible etiologies of glucocorticoid induced laminitis, Study 2 and 3 investigate the *in vivo* effects of dexamethasone treatment on the peripheral vasculature. Study 2 (chapter 6) reports on how vasoconstrictive responsiveness is modulated by glucocorticoid therapy, while study 3 (chapter 7) describes the finding on vasodilatory responsiveness. The additional chapters function to put the results of these studies in a better perspective. They include literature discussions on the mechanisms of actions by glucocorticoids and the relationship between glucocorticoid use in the horse and laminitis.

CHAPTER 1

INTRODUCTION TO GLUCOCORTICOIDS

Nomenclature

Glucocorticoids belong to a drug group that is used for their potent anti-inflammatory actions in medicine. They are structurally related to the bodies natural "stress hormone" cortisol. It was the publication in 1949 by Hench et al. (Hench, Kendall et al. 1949), in which they reported dramatic improvement of rheumatoid arthritis after administration of cortisone, which sparked the interest in the anti-inflammatory actions of these drugs. Cortisone is an inactive precursor form of the hormone cortisol. Cortisol has a steroid structure similar to cholesterol and is produced in the zona fasciculata of the cortex of the adrenal gland. Hence the initial name corticosteroid or corticoid for this drug group. Cortisol has many functions in the body. It appears however that the anti-inflammatory effect of corticosteroids parallels their key function involving the regulation of carbohydrate metabolism (gluconeogenic effect) (Schimmer and Parker 1995). A recent meeting of the European League Against Rheumatism (EULAR) regarding nomenclature and treatment regimes suggested therefore that the name glucocorticoid should be preferred over corticosteroid in order to avoid any confusion with the other adrenal steroid hormones (aldosterone, androgens) that are produced in the adrenal cortex (Buttgereit, da Silva et al. 2002) but which have different functional properties.

Structure, activity, and metabolism

Glucocorticoids are synthesized in the cortex of the adrenal gland from modifications of cholesterol. As such, glucocorticoids consists of a similar 4-ring, 21-carbon atom

framework. The rings are called A, B, C and D. Modifications to this framework determines the level of glucocorticoid and mineralocorticoid activity, relative potencies, and pharmacokinetic features of a glucocorticoid. Several of the carbon atoms have shown to be key positions regarding these characteristics (Figure 1). The 4,5 double bond and 3-keto group on ring A are an absolute requirement for glucocorticoid activity. The hydroxyl group (OH-group) on C-21 of ring-D is an absolute requirement for mineralocorticoid activity. On the other hand the presence of a hydroxyl group on the 11-carbon atom (C-11) is important for glucocorticoid activity at local tissue level. It appears that the configuration of this group is determined by the local balance in activities of two isoforms of the enzyme 11-beta-hydroxysteroid dehydrogenase (respectively abbreviated as 11-β- HSD-1 and 11-β- HSD-2). Oxidation (also referred to as dehydrogenation) of this C-11 hydroxyl group results in the inability of the glucocorticoid to bind to the cytoplasmic glucocorticoid receptor (GR). The glucocorticoid will as a consequence be less active in that tissue. The picture is complicated by the fact that the two isoforms of 11-β- HSD have bidirectional action with distributions and equilibriums that are tissue specific. For example, 11-β- HSD-2 is more prevalent in epithelial tissues such as tubular epithelium of the kidney, colonic mucosa, skin, epithelium of the airways as well vascular smooth musculature (Smith, Maguire et al. 1996) with a primarily oxidative action (leading to inactivation). 11 -β- HSD-1 is, however prevalent in most tissues, including liver and fat tissue, and has an equilibrium to the reductive side (leading to activation)(Li, Obeyeserkere et al. 1997; Brereton, van Driel et al. 2001). Examples of this prereceptor metabolism are the inactivation of prednisolone into inactive prednisone by 11-β- HSD-2 in the kidney and conversion of inactive cortisone into active cortisol

(hydrocortisone) by 11-β- HSD-1 in the liver. Consequently, renal tubular epithelium is therefore highly insensitive for prednisolone, while liver and fat tissue contribute to maintenance of systemic and tissue levels of cortisol (Rask, Olsson et al. 2001). Introduction of a double bond at the 1,2 position of the A-ring increases glucocorticoid potency and causes an enhanced glucocorticoid to mineralocorticoid potency ratio. Halogenation, of the C-9 group results in additional increases in glucocorticoid as well as mineralocorticoid potency. These halogenations, usually with fluorine, tilt the equilibrium for 11-β- HSD-1 more to the reductive side and make glucocorticoids more resistant to 11-β- HSD-2 (Oelkers, Buchen et al. 1994; Ferrari, Smith et al. 1996; Li, Obeyeserkere et al. 1997; Diederich, Hanke et al. 1998). As a result the halogenated glucocorticoids remain mainly in the activated form in tissues, including that of the renal tubular epithelium. (Diederich, Hanke et al. 1996; Diederich, Hanke et al. 1998). However, it appears that the unwanted improvement in mineralocorticoid potency can be eliminated by adding substitutions on C-16. Consequently almost all modern glucocorticoids are therefore halogenated (budesonide is an exception) and have additions to C-16. Additionally substitution and esterification at C-16, but also at C-17 or C-21 with an acetate, propionate or valate group will result in an increase in lipophility and local tissue availability of the glucocorticoid.

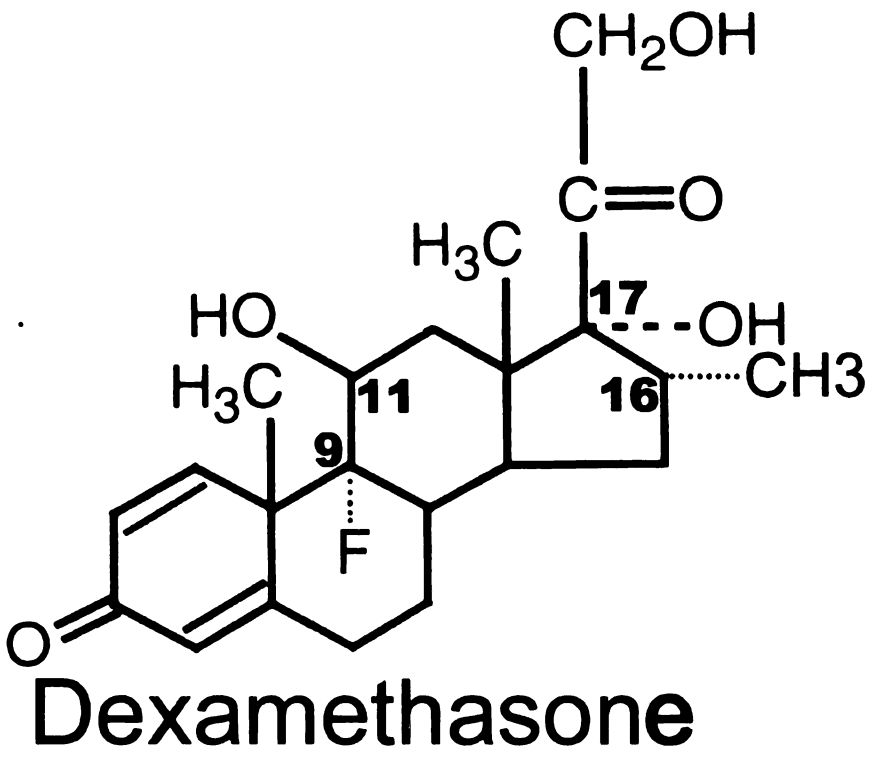


Figure 1: Chemical structure of dexamethasone. Compared to cortisol a double bound has been added between C1 and C2. C-9 is halogenated with fluorine and a methyl group is present on C-16.

These substitutions, therefore, also optimize glucocorticoids for use in long acting depot formulations (Dahlberg, Thalen et al. 1983). Final elimination of glucocorticoids is accomplished on basis of reduction of the 4,5 double bonds in hepatic and extrahepatic tissues followed by final reduction of the 3-keto group in the liver. In the liver and kidney these A-ring reduced steroids are subsequently conjugated with sulfate or glucuronide to the 3-hydoxyl group, and excreted with the urine (Liddle 1961; Dahlberg, Thalen et al. 1983; Schimmer and Parker 1995).

Mechanism of action

The biological actions of glucocorticoids, especially in relation to their anti-inflammatory properties, have been attributed to modulation of gene transcription. This involves a pathway that starts with the lipophilic glucocorticoid hormone acting as an intracellular ligand by binding to a cytoplasmic glucocorticoid receptor (GR). The ligand-bound GR translocates to the nucleus and activates a glucocorticoid response element (GRE) on the genome. The activated GRE subsequently influences transcription of nearby genes by either activating (transactivation) or repressing (transrepression) their transcription. This results in respectively more or less messenger RNA (mRNA) and therefore ultimately more or less protein products from mRNA-translation (Baxter and Tomkins 1971; Merkulova, Merkulov et al. 1997). This classical pathway (figure 2) from GR-occupation to alterations in transcription of the genome needs time, explaining why the first effects with most application of glucocorticoids are not observed before 2 to 4 hours after administration. Recent studies indicate that this view of the genomic pathway of glucocorticoid effects is too simplistic. It appears that the GR exists as GRα or GRβ splice variants that differ in the last 50 C-terminal amino acids. As a consequence GRβ is unable to bind with a glucocorticoid. GRβ also exists mainly as a free monomer in the cytoplasm and nucleus while GRα is sequestered in the cytoplasm as a multi-protein complex that includes heat-shock chaperone molecules (hsp-90 and hsp-70) and src, a member of the MAP-kinase family. Binding of a glucocorticoid to GRα results in dissociation of the multi-protein complex followed by dimerization of GRs. The dimers can be in GRα/GRα, GRα/GRβ or GRβ/GRβ formats. These GR-dimers translocate in the nucleus and bind to selective DNA sequences called glucocorticoid response elements

(GREs). However only GRα/GRα-dimer binding results in full transactivation or repression, while dimers containing GRβ have impaired activity (Bamberger, Schulte et al. 1996). The ratio GRα to GRβ appears therefore to influence the level of glucocorticoid activity. The effect on transactivation and transrepression by the ligand bound GRα/GRα-dimers is modulated in several ways.

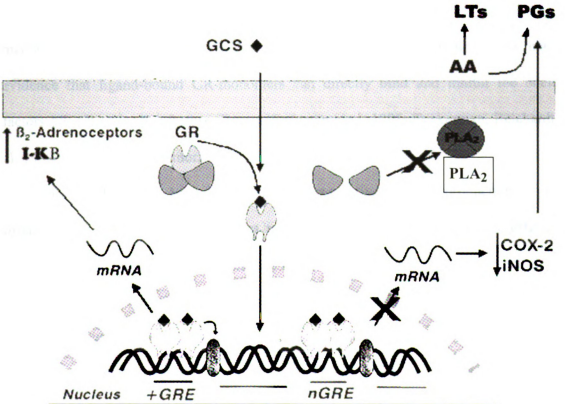


Figure 2. Genomic and non-genomic pathways by glucocorticoids. See text for abbreviations

For a start, the dimers can with the help of additional coactivator complexes (e.g. CBP, p300, P/CAF etc.), switch on nearby genes (Merkulova, Merkulov et al. 1997; Pelaia, Vatrella et al. 2003). The result is newly formed mRNA that will be translated into protein products within the cytoplasm. These products have specific anti-inflammatory

actions (e.g. IL-10 and lipocortin inhibit cPLA2) and additional effects (e.g. a new β-adrenergic receptor). Transactivation by ligand bound GR-dimers can also result in the production of a specific inhibitor (IκB) for the nuclear factor NF-κB. NF-κB appears to have a key role in the transcription of mediators of inflammation (Barnes, Pedersen et al. 1998). Binding of cytosolic NF-κB by IκB results consequently in strong down regulation of transcription of inflammatory interleukins and cytokines (e.g. TNFα, RANTES) or mediator-synthesizing enzymes (COX-2, iNOS, cPLA2). As an alternate variant there is evidence that ligand-bound GR-monomers can directly bind and inhibit the nuclear factors NF-κB and AP-1 factors (Barnes, Pedersen et al. 1998; Tuckerman, Reichardt et al. 1999; Adcock and Caramori 2001).

It has, however, been suggested that transactivation and production of anti-inflammatory proteins only contributes in a small amount to anti-asthmatic effects of glucocorticoids (Pelaia, Vatrella et al. 2003). Direct repression of genes is considered a more important pathway via which glucocorticoids result in anti-inflammatory effects. Repression is thought to result from steric hindrance in cases where binding of the GR-dimers on the GRE also partially overlaps TATA boxes of other genes. The expression of NF-κB and AP-1 is indeed repressed in this manner (Hofmann, Hehner et al. 1998; Jaffuel, Demoly et al. 2000). Because steric hindrance is, compared to multi-factor transactivation, relatively easily achieved, it has been suggested that (direct) repression might require relatively lower concentrations of synthetic glucocorticoid to have effect (Adcock and Ito 2000).

There is evidence that glucocorticoids can have faster effects than would be expected from the 2-hour window of the transcription/repression machinery. Examples of

this are the fast lymphopenia after glucocorticoid administration, the fast relief of status asthmaticus with prednylidene, the fast resolution of an Addisonian crisis with fludrocortisone and treatment of spinal shock or cerebral edema with dexamethasone or methylprednisolone (Schmidt, Gerdes et al. 2000). These observations suggest therefore the existence of additional pathways besides the modulation of transcription. (Buttgereit, Brand et al. 1999; Croxtall, Choudhury et al. 2000; Buttgereit, da Silva et al. 2002; Buttgereit and Scheffold 2002; Croxtall, van Hal et al. 2002). It appears that these rapid effects are more prominent in higher dose ranges, what has been postulated to be the result of an overflow effect after all GRs have been saturated with GC (Buttgereit, Brand et al. 1999; Buttgereit and Scheffold 2002). Recent studies have uncovered some of the non-genomic effects of glucocorticoids. For instance src, liberated from the GR-chaperone multi-protein complex after GC binding, will activate cytoplasmic lipocortin-1. This activated lipocortin-1 will bind and inactivate Grb2, a protein that plays a central role in the cellular signal transduction via MAPK-kinase pathways after, for instance, epidermal growth factor receptor (EGF-R) activation. Normally the activated MAPK-kinases result in phosphorylation and activation of cPLA2, an enzyme that catalyses the release of arachidonic acid from membrane phospholipids. These phospholipids are subsequently converted into inflammatory leukotrienes and prostaglandin by enzymes that include COX-2. The blocking of the MAPK-kinase pathways therefore causes cPLA2 to remain in its inactive form (figure 2b) and results in a decrease of leukotriene and prostaglandin production (Croxtall, Choudhury et al. 2000; Croxtall, van Hal et al. 2002). Another non-genomic pathway of glucocorticoid action might be the influence on calcium and sodium cycling across cell membranes and the increased proton permeability

across the inner mitochondrial membrane. It is thought that the basis for these effects is the insertion of the lipophilic glucocorticoid molecules in the membranes with consequently a change in physicochemical membrane properties. The clinical effects likely arise from decreased cytosolic calcium availability and energy production and thus influence on cell function and protein synthesis. A decreased ion cycling makes cells also less prone to development of edema. (Buttgereit, Brand et al. 1999; Buttgereit and Scheffold 2002).

Intrinsic glucocorticoid potencies

Glucocorticoids are used for their anti-inflammatory and immunosuppressive actions. Many clinical studies have indeed reported on dosage, administration and efficacy of glucocorticoids for treating inflammatory disease in human beings (Bolland and Liddle 1958; Liddle 1961; Derendorf, Hochhaus et al. 1993). However good comparable data on anti-inflammatory potencies between glucocorticoids, and for the species and clinical indications they are used for is still lacking. Some data involved studies on relative eosinopenic potencies or adverse effects in human beings (Derendorf, Hochhaus et al. 1993). However most comparative data studied bioassays on rodents or in vitro lymphocyte proliferation. General inferences from these investigations is now known as the classical table for comparative anti-inflammatory effects of glucocorticoids (table 1) (Liddle 1961; Melby 1977; Schimmer and Parker 1995).

Unfortunately there are some clinical observations that are not completely in line with this classical classification. For instance, why is it that a less potent glucocorticoid can have as strong side effects as one that is regarded as more potent? Or why, in case of two

equipotent glucocorticoids, is one more effective in treating obstructive airway disease or spinal injury? It is likely that some of these clinical discrepancies are based on differences between species and differences between the inflammatory models used. For instance some bioassays studied the effect of topical glucocorticoid application on granuloma inhibition or experimental induced edema in extremities of rats while other assays investigated thymolytic activity (Lerner, Turkheimer et al. 1964; Tonelli, Thibault et al. 1965; Dahlberg, Thalen et al. 1983).

Table 1: Classical and updated anti-inflammatory potencies as determined by inhibition in a lymphocyte proliferation test. Mineralocorticoid activity (MA) and relative receptor affinities (RRA) are provided as well. Data were obtained from [a] (Schimmer *et al.* 1995) and modified from [b] (Mager *et al.* 2003).

Glucocorticoid	classic[a]	updated[b]	MA[a]	RRA[b]
Fluticasone		156.2	0.0	202.3
Budesonide		32.1	0.0	94.6
Beclomethasone		3.6	0.0	5.8
Dexamethasone	20-30	9.3	0.0	11.1
Betamethasone	20-30	7.6	0.0	6.4
Triamcinolone	5.0	13.9	0.0	26.1
prednisolone	4.0	1.8	0.8	1.8
Cortisol	1.0	1.0	1.0	1.0

The in vitro studies on lymphocytes in turn focused on the ability of glucocorticoids to inhibit phytohemagglutinin (PHA) induced lymphocyte proliferation (Cantrill, Waltman et al. 1975; Langhoff and Ladefoged 1983; Spahn, Landwehr et al. 1996; Mager, Moledina et al. 2003).

In vivo, the response of cells to a glucocorticoid is the result of the interplay between 1) local concentration of free active glucocorticoid, 2) the ability of the cell to

transduce the glucocorticoid signal and 3) the genomic effects by the glucocorticoid. (Bamberger, Schulte et al. 1996). The concentration of free and active glucocorticoid is dependent on the pharmacokinetic properties and metabolism of the glucocorticoid. Thus for instance acetate esterifications will enhance lipophility and local tissue availability (Dahlberg, Thalen et al. 1983). Similarly, fluorination of glucocorticoids causes decreased binding to plasma proteins like corticosteroid binding globulin (CBG), decreased metabolism by the liver and decreased inactivation at local tissue level by the 11-β- HSD-isotypes. As a consequence, halogenation results in higher free and active glucocorticoid concentrations at the local tissue level.

The relative ability to transduce the glucocorticoid signal in the cell depends on the affinity of the GR for the glucocorticoid, and the subsequent ability of the activated GR to modify transcription. Glucocorticoids need to bind to the GR in order to activate it. The number of activated GRs is therefore an very important factor for the level of influence on the genome (Baxter and Tomkins 1971). Although the GR number per cell can differ between tissues (Damon, Rabier et al. 1985), it appears to be within a 1/2 log order. Therefore, unless large numbers of modified (=unresponsive) GRs are present, GR number per cell is a relatively minor factor for explaining differences in potency between glucocorticoids. It is therefore possible to compare relative glucocorticoid potencies on basis of receptor affinity for a GC between different tissues or species. Affinity is a net result from an equilibrium between the rate of association between GC and GR (defined as constant K_{ASS}) and the rate of dissociation of the GC-GR complex (defined as constant K_{DISS}). The net result, is reflected in the equilibrium dissociation constant K_D ($K_D=K_{DISS}/K_{ASS}$), which is an indicator for GC–GR complex stability and half life. Thus

an increased rate of association or a slower dissociation will result in longer GR occupation by glucocorticoids. Consequently relatively less glucocorticoid should be required to accomplish the same effect. K_D is, therefore inversely correlated with affinity of the GR for the GC. The number of occupied GRs translates in genomic effects, and thus a glucocorticoid for which the GR has a low K_D will have more occupied receptors at a lower concentration, and thus genomic effects at lower concentrations. Therefore GC receptor affinity translates in potency of the glucocorticoid. Indeed a linear relationship has been established between glucocorticoid affinity for the GR (as determined by glucocorticoid-GR half life) and glucocorticoid potency (Dahlberg, Thalen et al. 1983; Spahn, Landwehr et al. 1996; Brattsand and Axelssson 1997; Mager, Moledina et al. 2003). It appears that the K_{ASS} is almost equal amongst all glucocorticoids and therefore it is the K_{DISS} that mainly determines the stability of the activated glucocorticoid receptor complex, and thus the K_D (Yeakley, Balasubramanian et al. 1980; Rohdewald, Mollmann et al. 1985). Despite these differences in K_D between the glucocorticoids, the binding studies also show a maximal receptor occupation (saturation) for fluoroglucocorticoids between 10^{-7} to 10^{-6} M (Chen, Kohli et al. 1994) while that for cortisol and prednisolone is approximately an order higher at 10^{-5} M. Acetylations and modifications to the D-ring that cause enhanced lipophility is also one of the factors that cause differences in GR affinity and consequently explain some of the increased potencies of drugs like triamcinolone and dexamethasone (Manz, Grill et al. 1982). The result is also the existence of a correlation between lipophility and potency.

Recently it has become possible to compare glucocorticoids on basis of their ability to modify transcription with the help of gene expression assays. Although not

completely independent of GR-affinities these genomic expression assays allow distinction between either agonist or antagonist activated GRs and integrate this effect with cell line specific metabolism (Jaffuel, Roumestan et al. 2001). The genomic effects can be plotted as effective dose-response curves (Jaffuel, Roumestan et al. 2001) from which a more cell type specific trans-activating and trans-repressive effect of glucocorticoids can be compared (table 2). Comparative potencies can be inferred from these curves as the EC_{50} which is the effective dose that results in a 50% change of transcription. For instance in the A549 human lung carcinoma cell line and the HeLa human epithelioid cervix cell line it appears that triamcinolone is actually a more potent transactivator of a GRE-dependent luciferase gene than dexamethasone and betamethasone. In the classical order triamcinolone is however 5 fold weaker compared to dexamethasone and betamethasone. Similarly, in a HTC rat hepatoma cell line, triamcinolone is at least as equipotent as dexamethasone and betamethasone in the induction of the gluconeogenic enzyme tyrosine aminotransferase (TAT) (Jaffuel, Demoly et al. 2000; Jaffuel, Roumestan et al. 2001). On the other hand beclomethasone-diproprionate (BDP), a popular anti-asthmatic glucocorticoid used for inhalation treatment, has one of the weakest intrinsic potencies (table 2).

Table 2: Genomic potencies of glucocorticoids on transactivation and transrepression. The data are calculated from EC_{50} – values that were reported in gene expression assays (Jaffuel D. et al, Am J Respir Crit Care Med 2000; 57-63)

Glucocorticoid	Trans-activation		Repression (A549 cells)		
	Luciferase HeLa cells	TAT HTC-cells	AP-1 activation	NF-κB activation	RANTES production
FP	66	12.5	50	10	12.5
Budesonide	27	1	8.3	2.2	3.3
TAA	19	1	*1*	*1*	*1*
Dexamethasone	11			*1*	
Betamethasone	9			*1*	
BDP	6	NM	1.3	0.5	0.5
Prednisolone	3				
Cortisol	*1*				

Also marked differences between transrepression potencies of glucocorticoids have been reported with these gene expression assays. So in a HeLa and A549 cell lines, fluticasone propionate (FP) is the most potent repressor for AP-1 and NF-κB, and this correlates well with the ability of this drug to suppress RANTES transcription. Beclomethasone-diproprionate on the other hand is surprisingly weaker than triamcinolone or budesonide (table 2) (Jaffuel, Demoly et al. 2000). RANTES is an important cytokine in the pathogenesis of human asthma and therefore fluticasone seems an excellent choice as a therapeutic agent against asthma. Unfortunately fluticasone also has the strongest potential to induce gluconeogenic adverse effects (table 2). The gene expression studies indicate that different glucocorticoids have different EC_{50} for different cellular effects whether it is transactivation or transrepression. However at least for the repression of AP-1, it appears that the relative potencies between glucocorticoids is independent even of cell type (Jaffuel, Demoly et al. 2000). It also appears that the approximate maximal difference in an EC_{50} for an effect between the modern

glucocorticoids is slightly more than 1 log ($\approx$10-15x). For most effects this will translate into glucocorticoid concentrations between 10^{-9} and 10^{-7} M. The reported concentration necessary for maximal genomic effects is 10^{-7} to 10^{-6} M, which is similar to the earlier discussed values obtained from glucocorticoid receptor affinity (KD) studies. Overall no transactivation/repression activity is seen at glucocorticoid concentrations below 10^{-10} to 10^{-12} M (Jaffuel, Roumestan et al. 2001).

The genomic data are a helpful aid in better understanding certain clinical phenomena. For instance, inhalation therapy of glucocorticoids results in local tissue levels that are in the nanomolar range while systemic concentrations are around 10^{-10} M. Comparing the EC_{50}'s for the repression of RANTES, AP-1 and NF-κB suggests that FP is likely to be a far more effective glucocorticoid than BDP to treat airway inflammation with such low local concentrations. However, unlike BDP it is also likely that FP will cause unwanted systemic gluconeogenic effects. (Roumestan, Henriquet et al. 2003). The K_D and EC_{50} values for glucocorticoids show that genomic effects of glucocorticoids manifest themselves for concentrations between 10^{-10} and 10^{-6} M. Additionally at high doses with complete receptor saturation a trans-activating or trans-repressing effect willbe of similar magnitude between different glucocorticoids. However duration of actioncan still differ and non-genomic effects could become an important factor as well. Indeed, and as discussed before, non-genomic mechanisms of a glucocorticoid can explain some of its specific individual clinical effects. For instance betamethasone, which is equipotent to dexamethasone in the classical order, appears to have far less non-genomic potency, as measured by its ability to inhibit convalin-A stimulated respiration. The result is therefore a slower effect. Similarly prednilydene, a weak glucocorticoid in

the classical order, has stronger non-genomic potency than dexamethasone (figure 3) (Buttgereit, Brand et al. 1999).

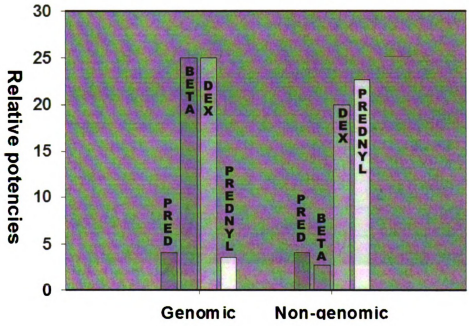


Figure 3: Comparison of relative genomic and non-genomic effects by the glucocorticoids prednislone (PRED), betamethasone (BETA), dexamethasone (DEX) and prednylidene (PREDNYL).

The non-genomic effects can also differbetween glucocorticoids. For instance even in high doses, potent glucocorticoids like beclomethasone-dipropionate, budesonide, fluticasone propionate dexamethasone and triamcinolone (table 3) (Croxtall, Choudhury et al. 2000; Croxtall, van Hal et al. 2002). Therefore drugs like fluticasone propionate that have strong genomic down regulation of cell growth and COX-2 production can still be relatively ineffective in blocking the production of specific mediators like LTD4, a potent bronchoconstrictor. It is thus interesting to note that fluticasone propionate recently has

become available as a compounded combination (Advair Diskus®) with the bronchodilator salmeterol for the treatment of human asthma.

Table 3: Relative glucocorticoid potencies on PGE2 production via genomic COX-2 repression and non-genomic cPLA2 inhibition (NS=no significant effect), Based on EC50 values as reported by croxtall et al. Br. J. Pharmacol. 2002; 135; 135:511-519

Glucocorticoid	$cPLA_2$ activity	COX-2 repression	PGE_2 Release
FP	*NS*	150	150
BDP	*NS*	100	100
Budesonide	*NS*	75	75
Prednisolone	*NS*	1.5	1.5
Cortisol	1	1	1
Dexamethasone	3.75	0.75	3.75
TAA	0.38	0.38	0.38

The new insights into genomic and non-genomic potencies have lead to a review of the standardized nomenclature and dosage regimens for glucocorticoids. A recent EULAR convention (Buttgereit, da Silva et al. 2002) produced a dosage advisory that is based on the fact that 100 mg prednisolone results in virtually a complete GR saturation in a normal adult person, or equal to a tissue concentration in the order of 10^{-5} M. The recommended doses are:

Low dose: < 7.5 mg prednisolone/day, equal to 42 % receptor occupation

Medium dose: > 7.5 but ≤30 mg prednisolone/day, equal to > 63 % receptor occupation.

High dose: > 30 mg but ≤100 mg prednisolone/day, equal to 100% receptor saturation.

Very high dose: ≥100 mg prednisolone/day.

Extrapolation from this recommendation suggests that, according the classical order of glucocorticoids, dexamethasone therapy in horses at a dose of 0.1 mg/kg would be categorized as a high dose equal to 35-42 mg prednisolone per person. For several

glucocorticoids, plasma and local tissue concentrations in horses have been reported (table 4). Even when adjustment for molar mass is taken into account, it appears that intra-articular concentrations exceed for a considerable time the KD and EC_{50} concentration ranges that produce genomic effects. From the potencies for non-genomic effects, methylprednisolone acetate would therefore likely be a better clinical choice than triamcinolone acetonide. Although plasma concentrationss are at best in a low activity range, it is likely that lipophility of these drugs causes adequate tissue concentrations and receptor occupation.

Table 4: Serum and intra-articular concentrations of glucocorticoids in horses. Serum and local concentrations are in ng/ml. Molar concentrations can be estimated by dividing the values with the molar masses Mw. The Mw for these glucocorticoids is in the range of 392-434.

DRUG	DOSE	APPLICATION	CONCENTRATION (ng/ml)				
			serum			Intra-articular	
			Peak	24-h	48-h	24-h	96-h
DEX[1,2,3]	0.01 mg/kg	IV once	20-35	<2.5			
	0.04 mg/kg	IV q24h		1.9			
	0.27 µg/kg	Eye q8h	0.1-0.5	<0.06			
TAA[4,5,6]		IV once					
	3 x 6 mg	IA once	4.3	2	<10	7500	10
	30 mg	IA once	2.4	1.8			
PREDNSL[7]	2.2 mg/kg	IV once	1700	<10			
MPA[8]	100 mg	IA once	<10	<10		20000	<100

Data interpretated from 1(Chen, Zhu et al. 1996), 2(Hoffsis and Murdick 1970),3(Spiess, Nyikos et al. 1999),4 (French, Pollitt et al. 2000), 5(Chen, Sailor et al. 1992), 6 (Koupai-Abyazani, Yu et al. 1995),7(Peroni, Stanley et al. 2002) and 8(Lillich, Bertone et al. 1996)

Summary

Glucocorticoids are potent anti-inflammatory drugs. Recent research shows that their mode of action is via genomic and non-genomic pathways. Genomic pathways are the result of transactivation or transrepression of transcription and take several hours to become effective. Non-genomic effects involve post translational modifications of cellular proteins and have rapid effects. The potency of glucocorticoids is associated with

affinities of the glucocorticoid receptor for the glucocorticoid and lipophility of a glucocorticoid.

CHAPTER 2

GLUCOCORTICOID THERAPY FOR RECURRENT AIRWAY OBSTRUCTION IN THE HORSE

Recurrent airway obstruction

Recurrent airway obstruction (RAO) in the horse is a lower airway disease of the middle-aged and geriatric horse (Couetil and Ward 2003). Similar to human asthma a genetic predisposition is suspected (Gerber and Bailey 1995; Marti *et al.* 1991; Marti *et al.* 2003). The disease is characterized by recurrent attacks of respiratory distress when horses are stabled indoors for considerable periods of time (Thomson and McPherson 1984; Vandenput *et al.* 1998). The clinical signs during such an RAO crisis include coughing, pronounced nostril flaring, an increased abdominal component to the respiration and an increased respiratory rate. Crackles and wheezes are audible upon auscultation of thorax (Dixon *et al.* 1995; Gerber 1968; Robinson *et al.* 2003; Robinson *et al.* 2001; Robinson *et al.* 2000). In more severe cases, the breathing can become very labored with an obvious expiratory push and horses can become hypoxemic (Dixon 1978; Vandenput *et al.* 1998). The association with a challenging stall environment suggests a hypersensitive inflammatory response to inhaled allergens that are present in this indoor milieu. This is supported by studies that show that organic substances such as thermophilic spores and endotoxin indeed can trigger clinical exacerbations of RAO (Derksen *et al.* 1988; Pirie *et al.* 2003a; Pirie *et al.* 2003b; Pirie *et al.* 2002; Robinson *et al.* 1996; Woods *et al.* 1993). Straw bedding and roughage are the primary sources for organic dusts and therefore control of the environment is mandatory. Indeed changing the bedding to wood shavings

or newspaper shred, and wetting the hay can decrease the amount of exposed allergens with consequent attenuation or subsidence of the clinical signs (Kirschvink *et al.* 2002; Thomson and McPherson 1984; Vandenput *et al.* 1998; Woods *et al.* 1993). Similarly removal of these horses to an outdoors environment can cause the horses to go into remission with complete resolution of the signs within 3 days (Jackson *et al.* 2000; Vandenput *et al.* 1998). New exposure to a challenging stall environment or hay will however result in rapid reoccurrence of the signs.

Inflammatory mediators in RAO

The inhaled allergens are known stimuli to epithelial cells and local resident macrophages to produce inflammatory cytokines (Hammond *et al.* 1999; Jackson *et al.* 2004; Liden *et al.* 2003; MacKay *et al.* 1991). These mediators of inflammation cause a massive neutrophil influx into the lower airway of the horse (Fairbairn *et al.* 1993) accompanied by airway bronchoconstriction and increased mucus production (Robinson *et al.* 2003; Robinson *et al.* 2001; Robinson *et al.* 1996). The bronchoconstriction and mucus production cause a narrowing of the airway lumen, with an increase in airway resistance as a result, hence the respiratory distress. Increased airway wall thickening due to inflammatory edema or mucus cell hyperplasia/metaplasia are considered additional causes for airway lumen obstruction (Robinson *et al.* 2003; Robinson *et al.* 1996). It is also thought that repeated episodes eventually can result in remodeling and scarring of the airway and lung tissue (Nevalainen *et al.* 2002). The production of the inflammatory mediators is the result from up regulated expression of their genes. The activation of these genes is under control of transcription factors such as NF-κB and AP-1. Indeed in

recent studies on RAO-affected horses the level of NF-κB activity in cells from bronchial epithelial brushings and in cells from bronchoalveolar fluid (BALF) was higher compared to that of healthy horses exposed to the same environmental challenges during crisis as well as remission (Bureau *et al.* 2000b; Sandersen *et al.* 2001). The level of NF-κB activity in turn correlated well with the level of clinical airway obstruction (Bureau *et al.* 2000a). In addition a recent study reported that during the acute phase of a crisis (table 1) the expression and concentrationl of the key regulatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) is significantly higher in the BALF of RAO horses compared to BALF of healthy horses (Giguere *et al.* 2002). Additionally, depending on the timing of the BALF samples, a significantly greater expression of the interleukins IL-4, IL-8, IL-13 and interferon-γ (INF-γ) in RAO-affected horses has been reported (table 2.1) (Ainsworth *et al.* 2003; Bowles *et al.* 2002; Giguere *et al.* 2002).

Tabel 2.1: Expression and levels of cytokines in BALF cells over time compared between control and RAO-affected horses

EXPRESSION[1,2]	LEVEL[2]	EXPRESSION[1]		EXPRESSION[2]		LEVEL[2]		EXPRESSION[1]
TNF-α				TNF-α	▲	TNF-α	≈	
IL-1β				IL-1β	▲	IL-1β	≈	
IL-4		IL-4	≈	IL-4		IL-4	≈	IL-4
IL-5						IL-5	▲	
IL-8	IL-8	IL-8	≈	IL-8	≈	IL-8	≈	IL-8
IL-13		IL-13	≈	IL-13	▼			IL-13
INF-γ		INF-γ	≈	INF-γ	▲			INF-γ
BASELINE		1 DAY		5 DAYS				5 WEEKS

[1] Data from Ainsworth D.M. et al Vet Immunol Immunolopathol 2003;83-91

[2] Data from Giguere S. et al Vet Immunol Immunolopathol 2002;147-158

▼▲≈ Significant change within RAO horses compared to baseline

Long term exposure to a challenging environment results in increased expression of INF-γ and IL-8. However the elevations in expression are not always significantly higher compared to initial remission values (e.g. IL-8, IL-13), or do not always result in significantly increased levels of active protein product (e.g. TNF-α, IL-1β, IL-4) in the

BALF (table 1). Overall during remission or crisis it appears that in RAO-affected horses only INF-γ is persistently elevated when compared to healthy control horses, while the expression of other cytokines such as IL-13 actually is decreased (table 2.1). Therefore up regulation of expression of other cytokines such as RANTES or inflammatory products like leukotriene LTB_4 (Lindberg *et al.* 2002; Lindberg *et al.* 2004), nitric oxide (Costa *et al.* 2001; Hammond *et al.* 1999) or matrix metalloproteinase MMP-9 (Nevalainen *et al.* 2002; Raulo *et al.* 2000) might have more pathophysiologic significance.

Treatment of allergic airway disease: Bronchodilators

Lower airway inflammation, bronchoconstriction, increased mucus production and airway remodeling are also clinical features of human asthma. It is thus no surprise that the treatment of RAO in horses has been following the many of the methods used to combat human asthma. Modern treatment of RAO consequently focuses on bronchodilation for improvement of ventilation during crisis, and control of the inflammatory cascade in order to prevent new attacks and stop airway remodeling (Robinson *et al.* 2001; Tesarowski *et al.* 1994; Thomson and McPherson 1983). Additionally mucolytics and expectorantia have been used in order to break up and increase the clearance of mucus. Anticholinergic drugs such as atropine (0.02 mg/kg IV), glycopyrolate (0.007 mg/kg IV) or ipratropium (360 µg/kg IH q6h) and methylxanthine derivates have shown to be successful bronchodilators for treating exacerbations of RAO in the horse (Robinson *et al.* 2001). However β_2-agonists, likely as a result of their massive use in the treatment of human asthma, have become a more popular application.

Various β_2-agonists have been used with success in the treatment of RAO in horses. The reports include oral (clenbuterol; 0.8-3.2 µg/kg q12h), intravenous (clenbuterol; 0.8-3.2 µg/kg q12h) and inhalation (albuterol 360-720 µg/kg q3h, fenoterol;1000 mg, pirbuterol 600-1200 µg/kg q12h) applications (Derksen *et al.* 1999; Erichsen *et al.* 1994; Mair 1996; Mazan *et al.* 2003; Robinson *et al.* 2000; Rush *et al.* 1999; Tesarowski *et al.* 1994; Torneke *et al.* 1998). As an additional benefit it appears that β_2-agonists stimulate the mucociliary escalator (Dixon 1992; Turgut and Sasse 1989). It is likely that the resulting improvement in clearance of obstructive mucus aids in improvement of ventilation. The continued use of β_2-agonists has been associated with a decrease in the number of β_2-agonist receptors on equine lymphocytes (Abraham *et al.* 2002). It is thus likely that, similar with the use of these drugs in human asthma (Nishikawa *et al.* 1996), this also happens in the equine lower airway and renders these drugs less effective on the long term.

Treatment of allergic airway disease: Glucocorticoids in Asthma

Glucocorticoids were introduced for the treatment of human asthma in the late fifties (Blanchon *et al.* 1956; Turiaf *et al.* 1956). However it was the introduction of inhalation therapy one decennium later (Kravis and Lecks 1966), in combination with a better understanding of these drugs, that resulted in their exponential use. Genomic and non-genomic mechanisms form the basis of the potent anti-inflammatory actions of glucocorticoids. The magnitude of these effects, as discussed earlier in chapter 1, are dependent on many factors including the intrinsic properties of the glucocorticoid, the number and affinity of the glucocorticoid receptors (GR) in the tissue, the genome of the

tissue type and the local tissue concentrations of the glucocorticoid. It appears that number and affinity of GRs between tissues or species is relatively constant (Damon *et al.* 1985; Rohdewald *et al.* 1985), as is the affinity of the GRs for an individual glucocorticoid (table 1, chapter 1). In addition, with the exception of cortisol and prednisolone, it appears that the modern glucocorticoids are insensitive to metabolism and inactivation by 11-β-HSD-2 (Diederich *et al.* 1998; Feinstein and Schleimer 1999; Li *et al.* 1997). Therefore the type of airway cell, the intrinsic potency of a glucocorticoid for a desired effect (e.g. inhibition of LTB_4, , table 2 chapter 1), dose and the pharmacokinetics of that glucocorticoid (e.g. inhalation vs. oral) are the main factors that determine the success of a chosen glucocorticoid in the treatment of asthma. Indeed reported anti-inflammatory effects by glucocorticoids in human asthma include **1)** increased apoptosis and a lower influx of eosinophils, the key inflammatory cell type in asthma (Debierre-Grockiego *et al.* 2003; Druilhe *et al.* 2003; Walsh *et al.* 2003) **2)** decreased NF-κB expression in lung (A549) epithelial cells (Roumestan *et al.* 2003) **3)** decreased expression of mucus genes (MUC-5) in goblet cells or lung (A549) epithelial cells (Fergie *et al.* 2003; Lu *et al.* 2005) **4)** decreased cytokine production by stimulated alveolar macrophages (Leung *et al.* 2004) and **5)** decreased levels of cytokines and other inflammatory mediators such as NO in tracheal epithelial lining fluid (TELF), BALF or breath condensate (Eum *et al.* 2003; Gelb *et al.* 2004; Hoshino *et al.* 1999; Lehtimaki *et al.* 2001; Leung *et al.* 2004; Pelaia *et al.* 2003; Umland *et al.* 2002; Yuan *et al.* 2003) and **6)** decreased MMP-9 expression in bronchial biopsies of asthmatics (Hoshino *et al.* 1999). On the other hand glucocorticoids have been ineffective in down regulating expression of **1)** NF-κB in bronchial biopsies and alveolar macrophages of asthmatic

patients or human airway (BEAS-2B) epithelial cells (Hart *et al.* 2000; Newton *et al.* 1998) **2)** mucin expression (MUC-5) in bronchial biopsies from asthmatics (Groneberg *et al.* 2002) **3)** iNOS in human bronchial epithelial cells (Donnelly and Barnes 2002) and 4) MMP-9 expression in human alveolar neutrophils (Cundall *et al.* 2003). Thus while it is clear that glucocorticoids down regulate key pathways relevant to the inflammation in human asthma, the exact interaction between them and different cell types still needs further study. Nevertheless several important clinical observations regarding glucocorticoid therapy for asthma have been made. For instance, it appears that the addition of a glucocorticoid to β_2-agonist therapy results in additional improvement of the airway resistance compared to the use a β_2-agonist alone. This effect is significant within 1-2 hours, has a duration of 10 hours (Storr *et al.* 1987) and results in lower hospital readmission rates (Littenberg and Gluck 1986; Scarfone *et al.* 1993). It is likely that this additive effect can be explained by lesser degrees of mucus production and inflammatory airwall thickening and based, on the speed of onset, might include non-genomic actions. The addition of glucocorticoids to β_2-agonist therapy also antagonizes β_2-agonist induced reduction in β_2-agonist membrane receptors (Mak *et al.* 1995a; Mak *et al.* 1995b). Glucocorticoid therapy, therefore contributes to successful β_2-agonist therapy with the use of lower needed doses. Despite apparent poor suppression of MMP-9 expression of alveolar neutrophils it appears that longer term inhalation glucocorticoid therapy still results in decreased remodeling and scarring of the lung (Lee *et al.* 2004; Trigg *et al.* 1994).

Treatment of allergic airway disease: Glucocorticoids in RAO

The successful use of glucocorticoids in the treatment of human asthma was soon followed by the introduction of these drugs for the treatment of RAO in horses. The first studies reported on the application 25 mg dexamethasone-alcohol via repeated intramuscular injections (Gerber 1969; Muylle and Oyaert 1973). Much later this was followed by clinical efficacy studies on the intramuscular application of the longer acting formulations of dexamethasone-21-isonicotinate (Robinson *et al.* 2002) and triamcinolone acetonide (Lapointe *et al.* 1993), oral applications of prednisone (Jackson *et al.* 2000; Robinson *et al.* 2002; Traub-Dargatz *et al.* 1992) and inhalation therapy with beclomethasone (Ammann *et al.* 1998; Rush *et al.* 1998a; Rush *et al.* 1998b) and fluticasone-diproprionate (FP)(Giguere *et al.* 2002). All these studies, with the exception of all reports regarding oral application of prednisone, showed significant improvement in parameters of lung functions after at least 3 days of treatment. The improvement was often as good as remission values [FP, 2000 µg, IH, q12h for 21 days; (Giguere *et al.* 2002)], [DEX, 0.1 mg/kg, IV q24h for 3 days; (Robinson *et al.* 2002)] or when compared to maximal bronchodilation with atropine [DEX, 0.04 mg/kg, IV q24h for 7 days; (Picandet *et al.* 2003)]. Recently dexamethasone treatment (0.1 mg/kg q24h, 7 days) was reported to result in significant improvement of mucus production and cough frequency (Robinson *et al.* 2003). The failure of prednisone has been attributed to the poor oral availability and the lack of conversion of this drug to the active metabolite, prednisolone, in the liver (Peroni *et al.* 2002).

The improvements in lung function, mucus production and cough by glucocorticoids in horses with RAO are likely the result of down regulation of the inflammatory response.

However similar to the studies of human asthma at the moment it is unclear which inflammatory pathways are down regulated. Currently it is not known what the effect of glucocorticoid therapy is on the up regulation of expression of the transcription factor NF-κB during an exacerbations of RAO. So far only one study has reported on cytokine profiles in BALF after glucocorticoid therapy in horses with exacerbations of RAO (Giguere *et al.* 2002). In this study 2000 µg fluticasone propionate q12h for 3 weeks resulted in a decreased expression of INF-γ compared to healthy control horses or RAO horses that received no treatment (table 2.2). Additionally IL-4 was significantly decreased compared to no treatment. The decrease in INF-γ and IL-4 did correlate with the improvement of lung function, however many of the other up regulated cytokines were not down regulated. Similarly glucocorticoid therapy of horses in a RAO crisis has not consistently resulted in a decrease of neutrophilic inflammation in the lower airway. Most studies report significant improvement of neutrophil percentages in BALF of RAO horses in crisis when compared to untreated RAO controls (Giguere *et al.* 2002; Lapointe *et al.* 1993; Robinson *et al.* 2003; Robinson *et al.* 2002; Rush *et al.* 1998a). However in most of these reports, the earlier improvement in respiratory parameters did not associate with a significant temporal decrease of the neutrophil response (Giguere *et al.* 2002;

Tabel 2.2: Effect of fluticasone-diproprionate treatment (TX) on cytokine expression in cells from BALF of horses in crisis of RAO compared to no treatment (NO-TX) or healthy controls.

CYTOKINE EXPRESSION	
TX vs CONTROL	TX vs NO-TX
TNF-α	TNF-α
IL-1β	IL-1β
IL-4	IL-4
IL-5	IL-5
IL-8	IL-8
IL-13	IL-13
IFN-γ	IFN-γ
POST 3 WEEKS FP[1]	

[1] FP 2000 µg IH Q12H

Lapointe *et al.* 1993; Robinson *et al.* 2003; Robinson *et al.* 2002; Rush *et al.* 1998a). Contrary to their effect on eosinophils in human asthma, it appears that glucocorticoids actually inhibit apoptosis of neutrophils (Cox 1995; Liles *et al.* 1995; Nittoh *et al.* 1998; Zhang *et al.* 2001; Zhang *et al.* 2002). This effect is dose-dependent and varies in potency amongst different glucocorticoids (table 2.3).

Table 2.3: Glucocorticoid potencies for apoptosis

HUMAN NEUTROPHIL APOPTOSIS	
GLUCOCORTICOID	EC_{50} (10^{-9} M)
	(10^{-9} M)
CORTISOL[1]	38 ± 5
PREDNISOLONE[1]	13 ± 15
DEXAMETHASONE[1]	8 ± 4
BECLOMETHASONE[1]	20 ± 0.3
BUDESONIDE[1]	0.8 ± 0.2
FLUTICASONE[1]	0.6 ± 0.2
MOMETHASONE[2]	0.17 ± 0.03

[1] Zhang, X. et al: Life sciences 2002 (71) ; 1523-1534

[2] Zhang, X. et al: Eur J Pharmacol 2001 (431); 365-371

Maximal inhibition is achieved at concentrations of 10^{-6} M and in the order of 50 to 90 % (Liles *et al.* 1995; Zhang *et al.* 2001; Zhang *et al.* 2002). As a consequence neutrophils will reside longer in sites of inflammation. Although reports on apoptosis of equine neutrophils by glucocorticoids are lacking, it is likely that inhibition of apoptosis is a key factor for the poor temporal decrease of BALF neutrophils. This delay of neutrophil apoptosis is also associated with prolonged or increased activity of these cells. So in human neutrophils the MMP-9 activity is poorly inhibited (Cundall *et al.* 2003) while superoxide production actually is up regulated (Cox 1995). Functional changes have also been documented for equine neutrophils after exposure to glucocorticoids and include enhanced stimulated migration (Morris *et al.* 1988) and increased superoxide production (Guelfi and Kraouchi 1989). Based on the dramatic improvement in clinical response it is therefore likely that

yet to define down regulation of other cell types, or inflammatory pathways, by glucocorticoids have more relevance in RAO.

Clinical efficacy of glucocorticoids in RAO

The clinical data indicate that glucocorticoids are valuable drugs for the treatment of RAO in the horse. Despite these data, comparative reports on ideal dose and dose-interval are still lacking. So far the improvement of lung function in RAO affected horses by glucocorticoids has only been reported from 24-h on after inhalation therapy (Rush *et al.* 1998b) or 3 days or more after parenteral application of various glucocorticoids in different doses (Ammann *et al.* 1998; Giguere *et al.* 2002; Lapointe *et al.* 1993; Picandet *et al.* 2003; Robinson *et al.* 2003; Robinson *et al.* 2002). However genomic and non genomic mechanisms, and human clinical studies (Storr *et al.* 1987) indictate that a faster response is more than likely. Ideally the therapy should be at a low dose to minimize adverse effects, be rapidly effective and easily applicable with a view towards maintenance therapy. Additionally the dose and dose interval should be flexible so it can be adjusted pending clinical improvement and environmental situation. In human medicine these requirements are all easily met by inhalation therapy through which a relativly small amount of potent glucocorticoid can be deposited at the needed location, and with minimal induction of systemic side effects. Although reported successful in the treatment of RAO, inhalation therapy of glucocorticoids in horses is cumbersome, as well as relatively expensive, due to the need of special delivery devices. It also appears that even low doses of the relativly weak inhalation steroid beclomethasone still results in systemic adrenocortical depression (Rush *et al.* 1998c), thereby taking away an important

rational for this type of application. Contrary to prednisone, the reported oral bioavailability in fasted horses for the glucocorticoids prednisolone and dexamethasone is quite good and in the order of 61% (Cunningham *et al.* 1996; Peroni *et al.* 2002). Therefore this way of application should have promise as a flexible, easily administrable and cheap method of treatment for RAO in the horse.

Summary chapter 2

Based on the previous discussion of the literature on glucocorticoid treatment of RAO in the horse it seems important that future studies focus on: **1)** which cell types and mediators are down regulated and correlate with clinical improvement; **2)** how fast do glucocorticoids result in clinical improvement of lung function; **3)** is oral application as efficacious a parenteral application, and what is the influence of roughage on this; and **4)** what is the ideal dose for achieving all these effects. Therefore the following chapter will report on a study to address points 2,3,4 regarding the treatment of RAO in horses with dexamethasone.

CHAPTER 3:

EFFICACY OF ORAL AND INTRAVENOUS DEXAMETHASONE IN HORSES WITH RECURRENT AIRWAY OBSTRUCTION

Cornelis J. Cornelisse, N. Edward Robinson, Cathy E.A. Berney, Christina A. Kobe, Daniel T. Boruta and **Frederik J. Derksen**

Pulmonary Laboratory, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824-1314 USA.

Corresponding author:

Cornelis Jan Cornelisse

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

East Lansing, MI 48824-1314

Telephone: 517-353-9710

Fax: 517-432-1042

email: cornelis@cvm.msu.edu

Keywords: horse; dexamethasone; RAO; pulmonary function

Word count: 4324 (including title page and summary)

Summary

Reasons for performing the study: **Although the efficacy of dexamethasone for the treatment of recurrent airway obstruction (RAO) has been documented, the speed of onset of effect and its duration of action are unknown, as is the efficacy of orally administered dexamethasone with or without fasting.**

Objectives: **To document the time of onset of effect and duration of action of a dexamethasone solution when administered intravenously or orally with and without fasting.**

Methods: **Protocol 1 used eight RAO-affected horses with airway obstruction in a crossover design experiment that compared the effect of intravenous (IV) administration of saline and dexamethasone (0.1 mg/kg) on pulmonary function over 4 hours. Protocol 2 used six similar horses to compare, in a crossover design, the effects of dexamethasone (0.1 mg/kg) IV, dexamethasone (0.164 mg/kg) PO with and without prior fasting, and dexamethasone (0.082 mg/kg) PO with fasting.**

Results: **Dexamethasone IV caused significant improvement in lung function within 2 hours with a peak effect in 4-6 hours. Similarly oral dexamethasone was effective within 6 hours with a peak effect at 24 hours when administered orally at a dose of 0.164 mg/kg prior to feeding. The duration of effect was for all dexamethasone treatments statistically significant for 30 hours when compared to saline and tended to longer duration of effect when used orally.**

Dexamethasone given orally at a dose of 0.164 mg/kg to fed horses had comparable mean effects as dexamethasone at a dose of 0.082 mg/kg PO given to fasted horses indicating that feeding decreases bioavailability.

Conclusions: **Intravenously administered dexamethasone has a rapid onset of action in RAO-affected horses. The oral administration of a bioequivalent dose of the same solution to fasted horses is as effective as IV administration and tends to have longer duration of action. Fasting horses before oral administration of dexamethasone improves the efficacy of the treatment.**

Potential relevance: **The oral administration to fasted horses of a dexamethasone solution intended for IV use provides an effective treatment for RAO-affected animals.**

Introduction

Recurrent airway obstruction (RAO) in horses is caused by exposure to organic dust that can be present in the horse's environment. Because stables contain a great deal of organic dust, stabling horses is one of the major predisposing factors associated with this disease. The exposure to organic dust can result in a lower airway inflammation characterized by increased mucus production, contraction of the bronchial smooth muscle and thickening of the airway wall. Airway obstruction that impairs breathing is the result. Glucocorticoids (GCs) have been used successfully to relieve the signs of RAO in horses (Lapointe *et al.* 1993, Ammann *et al.* 1998, Rush, *et al.* 1998, Robinson *et al.* 2002, Robinson *et al.* 2003). Most of the effects of GCs are attributed to the down-regulation of the cellular transcription machinery for mediators of inflammation, either directly via glucocorticoid response elements (GREs) or indirectly via NF-κB pathway (Jaffuel *et al.* 2000, Sandersen *et al.* 2001, Giguere *et al.* 2002). The down-regulation of inflammation results in reduction of excess fluid and secretions, as well as decreased production of bronchoconstrictor mediators.

An easy and economical way for the horse owner to administer GCs would facilitate treatment of horses known to be suffering from RAO. In general, intermittent bolus dosing and applications that bypass the systemic circulation are preferred over systemically long-acting formulations of GCs in order to minimize potential metabolic side effects. Oral GC administration provides an inexpensive and convenient alternative for therapy of RAO and allows flexibility and control over dose and dose interval. Oral bioavailibility for GCs in fasted horses has been reported for prednisolone and

dexamethasone and is in the order of 61% of the total administered dose (Cunningham *et al.*, 1996, Peroni *et al.* 2002). Despite the widespread use of parenteral and oral dexamethasone for treatment of RAO, the earliest measurements of its efficacy have been after 3 days of administration. The speed of onset and duration of action of dexamethasone in the equine airways has not been determined. Furthermore, feeding could negatively influence oral bioavailability and consequently efficacy. For these reasons, we investigated the following questions: 1) How rapid is the onset of action of dexamethasone? 2) Is a single oral dose (PO) of a dexamethasone solution as efficacious as a single intravenous (IV) dose in the treatment of RAO in horses? 3) Is the efficacy of orally administered dexamethasone affected by roughage feeding?

Increasingly veterinary clinicians are using a dexamethasone solution intended for intravenous use as an oral application because it is convenient to administer and inexpensive. Although owner and veterinary reports suggest satisfactory results there are no clinical studies of efficacy. Therefore the efficacy of this dexamethasone solution was investigated.

Materials and methods

The study used two protocols, which were approved by the All-University Committee for Animal Use and Care at Michigan State University. The aim of protocol 1 was to examine the speed of onset of action of dexamethasone on change in pleural pressure (ΔPpl_{max}) while protocol 2 studied the efficacy of orally and intravenously administered dexamethasone as measured by the onset, duration and magnitude of effect on several

respiratory parameters. Both protocols used RAO-affected horses from the Pulmonary Laboratory herd that normally are kept at pasture.

Protocol 1

Eight horses (seven mares, one gelding, 13 to 31 years old, 384 to 583 kg) affected with RAO were used in the protocol. These included six Quarter Horses, one Hanoverian and one Arabian. At the start of the experiment the horses were brought in from pasture, bedded with straw and fed hay that was of poor quality in order to induce lower airway obstruction. When horses started to show clinical signs of lower airway obstruction, pulmonary function was measured. When the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}) was 25 cm H_2O or more (measured without a facemask), the horses were randomly assigned to one of the following treatments: 1) saline IV or 2) 0.1 mg/kg bwt dexamethasone IV. The horses remained instrumented and cross-tied in the stall over a 4-hour period, during which ΔPpl_{max} was measured at 10, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after injection. Immediately after the 240-minute measurement, atropine (0.02 mg/kg bwt. IV) was given to induce maximal bronchodilation. Fifteen minutes later a final pulmonary function measurement was made. The treatments were separated by a minimum of 14 days and followed a crossover study design.

Protocol 2

Six horses (five Quarter Horses and one Hanoverian) that were affected with RAO were used. All were mares that were 13 to 31 years old and weighed between 404 and 555 kg. At the start of the experiment the horses were brought in from pasture and lung function was measured to confirm that they were in remission (ΔPpl_{max} < 15 cm H_2O with facemask). The horses were thereafter bedded on straw and fed poor quality hay. Prior to feedings in the morning, pulmonary function measurements were made if clinical signs suggested an acute exacerbation of RAO. When the ΔPpl_{max} was 25 cm H_2O or more (with facemask), the horses were randomly assigned to one of the following five treatments: 1) 0.1 mg/kg bwt. dexamethasone IV, 2) an equivalent volume of saline IV, 3) 0.082 mg/kg bwt. dexamethasone PO (low-dose non-fed; LDNF), 4) 0.164 mg/kg bwt. dexamethasone PO (high-dose, non-fed; HDNF) or 5) 0.164 mg/kg bwt. dexamethasone PO 1 hour after roughage feeding (high-dose fed; HDF). The intravenous dexamethasone and saline groups were fed roughage 1 hour prior to treatment while for the HDNF and LDNF groups a minimum of 1 hour was allowed after treatment before the horses were fed. Subsequently pulmonary function was measured at 6, 24, 30, 48 and 72 hours after treatments. The horses remained in the stall during that period. The treatments were separated by a minimum of 14 days and followed a randomized crossover design.

Instrumentation and measurements

For both protocol 1 and 2, ΔPpl_{max} was estimated by measurement of esophageal pressure with a latex condom sealed over the distal end of a polypropylene catheter (3 mm inside

diameter, 4.4 mm outside diameter, 240 cm long). The balloon was passed via the nares to the distal third of the esophagus and connected via a pressure transducer (Validyne[1], Model DP/45-35) to either a portable physiograph (Dash II[2]) in protocol 1 or a respiratory function computer with data acquisition software (Buxco BioSystem XA[3]) for protocol 2. The final position of the catheter was adjusted to obtain the maximal ΔPpl_{max} during tidal breathing and was thereafter secured by taping it to the halter. For protocol 2 airflow rate also was measured by use of a pneumotachograph (No. 5 Fleisch[4]) fitted in a facemask that was placed over the horse's muzzle. The system was sealed with a rubber shroud and tape. The pneumotachograph was connected to a transducer (Validyne[1], Model DP/45-22) and the respiratory function computer. The flow signal was integrated to provide tidal volume. Prior to measurements, volume was calibrated by means of a 2-liter syringe and pressure by a water manometer.

Drugs

A commercial solution of dexamethasone[5] intended for IV use was chosen for both oral and IV administration. The IV dose (0.1 mg/kg) was chosen because it has been demonstrated to be effective for treatment of RAO (Rush, Raub *et al.* 1998; Robinson, Jackson *et al.* 2002). The higher oral dose (0.164 mg/kg) was based on the IV dose adjusted to be equipotent based on published bioavailability. The lower oral dose was 50 percent of the high dose. The saline dose was standardized to a volume equal to that of the injected dexamethasone formulation.

Data analysis

Data were collected over a 2-minute period or a minimum of 10 breaths. For protocol 1 the ΔPpl_{max} was calculated from the difference between peak inspiratory and peak expiratory pressure of at least 10 breaths. Swallows and sighs were not included. Data of at least 10 breaths were averaged in protocol 2. The measured parameters included ΔPpl_{max} , tidal volume (V_T), respiratory rate (RR), minute ventilation (V_{min}), peak inspiratory flow (PIF) and peak expiratory flow (PEF). The ΔPpl_{max}, flow and V_T during breathing were additionally processed to calculate pulmonary resistance (R_L) and dynamic compliance (C_{dyn}).

Statistical analysis

Data were analyzed with the aid of statistical software[6] by two-way ANOVA for repeated measures with time and treatment as main effects. The Student Newman-Keul's test was used for post hoc comparison between means. A significance level of 0.05 was chosen.

Results

Protocol 1

There was no significant difference between the baseline values of ΔPpl_{max} for the 2 treatments, indicating comparable levels of obstructive disease. There were significant effects of time and treatment as well as a significant time x treatment interaction. Post hoc comparison showed significant differences in ΔPpl_{max} between dexamethasone IV and

saline that occurred between 120 and 240 minutes after treatment. Additionally, at these times, ΔPpl_{max} was also significantly less than at baseline (Fig 3.1). Following dexamethasone IV the decrease in ΔPpl_{max} corresponded to an improvement of 36% and 59% at 120 min and 240 min, respectively. The ΔPpl_{max} after atropine treatment was significantly different from 240 minutes in both the saline as well as the dexamethasone treated group.

Protocol 2

Baseline values (0 hours) were not significantly different among all treatment groups, indicating a similar level of airway obstruction at the start of the experiments. During remission, there were no differences among treatments with the exception of C_{dyn}, which was significantly greater for HDF than for saline or HDNF.

There were significant main effects of treatment on ΔPpl_{max}, PIF, C_{dyn} and RR and significant effects of time were on ΔPpl_{max}, C_{dyn}, PIF, PEF, RR, TV and V_{min}. Significant treatment x time interactions were detected for ΔPpl_{max}, C_{dyn}, R_L, PIF, PEF and RR (Fig 3.2, Table 3.1). For all variables measured, there were differences between saline and dexamethasone treatments but no differences among the treatments themselves.

All of the dexamethasone treatments resulted in a significant decrease of ΔPpl_{max} at 6, 24 and 30 hours when compared to saline. At 48 and 72 hours, the ΔPpl_{max} after HDNF continued to be statistically significantly better than after saline administration . Compared to saline treatment, all dexamethasone treatments resulted in a significant improvement of C_{dyn} at 6 hours. This continued to be the case for dexamethasone IV and

HDNF at 24 hours, while HDNF was still effective at 30 hours. Treatment with dexamethasone IV resulted in a significant reduction of R_L at 6 and 24 hours. Treatments HDF and HDNF caused significant improvement in R_L at 30 hours while HDNF was also effective at 48 hours. All dexamethasone treatments resulted also in significantly lower PIF at 6 hours when compared to the saline treatment. This effect continued at 24 hours for HDNF and LDNF, whereas at 30 hours only HDNF caused a significant improvement over saline. All dexamethasone treatments resulted in a significant improvement of PEF at 6 hours when compared to saline. Treatment with dexamethasone IV, HDF and LDNF caused significant improvement of the respiratory rate (RR) at 6 hours. All treatments were better than saline at 24 and 30 hours. HDNF and LDNF as well HDF, HDNF and LDNF were still effective at 48 and 72 hours, respectively.

In the time within treatment comparisons (Fig 3.2, Table 3.1), the ΔPpl_{max} was, in the case of dexamethasone IV, significantly less than baseline values at 6 and 24 hours and also not significantly different from remission values at these times. Additionally, the ΔPpl_{max} after HDNF was significantly less than baseline at 24 hours and it was also not different from remission at this time. Also in the case of R_L, dexamethasone IV at 6 and 24 hours resulted in values similar to those in remission and significantly different from baseline. At 6 hours the treatments dexamethasone IV and LDNF resulted in PEF values as good as remission and significantly different from baseline.

Discussion

Glucocorticoids are well accepted as treatment for exacerbations of asthma in people. They additionally appear to have an important role in maintenance therapy and can prevent remodeling of the lung in the long term (Trigg *et al.* 1994, Hoshino *et al.* 1999). The effects of GCs are attributed to the down-regulation of the cellular transcription pathways responsible for inflammation, which is reported to take several hours to become fully effective (Schimmer *et al.* 1996). Despite a relative slower onset of action compared to bronchodilators, GCs are a very useful adjuvant therapy in treating exacerbations of asthma. In asthmatic patients the addition of prednisone or prednisolone to the initial bronchodilator therapy results in lower hospital admission rates compared to placebo when reassessed 4 hours after admission to an ER (Littenberg *et al.* 1986, Scarfone *et al.* 1993). The addition of a single treatment with an oral or intravenous GC to these patients results in increased peak expiratory flow rates within 1 to 2 hours compared to a placebo (Storr *et al.* 1987). The duration of this additive difference was approximately 10 hours.

Protocol 1 showed that dexamethasone in horses with RAO has a similar time course of the effect as GCs in human patients. Intravenous dexamethasone administration resulted in a significant improvement of ΔPpl_{max} beginning 120 minutes after administration. The average improvement at this time was 36.5%. After 4 hours, the ΔPpl_{max} showed approximately 59% improvement to a ΔPpl_{max} of 17.71 cm H_2O. This improvement is comparable to the lowest value after dexamethasone IV in protocol 2 found at 6 hours (56% compared to the within-treatment baseline). These observations suggest therefore that significant effects of down-regulation of transcription of

inflammatory mediators occurs within 2 to 6 hours after dexamethasone administration in the horse. Although maximal effect was achieved between 4 to 6 hours, atropine given at the end of protocol 1 still resulted in improvement of ΔPpl_{max}, indicating that maximal bronchodilation had not been achieved.

Protocol 2 showed that orally administered dexamethasone solution was as effective as dexamethasone IV. The oral bioavailability of GCs is around 61 % in horses that are fasted or receive the drug with some grain. This results in peak blood levels within 1-2 hours (Cunningham *et al.* 1996, Peroni *et al.* 2002). This rapid absorption is the reason that all oral treatments caused significant improvement in ΔPpl_{max} by 6 hours. Based on the measurement of ΔPpl_{max}, all dexamethasone treatments were effective for up to 30 hours. Our data further suggest that 0.164 mg/kg dexamethasone administered before feeding had beneficial effects for up to 72 hours. Although no significant differences were found between the dexamethasone treatments themselves, the means of our data suggest that oral applications of dexamethasone, especially HDNF, prolongs duration of action in comparison to IV administration. Indeed, if a $p < 0.1$ is chosen in the case of ΔPpl_{max} the HDNF would be better than HDF and LDNF at 24 hours, and better than HDF or DEX IV at 30 and 48 hours. This therefore strongly suggests that oral application results in a longer duration of action and that feeding impairs bioavailability. Although prolonged benefits of orally administered dexamethasone were observed for up to 72 hours, some of these effects would not be clinically visible to the horse owner. Published studies from our laboratory show that at a clinical score > 5 clinicians can confidently detect signs of RAO. This clinical score of 5 correlates with a ΔPpl_{max} of 24.5 ± 1.1 cm H_2O when

measured while horses wore a facemask (Robinson *et al.* 2000). Translated to an owner's point of view, our data suggest that dexamethasone IV and HDNF provide acceptable improvement for up to 24 hours in horses that are in a severe exacerbation of RAO.

The ΔPpl_{max} is determined by changes in both airway caliber (indicated by C_{dyn} and R_L) and breathing strategy (indicated by PIF, PEF and RR) (Robinson *et al.* 1999). Both C_{dyn} and R_L are indicators of the severity airway obstruction, with C_{dyn} reflecting inhomogeneous ventilation of the more peripheral airways, and R_L the resistance in the larger airways. Consistent with previous reports (Lapointe *et al.* 1993, Ammann *et al.* 1998, Rush *et al.* 1998, Robinson *et al.* 2002) this study showed that dexamethasone improved C_{dyn} as well R_L in horses with RAO. The additive beneficial effects of dexamethasone on C_{dyn} and R_L contributed to the significant overall effect on ΔPpl_{max}. During airway obstruction, horses with heaves try to maintain V_{min}. For this they alter breathing strategy, either via changes in V_T, RR, ΔPpl_{max} or combinations thereof and this consequently affects flow rates (Petsche *et al.* 1994). Protocol 2 showed that horses in all treatment groups maintained constant V_{min} and V_T. However, after the dexamethasone treatments similar V_{min} and V_T could be generated with lower RR, PIF, and PEF. Hence the reductions in respiratory rate and airflow added to the significant reduction in ΔPpl_{max}. Our investigation did not identify the reasons for the lower respiratory rate. One possibility is improved gas exchange and a reduction in the severity of hypoxemia. However, Derksen *et al.* (1982) demonstrated that the increased respiratory rate that followed aerosol antigen challenge of sensitized ponies was due to vagal inputs from the inflamed lung. It is possible therefore that dexamethasone, by reducing inflammation,

decreased the activation of pulmonary vagal afferents that participate in the regulation of respiratory rate.

Conclusion

Intravenous and oral administration of a dexamethasone solution is effective in treating exacerbations of RAO in horses. The effect of dexamethasone becomes clinically relevant within 6 hours post administration. An oral dose of 0.164 mg/kg is clinically effective for up to 24 hours in horses that have not eaten roughage in the previous 12 hours.

Table 3.1. Effect of intravenous and orally administered dexamethasone on pulmonary function parameters in RAO-affected horses. Measurements were made before horses were stabled (-1), at baseline when horses had airway obstruction (0), and 6, 24, 30, 48 and 72 hours after treatment. Data are mean ± se. n = 6. C_{dyn} = dynamic compliance (l/cm H_2O), R_L = pulmonary resistance (cm H_2O/l/s), PIF = peak inspiratory flow (l/s), PEF = peak expiratory flow (l/s), RR = respiratory rate (breaths/min). See text for definition of treatments. Treatment (T_X) within time comparison: ($p<0.05$). [a] significantly different from saline IV. Time within treatment comparison: [b] significantly different from baseline (0 h) and not significantly different from remission (REM).

Table 3.1.

TIME	Tx	C_{DYN}	R_L	PIF	PEF	RR
REM	SAL IV	0.97 ± 0.14	0.95 ± 0.11	3.72 ± 0.33	3.98 ± 0.47	12.65 ± 1.67
	DEX-IV	1.11 ± 0.10	0.76 ± 0.19	4.29 ± 0.62	4.27 ± 0.61	13.39 ± 2.11
	H D F	1.32 ± 0.08[a]	0.80 ± 0.12	4.34 ± 0.78	4.45 ± 0.65	13.32 ± 2.21
	HDNF	0.94 ± 0.05	0.85 ± 0.12	4.22 ± 0.54	4.35 ± 0.37	14.49 ± 1.51
	LDNF	1.17 ± 0.22	1.07 ± 0.19	3.60 ± 0.30	3.40 ± 0.21	12.35 ± 1.83
0	SAL IV	0.36 ± 0.11	2.22 ± 0.11	7.21 ± 0.72	6.47 ± 0.54	23.96 ± 2.31
	DEX-IV	0.40 ± 0.06	2.36 ± 0.64	6.20 ± 0.81	6.82 ± 0.39	23.05 ± 1.30
	H D F	0.41 ± 0.06	2.03 ± 0.24	5.80 ± 0.92	6.30 ± 0.30	21.62 ± 0.68
	HDNF	0.39 ± 0.09	2.35 ± 0.34	6.21 ± 0.89	6.31 ± 0.49	20.35 ± 2.68
	LDNF	0.36 ± 0.07	2.51 ± 0.50	6.46 ± 0.76	6.49 ± 0.89	20.40 ± 1.34
6	SAL IV	0.24 ± 0.05	2.81 ± 0.35	8.80 ± 0.77	6.96 ± 0.62	26.36 ± 2.21
	DEX-IV	0.61 ± 0.09[a]	1.37 ± 0.18[a,b]	3.96 ± 0.20[a]	4.72 ± 0.24[a,b]	20.02 ± 2.26[a]
	H D F	0.57 ± 0.11[a]	1.62 ± 0.18	4.48 ± 0.70[a]	5.01 ± 0.33[a]	19.32 ± 1.12[a]
	HDNF	0.57 ± 0.10[a]	1.79 ± 0.32	5.04 ± 0.86[a]	5.55 ± 0.86[a]	21.45 ± 4.05
	LDNF	0.50 ± 0.06[a]	2.52 ± 0.61	4.17 ± 0.61[a]	4.59 ± 0.51[a,b]	17.00 ± 1.68[a]
24	SAL IV	0.24 ± 0.08	2.68 ± 0.49	8.57 ± 0.49	6.48 ± 0.36	28.04 ± 1.94
	DEX-IV	0.57 ± 0.08[a]	1.31 ± 0.19[a,b]	4.97 ± 0.35	6.06 ± 0.11	19.33 ± 1.50[a]
	H D F	0.45 ± 0.13	2.22 ± 0.84	6.79 ± 1.29	6.34 ± 0.67	20.63 ± 1.25[a]
	HDNF	0.66 ± 0.03[a]	1.53 ± 0.20	4.74 ± 0.72[a]	5.00 ± 0.64	16.94 ± 2.42[a]
	LDNF	0.51 ± 0.16	2.34 ± 0.59	4.87 ± 0.56[a]	5.30 ± 0.47	17.58 ± 1.18[a]
30	SAL IV	0.22 ± 0.07	3.47 ± 0.54	9.51 ± 0.92	6.71 ± 0.32	28.55 ± 3.14
	DEX-IV	0.47 ± 0.08	2.44 ± 0.90	7.09 ± 0.84	6.85 ± 0.37	20.10 ± 1.44[a]
	H D F	0.41 ± 0.11	2.16 ± 0.66[a]	6.87 ± 0.98	6.89 ± 0.47	21.26 ± 1.54[a]
	HDNF	0.56 ± 0.06[a]	1.75 ± 0.27[a]	5.16 ± 0.57[a]	5.39 ± 0.23	16.20 ± 1.05[a]
	LDNF	0.47 ± 0.11	2.43 ± 0.57	7.35 ± 1.01	7.11 ± 1.01	18.22 ± 1.14[a]
48	SAL IV	0.25 ± 0.09	3.06 ± 0.26	7.62 ± 1.32	6.40 ± 0.64	26.96 ± 3.53
	DEX-IV	0.38 ± 0.13	2.69 ± 0.71	6.93 ± 0.50	6.95 ± 0.24	23.02 ± 2.75
	H D F	0.43 ± 0.12	2.13 ± 0.44	7.81 ± 1.06	7.03 ± 0.61	23.77 ± 1.24
	HDNF	0.54 ± 0.14	1.56 ± 0.23[a]	6.06 ± 0.94	5.69 ± 0.61	19.29 ± 2.95[a]
	LDNF	0.46 ± 0.09	2.15 ± 0.31	6.47 ± 1.23	6.57 ± 0.80	18.77 ± 1.11[a]
72	SAL IV	0.15 ± 0.03	2.44 ± 0.44	8.68 ± 0.63	6.65 ± 0.31	29.53 ± 3.05
	DEX-IV	0.271 ± 0.07	2.50 ± 0.38	6.81 ± 0.72	6.49 ± 0.40	24.96 ± 3.22
	H D F	0.43 ± 0.11	2.32 ± 0.53	6.26 ± 0.78	6.65 ± 0.42	20.15 ± 1.55[a]
	HDNF	0.47 ± 0.12	1.99 ± 0.32	6.79 ± 1.56	6.00 ± 0.34	21.50 ± 4.45[a]
	LDNF	0.29 ± 0.08	2.60 ± 0.36	7.18 ± 0.88	6.23 ± 0.48	20.92 ± 1.87[a]

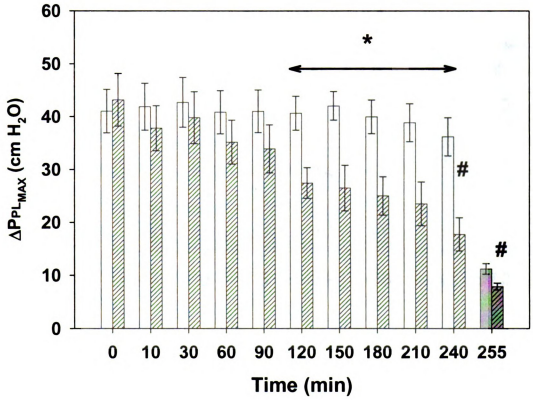


Fig 3.1: Effect of intravenous saline and intravenous dexamethasone on the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}) in RAO-affected horses. Saline or dexamethasone (0.1 mg/kg IV) was administered immediately after the time 0 measurement. Atropine (0.02 mg/kg IV) was administered immediately after the time = 240 min. measurement period. Data are mean ± se. n = 8. * = Significant difference between treatments within a measurement period. # = Significantly different from atropine within treatment. ($p<0.05$).

SALINE IV

DEX IV (0.1 mg/kg)

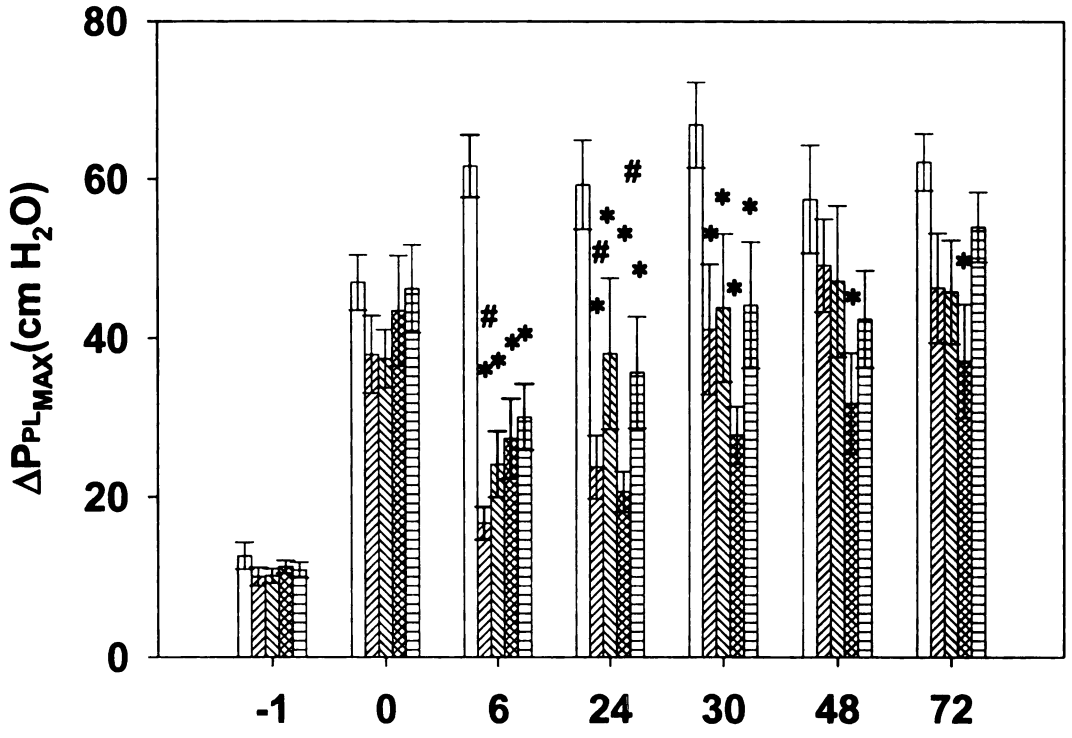


Fig 3.2. Effect of intravenous and orally administered dexamethasone on the maximal change in pleural pressure (ΔPpl_{max}) in RAO-affected horses. Measurements were made before horses were stabled (-1), at baseline when horses had airway obstruction (0), and 6, 24, 30, 48 and 72 hours after treatment. Data are mean ± se. n = 6. * significantly different from saline within a time

significantly different form baseline (0 h) and not significantly different from remission within a treatment. ($p<0.05$).

SALINE IV

DEX IV (0.1 mg/kg)

HDF (0.164 mg/kg, food)

HDNF (0.164 mg/kg, no food)

LDNF (0.082 mg/kg, no food)

CHAPTER 4

GLUCOCORTICOID THERAPY AND EQUINE LAMINITIS: FACT OR FICTION?

Cornelisse, C.J., Robinson, N.E.

Pulmonary Laboratory, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East-Lansing, MI 48824, USA

Introduction

Is administration of glucocorticoids associated with laminitis in horses? That is the core question in the paper by McCluskey and Kavenagh regarding the prevalence of equine laminitis after parenteral treatment of 205 cases with triamcinolone acetonide (TMC).

Is there clinical evidence for the connection between glucocorticoids and laminitis?

Since the introduction of glucocorticoids into equine medicine more than 30 years ago, their use has been accompanied by fear of induction of laminitis. Despite this fear, real information regarding glucocorticoid use and laminitis is scarce. In a handful of older reports in the veterinary and lay literature that implied an association between laminitis and prior glucocorticoid use, it was unclear if the glucocorticoid was really the cause of the problem. For instance, some reports included horses with chronic obstructive pulmonary disease (Gerber 1970; Muylle and Oyaert 1973), animals that are usually older and could have had Cushing's disease. Horses in other reports may have had coexisting gastrointestinal disease (Lose 1980) or what is now known as peripheral Cushing's syndrome (PCS) (Frederick and Kehl 2000). Both cushingoid disorders, as

well gastrointestinal diseases, are often associated with laminitis. The lack of credible reports makes it difficult to get a good impression of the risk of equine laminitis associated with glucocorticoid use. There is some information, however, that suggests that this risk is not very high. Out of a combined total of 526 horses from three studies that looked at *multiple risk factors* in referred cases of acute or chronic laminitis (Hunt 1993; Slater *et al.* 1995; Cripps and Eustace 1999), only three were attributed to glucocorticoid-induced laminitis. Even more convincing is the lack of support for a high prevalence of steroid induced laminitis in published reports of adverse drug reactions. For instance, the 1993 and 1994 reports of the Australian Veterinary Association Adverse Drug Reaction Subcommittee (Maddison 1994; Maddison 1996) did not mention any case of glucocorticoid-induced equine laminitis. Similarly, the current FDA-CVM Adverse Drug Experience web pages (FDA 2002) mention only 5 "possibly" drug-related cases of laminitis over the period 1987-2000. These were associated with a variety of parenterally used glucocorticoids, including dexamethasone (DEX), betamethasone, TMC, and methyl-prednisolone. The lack of information regarding actual numbers of sales for these products, as well as the likelihood that underreporting exists, make it difficult to determine the exact prevalence of glucocorticoid-induced laminitis. However, the inclusion of owner reports in the FDA cases does suggest that glucocorticoid-induced laminitis is a relatively rare occurrence.

In addition to the scarcity of reports of laminitis induction, there are the multiple studies in which glucocorticoids were administered for longer periods, or at higher doses without mention of the induction of laminitis. Laminitis was not reported as a complication during or after treatment with either parenteral administration of TMC (0.09

– 0.2 mg/kg) (LaPointe *et al.* 1993; Lepage *et al.* 1993; French *et al.* 2000) or DEX at clinical (0.1 mg/kg for up to 10 days) (Rush *et al.* 1998; Robinson *et al.* 2002) or even higher doses (1 mg/kg for 9 days) (Tumas *et al.* 1994).

How might glucorticoids predispose to the development of laminitis?

Despite the lack of credible clinical evidence that glucocorticoid therapy causes laminitis, the association of the condition with both Cushing's disease and peripheral cushingoid syndrome in obese horses (Johnson 1999) suggests that *chronic exposure* to glucocorticoids may be an important modulating factor. The classic theories on the pathogenesis of laminitis focus on either **1)** a primary disturbance of perfusion of the laminae followed by secondary events that lead to laminar injury, or **2)** a disturbance in the proliferation and differentiation of the keratinocyte by toxic or metabolic substances, or **3)** increased matrix metallo-protease (MMP) activity induced, for example, by endotoxins or streptococcal exotoxins (Grosenbaugh *et al.* 1991; Weiss 1997; Pollitt and Daradka 1998; Adair *et al.* 2000; Mungall *et al.* 2001; Wattle 2001). Current understanding of the molecular mechanisms of glucocorticoid action can be implicated in any of the above theories.

The activation of the cellular glucocorticoid receptor is dependent on cortisol, the active form of the glucocorticoid molecule. The net balance of the combined activity of two kinetically distinct isoforms of the enzyme 11β-hydroxy steroid hydrogenase (11-β HSD) determines the local tissue level of this cortisol. The 11-β HSD-1 isoform primarily converts inactive cortisone into cortisol whereas 11-β HSD-2 catalyzes the reverse reaction. It appears that the net balance between the 11-β HSD isoforms for 9-alpha fluoro glucocorticoids like DEX and TMC favors a decreased breakdown of these

drugs (Best *et al.* 1997; Diederich *et al.* 1998) and hence a prolonged tissue activity. In certain types of obesity in humans increased activity of 11-β HSD-1 is associated with insulin resistance (Masuzaki *et al.* 2001; Paulmyer-Lacroix *et al.* 2002), which in turn is associated with an impaired production of nitric oxide (NO) by endothelial cells (Masuzaki *et al.* 2001; Paulmyer-Lacroix *et al.* 2002; Steinberg and Baron 2002; Trovati and Anfossi 2002). Production of NO has a direct vasodilator effect on equine digital arterial vascular smooth muscle and impairment of its production could cause reduced blood flow to the foot (Baxter 1995; Cogswell *et al.* 1995; Schneider *et al.* 1999). However, regulation of digital blood flow is not simply under the control of one mediator but is due to a complex interplay between vasoconstrictors such as endothelin (ET) and vasodilators such as NO (Cardillo *et al.* 1999; Miller *et al.* 2002). Tipping the balance toward vasoconstriction by reduction of NO production could play a part in the onset of laminitis. A similar combination of mechanisms is thought to be responsible for the high prevalence of peripheral vascular disease in humans with obesity and diabetes (Miller *et al.* 2002).

In rats, chronic administration of low doses of dexamethasone induces hypertension on the basis of decreased NO production. The hypertension is preceded by a decreased sensitivity to insulin, and both can be blocked by metformin, a drug that increases peripheral insulin sensitivity and cellular glucose uptake (Severino *et al.* 2002). Thus, glucocorticoids decrease NO production and cause an impaired vasodilator reserve. The observations from obese insulin-resistant humans and rats could be in agreement with 1) a decreased lamellar perfusion model as the initiating event of laminitis, 2) exaggerated contractile responses of equine digital blood vessels after administration of

glucocorticoids (Eyre *et al.* 1979), and 3) a reduced production of endothelium derived nitric oxide that contributes to an impaired vasodilator reserve (Baxter 1995; Schneider *et al.* 1999). Additionally endotoxins impair the vasodilator reserve of equine digital arteries (Baxter 1995) and increased ET expression has been documented in laminitic hoof tissue (Katwa *et al.* 1999). It could therefore be the case that glucocorticoids potentially function as modulators of laminitis induction in endotoxemic diseases and in cases of chronic laminitis. However, in two large studies of horses with Cushing's disease, of which a large percentage also had laminitis, a single intramuscular dose of DEX (10 mg) did not induce or exacerbate the laminitis (Dybdal *et al.* 1994; Schott personal communication 2002).

How do glucocorticoids affect keratinocytes and how might that be a factor in laminitis? Keratinocytes and fibroblasts are responsible for the production and quality of the connective tissues in the skin whereas the spatial and temporal activity of MMPs is important in wound healing. Glucocorticoids can alter proliferation and differentiation properties of keratinocytes and fibroblasts, resulting in decreased collagen synthesis and thus a weakened structural supporting matrix. Therapy with glucocorticoids leads to skin atrophy and impaired wound healing. Indeed, 3 days of topical glucocorticoid treatment results in down regulation of the collagen synthesis in human skin (Haapasaari *et al.* 1996; Oikarinen *et al.* 1998) and clinically significant thinning of the skin can be found after 1-2 weeks of daily topical fluorinated glucocorticoids (Korting *et al.* 2002). Because alterations in the proliferation and differentiation of keratinocytes are features of equine laminitis, it is therefore possible that glucocorticoids can influence the integrity of the connective tissues and contribute to the onset of laminitis, especially if used long term. It

seems unlikely however that increased basement membrane degradation due to increased MMP activity per se contributes to this since there is no evidence that glucocorticoids up regulate MMP activity (Kylmaniemi *et al.* 1996; Madlener *et al.* 1998).

Based on data obtained from horses with cushingoid diseases, it seems likely that chronic exposure to glucocorticoids at the local tissue level could be a very important factor in the induction of laminitis. The relative refractoriness of fluoro-glucocorticoids like TMC and DEX to 11-β HSD-2 inactivation could therefore mean that basement membrane keratinocytes are always exposed to an activated form of glucocorticoids. Although the reported half-life of intravenous TMC in the horse is shorter (1.39 –1.58 h) (French *et al.* 2000) than that of DEX (2.63 h) (Cunningham *et al.* 1996), TMC appears to have a longer GR-binding half-life (Rohdewald *et al.* 1986). Additionally, different glucocorticoids have different transcriptional potencies in different tissues. This consequently results in effects of different magnitude in different tissues, and bypasses the classical order of glucocorticoid potencies (Jaffuel *et al.* 2001). For example, TMC actually has equipotent diabetogenic effect as dexamethasone by inducing similar increases in the activity of the gluconeogenic enzyme tyrosine aminotransferase (TAT) in a hepatoma tissue cell line.

Drug formulation and mode of application have significant effects on elimination rate and final tissue levels. Acid esterification of drugs (e.g., acetate, propionate) results in a slower systemic absorption of the drug compared to the alcohol or sodium phosphate/succinate analogs (Schimmer and Parker 1996). Current formulations of TMC in use in veterinary medicine are the long-acting acetonide esters.

The 205 cases reported by McCluskey and Kavenagh were treated by local application in the joints, tendon sheaths, and bursae. This is likely associated with low, short duration systemic levels of glucocorticoids, and thus poses a smaller risk for additional side effects compared to systemic application. Indeed, 18 or 30 mg TMC by the intra-articular route results in serum levels that are detectable for less than 48-102 hours with peaks between 4 and 12 hours respectively. The lower dose results in cortisol suppression for less than 120 hours (Chen *et al.* 1992; Koupai-Abyazani *et al.* 1995), compared to 336 hours (Slone *et al.* 1983) after a similar intramuscular dose. Additionally, in reports spanning a total of 472 horses that were treated intra-articularly or intra-bursally with different glucocorticoids, including TMC, and followed over a longer period after administration, no cases of laminitis were mentioned (Verschooten *et al.* ; Houdeshell 1969; Swanstrom and Dawson 1974; Vernimb *et al.* 1977; Frisbie *et al.* 1997). This therefore supports the relative safety of intra-articular application of glucocorticoids in the horse.

In conclusion, current research is getting closer to unraveling the mechanisms by which glucocorticoids work. Besides the effects of formulation and route of administration, it appears that the newer glucocorticoids have long activity at the tissue level. Their genomic effects are tissue dependent and do not necessarily follow the classical ordering of glucocorticoids. Their effects can be associated with vascular dysfunction and interfere with keratinocyte proliferation/differentiation as well as matrix integrity, all mechanisms that possibly can initiate laminitis. A summation of factors suggests that TMC might have longer tissue activity and have stronger diabetogenic effects than DEX. However, regarding the induction of laminitis, a paucity of reported

cases and a multitude of clinical studies suggests that the risk for this after parenteral, especially intra-articular use, is not high. Based on experiences of horses with a cushingoid disorder, it seems that the most likely risk factor might be prolonged treatment. Additionally prior diseases such as endotoxemia, prior laminitis that can affect vascular function and/or keratinocyte proliferation/differentiation might modulate this risk as well. However evidence for this based on sufficient numbers is currently lacking.

CHAPTER 5

EFFECTS AND MECHANISMS OF GLUCOCORTICOID ACTIONS ON MICROCIRCULATION

The microcirculation

The viability and function of cells in the tissues is dependent on an adequate blood supply for the delivery of nutrients, hormones and oxygen, and the uptake and removal of cellular products. The blood supply at the tissue level is regulated by the microcirculation, i.e. the blood vessels from the first order arterioles via capillaries into first order venules (Boron and Boulpaep 2003). The activity level of the cells fluctuates, and therefore an adaptive microcirculation is needed to guarantee an optimal blood flow. Regulation of blood flow is via alteration in the tone of vascular smooth muscle cells (VSMC). These VSMC are distributed throughout the wall of the arterioles and venules. Of particular importance are the precapillary sphincters, i.e. small conglomerates of VSMC, which strategically are placed in front of the capillary entrance. The tone of the precapillary sphincters determines the amount of blood flow to the downstream capillary bed. In some vascular beds, like the skin and laminae of the equine hoof, direct arterio-venous shunts (A-V shunts) can short circuit blood supply if needed (Hood, Amoss et al. 1978; Robinson 1990; Hood, Grosenbaugh et al. 1993). Strong precapillary tone with additional bypass of the blood via A-V shunts will consequently minimize the capillary blood flow. The tone of VMCS is under control of systemic and local regulatory systems that work together to adjust and optimize the blood flow to the tissues.

Systemic regulation

Systemic regulation of the microvascular blood flow is under control of the autonomic nervous system. Sympathetic and some parasympathetic nerve endings that are enmeshed around the blood vessels walls communicate on a local level with VSMC and endothelial cells. The sympathetic nerve endings release norepinephrine, a catecholamine that activates adrenergic α_1-receptors on VSMC. Transduction of the α_1-receptor signal is through a G-protein coupled pathway and results in contraction of the VSMC, hence vasoconstriction. Activation of the enzyme myosin light chain kinase (MLCK) plays a central role in VSMC contraction (Boron and Boulpaep 2003). Sympathetically controlled release of epinephrine into the blood stream can on the other hand cause VSMC relaxation via an adrenergic β_2-receptor mediated decrease in MLCK activity. The parasympathetic regulation of blood flow is under control of 1) acetylcholine (ACH), a muscarinic agonist and 2) nitric oxide (NO) which acts as an activator for soluble guanylyl cyclase in VSMCs. Both are released from the terminal nerve endings (Boron and Boulpaep 2003). ACH causes vasodilation via two pathways. Activation of M_2-receptors on VSMCs in the vascular wall, but not the precapillary sphincters, results in a G-protein coupled pathway that results in a decrease of MLCK activity. ACH additionally activates M_3-receptors on endothelial cells, which results in the production of various vasoactive substances by these cells. This includes a G-protein mediated increase in activity of the enzyme nitric oxide synthase (NOS). NOS catalyzes the conversion of arginine into citrulline and NO. The nitric oxide from the terminal nerve ending and that of the endothelial cell diffuse into nearby VSMC and activates a soluble guanylyl cycle. A downstream pathway causes a decrease in MLCK activity with vasodilation as a result.

Other vasodilators that are released by the endothelial cell in response to ACH stimulation include prostaglandins (PGI_2) (Kellogg, Zhao et al. 2005) and endothelium-derived hyperpolarizing factor (EDHF) (Buus, Simonsen et al. 2000). Additional systemic regulation of vascular tone includes hormones like angiotensin II.

Local regulation

The local adjustment of blood flow is regulated via several mechanisms that involve a complex interplay between local stimuli, VSMCs and endothelial cells. This autoregulatory system on vascular tone can override that of the autonomic system, and therefore optimize blood supply to local demand. The VSMCs respond to local changes in PO_2, CO_2, pH and shear stress. Changes that accompany increases in metabolism (e.g. increased PCO_2, decreased PO_2) are associated with vasodilation, while increases in shear stress cause vasodilation via stress sensitive channels on the VSMCs (Boron and Boulpaep 2003). However the local stimuli also influence the production and paracrine release of several vasoactive compounds by endothelial and vascular smooth muscle cells (Morawietz, Talanow et al. 2000). These include the earlier discussed vasodilators NO, PGI_2 and EDHF by endothelium, but also the potent vasoconstrictor endothelin-1 (ET-1) by endothelial cells and VSMCs. The net production of endothelin-1 is dependend on the intracellular expression of preproendothelin, a precursor molecule. Preproendothelin is intracellularly converted by endothelin converting enzyme into the smaller active endothelins (ET-1, ET-2, and ET-3). The endothelins activate ET_A- and ET_B-receptors on VSMCs and ET_B receptors on endothelial cells. Their activation involves a G-protein coupled pathway. Activation of ET_A- and ET_B-receptors on VSMC by ET-1 results in

vasoconstriction. On the other hand the activation of ET_B-receptors on endothelial cells results in production and release of NO, which consequently is associated with relaxation of nearby VSMCs, and thus vasodilation (Cardillo, Kilcoyne et al. 2000). Normally in the vasculature the ET_A- receptors outnumber ET_B-receptors, but receptor type and density differs between vascular beds (e.g. systemic vs. pulmonary), gender and species (Brain, Crossman et al. 1989; Crossman, Brain et al. 1991; Warner 2001). The endothelin-1 induced vasodilation is usually seen at lower ET-1 concentrations and more common in females (Kellogg, Liu et al. 2001). The overall response of ET-1 is however a prolonged vasoconstriction of arteries and veins (Haynes, Clarke et al. 1991; Warner 2001; Katz, Marr et al. 2003). The function of ET-2 and ET-3 is less clear. They are produced in much smaller amounts and have less affinity for ET_A-receptors and equipotent affinity for ET_B-receptors. Their role seems less prominent. The endothelins might additionally be involved in potentiation of angiotensin-II and norepinephrine mediated vasoconstriction (Webb, Dickinson et al. 1992; Wenzel, Ruthemann et al. 2001). ET_A receptors have been detected in equine digital, intestinal and pulmonary vasculature (Benamou, Marlin et al. 2001; Venugopal, Holmes et al. 2001; Benamou, Marlin et al. 2003; Katz, Marr et al. 2003). The detection of ET_B-receptors in equine digital vasculature so far has been unsuccessful. There is some evidence that NO also can influence ET-1 production. It appears that blocking of NOS with inhibitor L-NAME abolishes a decrease in preproendothelin expression from shear stress compared to no treatment (Morawietz, Talanow et al. 2000). In this way NO would also stimulate vasodilation.

Vascular effects of glucocorticoids.

The genomic and nongenomic effects of glucocorticoids form the basis for their broad range of effects. This includes modification of the vascular tone. For instance topical application of glucocorticoids on human skin results in local blanching (Ahluwalia and Flower 1993; Goldsmith, Bunker et al. 1996). Studies with laser Doppler imaging associated the blanching with a decrease in dermal blood flow (Sommer, Veraart et al. 1998)and venoconstriction (Andersen, Milioni et al. 1993). Similarly systemic administration of glucocorticoids has been associated with hypertension (Wallerath, Witte et al. 1999; Severino, Brizzi et al. 2002; Di Filippo, Rossi et al. 2004; Wallerath, Godecke et al. 2004). Some of these effects could be related to changes in electrolyte balance and plasma volume from residual mineralocorticoid action of some glucocorticoids (Kenyon, Brown et al. 1990). However modification of the local vascular regulation appears to be the major mechanism by which glucocorticoids alter vascular tone. Increased vasomotor tone is induced via several mechanisms.

1) Glucocorticoids up regulate the expression of alpha and beta adrenergic receptors in many tissues, including on the surface of VSMC (Haigh and Jones 1990; Mak, Nishikawa et al. 1995). This results in a greater and potentiated reaction to the catecholamines norepinephrine and epinephrine (Eyre, Elmes et al. 1979; Stepp and Frisbee 2002)
2) Glucocorticoid therapy results in higher circulating levels of ET-1 (Di Filippo, Rossi et al. 2004), and this has been associated with hypertension. A glucocorticoid induced increase in expression of preproendothelin forms the basis for this effect (Koshino, Hayashi et al. 1998; Morin, Asselin et al. 1998;

Provencher, Villeneuve et al. 1998). In vitro studies suggest that VSMC, and not endothelial cells, are the primary source for this rise in ET-1 levels (Kanse, Takahashi et al. 1991). These studies also showed that the rise of ET-1 is associated with a decrease in ET_A-receptor numbers on the VSMCs(Clozel, Loffler et al. 1993; Koshino, Hayashi et al. 1998). This negative feedback mechanism resulted consequently in an attenuated vasoconstrictive response by exogenous ET-1. (Nambi, Pullen et al. 1992; Roubert, Viossat et al. 1993; Telemaque-Potts, Kuc et al. 2002).

3) Glucocorticoid therapy has been associated with decreased circulating levels of nitrate, a breakdown product of NO (Wallerath, Witte et al. 1999; Rogers, Bonar et al. 2002; Severino, Brizzi et al. 2002; Di Filippo, Rossi et al. 2004; Wallerath, Godecke et al. 2004). This effect is mediated through a decrease in NOS-expression (Kunz, Walker et al. 1996; Wallerath, Witte et al. 1999; Rogers, Bonar et al. 2002; Wallerath, Godecke et al. 2004) and an increased post translational degradation of this enzyme (Rogers, Bonar et al. 2002).
4) Glucocorticoids increase the number of angiotensin-II receptors (AT-1) on VSMCs (Sato, Suzuki et al. 1994; Provencher, Saltis et al. 1995; Ullian, Walsh et al. 1996) and increases pulmonary angiotensin converting enzyme (ACE) activity (Ialenti, Calignano et al. 1986; Dasarathy, Lanzillo et al. 1992). Angiotensin-II is a potent vasoconstrictor. Consequently these effects have been associated with glucocorticoid induced hypertension (Ullian, Walsh et al. 1996).
5) Glucocorticoids are associated with the induction of type II insulin resistance (Severino, Brizzi et al. 2002; Binnert, Ruchat et al. 2004). Clinical Type II

diabetes is associated with hypertension, increased vascular complications (Tooke 1999; Chaturvedi, Stevens et al. 2001), and a rise in non esterfied fatty acids (NEFA) (Barbera, Fierabracci et al. 2001). Insulin appears to have a vasodilatory action that primarily is mediated via NO production (Steinberg, Brechtel et al. 1994; Steinberg and Baron 2002). The downstream insulin receptor pathway includes the activation of insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI3K) and the serine/threonine protein kinase AKT/PKB. Blocking of PI3K abolishes insulin mediated NO production, while AKT has shown to be important in the activation of NOS. Glucocorticoids cause a decrease in the expression of IRS-1, PI3K and PKB (Ducluzeau, Fletcher et al. 2002). It could therefore be possible that this consequently results in an impairment of insulin mediated NO production, and hence an augmented impairment in NO mediated vasodilatation. Additionally recent research indicates that elevated levels in NEFAs could cause impairment of a vasodilatory response of the vasculature. This is hypothesized to involve NO and NO-independent pathways including interference with PI3K, shear stress induced NO production and muscarinic receptors (Steinberg and Baron 2002). Hyperinsulinemia also results in increased ET-1 levels and ET_A-receptor numbers (Piatti, Monti et al. 1996; Piatti, Monti et al. 2000; Katakam, Pollock et al. 2001). It appears that blocking of ET_A-receptors normalizes the vasodilator responses to insulin in insulin resistant subjects (Miller, Tulbert et al. 2002). This therefore indicates that hyperinsulinemia is associated with enhanced endothelin activity.

From the above it is clear that glucocorticoids alter both the vasoconstrictive and vasodilating side of the vasomotor balance. Recent research indicates that the glucocorticoid-mediated alterations on both systems are closely related. For instance studies in rats showed that glucocorticoid (prednisolone) induced hypertension can be blocked by the simultaneous administration of a nitric oxide donor (Severino, Brizzi et al. 2002; Di Filippo, Rossi et al. 2004). This effect was associated with an absence in rise of ET-1 levels (Di Filippo, Rossi et al. 2004). On the other hand the administration of an ET_A-receptor antagonist during glucocorticoid therapy was also successful in blocking the induction of hypertension (Di Filippo, Rossi et al. 2004). Additionally in genetically altered mice that are homozygotus negative for NOS, no changes in blood pressure are observed with glucocorticoid therapy (Wallerath, Godecke et al. 2004). Therefore current literature suggests that glucocorticoid induced reductions in NO partially are related to increased ET-1 levels. The increased ET-1 results in a greater vasoconstrictor response that is not compensated by a properly working vasodilator system. An autoregulated feedback mechanism that causes a decrease in the ET_A-receptors numbers on VSMC attempts to attenuate this effect instead.

Implications

Glucocorticoids have vascular effects that interfere with both the autonomic (systemic) and local regulation of vascular tone. Both the vasoconstrictive and vasodilatory responsiveness are affected. The net effect of glucocorticoids therapy is an increased vascular tone. It is likely that such an effect in tissues with A-V shunts

will result in an even more significant decrease of capillary blood perfusion. Bypass of blood flow through A-V shunts has been implicated as cause for clinical laminitis in the horse. Therefore a potentiated vasoconstrictor response, or impaired vasodilatory response, or both from glucocorticoid administration to horses could be a vascular mechanism that leads to laminitis. The *in vivo* vascular responsiveness during glucocorticoid therapy has not been investigated in the horse. The following two studies were designed to answer the question if glucocorticoid therapy in horses alters local vasoconstrictior and vasodilator responsiveness in the horse.

CHAPTER 6

A THERMOGRAPHIC STUDY OF THE *IN VIVO* MODULATION OF VASCULAR RESPONSES TO PHENYLEPHRINE AND ENDOTHELIN-1 BY DEXAMETHASONE IN THE HORSE

C.J. Cornelisse*, N.E. Robinson, C.A.E. Berney, S. Eberhardt, J.G. Hauptman and **F.J. Derksen**

Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48823-1314, USA.

Corresponding author:

Dr. N. Edward Robinson

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

East Lansing, MI 48824-1314

Telephone: 517-353-5978

Fax: 517-353-2935

Email: robinson@cvm.msu.edu

Keywords: horse; dexamethasone; endothelin; intradermal; phenylephrine; thermography; vascular function

Word count: 5,962

Summary

Reasons for performing the study: Glucocorticoids modulate many physiologic mechanisms, including those hypothesized to be involved in the development of laminitis. A vascular etiology has been implicated as a cause for laminitis and *in vitro* studies of equine digital vessels have shown an augmented constrictive response to catecholamines after addition of a glucocorticoid to the medium. However *in vivo* compensatory mechanisms can still be present and the net responses could therefore be different. Up to now the *in vivo* modulation of constrictor responses of vasculture by glucocorticoids has not been investigated in the horse.

Objectives: To study the *in vivo* effects of glucocorticoid therapy on vasoconstrictor responsiveness in the horse. For this purpose the skin was used as a model for the laminar vascular bed of the equine hoof. The vascular responses to intradermal injections of various concentrations of vasoconstrictors were investigated by means of thermography in horses treated with either dexamethasone or saline.

Methods: In a blinded randomized cross-over design, nine horses were treated with either dexamethasone (0.1 mg/kg IV q24h) or saline IV for six days. On day six, the changes in local average skin temperature before (baseline) and after intradermal injections (at times 20, 60, 120 and 180 minutes) of the catecholamine phenylephrine (PHE; at 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M), endothelin-1 (ET-1; at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M) or endothelin-1 plus a

blocker (BQ-123, RES-701 and L-NAME) were investigated with a thermograph.

Results: **Baseline skin temperatures were significantly lower in the dexamethasone group, suggesting an increase in vasomotor tone. This was accompanied by a greater and potentiated effect of PHE as demonstrated by the left shift in both the dose-response curve and 50% decrease in EC_{50}. DEX did not potentiate ET-1, but the interplay with the lower baseline temperature still resulted in significantly lower skin temperatures at each ET-1 concentration. The different ET-1 blockers had no effect on the ET-1 decrease in temperature in either treatment group.**

Conclusions: **Our results show that dexamethasone therapy in horses causes a significant decrease in skin perfusion, most likely due to an increase in basal vasoconstrictor tone. This is accompanied by a potentiated vasoconstrictor response to catecholamines. As a consequence of the increase in basal vascular tone, the responses to other potent vasoconstrictors such as ET-1 can be greater without the need for potentiation of the response to these substances. Thermography proved to be a useful technique to study in vivo vascular dose-responses in the skin, and their modulation by the application of a systemic drug.**

***Potential relevance*: Based on the similarities between blood flow in the skin and the laminar vascular bed it seems likely that glucocorticoid therapy can result in additional compromise of perfusion of the equine hoof during disease states that are already characterized by hypoperfusion and/or increased levels of**

circulating catecholamines. Thus, during such pathophysiologic states, glucocorticoid therapy could, according to the vascular model of laminitis, tilt the balance in favor of laminitis.

Introduction

Glucocorticoids are potent anti-inflammatory drugs that are used successfully in equine medicine for treatment of various diseases, including inflammatory/allergic airway disease, lameness and immune-mediated syndromes. Unfortunately, laminitis has been implicated as a possible severe adverse effect from the use of these drugs in horses (Gerber 1970; Ryu *et al.* 2004). Several mechanisms regarding glucocorticoid-induced laminitis have been proposed (Eyre *et al.* 1979; Pass *et al.* 1998; French *et al.* 2000; Cornelisse and Robinson 2004), including the derailment of vascular function and disturbances in energy and metabolic pathways.

In the vascular model, enhanced constriction of pre- and/or post-capillary sphincters is thought to cause decreased blood flow to the laminae, with most of the blood bypassing this tissue via arterio-venous shunts and thereby predisposing the hoof to laminitis (Robinson *et al.* 1976; Hood *et al.* 1978). Support for vascular effects by glucocorticoids being the cause can be inferred from several observations: 1) topical application of glucocorticoids on human skin causes vasoconstriction as indicated by blanching in the skin (Brown *et al.* 1991; Andersen *et al.* 1993; Noon *et al.* 1996; Sommer *et al.* 1998), 2) glucocorticoids potentiate vasoconstrictor responses to catecholamines (Eyre *et al.* 1979), and 3) glucocorticoids induce insulin resistance (French *et al.* 2000) and insulin resistance has been associated with an increased risk for vascular disease as well as with potentiated responses to catecholamines (Tooke 1999; Tooke and Goh 1999; Stepp and Frisbee 2002).

Despite these observations, so far only one study has reported on the direct effects of glucocorticoids on equine vasculature. The *in vitro* study reported additional

vasoconstriction of partly precontracted equine digital blood vessels (arteries and veins) immediately after addition of a glucocorticoid to the medium, and detected enhanced contraction to the catecholamines epinephrine and norepinephrine 30 minutes later (Eyre *et al.* 1979). However, *in vivo* the vascular tone is the net result of a balance between vasodilator and vasoconstrictor mechanisms, both regulated by a variety of different local vasoactive mediators (Hocher *et al.* 2004; Mather *et al.* 2004). Therefore, conclusions regarding vascular tone on the basis of enhancement of one system without knowing the status of the opposite system can be misleading.

Recent studies in human subjects have indicated complex interactions at the local vascular level between vasoconstrictors such as norepinephrine and endothelin-1 (ET-1), and the vasodilator nitric oxide (NO). It appears that stimulation of the ET_A- or ET_B-type receptors that are present on vascular smooth muscles cells results in contraction, whereas stimulation of ET_B receptors on endothelial cells results in NO production with subsequent vascular smooth muscle cell relaxation. Additionally, blockade of ET_A receptors attenuates the vasoconstrictor response to norepinephrine and angiotensin II (Wenzel *et al.* 2001). Glucocorticoid therapy is associated with hypertension (Severino *et al.* 2002; Di Filippo *et al.* 2004) and affects all the local systems that are involved in the regulation of vascular tone. This includes the up-regulation of pulmonary angiotensin converting enzyme (Ialenti *et al.* 1986), increases in vascular smooth muscle angiotensin-I receptor density (Provencher *et al.* 1995) and the down-regulation of transcription of endothelial nitric oxide synthase (Di Filippo *et al.* 2004; Wallerath *et al.* 2004), the key enzyme involved in NO production. Glucocorticoids have also been associated with increased levels of ET-1 (Kanse *et al.* 1991; Roubert *et al.* 1993; Kato *et al.* 1995;

Koshino *et al.* 1998; Morin *et al.* 1998; Di Filippo *et al.* 2004). All of these effects suggest a net shift towards increased vasoconstriction. However, glucocorticoids cause a 50 to 60% decrease in ET_A receptor density on vascular smooth muscle cells (VSMC), resulting in an attenuated response to ET-1 (Nambi *et al.* 1992; Roubert *et al.* 1993; Provencher *et al.* 1995; Koshino *et al.* 1998) and suggests the presence of autocrine feedback mechanisms that modulate the *in vivo* responses.

Direct *in vivo* interventional studies on the local vasculature of the laminae in hoof are restricted by the limited access to this tissue. However, skin tissue has the same ontogenic origin and has many identical vascular properties as the laminar tissue in the hoof, including A-V shunts (Robinson *et al.* 1975; Johnson *et al.* 2004). The aim of the present study was to investigate the *in vivo* vascular effects of glucocorticoid therapy in the horse with the skin as a model for local circulation in the hoof. The responses to local intradermal injections of the vasoactive substances phenylephrine (PHE; vasoconstrictor), endothelin-1 (ET-1;vasoconstrictor), BQ-123 (ET_A-receptor antagonist), RES-701 (ET_B-receptor antagonist) and the nitric oxide synthase antagonist L-NAME were investigated in horses treated with either dexamethasone or saline. The local perfusion in the skin is reflected in the skin surface temperature. The change in local dermal circulation by the injected substances was therefore measured as changes in surface temperature with a thermograph. Our hypothesis was that glucocorticoid therapy enhances *in vivo* vascular responsiveness to vasoconstrictors.

Material and methods

Preparation of solutions for intradermal injections

Stock solutions were prepared freshly every 48 hours out of weighed amounts of endothelin-1 (ET-1[a]), endothelin receptor antagonists (BQ-123[a], RES-701[a]) and the nitric oxide synthase antagonist L-NAME[b]. The drugs were dissolved in DMSO[c] and subsequently further diluted in phosphate-buffered saline (PBS). The stock solutions were refrigerated at 10°C until further processing into solutions of ET-1, antagonists alone, or ET-1 plus an antagonist. The final working solutions (RX) therefore contained ET-1 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) with or without one of the antagonists (BQ-123, RES-701 [10^{-6} M] or L-NAME [10^{-4} M]). Control solutions included either BQ-123, RES-701 (10^{-6} M) or L-NAME (10^{-4} M). The final dilution of the solvent DMSO in all solutions was 1:10,000. Additionally, fresh solutions of phenylephrine[d] (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} to 10^{-4} M) in PBS were prepared. All solutions were equilibrated to room temperature (22°C) prior to injection. The concentrations of ET-1, antagonists, and PHE were based on *in vitro* and *in vivo* vascular dose-response studies (Elliott *et al.* 1994; Lawrence and Brain 1994; Pang *et al.* 1998; Lipa *et al.* 1999; Katugampola *et al.* 2000; Venugopal *et al.* 2001; Benamou *et al.* 2003; Katz *et al.* 2003; Bowen *et al.* 2004; Wilson *et al.* 2004).

Horses

Nine recurrent airway obstruction-affected mares (age 16-27 years, weight 442-571 Kg) (Robinson *et al.* 1999) were used in the study. During the study, horses were in clinical remission. While on pasture the horses were treated at 08.00 with either dexamethasone[e] (0.1 mg/kg IV q24h) or an equivalent volume of saline (SAL IV q24h) for 6 consecutive days. The treatments were administered according to a randomized cross-over design and were blinded to the investigator. The wash-out period between treatments was at least 2

weeks. The horses were stabled on shavings in a temperature-controlled environment 18 hours prior to the start of the experiments in order to acclimatize, and were fed a commercial pelleted diet. Immediately after stabling, skin test areas (25 by 20 cm) over both lateral shoulders were clipped and subsequently cleaned with alcohol. A 5 by 5 grid was drawn on the area with a silver sharpie pen so that the lines were visible on thermography images.

Study protocol

During the experiments, the horses were free standing in their temperature-controlled stall (22°C). In each test area, a top, middle and ventral row of the grid was used for intradermal injections, leaving an unused row between each treatment row. In each of the three treatment rows one of the solutions of ET-1, ET-1 plus antagonist, PHE, or controls was injected craniad to caudad in decreasing concentration (CONC). The control row additionally included DMSO 1:10000 and an empty needle stick. The order of the rows was randomized between horses, but identical within a horse between the treatments. A total volume of 0.1 ml was injected intradermally. A 24-mm diameter circular adhesive bandage[f] was applied between two of the rows for purposes of distance calibrations. Earlier pilot studies had determined that the bandage size was similar to the vasoconstricted area of 0.1 ml intradermal PHE at 10^{-4} M. A mounted thermal imager (Microscan 7515[g]) was used to measure the skin temperature in the injected areas. The emissivity of the imager was set at 0.98 with a sensitivity of 0.3°C, and the camera was adjusted for background temperature and humidity. The imager was kept at a distance of 70 cm perpendicular to the study area. Digital temperature images were captured before

(baseline at TM = 0 min) and 20, 60, 120 and 180 minutes after intradermal injections. During the study, environmental temperature and humidity data also were collected. The study was approved by the All-University Committee for Animal Use and Care at Michigan State University.

Image and data analysis

The digital images were analyzed with complementary software (Microspec 2.7)[g]. First, the total number of pixels in the area covered by the bandage was determined. Subsequently, the average temperature (T_{AVG}) of circular areas of similar pixel size was measured over the individual injection locations. A mixed factorial procedure for repeated measures (SAS statistical software[h]) was used for analysis of 1) baseline skin temperature between treatments; 2) PHE and the needle stick as control; and 3) ET-1 with its antagonists and DMSO. When significant effects ($p < 0.05$) were observed for the main effects and their interactions, the Bonferoni test was used to determine differences between means. Additionally, at each time in a series, the temperature difference between the control and a given concentration of agonist was calculated and this difference was used to construct a concentration-response curve. T-tests were then used to compare these temperature differences between saline and dexamethasone treatments at each agonist concentration. Similarly, in each horse the estimated dose of ET-1 and PHE that produced 50% of the maximal response (EC_{50}) was determined by iteration for times 60 and 120 minutes. From the mean EC_{50} the clinical potency for ET-1 and PHE was compared within the SAL or DEX treatment with a paired t-test. The environmental data were compared with a t-test.

Results

Baseline skin temperature

A mixed procedure for repeated measures showed that the baseline skin temperature after DEX (33.58 ± 0.05 °C) was significantly ($P < 0.0001$) lower compared to baseline values after SAL (34.33 ± 0.05 °C) (Figure 6.1 and Figure 6.3).

PHE-series

Significant differences were found for main effects of treatment (TX; $p = 0.0004$), time (TM; $p < 0.001$) and PHE concentration (CONC; $p < 0.0001$) (Table 6.1). Additionally, significant differences were detected for the TM*CONC ($p < 0.0001$), TX*CONC ($p = 0.0002$) and TX*TM*CONC ($p < 0.0001$) interactions. Within DEX and SAL treatments there was no difference in local skin temperature prior to PHE injections. PHE injections caused a concentration-dependent decrease in skin temperature that was maximal 60-120 minutes after injection. The decrease became significant at 10^{-5} M and 10^{-4} M (Table 6.1). Post hoc analysis of the TX*CONC interactions showed that at PHE concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M, skin temperature was significantly lower after DEX than after SAL treatment (Figure 6.1). Figure 6.2 shows the concentration-response curves to PHE at 20, 60 and 120 minutes after injection. DEX treatment shifted the concentration-response curve to the left with a significant greater efficacy at 10^{-5} M PHE.

ET-1, antagonists and ET-1 plus antagonist combination (RXs) series

Statistical analysis showed significant main effects of treatment (TX; $p = 0.0043$), time (TM; $p < 0.001$) and concentration (CONC; $p < 0.0001$) (Table 6.2). Significance was detected for the TX*CONC ($p = 0.001$), TM*CONC ($p < 0.0001$) and TX*RX*TM*CONC ($p < 0.0003$) interactions. Antagonists alone had no effect on skin temperature ($p = 0.33$). The ET-1 and ET-1 plus antagonist combinations caused a concentration-dependent decrease in local skin temperature that was maximal 60-120 minutes after injection (Figure 6.3). None of the antagonists, BQ-123, RES-701 or L-NAME had any effect on the reduction in skin temperature caused by ET-1. Dexamethasone treatment significantly reduced skin temperature at each concentration of ET-1 but did not potentiate the response to ET-1 or any of the antagonist combinations.

Relative clinical potency of PHE and ET-1

During saline treatment at 60 minutes, the EC_{50} (in –LOG[M]) for ET-1 and PHE were 6.87 and 4.67, respectively, and were significantly different from each other ($p < 0.0001$). Treatment with dexamethasone potentiated the effect of PHE significantly ($p = 0.0019$), as demonstrated by the left shift of the EC_{50} to 5.68, whereas the EC_{50} for ET-1 was unchanged at 7.09 ($p = 0.29$) (Figure 6.4[a,b]). Similarly, during saline treatment at 120 minutes the EC_{50} for PHE was 4.49 and was significantly ($p = 0.00002$) different from that of ET-1 at 6.88. Dexamethasone treatment shifted the EC_{50} of PHE significantly ($p = 0.039$) to 5.33 while that for ET-1 was unchanged at 6.71 ($p = 0.49$).

Environment

Statistical analysis detected no significant difference between the treatments for environmental temperature (DEX: 22.4 ± 0.1 °C, SAL: 22.1 ± 0.2 °C) and humidity (DEX: 73.3 ± 1.7 %, SAL: 69.6 ± 1.7 %). These values were within those reported for the thermoneutral zone of the horse (Morgan 1998).

Discussion

Thermography proved to be a successful technique to measure the *in vivo* dose response of vasoconstrictor substances that were injected in the skin. Because these responses reflected the net result between vasoconstricting and vasodilating systems it was possible with this technique to investigate effects that are closer to the net physiological outcome. It was therefore also possible to study the modulation of the responses by systemic dexamethasone treatment.

The treatment with dexamethasone resulted in significantly lower baseline temperatures and significantly lower temperatures of the skin after intradermal injections of PHE and ET-1. This was accompanied by potentiation of PHE with a shift of 1 log concentration to the left for the EC_{50} of PHE (Figure 6.2, 6.4a). PHE had additionally a greater maximal effect compared to ET-1 (Figure 6.1b, 6.3b). Recent studies on vascular smooth muscle cells have shown that dexamethasone causes an increase in α_1-adrenoceptor gene transcription and α_1-adrenoceptor second messenger production. The result, respectively a greater α_1-adrenoceptor density and stronger coupling to G-proteins, would consequently cause an increased sensitivity of vascular smooth muscle to catecholamines (Haigh and Jones 1990; Sakaue and Hoffman 1991; Liu *et al.* 1992) . It is

likely that this effect therefore explains the potentiated effect for PHE in our dexamethasone group. The increased vasomotor tone would subsequently have resulted in a lower baseline skin temperature. Increased vasomotor tone has indeed been implicated as the cause for dermal blanching after topical application of glucocorticoids (Brown *et al.* 1991; Andersen *et al.* 1993; Noon *et al.* 1996; Sommer *et al.* 1998).

While it is clear that DEX potentiated the effect of the catecholamine PHE, that may not be the only cause of the decrease in skin temperature. For example, recently it was shown that glucocorticoid-associated hypertension in rats is associated with decreased NO production (Wallerath *et al.* 1999; Severino *et al.* 2002) and increased plasma ET-1 concentrations (Di Filippo *et al.* 2004). Pretreatment with an ET-antagonist (Di Filippo *et al.* 2004) or NO donor (Severino *et al.* 2002; Di Filippo *et al.* 2004) abolishes this hypertensive effect, indicating the importance of these systems. Although we did not measure ET-1 and NO concentrations in the blood it could be possible that an increase in ET-1 or decrease in NO concentrations did contribute to an increase in vasomotor tone. Our study did show the presence of endothelin receptors in the equine dermal vasculature. In vascular smooth muscle cells of rats and rabbits the *in vitro* glucocorticoid-induced increase of ET-1 production was accompanied by a decrease in ET-1 receptor density. This consequently resulted in an attenuated response to exogenous ET-1 (Nambi *et al.* 1992; Roubert *et al.* 1993; Provencher *et al.* 1995; Koshino *et al.* 1998). We explored the effect of DEX on the response to ET-1 and found neither attenuation nor potentiation of its effect (Figure 6.3, Figure 6.4). The reason for this is unclear but could be similar to that for bovine smooth vascular muscle cells for which *in*

vitro no change in endothelin receptor density could be demonstrated after glucocorticoid addition to the medium (Kanse *et al.* 1991).

We were unable to detect specific effects related to the coinjection of endothelin antagonists BQ-123 and RES-701, or the NOS blocker L-NAME. This was surprising because, in other species, although measured with different techniques, the coinjection of these drugs caused changes in dermal perfusion (Clough 1999; Katugampola *et al.* 2000; Shastry *et al.* 2000; Wenzel *et al.* 2001). It is possible that differences between species or technique could account for our inability to detect antagonism by these drugs. Indeed, up to now, blocking of ET_B-receptors in the different vascular beds of the horse has been unsuccessful (Benamou *et al.* 2003; Katz *et al.* 2003). It has also been reported that the blockade of ET_B-receptors in other species did not always result in an alteration of vasomotor tone (Pang *et al.* 1998; Lipa *et al.* 1999; Leslie *et al.* 2004). It could on the other hand be possible that the alteration in vasomotor tone caused by the blockers resulted in temperature changes that were below the limit of detection of thermography. Coinjection of endothelin antagonists or L-NAME, even when injected in surplus to ET-1, resulted in maximal dermal flow changes in the order of 40 to 50% (Clough 1999; Katugampola *et al.* 2000; Shastry *et al.* 2000; Wenzel *et al.* 2001). With a reported correlation range from 0.3 to 0.79 between the change in dermal perfussion and a change in skin temperature (Harrison *et al.* 1994; Bornmyr *et al.* 1997; Clark *et al.* 2003; Yosipovitch *et al.* 2004) it is thus possible that the antagonistic effects of BQ-123, RES-701 and L-NAME were not detected with thermography. A slower diffusion into the skin or a different time to become effective as causes for the failure to detect antagonism by these blockers seems however unlikely because we used doses and time windows similar

to those used in other coinjection protocols (Clough 1999; Katugampola *et al.* 2000; Shastry *et al.* 2000; Wenzel *et al.* 2001).

This study was conducted because of the common belief that dexamethasone treatment can be complicated by the development of laminitis, and our findings support that hypothesis. Dexamethasone caused an *in vivo* decrease of baseline dermal temperature that was likely due to reduced perfusion and this effect was accompanied by a potentiated vasoconstrictor response to catecholamines. The combination of reduced blood flow and a magnified vasoconstrictor response to activation of alpha-adrenoceptors could lead to a reduction in blood flow in situations in which circulating catecholamines concentrations are increased. Even though the response to ET-1 was not potentiated by dexamethasone, the additive effect of a DEX-induced reduction in blood flow to the vasoconstriction caused by ET could still result in a clinically significant hypoperfusion. The many anatomic and physiologic similarities between the dermal and laminar vascular beds suggest that dexamethasone therapy could cause a significant decrease in the laminar perfusion during disease states that are characterized by hypoperfusion and/or increased circulating levels of catecholamines. Thus, during pathophysiologic states such as colic or septic metritis, glucocorticoid therapy could, according to the vascular model of laminitis, possibly tilt the balance towards the direction of laminitis. However, the vascular changes induced by DEX may be of little clinical significance for diseases such as heaves, or skin and joint disorders which have minimal involvement of the systemic circulation or are unaccompanied by increased concentrations of circulating catecholamines.

Table 6.1: Average temperatures (T_{AVG}) of local skin areas injected with various concentrations (CONC) of phenylephrine over TIME in horses treated with either dexamethasone (DEX) or saline (SAL). [a-e] Same scripts are significantly different within main effect.

MAIN EFFECT	VALUE	(T_{AVG} in ^{0}C) Mean ± se	P < 0.05
TREATMENT	DEX	32.85 ± 0.12	a
TREATMENT	SAL	33.89 ± 0.12	a
TIME (MIN)	0	33.85 ± 0.14	a,b
TIME (MIN)	20	33.37 ± 0.14	a
TIME (MIN)	60	33.06 ± 0.14	b,c
TIME (MIN)	120	33.02 ± 0.14	a,d
TIME (MIN)	180	33.56 ± 0.14	c,d
CONC (M)	control	33.78 ± 0.13	a
CONC (M)	10^{-8}	33.79 ± 0.13	b
CONC (M)	10^{-7}	33.70 ± 0.13	c
CONC (M)	10^{-6}	33.56 ± 0.13	d
CONC (M)	10^{-5}	33.20 ± 0.13	a,b,c,d,e
CONC (M)	10^{-4}	32.21 ± 0.13	a,b,c,d,e

Table 6.2: Average temperatures (T_{AVG}) of local skin areas injected with various concentrations (CONC) of ET-1 over TIME in horses treated with either dexamethasone (DEX) or saline (SAL). [a-c]Same scripts are significantly different within main effect.

MAIN EFFECT	VALUE	(T_{AVG} in ^{o}C) Mean ± se	p < 0.05
TREATMENT	SAL	34.03 ± 0.32	a
TREATMENT	DEX	33.08 ± 0.32	a
TIME (min)	0	33.99 ± 0.31	a,b,c
TIME (min)	20	33.62 ± 0.31	b
TIME (min)	60	33.38 ± 0.31	a
TIME (min)	120	33.14 ± 0.31	b,c
TIME (min)	180	33.66 ± 0.31	c
CONC (M)	0	33.86 ±0.30	a
CONC (M)	10^{-9}	33.81 ± 0.30	b
CONC (M)	10^{-8}	33.76 ± 0.30	c
CONC (M)	10^{-7}	33.50 ± 0.30	a,b,c
CONC (M)	10^{-6}	33.31 ± 0.30	a,b,c
CON (M)	10^{-5}	33.10 ± 0.30	a,b,c
RX	DMSO	33.57 ± 0.30	
RX	BQ-123	33.59 ± 0.30	
RX	RES-701	33.46 ± 0.30	
RX	L-NAME	33.61 ± 0.30	

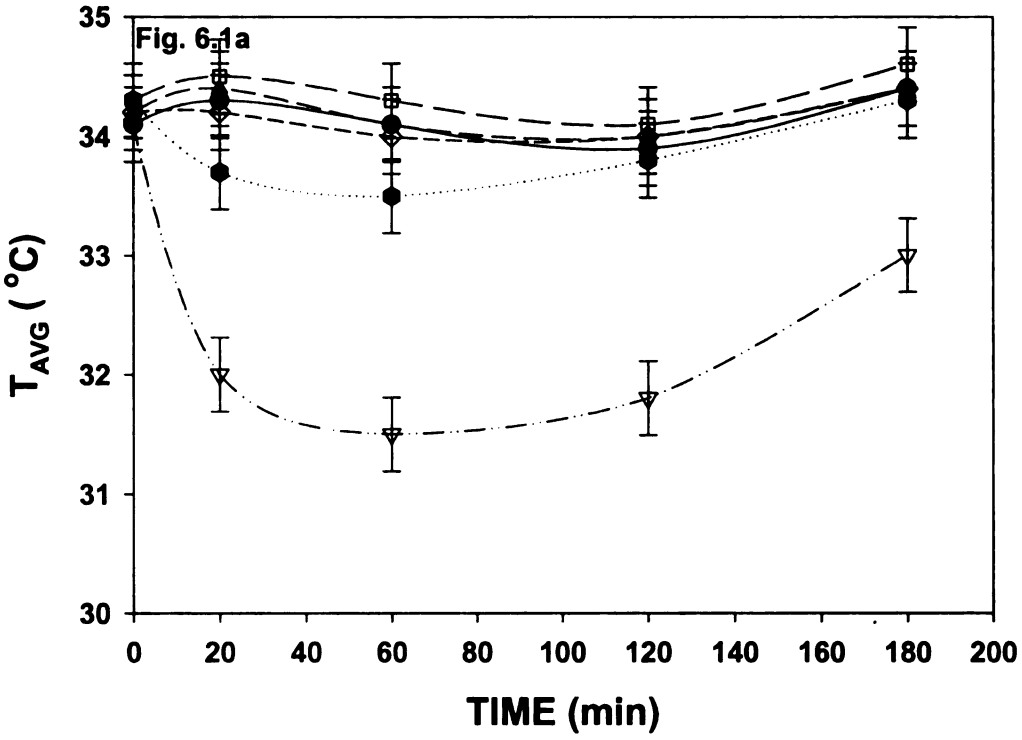
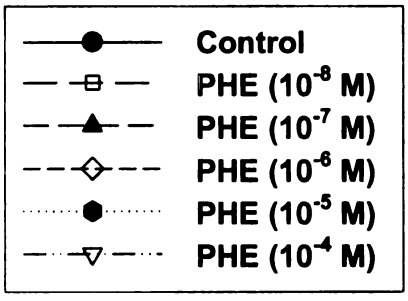


Figure 6.1a: Temperatures (T_{AVG}; mean and sem) of local skin areas injected with a control (needle stick) or various concentrations of phenylephrine (PHE) over TIME in horses treated with either saline (1a) or dexamethasone (1b). The sem represents the comparisons between TX but within TM x CONC.



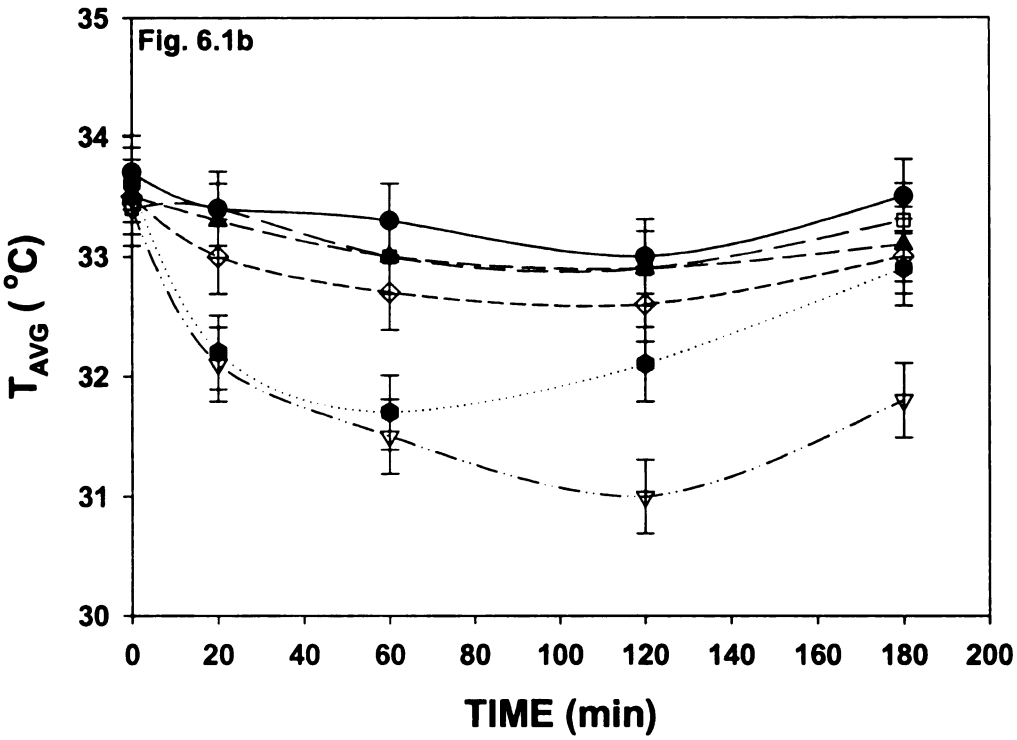
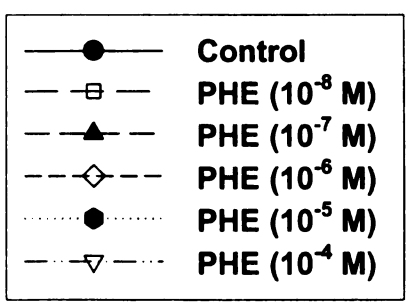


Figure 6.1b: Temperatures (T_{AVG}; mean and sem) of local skin areas injected with a control (needle stick) or various concentrations of phenylephrine (PHE) over TIME in horses treated with either saline (1a) or dexamethasone (1b). The sem represents the comparisons between TX but within TM x CONC.



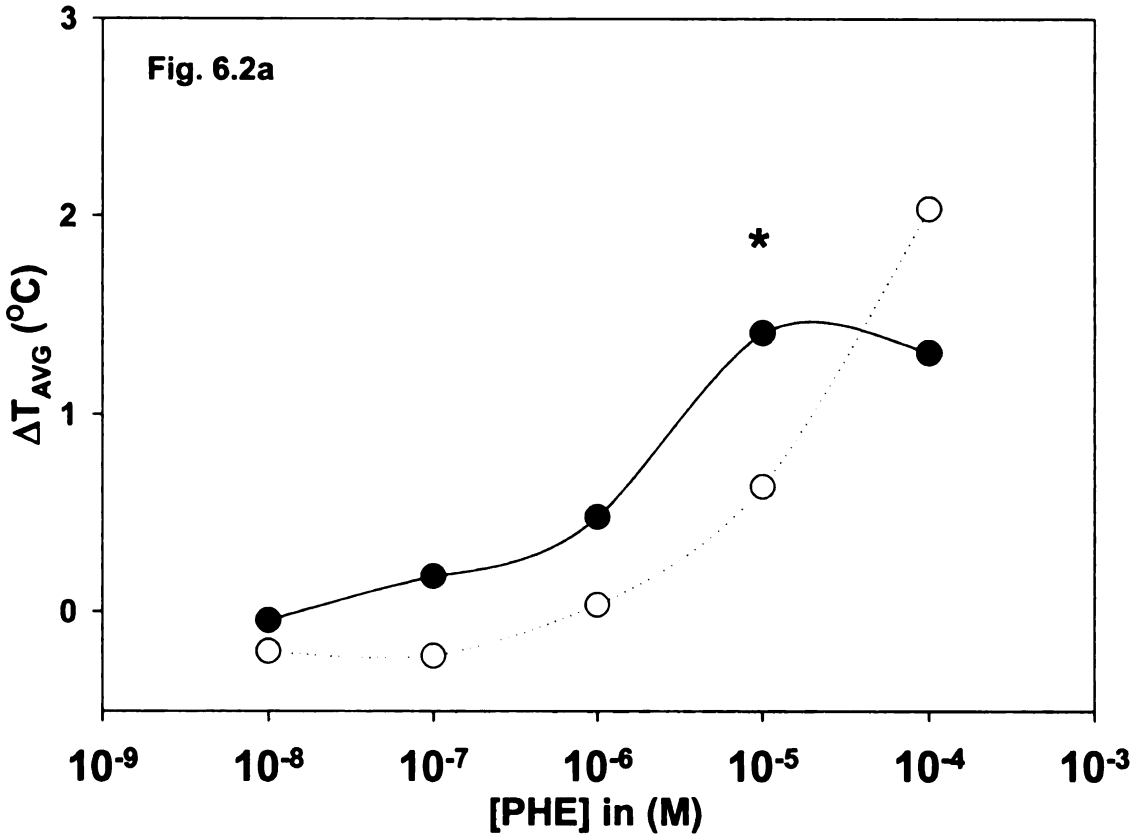


Figure 6.2a: Temperature differences (ΔT_{AVG}) between control (needle stick) and various concentrations of phenylephrine [PHE] in horses treated with saline (open circle, dotted line) or dexamethasone (closed circle, solid line) at time 20 minutes. * significantly different ($P < 0.05$).

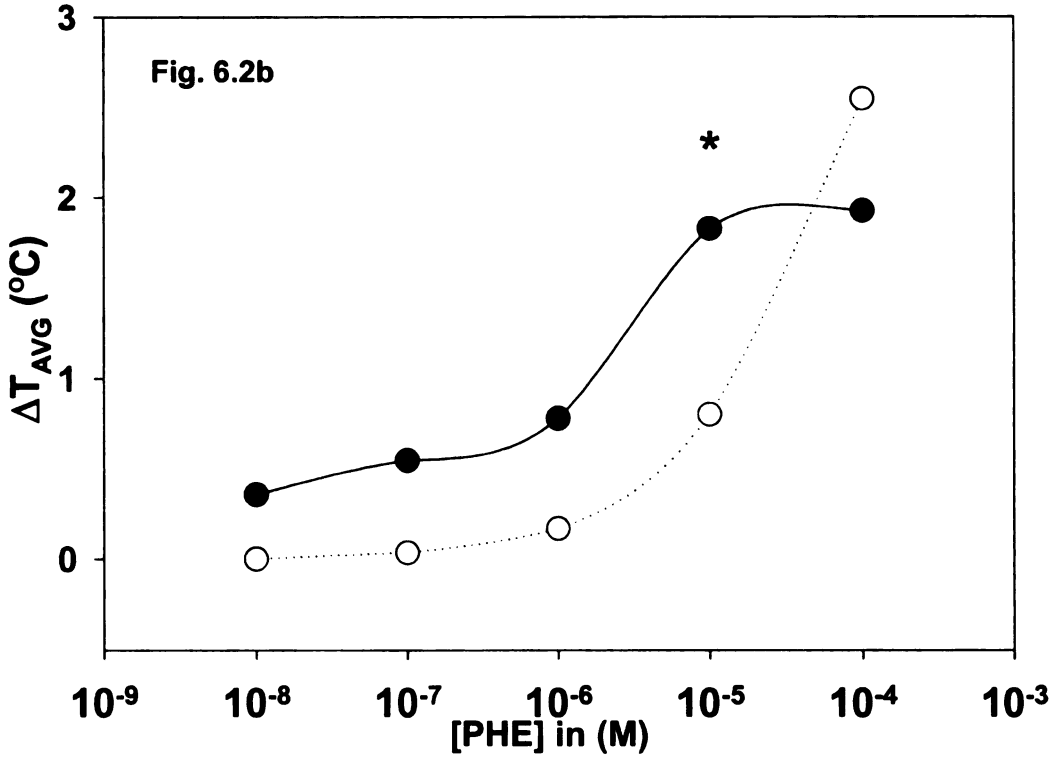


Figure 6.2b: Temperature differences (ΔT_{AVG}) between control (needle stick) and various concentrations of phenylephrine [PHE] in horses treated with saline (open circle, dotted line) or dexamethasone (closed circle, solid line) at time 60 minutes.
* significantly different ($P < 0.05$).

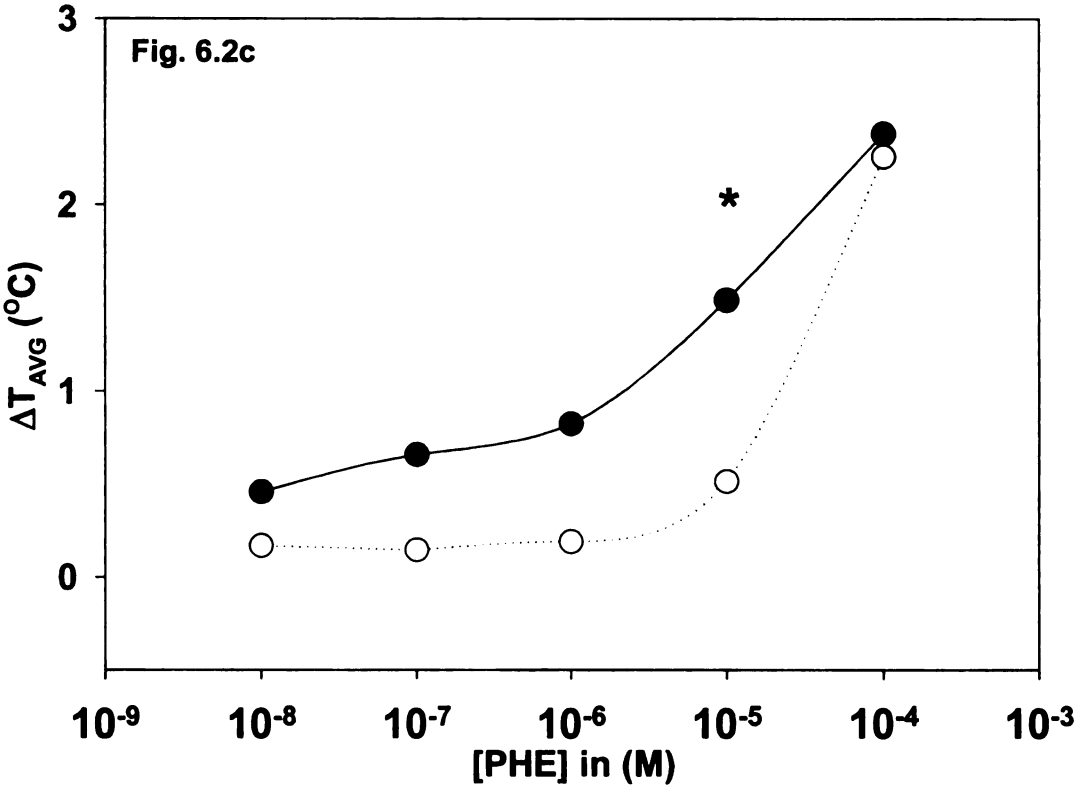


Figure 6.2c: Temperature differences (ΔT_{AVG}) between control (needle stick) and various concentrations of phenylephrine [PHE] in horses treated with saline (open circle, dotted line) or dexamethasone (closed circle, solid line) at time 120 minutes.* significantly different ($P < 0.05$).

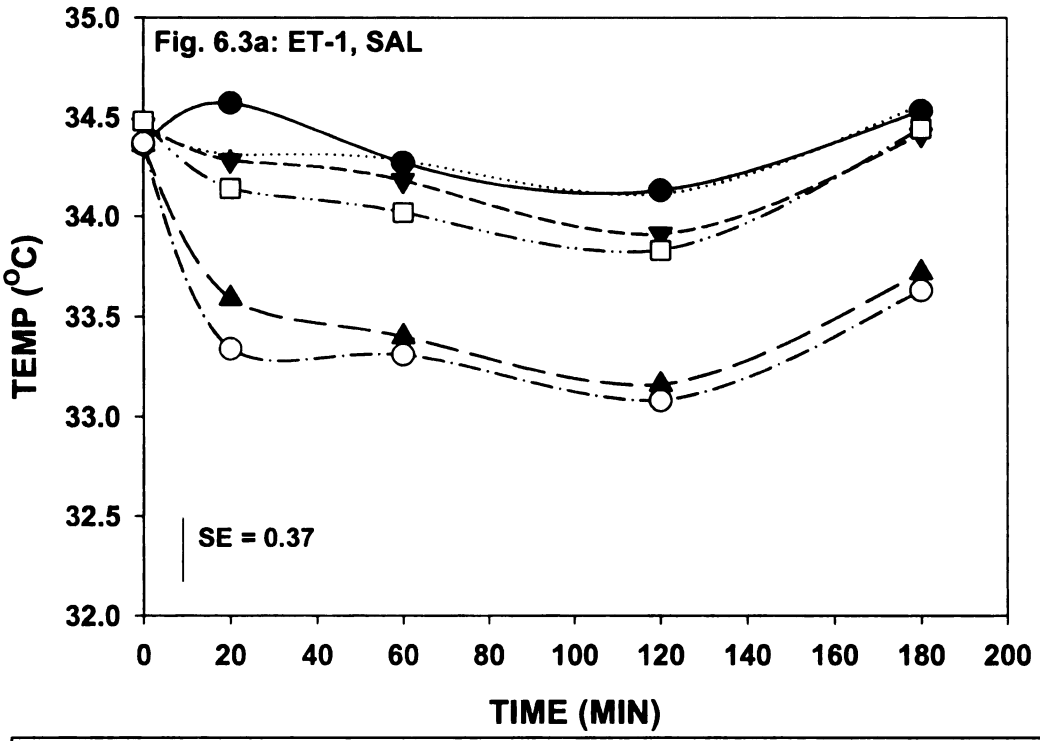
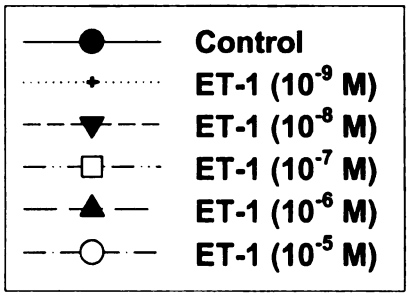


Figure 6.3a: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



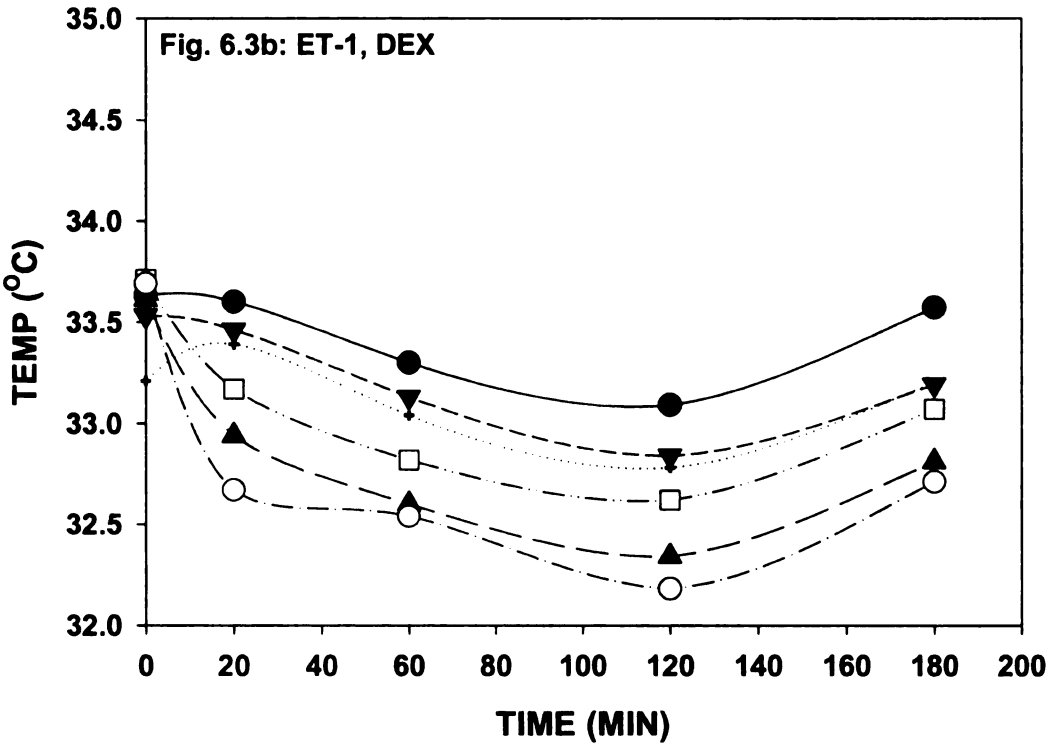
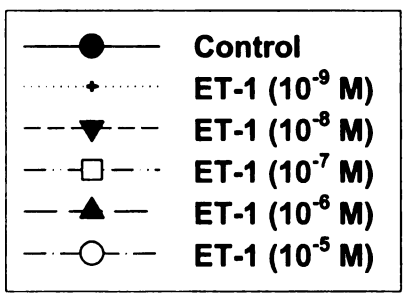


Figure 6.3b: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



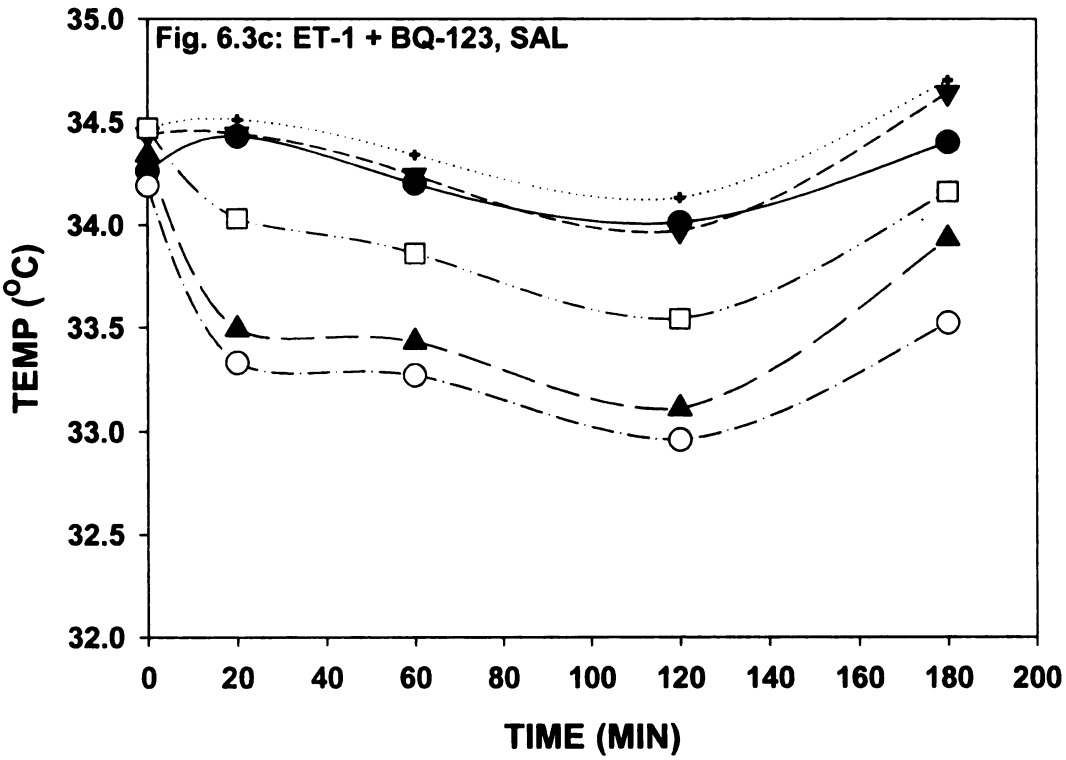
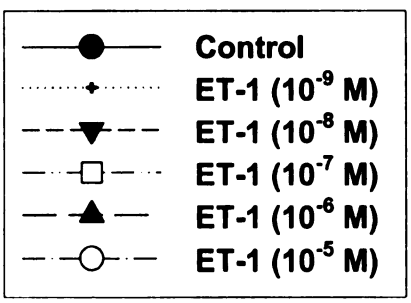


Figure 6.3c: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



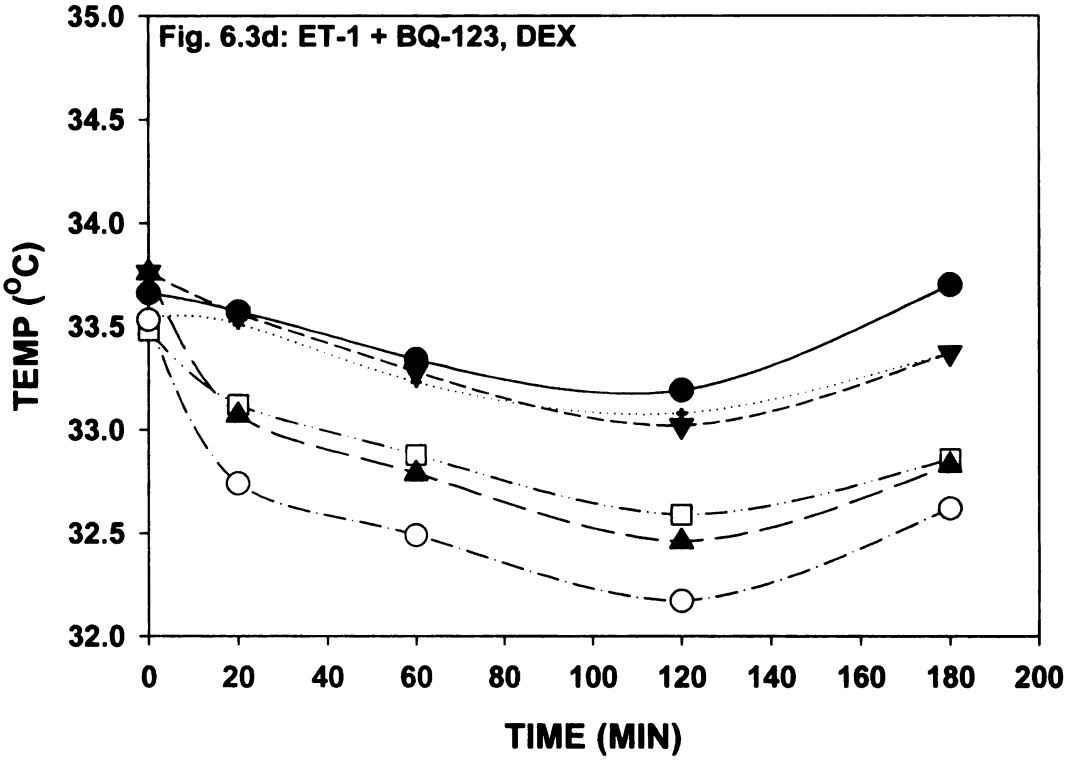
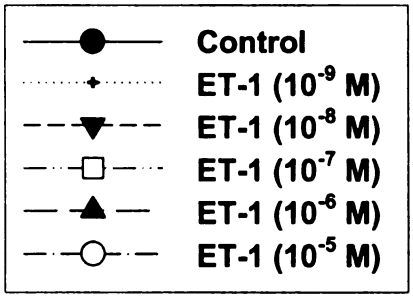


Figure 6.3d: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



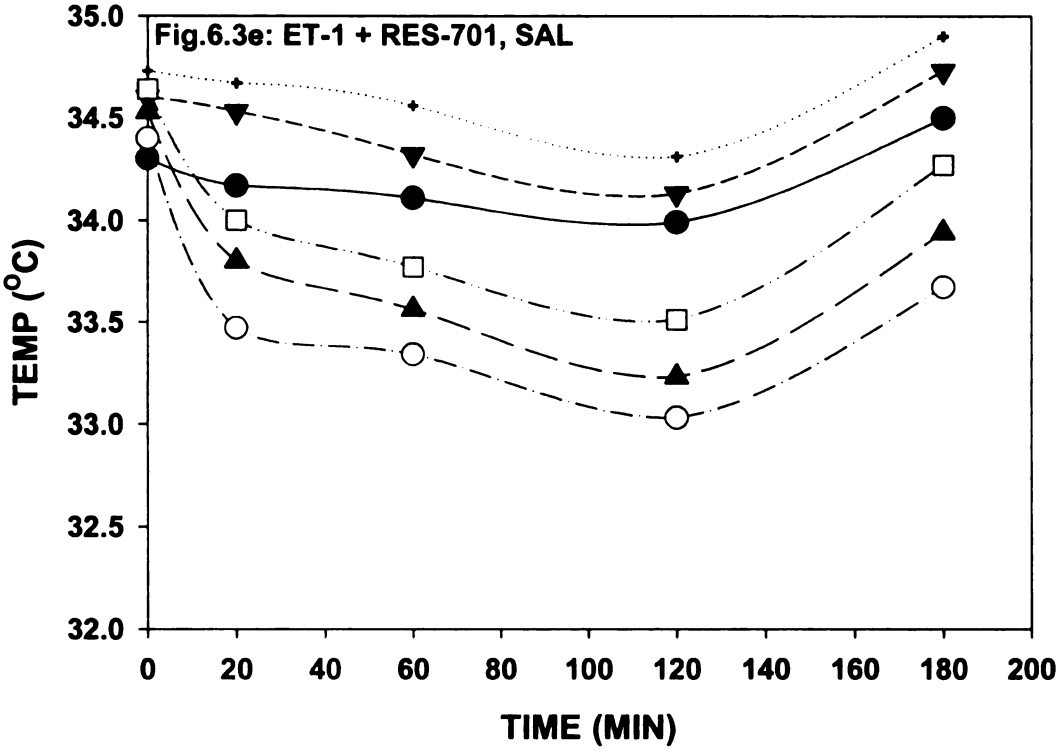
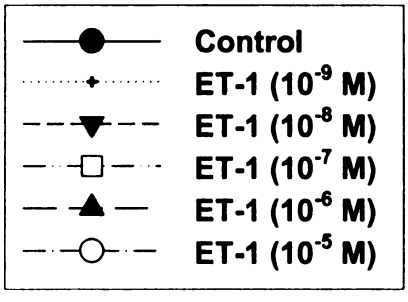


Figure 6.3e: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



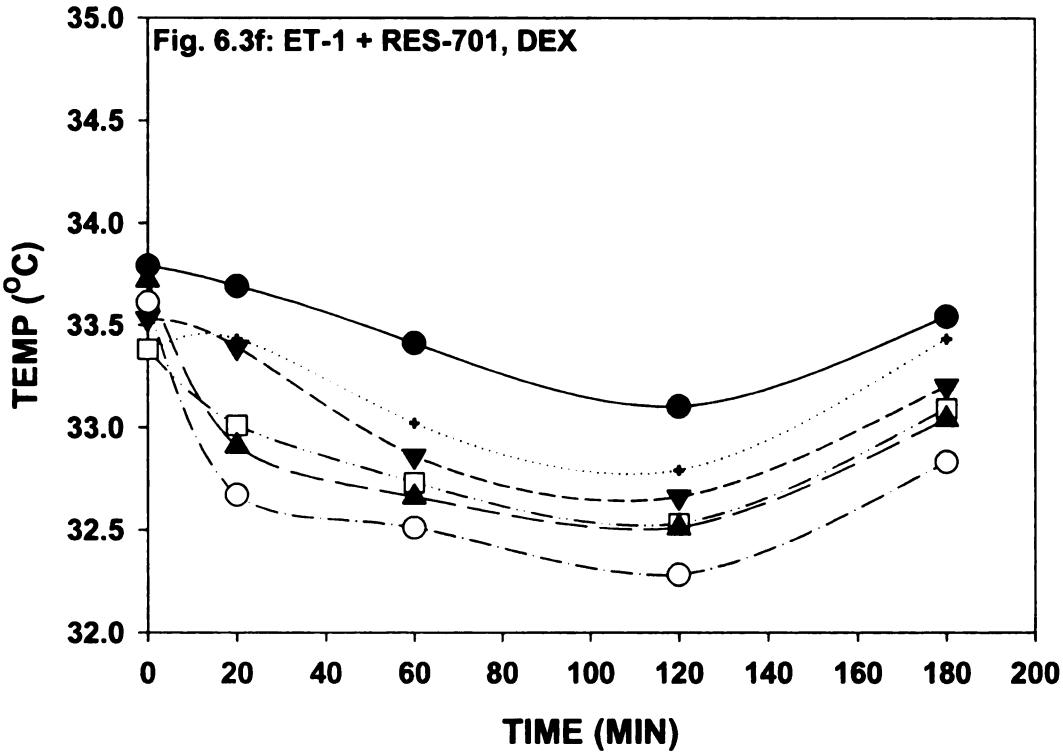
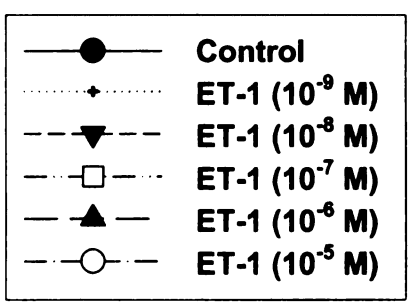


Figure 6.3f: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



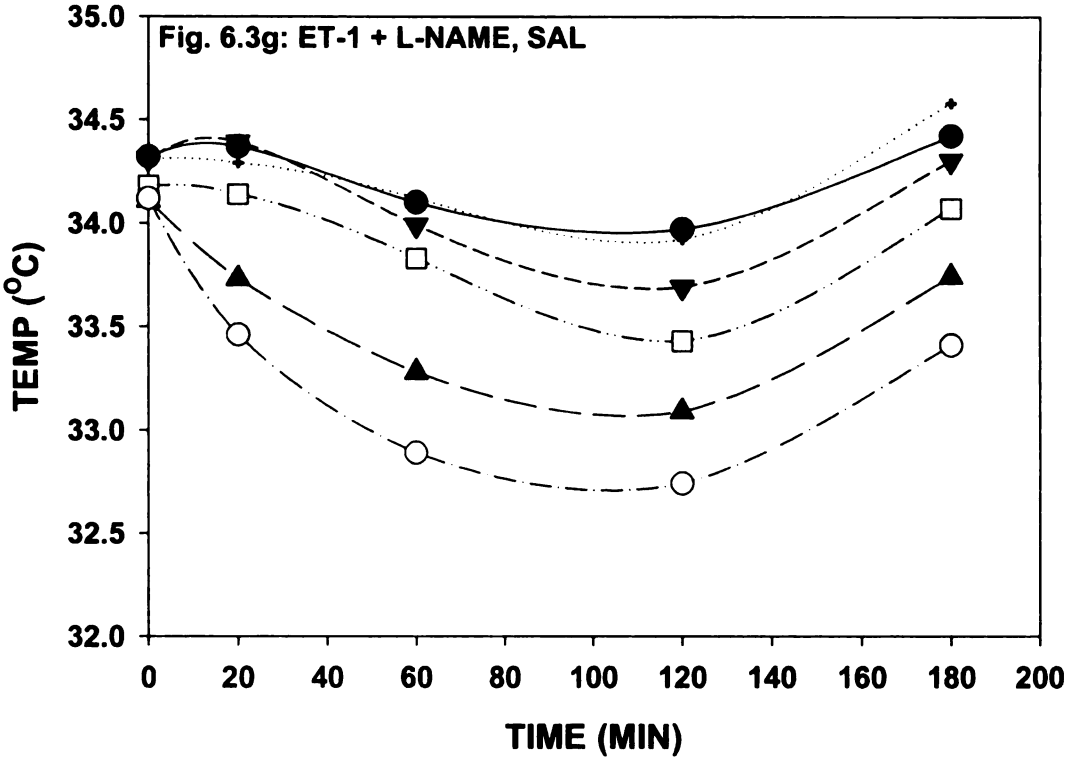
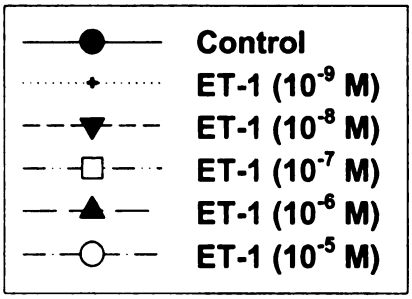


Figure 6.3g: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



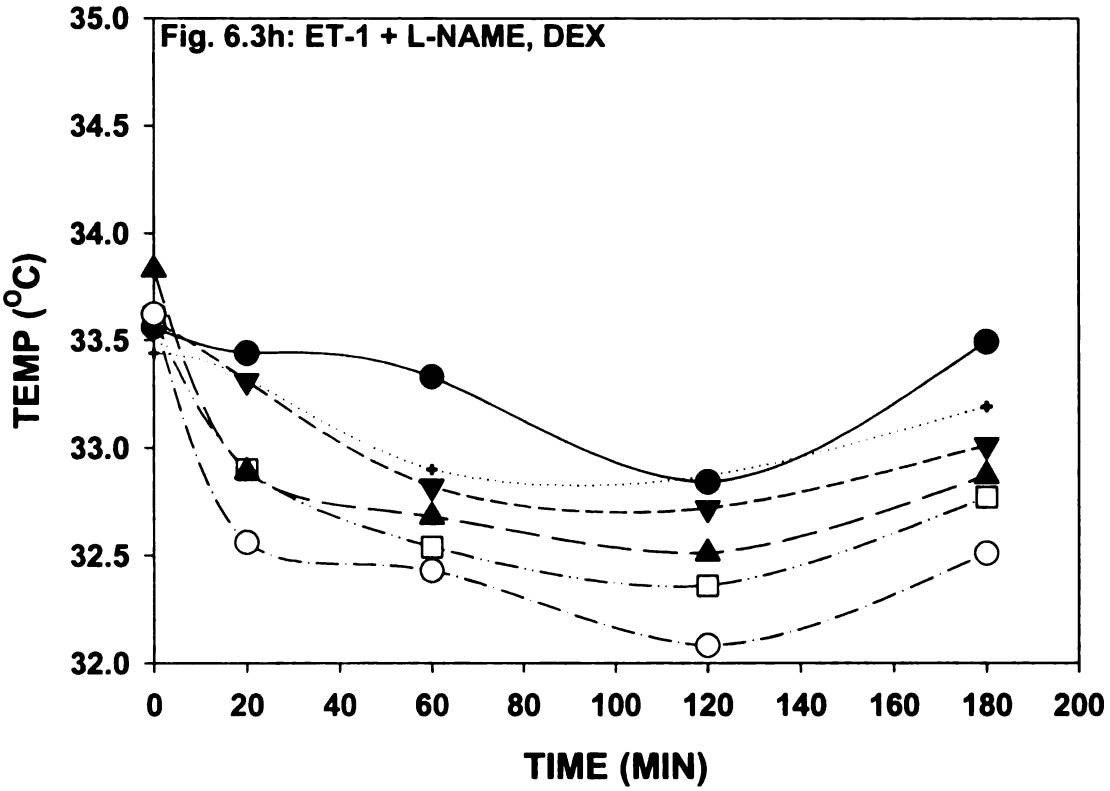
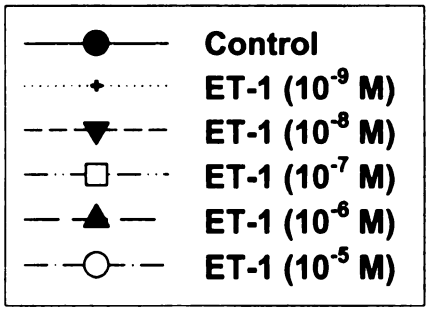


Figure 6.3h: Average local skin temperatures (T_{AVG}) of areas injected with various concentrations of ET-1 (see legend) without (a,b) or with either antagonists BQ-123 (c,d) or RES-701 (e,f) or L-NAME (g,h) over TIME in horses treated with either saline (SAL) or dexamethasone (DEX). The sem for comparison between TX but within RX xTM x CONC values is 0.35.



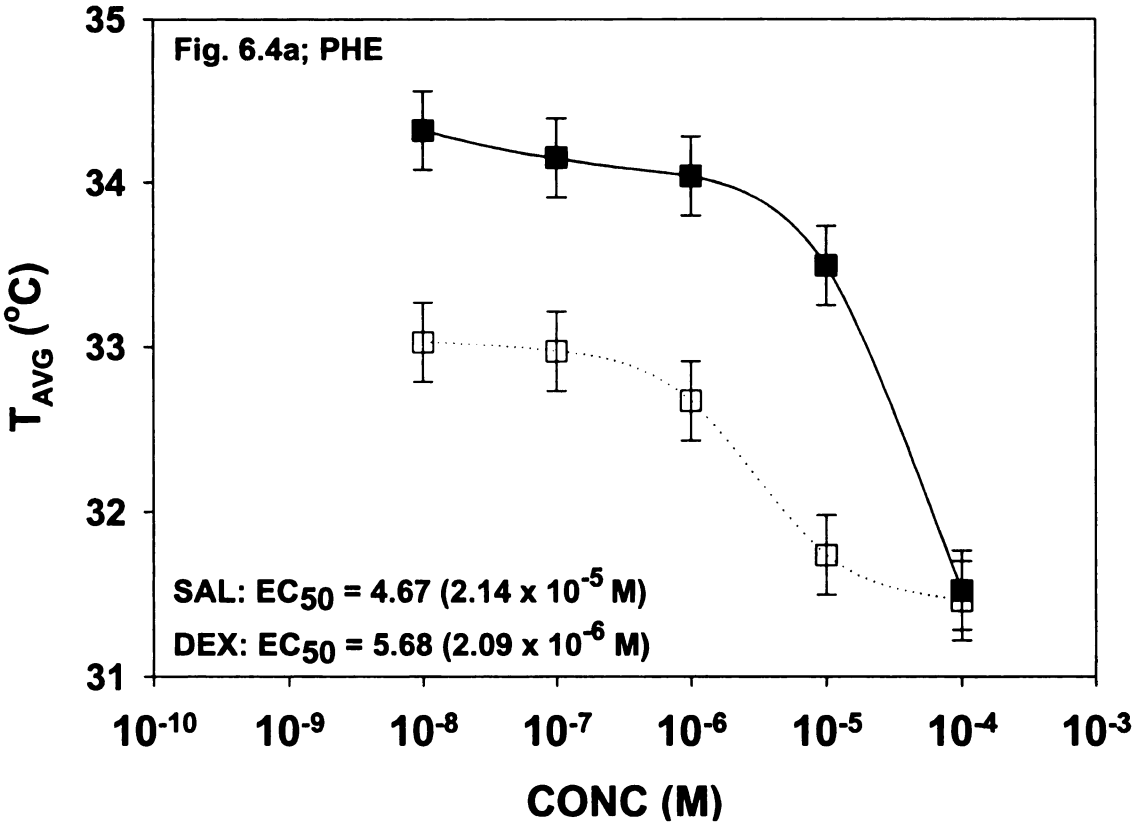


Figure 6.4a: Dose-temperature (T_{AVG}) curves of PHE 60 minutes after intradermal injection in horses that are treated with either saline (SAL; solid symbol and line) or dexamethasone (DEX; open symbol, dotted line). Effective concentration required to produce 50% of the maximal response.

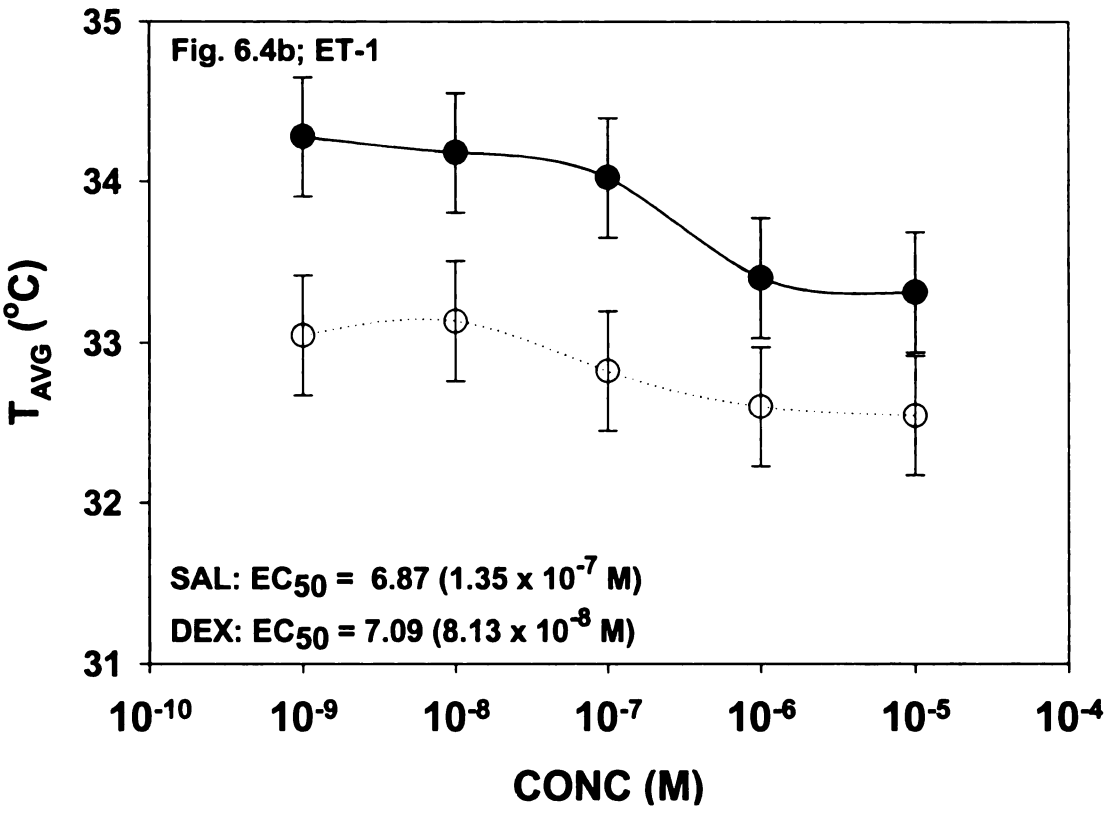


Figure 6.4b: Dose-temperature (T_{AVG}) curves of ET-1 60 minutes after intradermal injection in horses that are treated with either saline (SAL; solid symbol and line) or dexamethasone (DEX; open symbol, dotted line). Effective concentration required to produce 50% of the maximal response.

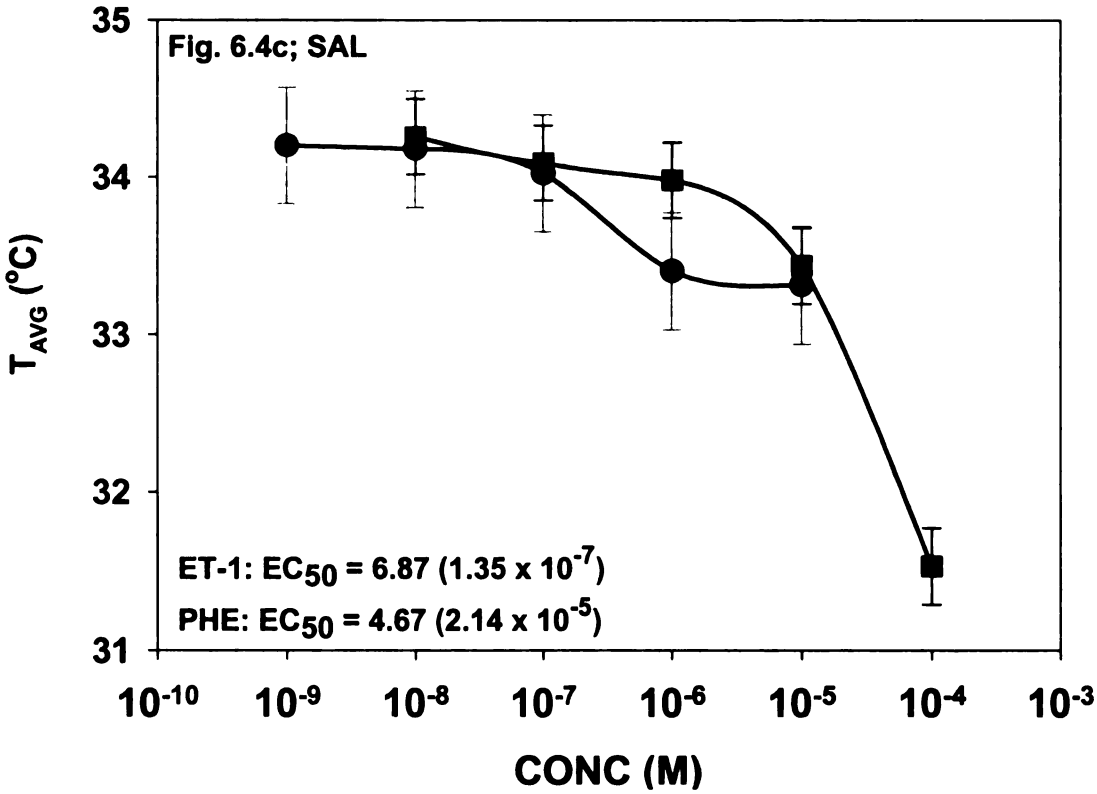


Figure 6.4c: The relative potencies between PHE and ET-1 are depicted as dose-temperature (T_{AVG}) curves of PHE 60 minutes after intradermal injection in horses that are treated with saline (solid symbol and line). EC_{50}: Effective concentration required to produce 50% of the maximal response.

CHAPTER 7

A THERMOGRAPHIC STUDY OF THE *IN VIVO* MODULATION OF METHACHOLINE INDUCED VASODILATION BY DEXAMETHASONE IN THE HORSE.

Summary

***Reasons for performing the study*: Glucocorticoids modulate many physiologic pathways, including those that are hypothesized to be involved in the development of laminitis. A vascular etiology has been implicated as a cause for laminitis. In vitro studies of equine digital vessels and studies in other species indicate that glucocorticoids can impair endothelial production of nitric oxide, a potent local vasodilator. Neuronal acetylcholine, an endothelial M_3-receptor agonist, is considered the primary stimulus for local nitric oxide production. An impaired production of nitric oxide can tilt the vascular balance towards increased vasomotor tone. In horses such an effect could therefore result in decreased perfusion of the laminar tissue of the hoof. Up to date no *in vivo* vascular studies on the modifying effect of vasodilation by glucocorticoids has been investigated in the horse.**

***Objectives:* To study the *in vivo* effects of glucocorticoid therapy on nitric oxide induced vasodilation in the horse, and its possible implications for laminitis. For this purpose the skin was used as a model for the laminar vascular bed of the equine hoof. Responses to intradermal injections with synthetic M_3-agonist methacholine were investigated by means of thermography in horses that were treated with either dexamethasone or saline. The effect of the treatments on MCH was further studied with the coinjection of substances that either attenuate (L-NAME) or aggravate effects of MCH (L-arginine, BQ-123). A nitric oxide donor (SNAP) was studied as well.**

Methods: **In a blinded randomized cross over design, ten horses were treated with either dexamethasone (0.1 mg/kg IV q24h) or saline IV for six days. On day six, the changes in local average skin temperature before (baseline) and after intradermal injections (20 minutes) that contained MCH (5 x 10^{-4}M), L-arginine (10^{-3} M), L-NAME (10^{-3} M), BQ-123 (10^{-4} M), or phenylephrine (PHE; 10^{-4} M). A nitric oxide donor (SNAP; 3.75 x 10^{-4} M), saline and placebo needle stick were injected as well. The horses were studied in a cold environment in order to induce baseline vasoconstriction. Skin on both shoulder areas was studied for duplicate measurements.**

Results: **Baseline skin temperatures were significantly lower in the dexamethasone group indicating an increase in vasomotor tone. There was a significant increase in skin temperature over time which affected the overall sensitivity of the study. Some significant effects were detected from various intradermal injections. The data were, however, inconsistent and difficult to interpret.**

Conclusions: **Our results show that dexamethasone therapy in horses causes a significant decrease in skin perfusion. This effect appears greater than under normal room temperatures. The study was unable to answer the question if dexamethasone impairs nitric oxide mediated vasodilation.**

Introduction

Glucocorticoids are potent drugs that with great success have been used for allergic and inflammatory airway diseases in the horse. However glucocorticoid therapy in the horse has been associated with laminitis, a debilitating disease of the equine hoof (Cornelisse and Robinson 2004). Several pathophysiologic mechanisms have been proposed in the etiology of clinical laminitis. This includes an vascular model with decreased perfusion of the laminar tissue in the hoof (Robinson, Scott et al. 1976; Hood, Amoss et al. 1978).

Vascular tone at local tissue level is the net result from a delicate interaction between parasympathetic and sympathetic tone, and the local production of vasoactive substances such as nitric oxide (NO) and endothelin-1 (ET-1). Increased acetylcholine release from parasympathetic nerves stimulates M_3-receptors that are present on endothelial cells. In response endothelial cells produce via increased activation of the enzyme nitric oxide synthase increased amounts nitric oxide. The nitric oxide relaxes the locally present vascular smooth muscles cells, which results in local vasodilation. Glucocorticoids are known to modulate the production of nitric oxide and ET-1 (Morin, Asselin et al. 1998; Provencher, Villeneuve et al. 1998; Wallerath, Witte et al. 1999; Severino, Brizzi et al. 2002; Di Filippo, Rossi et al. 2004). Therefore these drugs can modulate the net balance between locally present vasodilators and vasoconstrictors. Clinically glucocorticoids have indeed been associated with hypertension due to change in vasomotor tone (Wallerath, Witte et al. 1999; Severino, Brizzi et al. 2002). A glucocorticoid induced imbalance between locally active levels of vasodilating and

vasoconstricting substances could therefore be a vascular mechanism that results in equine laminitis.

The aim of the present study was to investigate the *in vivo* effects of glucocorticoid therapy on vascular tone in the horse with emphasis on the vasodilatory action of nitric oxide. The skin was used as a vascular model because it has many anatomical similarities to the equine laminar vascular bed and is easy to study. Local nitric oxide production was stimulated with methacholine (MCH), a more stable form of M_3-agonist. Additional coinjection with other vasoactive substances was used to estimate the relative importance of nitric oxide in equine vascular tone. These substances included L-arginine (ARG; nitric oxide precursor), L-NAME (nitric oxide synthase antagonist), BQ-123 (ET_A-receptor antagonist) and phenylephrine (PHE; vasoconstrictor). The local perfusion in the skin is reflected in the skin surface temperature. The change in local dermal circulation by the injected substances was therefore measured as changes in surface temperature with a thermograph. Our hypothesis was that glucocorticoid therapy impairs *in vivo* mediated vasodilation of the MCH from impaired nitric oxide production.

Material and methods

Preparation of solutions for intradermal injections

Fresh stock solutions of weighed amounts of endothelin receptor-A antagonists BQ-123[a], nitric oxide precursor L-arginine[b] (ARG) and the nitric oxide synthase antagonist L-NAME[c] were prepared every 48 hours. For this the drugs were dissolved and further diluted in phosphate buffered saline (PBS). The stock solutions were refrigerated at 10 °C

until further processing. A solution of methacholine[d](MCH), a synthetic M_3- receptor agonist, was freshly prepared in PBS prior to the experiments. Fresh MCH and stock solutions were combined for intradermal injections. Final working solutions (RX) therefore included BQ-123 (10^{-4} M), ARG (10^{-3} M) and L-NAME (10^{-3} M) alone or with MCH (5 x 10^{-4} M). Additional RX included freshly prepared control solutions in PBS of nitric oxide donor SNAP[e] (3.75 x 10^{-4} M) and α_1-agonist phenylephrine[f] (PHE; 10^{-4} M). All solutions were kept in a water bath with a controlled temperature of 22°C prior to injection. The concentrations of MCH, BQ-123, ARG, L-NAME, SNAP and PHE were based on on expected maximal effects (Clough, Bennett et al. 1998; Lipa, Neligan et al. 1999; Katugampola, Church et al. 2000; Newton, Khan et al. 2001; Milosevic, Janosevic et al. 2002; Katz, Marr et al. 2003; Vivancos, Parada et al. 2003; Wilson, Monahan et al. 2004).

Horses

Ten recurrent airway obstruction- affected mares (age 16-27 years, weight 442-571 Kg) (Robinson, Derksen et al. 1999) were used in the study. During the study, horses were in clinical remission. They were fed a commercial pelleted diet. While on pasture the horses were treated (TX) at 08.00 am with either dexamethasone[g] (DEX 0.1 mg/kg IV q24h) or an equivalent volume of saline (SAL IV q24h) for 6 consecutive days. The treatments were administered according a randomized cross over design and were blinded to the investigator. The wash out period between treatments was at least 2 weeks. Horses were 18-hours prior to the start of the experiments transported to a separate outside pen that had free communication with a covered study facility that was open to the environment.

Immediately after arriving at this study facility, skin test areas (25 by 20 cm) over both lateral shoulders were clipped and subsequently cleaned with alcohol. A 3 by 4 grid was drawn on the area with a silver sharpie pen so that the lines were visible on thermography images.

Study protocol

The studies were performed in an cold environment in order to archieve baseline vasoconstriction. Therefore, during the winter months January and February, horses were studied while standing in an examination stock that was centrally placed in the covered study facility with open communication to the outdoor environment. Starting on the left side, 0.1 ml of solutions containing MCH alone or MCH with BQ-123, ARG or L-NAME, and control solutions containing BQ-123, ARG, L-NAME, SNAP and PHE were injected intradermally with 1-minute intervals between each injection. Additional controls included 0.1 ml PBS and an empty needle stick. A 24-mm diameter circular bandage[h] was applied for purposes of reference size. The location of all injections and circular bandage was the same amongst all horses and treatments (figure 7.1), but randomized in time order within treatment for each horse. Thermographic pictures of the injection sites were taken at before (TIME= baseline), and 20 minutes after each injection with a mounted thermal imager (Microscan 7515[i]). The emissivity of the imager was set at 0.98 with a sensitivity of 0.7 °C, and the camera was adjusted for background temperature and humidity. The imager was kept at a distance of 70 cm perpendicular to the study area. The injection protocol and thermographic data collection were repeated on the right side in an identical manner. Earlier pilot studies had determined that the circular

bandage size was similar to the vasoconstricted area of 0.1 ml intradermal PHE at 10^{-4} M. During the study, environmental temperature and humidity data at the open study facility were collected. The study was approved by the All University Committee for Animal Use and Care at Michigan State University.

Image and data analysis

The digital thermograph images were analyzed with complementary software (Microspec 2.7)[i]. First the total number of pixels in the area covered by circular bandage was determined. Subsequently the average temperature (T_{AVG}) of circular areas of similar size (CIRCLE-1) and 1.5 times the size (CIRCLE-1.5) were measured over each individual injection at baseline and 20 minute values. By electronically deleting CIRCLE-1 temperature pixels out of the CIRCLE-1.5 image it was additionally possible to calculate the T_{AVG} of the area between both circles ($T_{AVG1.5}$). In this area it was less likely to find residual effects related to injection or absorption of a small intradermal volume. A mixed factorial procedure for repeated measures (SAS statistical software[j]) was used for analysis of main effects TX, TM and RX on T_{AVG1} or $T_{AVG1.5}$ of the left or the right side, and for the averaged value of both sides combined . When significant effects ($p < 0.05$) were observed for the main effects and their interactions, the Bonferoni test was used to determine differences between means. The strength of association for T_{AVG} data (SAL and DEX combined) between the left side and right side was determined by simple linear regression at times 0 and 20 minutes. The environmental data were compared with a simple t-test.

Results

Intradermal injections (RX)

There were significant main effects of treatment (TX) and TIME on T_{AVG1} and $T_{AVG1.5}$ when analyzed for the left side, right side, or both sides combined (table 7.1). The dexamethasone treatment resulted on both sides, or when combined, in a significant decrease of skin temperature for both T_{AVG1} and $T_{AVG1.5}$ (table 7.2). On both sides, and when combined, T_{AVG1} as well T_{AVG15} increased significantly between baseline and 20 minutes later (table 7.2). A significant TIME x TX interaction was detected on the left side (table 7.1). T_{AVG1} as well T_{AVG15} were significantly lower with dexamethasone treatment than with saline. After 20 minutes, both temperatures had increased and were no longer different from one another i.e. temperature increased more with dexamethasone treatment (table 7.2). The various intradermal injections (RX) resulted in significant effects only on $T_{AVG1.5}$ and only on the right side (table 7.1). Post hoc analysis of RX showed that injections of MCH plus NAME (mean ± se: 30.81 ± 0.36) resulted in significantly higher $T_{AVG1.5}$ compared to injections with MCH (29.35 ± 0.36, p=0.006), NAME-C (29.47 ± 0.36, p=0.019), SAL (29.51 ± 0.36, p=0.027) or PHE (29.51 ± 0.36, p=0.020). On the right side TX x RX and TIME x RX interactions were additionally significant for $T_{AVG1.5}$. The post hoc analysis of TX x RX interaction during dexamethasone treatment revealed a significantly higher $T_{AVG1.5}$ for MCH plus NAME (29.81 ± 0.55) compared to SAL (27.14 ± 0.55, p=0.0015), SNAP (27.66 ± 0.55, p=0.049) or MCH (27.0 ± 0.55, p=0.0012). MCH and SAL were additionally lower than NDL (29.46 ± 0.55, p=0.013). The many TIME x RX interactions that were significant in the post hoc analysis showed that 20 minute values of $T_{AVG1.5\text{-}R}$ of MCH, MCH plus

NAME, MCH plus BQ, NDL, SAL and SNAP were significantly higher than their baseline values (table 7.3).

Strength of association between left and right side

Simple linear regression detected a poor association between left and right side at baseline time for both T_{AVG1} (r^2 = 0.0023, p=0.62) and $T_{AVG1.5}$ (r^2=0.0018, p=0.65). At baseline (mean ± sem) T_{AVG} was consistently lower on the left side (29.18 ± 0.17 °C) than on the right side (30.18 ± 0.14 °C) (Fig 7.4a, 7.4c). The association between the left and right side was much stronger at 20 minutes and significant for both T_{AVG1} (r^2 = 0.65, p=0.001) and $T_{AVG1.5}$ (r^2 = 0.66, p=0.001) (fig 7.4b, 7.4d).

Environment

There was a significant difference (p=0.007) in environmental temperature when horses were treated with dexamethasone (2.3 ± 1.4 °C, mean ± se) compared to when they were treated with saline (7.0 ± 0.53 °C). Differences in environmental humidities were not significant (p=0.07) (table 7.4)

Discussion

Dexamethasone resulted in a significantly lower baseline temperature of the skin. This effect was similar but more pronounced (approximately 2 °C difference between saline and dexamethasone treatment) compared to the findings in the vasoconstriction study (approximately 1 °C, chapter 5). Based on the observations in the vasoconstriction study it is likely that a greater response to various endogenous vasoconstrictors (ET-1, PHE)

resulted in a decreased of the baseline skin temperature observed here. Several studies have reported that exposure to cold results in increased sympathetic activity with higher circulating norepinephrine levels (Hiramatsu, Yamada et al. 1984; Jansky, Sramek et al. 1996). Clinically this is associated with hypertension and dermal vasoconstriction (Jansky, Sramek et al. 1996). A potentiated response to endogenous catecholamines by dexamethasone is thus likely to result in an even more increased basal vasomotor tone under cold environmental conditions.

Over TIME temperature increased significantly on both sides of the horse and this complicated the analysis of the data. On the left side this resulted in a significant interaction with treatment (table 7.1, table 7.2). The observations that the horses became more anxious and agitated (stamping, pushing, and attempts to back out the stock) during the experiments can possibly explain the overall general increase in temperature at 20 minutes. The result, an increase in body heat production likely caused skin temperature to rise over the study period on the left side and increased baseline temperature on the right side (table 7.2, figure 7.4a, figure 7.4c). Consequently a more pronounced temperature change by TIME was present on the left side that caused a blunting of the response to injections (RX) and a significant TX x TIME interaction. However ongoing anxiety had a smaller effect on the right side. This resulted for $T_{AVG1.5}$ in a significant effect by the injections (RX), and significant TX x RX and TIME x RX interactions. The effects of RX and the interactions on the right side for T_{AVG15} are however hard to explain because some of the significant findings were opposite to what was expected. For instance in RX, significantly higher temperatures were detected for MCH plus NAME and not MCH alone or MCH combined with BQ-123 (ET_A-blocker) or L-arginine (ARG: nitric oxide

donor). Similarly in the TX x RX interaction during dexamethasone treatment skn temperature afte MCH was also lower than MCH plus NAME while not different from saline but lower than NDL. MCH alone or when combined with ARG or BQ-123 had however a significant effects over TIME, as did controls SAL and NDL (table 3). Overall the significant findings in this study for only $T_{AVG1.5}$ and only on the right side suggest that besides TX and TIME other factors must have confounded the effects of RX. These factors remain unexplained from these experiments but likely related to study design (e.g. injections too close together, fixed injection locations, dose of MCH and the other substances) or assumptions that were in hindsight possibly different than expected (e.g. same baseline temperatures in different injection areas, same response of skin in different injection areas, a 20 minute interval for measurement, combination of substances does not interfere with ability of intradermal diffusion). Additionally it appeared that the environmental temperatures during the experiments on the horses that were treated with dexamethasone were significantly higher compared to experiments on horses treated with saline (table 6.4). Although this was a relative small difference it could have resulted in a different (underestimated) baseline perfusion of the skin with different responses to intradermal injection due to better diffusion and uptake by increased perfusion in the skin.

The aim of this study was to investigate the in vivo modulation of vasodilation by glucocorticoids, with emphasis on NO mediated effects of MCH. The study reinforced the findings of the earlier vasoconstriction study that dexamethasone treatment results in a lower baseline temperature. This effect appeared amplified in a cold environment, likely associated with increased levels of circulating catecholamines and increases in

sympathetic tone. Some significant effects were detected from intradermal injection of MCH alone or with other substances. The data were inconsistent and difficult to interpret likely due to confounding in the experimental design. The study was unable to answer the question if dexamethasone impairs nitric oxide production.

Table 7.1: Effects of treatment (TX), TIME, injections (RX) and their interactions on T_{AVG1} and $T_{AVG1.5}$ of the left (L) side, right side (R) or both sides combined (L+R).

Table 7.1 EFFECT	P-VALUE		
	T_{AVG1}		
	T_{AVG1}, L	T_{AVG1}, R	T_{AVG1}, L+R
TX	*0.0001*	*<0.001*	*<.0001*
TIME	*<.0001*	*<0.001*	*<.0001*
RX	0.465	0.477	0.131
TIME x TX	*0.015*	0.207	0.063
TX x RX	0.994	0.993	0.988
TIME x RX	0.212	0.206	0.503
TIME x TX x RX	0.986	0.987	0.934
EFFECT	$T_{AVG1.5}$		
	$T_{AVG1.5}$, L	$T_{AVG1.5}$, R	$T_{AVG1.5}$, L+R
TX	*<.0001*	*0.021*	*<.0001*
TIME	*<.0001*	*<.0001*	*<.0001*
RX	0.830	*<.0001*	0.845
TIME x TX	*0.031*	0.052	0.077
TX x RX	0.998	*0.002*	0.997
TIME x RX	0.488	*<.0001*	0.680
TIME x TX x RX	0.997	0.055	0.999

Table 7.2: Influence of main effects treatment (TX), TIME, injections (RX) and TIME xTX interactions on T_{AVG1} and $T_{AVG1.5}$ (both in ^{o}C) for left (L) side, right side (R) or both sides combined (L+R). $^{a,b,c,\#,\$}$ significantly different.

Table 7.2			T_{AVG1}								
			LEFT			RIGHT			LEFT + RIGHT		
EFFECT	VALUE		mean	error	p<0.05	mean	error	p<0.05	mean	error	p<0.05
TX	DEX		29.124	0.418	a	30.675	0.322	a	30.058	0.323	a
TX	SAL		31.069	0.418	a	32.468	0.322	a	31.806	0.323	a
TIME		0 MIN	28.066	0.421	b	30.788	0.322	b	29.623	0.323	b
TIME		20 MIN	32.127	0.414	b	32.356	0.322	b	32.241	0.323	b
TIME*TX	DEX	0 MIN	26.602	0.476	c,#	29.746	0.356	NA	28.492	0.365	NA
TIME*TX	SAL	0 MIN	29.530	0.476	c,$	31.829	0.356	NA	30.753	0.365	NA
TIME*TX	DEX	20 MIN	31.646	0.463	#	31.604	0.356	NA	31.625	0.365	NA
TIME*TX	SAL	20 MIN	32.608	0.463	$	33.107	0.356	NA	32.858	0.365	NA
Table 2			$T_{AVG1.5}$								
			LEFT			RIGHT			LEFT + RIGHT		
EFFECT	VALUE		mean	error	p<0.05	mean	error	p<0.05	mean	error	p<0.05
TX	DEX		29.215	0.394	a	31.548	0.723	a	30.143	0.309	a
TX	SAL		31.278	0.394	a	28.264	0.886	a	32.084	0.309	a
TIME		0 MIN	28.131	0.397	b	28.752	0.577	b	29.778	0.309	b
TIME		20 MIN	32.362	0.390	b	31.059	0.577	b	32.449	0.309	b
TIME*TX	DEX	0 MIN	26.694	0.454	c,#	30.568	0.730	NA	28.578	0.349	NA
TIME*TX	SAL	0 MIN	29.568	0.454	c,$	26.936	0.894	NA	30.977	0.349	NA
TIME*TX	DEX	20 MIN	31.736	0.441	#	32.527	0.730	NA	31.708	0.349	NA
TIME*TX	SAL	20 MIN	32.988	0.441	$	29.591	0.894	NA	33.191	0.349	NA

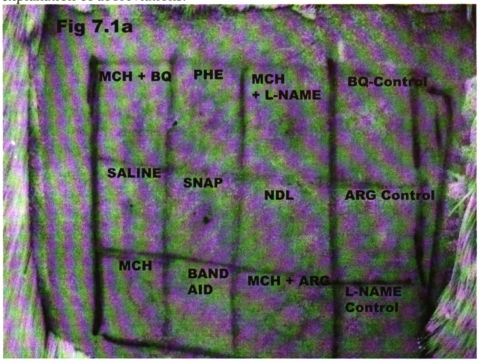
Table 7.3: Effect of TIME x RX interaction on T_{AVG15} (in ^{o}C) on the right side.

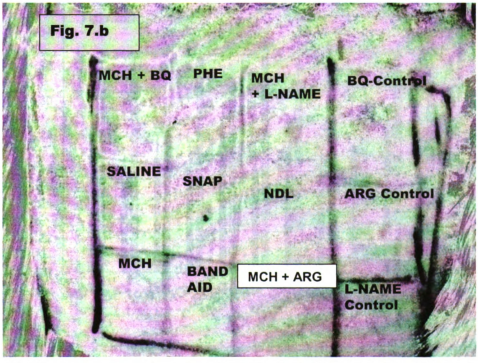
Table 7.3	BASELINE	TIME = 20	
RX	mean ± se	mean ± se	p-value
MCH	27.45 ± 0.53	31.26 ± 0.53	<.0001
MCH+ARG	28.35 ± 0.53	31.79 ± 0.53	<.0001
MCH+BQ	28.30 ± 0.53	32.12 ± 0.53	<.0001
NDL	29.38 ± 0.53	31.60 ± 0.53	0.01
SAL	28.44 ± 0.53	30.58 ± 0.53	0.01
SNAP	28.62 ± 0.53	30.63 ± 0.53	0.03

Table 7.4; Mean and se of the enviromental temperature (°C) and humidity (%) in the study facility when studying horses treated (TX) that were treated with dexamethasone (DEX) or saline (SAL).

Table 7.4	Temperature		Humidity	
TX	mean	se	mean	se
SAL	7.00	0.53	72.50	2.78
DEX	2.32	1.45	64.60	3.05
p-value	*0.0073*		0.0700	

Figure 7.1: a) Locations of intradermally injected vasoconstrictors 20 minutes post administration. b) Thermographic image of figure 1a. See main text for explanation of abbreviations.





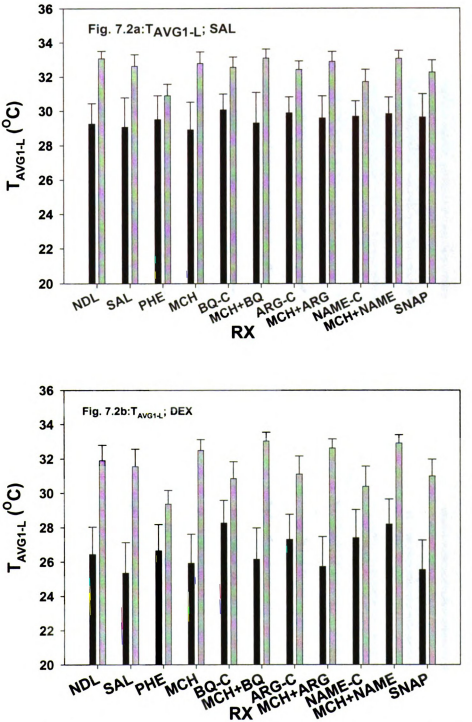
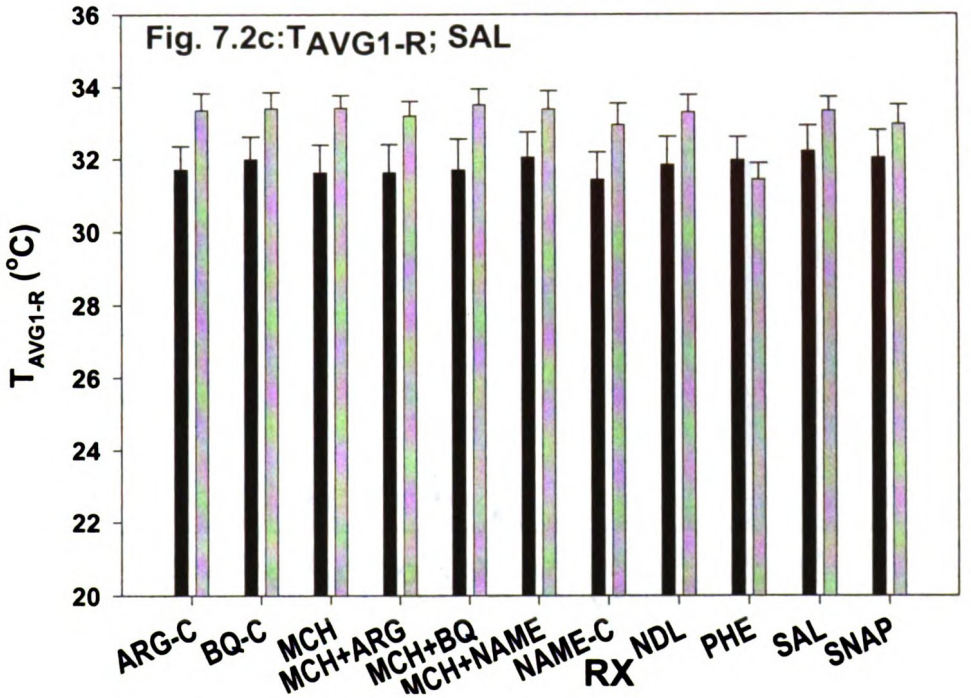


Figure 7.2a,b: Effect of the interaction between treatment (DEX or SAL) ,TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on T_{AVG1} of the left side (a:SAL, b:DEX). Data as mean and se. See text for abbreviations.



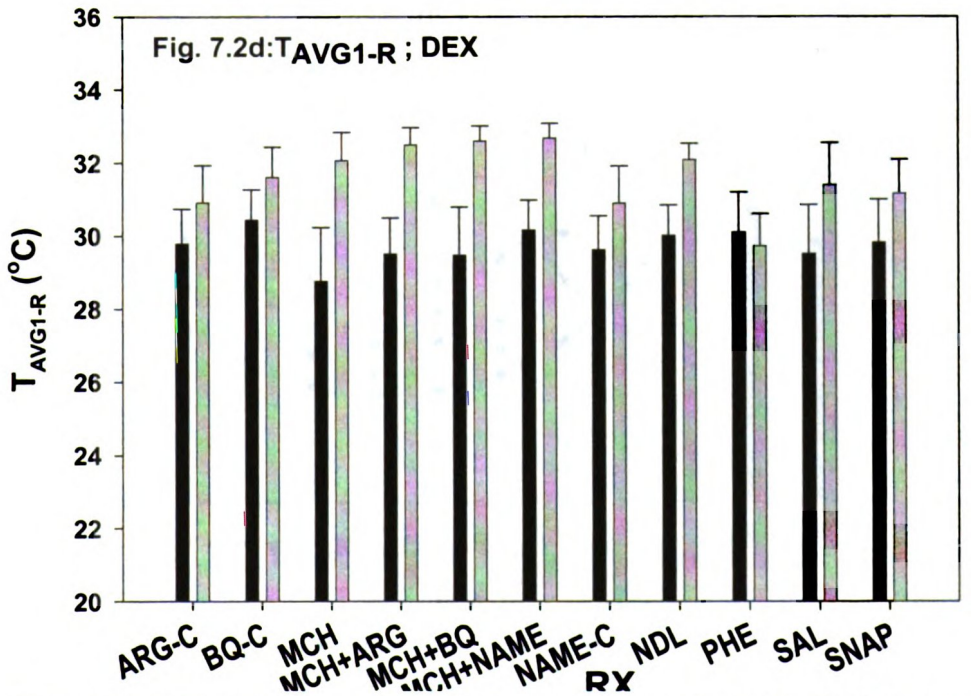


Figure 7.2c,d: Effect of the interaction between treatment (DEX or SAL) ,TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on T_{AVG1} of the right side (c:SAL, d:DEX). Data as mean and se. See text for abbreviations.

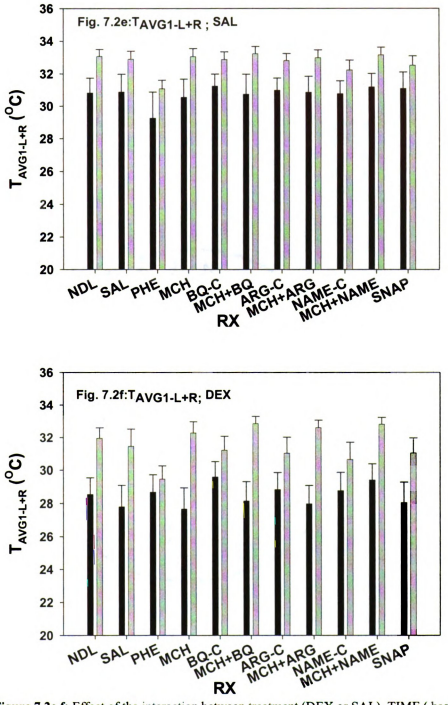


Figure 7.2e,f: Effect of the interaction between treatment (DEX or SAL) ,TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on T_{AVG1} of the left + right side (e: SAL, f: DEX. Data as mean and se. See text for abbreviations.

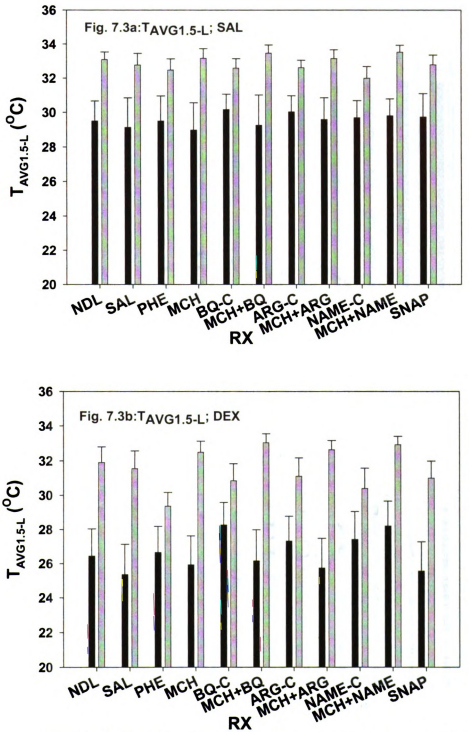
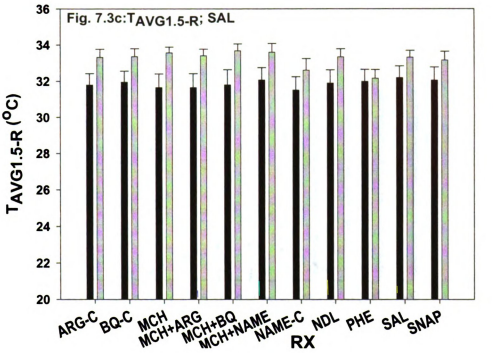


Figure 7.3a,b: Effect of the interaction between treatment (DEX or SAL) , TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on $T_{AVG1.5}$ of left side (a: SAL, b: DEX). Data as mean and se. See text for abbreviations



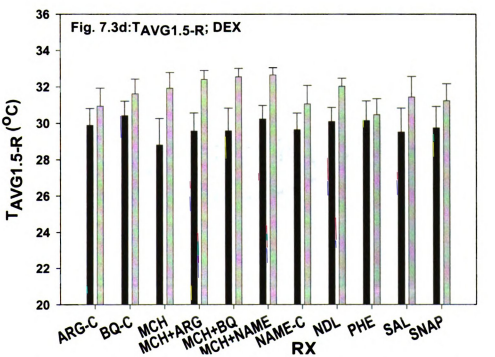


Figure 7.3c,d: Effect of the interaction between treatment (DEX or SAL) , TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on $T_{AVG1.5}$ of right side (c: SAL, d: DEX). Data as mean and se. See text for abbreviations

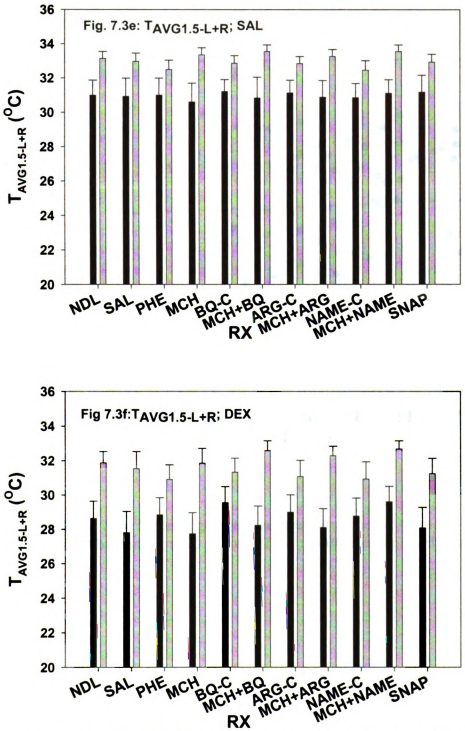


Figure 7.3e,f: Effect of the interaction between treatment (DEX or SAL) , TIME (baseline: Black bar, or 20 minutes: grey bar) and injected solutions (RX: MCH, MCH +ARG, MCH+BQ, MCH+NAME, ARG, BQ, NAME, PHE, SALINE, SNAP,NDL) on $T_{AVG1.5}$ of left + right side (e: SAL, f: DEX). Data as mean and se. See text for abbreviations

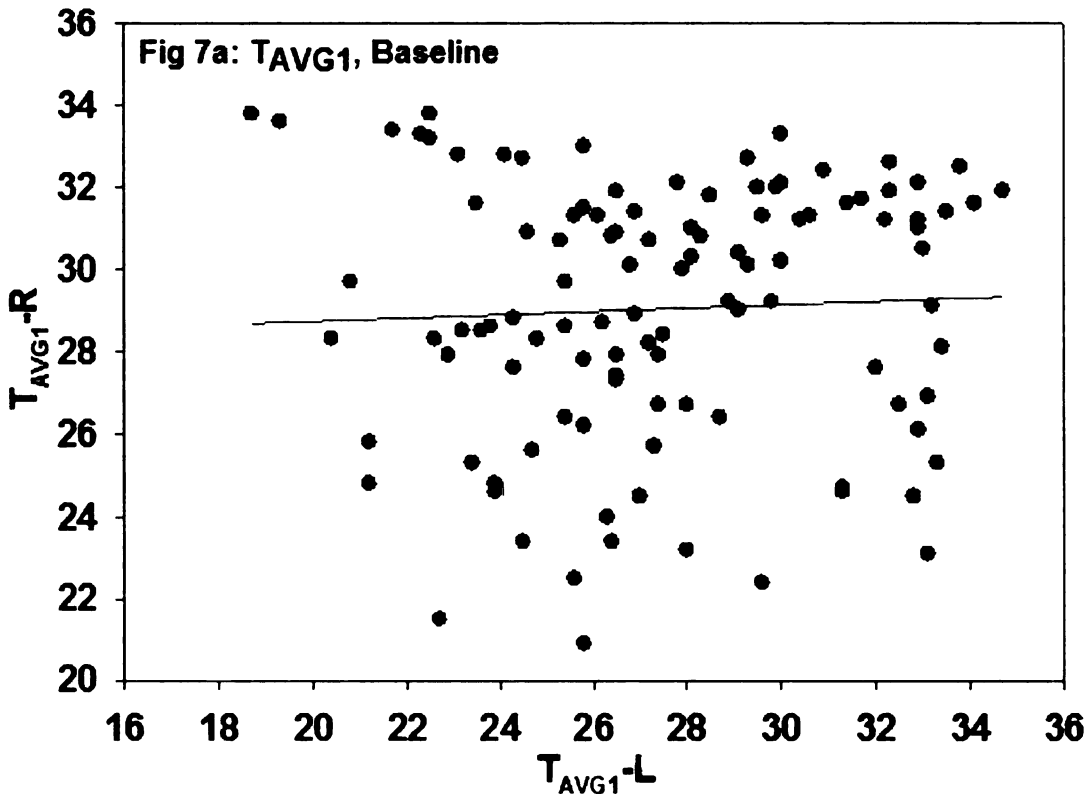


Figure 7.4a: Linear regressions between temperatures from left and right at either baseline or at 20 minutes. T_{AVG1}: a) Baseline or b) 20 minues.

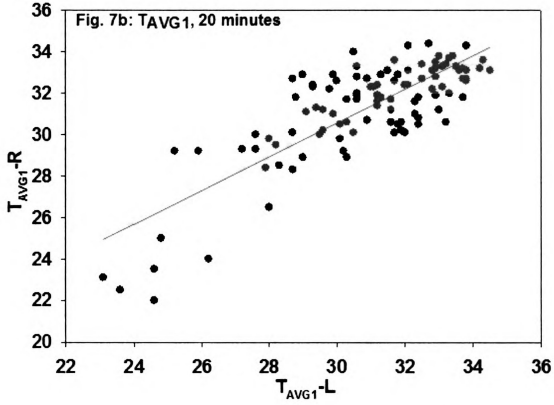


Figure 7.4b: Linear regressions between temperatures from left and right at either baseline or at 20 minutes. T_{AVG1}: a) Baseline or b) 20 minues.

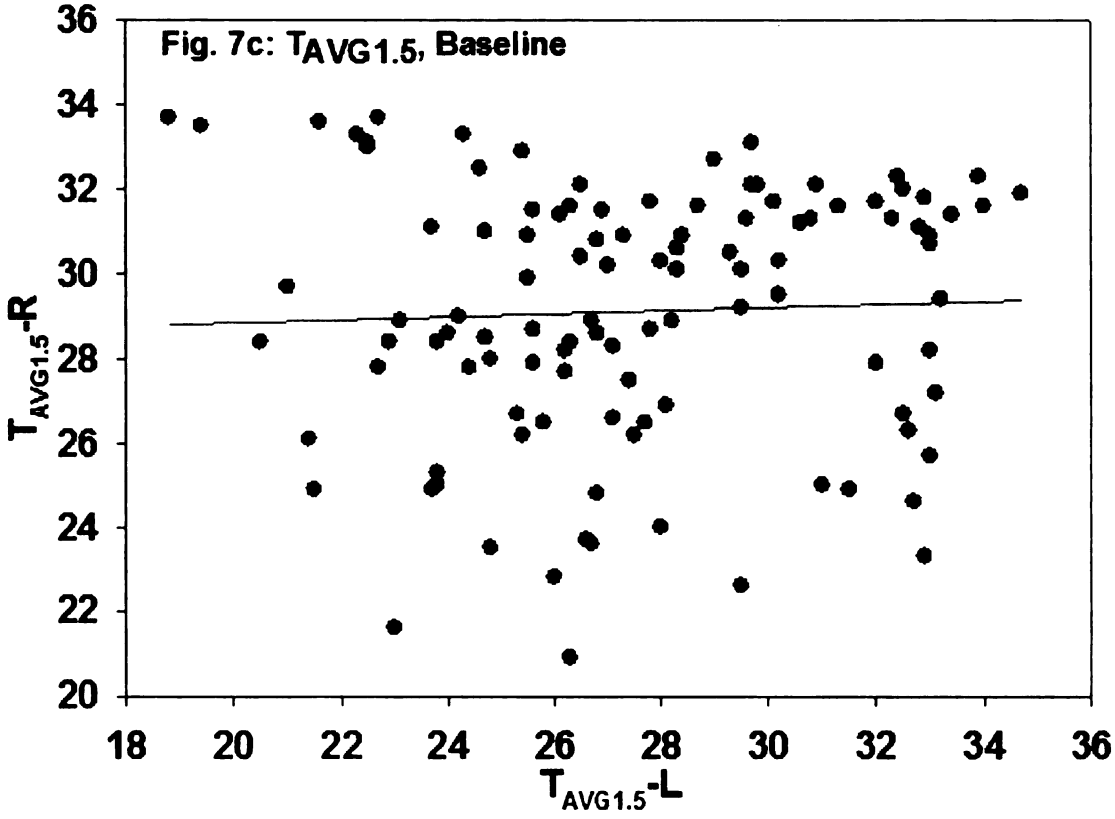


Figure 7.4c: Linear regressions between temperatures from left and right at either baseline or at 20 minutes.$T_{AVG1.5}$: c) Baseline or b) 20 minutes

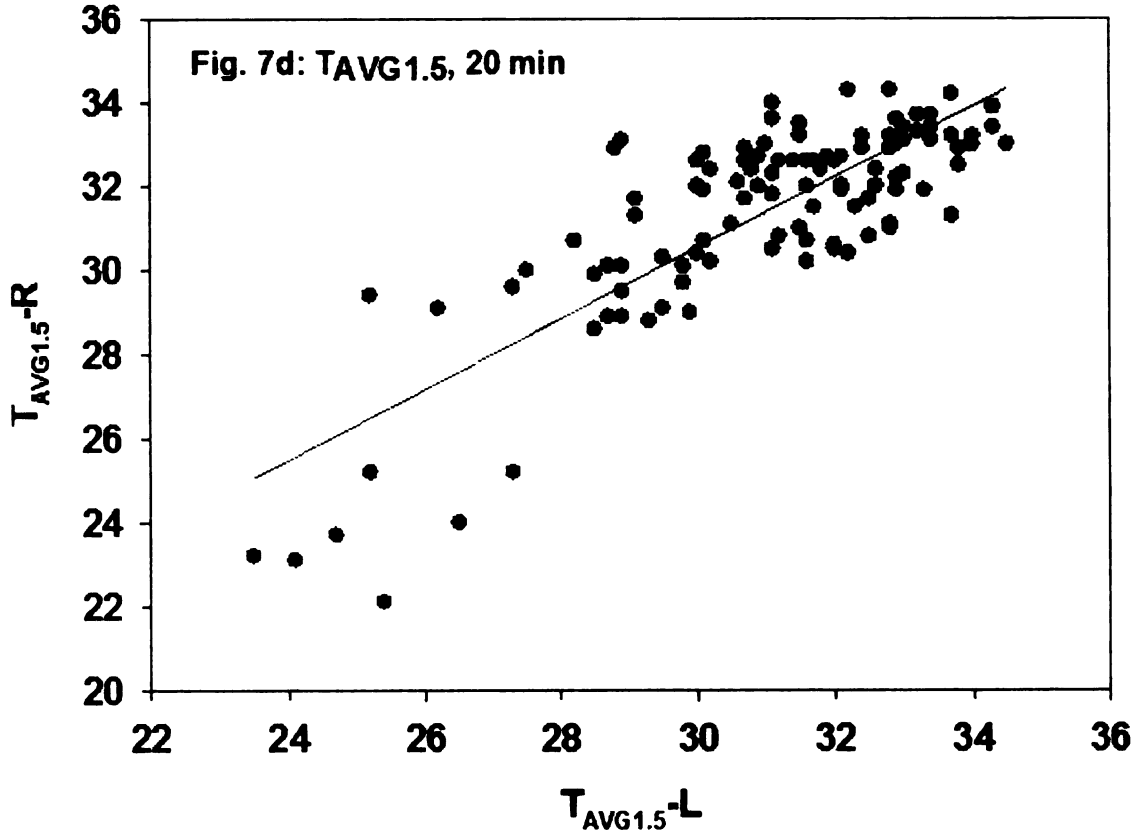


Figure 7.4e: Linear regressions between temperatures from left and right at either baseline or at 20 minutes. $T_{AVG1.5}$: c) Baseline or b) 20 minutes

GENERAL CONCLUSIONS

The aims of my Ph.D studies were to investigate the clinical effects of glucocorticoid therapy in the horse. My first study investigated the efficacy of oral and intravenous dexamethasone for the treatment of RAO in horses. The purpose of this study was to come up with a dose that would be efficacious, safe and easily applicable under practice conditions. This included keeping the horses in a challenging environment. The question of how fast these drugs result in clinical improvement, and thus owner satisfaction was also studied. My research on horses in RAO showed, based on analysis of many lung function parameters, that one time oral application of an injectable dexamethasone solution at doses of 0.164 mg/kg with or without food, or at a dose of 0.082 mg/kg without food, is at least as efficacious as the standard intravenous dose of 0.1 mg/kg when compared to placebo treatment. All these dexamethasone applications resulted in a statistical improvement of lung function within 6 hours and had effects for at least 30 hours (as determined by the intrapleural pressure change ΔP_{PLMAX}). Oral applications tended to have longer duration of effect. A clinical relevant effect based on the cut off ΔP_{PLMAX} for detection of heaves showed that dexamethasone IV and oral dexamethasone at a dose of 0.164 mg/without food were the most efficacious with duration of effect for up to 24 hours. Intravenous dexamethasone results in clinical and significant improvement within 4 hours, although its effect is not as good as complete bronchodilation by atropine. The practical implications of this study are therefore that an owner can treat an exacerbation with on oral dose of 0.164 mg/kg. They can expect clinical improvement within 4-6 hours and a dosing interval under challenging conditions

in the order of 24-30 hours. On a daily scheme when drug administration before food can be controlled, or in combination with improvements in the environment, interpolation suggests that even lower doses and dose intervals are likely to be beneficial.

Because laminitis has been implicated as an adverse effect of glucocorticoid therapy in the horse, my second and third study investigated the *in vivo* effects of dexamethasone treatment on local vascular function. In these studies the skin was studied as a representative vascular model with many similarities to that of the laminar vascular bed of the equine hoof. The *in vivo* responses to various intradermally injected concentrations of vasoconstricting (study 2) and vasodilating (study 3) substances were measured with a thermographic imager in horses that were treated either intravenous saline or dexamethasone. Dexamethasone treatment resulted in a significant lower baseline temperature compared to saline suggesting an increased vasomotor tone. This was accompanied by a greater and potentiated response to the α_1-agonist phenylephrine. Additionally, the interplay with the lower baseline temperature resulted in a greater response to vasoconstrictor endothelin-1. Study 3 confirmed and reemphasized the finding of the lower baseline temperature by dexamethasone. The greater decrease in baseline temperature was likely due to higher circulating levels of catecholamines under cold experimental conditions. Unfortunately the statistical analysis of the data on nitric oxide controlled vasodilation from intradermal injections of methacholine was difficult to interpret. The reasons for this were a rise in skin temperature over time due to anxiety of the horses, likely in combination with other experimental confounding factors. Conclusion regarding impaired vasodilatory capacity of the equine vasculature during glucocorticoid treatment could therefore not be made. Based on the similarities between

blood flow in the skin and the laminar vascular bed, study 2 and study 3 indicate that glucocorticoid therapy can result in additional compromise of perfusion of the equine hoof during disease states that are already characterized by hypoperfusion and/or increased levels of catecholamines. Thus during such pathologic states, glucocorticoid therapy could, according the vascular model of laminitis, tilt the balance in favor of laminitis.

The results of these studies, in combination with the literature reviews of glucocorticoid actions on RAO and the risk for laminitis, indicate that these drugs are of great benefit for the treatment of RAO, a disease not associated with circulatory risk factors.

APPENDIX A

FOOTNOTES

(Manufacturers'addresses)

Chapter 3

[1]Validyne, Northridge, California, USA

[2]Dash, Astro-Med Inc., West Warwick, RI, USA

[3]Buxco Electronics Inc., Sharon, Connecticut, USA

[4]Fleisch, OEM Medical, Richmond, Virginia, USA

[5]Dexamethasone solution (2 mg/ml): Vedco, Inc., St Joseph, Missouri, USA

[6]Sigmastat 2.0, SPSS Inc., Chicago, IL, USA

Chapter 6

[a] H-6995 (ET-1), H-1252 (BQ-123), H-2508 (RES-701): Bachem California, Torrance Ca 90505

[b] DMSO America, Lawrence, MA 01843

[c] N5751 (L-NAME): Sigma-Aldrich, St Louis, MO 63178

[d] Phenylephrine-HCL 1%: American Regent Laboratories, NY 11967

[e] Dexamethasone solution: Vedco, St Joseph MO 64507

[f] Nexcare™: 3M Health Care, St Paul, MN 55144

[g] Microscan 7515 and Microspec 2.7: Mikron^{R}, Oakland, NJ 07436

[h] SAS 9.0: SAS Institute Inc, Cary, NC 27513

Chapter 7

[a] H-1252 (BQ-123): Bachem California, Torrance Ca 90505

[b] (L-ARGININE): Sigma-Aldrich, St Louis, MO 63178

[c] N5751 (L-NAME): Sigma-Aldrich, St Louis, MO 63178

[d] Methacholine: Sigma-Aldrich, St Louis, MO 63178 Laboratories, NY 11967

[e] ALX-420-00 (SNAP) : Alexis,

[f] Phenylephrine-HCL 1%: American Regent

[g] Dexamethasone solution: Vedco, St Joseph MO 64507

[h] Nexcare™: 3M Health Care, St Paul, MN 55144

[i] Microscan 7515 and Microspec 2.7: Mikron^{R}, Oakland, NJ 07436

[j] SAS 9.0: SAS Institute Inc, Cary, NC 27513

BIBLIOGRAPHY

References chapter 1: Introduction to glucocorticoids

Adcock, I. M. and G. Caramori (2001). "Cross talk between pro-inflammatory transcription factors and glucocorticoids." Immunology and Cell Biology **79**: 376-384.

Adcock, I. M. and K. Ito (2000). "Molecular mechanisms of cortiocoid actions." Monaldi Archives for Chest Disease **55**: 256-266.

Bamberger, C. M., H. M. Schulte, et al. (1996). "Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids." Endocrine reviews **17**(3): 245-261.

Barnes, P. J., S. Pedersen, et al. (1998). "efficacy and safety of inhaled corticosteroids." Am J Respir Crit Care Med **157**: S1-S53.

Baxter, J. D. and G. M. Tomkins (1971). "Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells." Proc Natl Acad Sci U S A **68**: 936-946.

Bolland, E. W. and G. W. Liddle (1958). "16-alpha-methyl corticosteroids: A new series of anti-inflammatory compounds; clinical appraisal of their anti-rheumatic properites." California Med **88**: 417-422.

Brattsand, R. and B. I. Axelssson (1997). Basis of airway selectivity of inhaled glucocorticoids. Inhaled glucocorticoids in asthma: mechanisms and clinical actions. R. P. Schleimer, W. W. Busse and P. M. O'Byrne. New York, Marcel Dekker, Inc.: 351-379.

Brereton, P. S., R. R. van Driel, et al. (2001). "Light and electron microscopy localization of the 11-beta-hydroxysteroid dehydrogenase type I enzyme in the rat." Endocrinology **142**(4): 1644-1651.

Buttgereit, F., M. D. Brand, et al. (1999). "Equivalent doses and relative drug potencies for non-genomic glucocorticoid effects: A novel glucocorticoid hierarchy." Biochemical Pharmacology **58**: 363-368.

Buttgereit, F., J. A. P. da Silva, et al. (2002). "Standardized nomenclature for glucocorticoid dosages and glucocorticoid treatment regimens: Current questions and tentative answers in rheumatology." Ann Rheum Dis **61**: 718-722.

Buttgereit, F. and A. Scheffold (2002). "rapid glucocorticoid effects on immune cells." Steroids **67**: 529-534.

Cantrill, H. L., S. R. Waltman, et al. (1975). "In vitro determination of relative corticosteroid potency." J Clin Endocrinol Metab **40**: 1073-1077.

Chen, C. L., J. A. Sailor, et al. (1992). "Synovial and serum levels of triamcinolone following intra-articular administration of triamcinolone acetonide in the horse." J Vet Pharmacol Ther **15**(3): 240-6.

Chen, C. L., D. Zhu, et al. (1996). "Use of enzyme-linked immunosorbent assay and radioimmunoassay to determine serum and urine dexamethasone concentrations in thoroughbreds after intravenous administration of the steroid." Am J Vet Res **57**(2): 182-6.

Chen, D., K. Kohli, et al. (1994). "Phenylalanine-780 near the C-terminus of the mouse glucocorticoid receptor is important for ligand binding affinity and specificity." Mol Biol **8**: 422-430.

Croxtall, J. D., Q. Choudhury, et al. (2000). "Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism." Br J Pharmacol **130**(2): 289-98.

Croxtall, J. D., P. T. W. van Hal, et al. (2002). "Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A54 cells." Br J Pharmacol **135**: 511-519.

Dahlberg, E., A. Thalen, et al. (1983). "Correlation between chemical structure, receptor binding and biological activity of some novel, highly active, 16a,17a acetal substituted glucocorticoids." Molecular Pharmacology **25**: 70-78.

Damon, M., M. Rabier, et al. (1985). "Glucocorticoid receptors in cultured fibroblasts from inflammatory sites. Preliminary results." Agents and Actions **17**(3/4): 292-293.

Derendorf, H., G. Hochhaus, et al. (1993). "Receptor-based pharmacokinetic-pharmacodynamic analysis of corticosteroids." J Clin Pharmacol **33**: 115-123.

Diederich, S., B. Hanke, et al. (1996). "The metabolism of 9-alpha -fluorinated steroids in the human kidney." Endocrine Research **22**: 803-810.

Diederich, S., B. Hanke, et al. (1998). "Metabolism of synthetic corticosteroids by 11-B-hydroxysteroid-dehydrogenases in man." Steroids **63**: 271-277.

Ferrari, P., R. E. Smith, et al. (1996). "Substrate and inhibitor specificity of the cloned human 11-beta-hydroxysteroid dehydrogenase type 2 isoform." Am J Physiol Endocrinol Metab **33**: E900-E904.

French, K., C. C. Pollitt, et al. (2000). "Pharmacokinetics and metabolic effects of triamcinolone acetonide and their possible relationships to glucocorticoid-induced laminitis in horses." J Vet Pharmacol Ther **23**(5): 287-92.

Hench, R. F., E. C. Kendall, et al. (1949). "The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis." Proc. Staff. Meet. Mayo. Clin. **24**: 181-197.

Hoffsis, G. F. and P. W. Murdick (1970). "The plasma concentrations of corticosteroids in normal and diseased horses." J Am Vet Med Assoc **157**(11): 1590-4.

Hofmann, T. G., S. P. Hehner, et al. (1998). "Various glucocorticoids differ in their ability to induce gene expression, apoptosis and to repress NF-kB-dependent transcription." FEBS Lett **441**: 441-446.

Jaffuel, D., P. Demoly, et al. (2000). "Transcriptional potencies of inhaled glucocorticoids." Am J Respir Crit Care Med **162**: 57-63.

Jaffuel, D., C. Roumestan, et al. (2001). "Correlation between different gene expression assays designed to measure trans-activation potencies of systemic glucocorticoids." Steroids **66**: 597-604.

Koupai-Abyazani, M. R., N. Yu, et al. (1995). "Determination of triamcinolone acetonide in equine serum and urine by liquid chromatography-atmospheric pressure ionization mass spectrometry." J Anal Toxicol **19**(3): 182-6.

Langhoff, E. and J. Ladefoged (1983). "Relative immunosuppressive potency of various corticorsteroids measured in vitro." Eur J Clin Pharmacol **25**: 459-462.

Lerner, L. J., A. R. Turkheimer, et al. (1964). "Comparison of anti-granuloma, thymolytic and glucocorticoid activities of anti-inflammatory steroids." Proc Soc Exp Biol med **116**: 385-388.

Li, K. X. Z., V. R. Obeyeserkere, et al. (1997). "Oxoreductase and dehydrogenase activities of the human and rat 11-beta-hydroxysteroid dehydrogenase type II enzyme." Endocrinology **138**(7): 2948-2952.

Liddle, G. W. (1961). "Clinical pharmacology of anti-inflammatory steroids." Clinical pharmacology and therapeutics **2**(5): 615-635.

Lillich, J. D., A. L. Bertone, et al. (1996). "Plasma, urine, and synovial fluid disposition of methylprednisolone acetate and isoflupredone acetate after intra-articular administration in horses." Am J Vet Res **57**(2): 187-92.

Mager, D. E., N. Moledina, et al. (2003). "Relative immunosuppresive potency of therapeutic corticosteroids measured by whole blood lymphocyte proliferation." J Pharmacol Sci **92**(7): 1521-1525.

Manz, B., H. J. Grill, et al. (1982). "Steroid side chain modification and receptor affinity: Binding of synthetic derivates of corticoids to human spleen tumor and rat liver glucocorticoid receptors." J Steroid Biochem **17**: 335-342.

Melby, J. C. (1977). "Clinical pharmacology of systemic corticosteroids." Ann Rev Pharmacol **17**: 511-527.

Merkulova, T. I., V. M. Merkulov, et al. (1997). "Glucocorticoid regulation mechanisms and glucocorticoid-controlled gene regulatory regions: Description in the TRRD database." Mol Biol **31**(4): 605-615.

Oelkers, W., S. Buchen, et al. (1994). "Impaired renal 11-beta-oxydation of 9-alpha-fluorocortisol: An explanation for its mineralocorticoid potency." J Clin Endocrinol Metab **78**(4): 928-932.

Pelaia, G., A. Vatrella, et al. (2003). "Molecular mechanisms of corticoid actions in chronic inflammatory airway diseases." Life Sci **72**: 1549-1561.

Peroni, D. L., S. Stanley, et al. (2002). "Prednisone per os is likely to have limited efficacy in horses." Equine Vet J **34**(3): 283-7.

Rask, E., T. Olsson, et al. (2001). "Tissue-specific dysregulation of cortisol metabolism in human obesity." J Clin Endocrinol Metab **86**(3): 1418-1421.

Rohdewald, P., H. W. Mollmann, et al. (1985). "Affinities of glucocorticoids for glucocorticoid receptors in human lung." Agents and Actions **17**(3/4): 290-291.

Roumestan, C., C. Henriquet, et al. (2003). "Fluticasone proprionate and momethasone furoate have equivalent transcriptional potencies." Clin Exp Allergy **33**: 895-901.

Schimmer, B. P. and K. L. Parker (1995). Chapter 59: Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of the adrenocortical hormones. Goodman & Gilman's The farmacological basis of therapeutics. J. G. Hardman, L. L. Limbird, P. B. Molinoff and R. W. Ruddon. New York, McGraw-Hill: 1459-1485.

Schmidt, B. M., D. Gerdes, et al. (2000). "Non-genomic steroid actions: A new Age?" Front neuroendocrinol **21**: 57-94.

Smith, R. E., J. A. Maguire, et al. (1996). "Location of 11-beta-hydroxysteroid dehydrogenase type II in human epithelial tissues." J Clin Endocrinol Metab **81**(9): 3244-3248.

Spahn, J. D., L. P. Landwehr, et al. (1996). "Effects of glucocorticoid on lymphocyte activation in patients with steroid-sensitive and steroid-resistant asthma." J Allergy Clin Immunol **98**: 1073-1079.

Spiess, B. M., S. Nyikos, et al. (1999). "Systemic dexamethasone concentration in horses after continued topical treatment with an ophthalmic preparation of dexamethasone." Am J Vet Res **60**(5): 571-6.

Tonelli, G., L. Thibault, et al. (1965). "A bio-assay for the concominant assessment of the antiphlogistic thymolytic activitiesof topically applied corticoids." Endocrinology **77**(4): 625-634.

Tuckerman, J. P., H. M. Reichardt, et al. (1999). "The DNA binding-independent function of glucocorticoid receptor mediates repression of AP-1 dependent genes in the skin." J Cell Biol **147**(7): 1365-1370.

Yeakley, J. M., K. Balasubramanian, et al. (1980). "Comparison of glucocorticoid-receptor binding kinetics with predictions from a bimolecular model." J Biol Chem **255**: 4182-4188.

References **chapter 2: Glucocorticoid therapy for recurrent airway obstruction in the horse**

Abraham, G., Brodde, O.E. and Ungemach, F.R. (2002) Regulation of equine lymphocyte beta-adrenoceptors under the influence of clenbuterol and dexamethasone. *Equine Vet J* **34**, 587-593.

Ainsworth, D.M., Grunig, G., Matychak, M.B., Young, J., Wagner, B., Erb, H.N. and Antczak, D.F. (2003) Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. *Vet Immunol Immunopathol* **96**, 83-91.

Ammann, V.J., Vrins, A.A. and Lavoie, J.P. (1998) Effects of inhaled beclomethasone dipropionate on respiratory function in horses with chronic obstructive pulmonary disease (COPD). *Equine Vet J* **30**, 152-157.

Blanchon, P., Georges, R., Sauvan, R. and Turiaf, J. (1956) [Chronic cor pulmonale in asthma and its treatment with adrenal cortex hormones; ACTH, cortisone and derivatives.]. *Arch Mal Coeur Vaiss* **49**, 497-524.

Bowles, K.S., Beadle, R.E., Mouch, S., Pourciau, S.S., Littlefield-Chabaud, M.A., Le Blanc, C., Mistric, L., Fermaglich, D. and Horohov, D.W. (2002) A novel model for equine recurrent airway obstruction. *Vet Immunol Immunopathol* **87**, 385-389.

Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., Merville, M.P., Bours, V. and Lekeux, P. (2000a) Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am J Respir Crit Care Med* **161**, 1314-1321.

Bureau, F., Delhalle, S., Bonizzi, G., Fievez, L., Dogne, S., Kirschvink, N., Vanderplasschen, A., Merville, M.P., Bours, V. and Lekeux, P. (2000b) Mechanisms of persistent NF-kappa B activity in the bronchi of an animal model of asthma. *J Immunol* **165**, 5822-5830.

Costa, L.R., Seahorn, T.L., Moore, R.M., Oliver, J.L. and Hosgood, G.L. (2001) Plasma and bronchoalveolar fluid concentrations of nitric oxide and localization of nitric oxide synthesis in the lungs of horses with summer pasture-associated obstructive pulmonary disease. *Am J Vet Res* **62**, 1381-1386.

Couetil, L.L. and Ward, M.P. (2003) Analysis of risk factors for recurrent airway obstruction in North American horses: 1,444 cases (1990-1999). *J Am Vet Med Assoc* **223**, 1645-1650.

Cox, G. (1995) Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* **154**, 4719-4725.

Cundall, M., Sun, Y., Miranda, C., Trudeau, J.B., Barnes, S. and Wenzel, S.E. (2003) Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *J Allergy Clin Immunol* **112**, 1064-1071.

Cunningham, F.M., Rogers, S., Fischer, J.H. and Jensen, R.C. (1966) The pharmacokinetics of dexamethasone in the thoroughbred racehorse. *J Vet Pharmacol Ther* **19**, 68-71.

Damon, M., Rabier, M., Loubatiere, J., Blotman, F. and Crastes de Paulet, A. (1985) Glucocorticoid receptors in cultured fibroblasts from inflammatory sites. Preliminary results. *Agents and Actions* **17**, 292-293.

Debierre-Grockiego, F., Fuentes, V., Prin, L., Gouilleux, F. and Gouilleux-Gruart, V. (2003) Differential effect of dexamethasone on cell death and STAT5 activation during in vitro eosinopoiesis. *Br J Haematol* **123**, 933-941.

Derksen, F.J., Olszewski, M.A., Robinson, N.E., Berney, C., Hakala, J.E., Matson, C.J. and Ruth, D.T. (1999) Aerosolized albuterol sulfate used as a bronchodilator in horses with recurrent airway obstruction. *Am J Vet Res* **60**, 689-693.

Derksen, F.J., Robinson, N.E., Scott, J.S. and Stick, J.A. (1988) Aerosolized Micropolyspora faeni antigen as a cause of pulmonary dysfunction in ponies with recurrent airway obstruction (heaves). *Am J Vet Res* **49**, 933-938.

Diederich, S., Hanke, B., Burkhardt, P., Muller, M., Schoneshofer, M., Bahr, V. and Oelkers, W. (1998) Metabolism of synthetic corticosteroids by 11-B-hydroxysteroid-dehydrogenases in man. *Steroids* **63**, 271-277.

Dixon, P.M. (1978) Pulmonary artery pressures in normal horses and in horses affected with chronic obstructive pulmonary disease. *Equine Vet J* **10**, 195-198.

Dixon, P.M. (1992) Respiratory mucociliary clearance in the horse in health and disease, and its pharmaceutical modification. *Vet Rec* **131**, 229-235.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995) Equine pulmonary disease: a case control study of 300 referred cases. Part 1: Examination techniques, diagnostic criteria and diagnoses. *Equine Vet J* **27**, 416-421.

Donnelly, L.E. and Barnes, P.J. (2002) Expression and regulation of inducible nitric oxide synthase from human primary airway epithelial cells. *Am J Respir Cell Mol Biol* **26**, 144-151.

Druilhe, A., Letuve, S. and Pretolani, M. (2003) Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. *Apoptosis* **8**, 481-495.

Erichsen, D.F., Aviad, A.D., Schultz, R.H. and Kennedy, T.J. (1994) Clinical efficacy and safety of clenbuterol HCl when administered to effect in horses with chronic obstructive pulmonary disease (COPD). *Equine Vet J* **26**, 331-336.

Eum, S.Y., Maghni, K., Hamid, Q., Eidelman, D.H., Campbell, H., Isogai, S. and Martin, J.G. (2003) Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: role of eosinophils, IL-5, eotaxin, and IL-13. *J Allergy Clin Immunol* **111**, 1049-1061.

Fairbairn, S.M., Page, C.P., Lees, P. and Cunningham, F.M. (1993) Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure. *Clin Exp Allergy* **23**, 821-828.

Feinstein, M.B. and Schleimer, R.P. (1999) Regulation of the action of hydrocortisone in airway epithelial cells by 11beta-hydroxysteroid dehydrogenase. *Am J Respir Cell Mol Biol* **21**, 403-408.

Fergie, N., Guo, L., Sithole, J., Pearson, J.P. and Birchall, J.P. (2003) Influence of prednisolone on the secretion of mucin from the HT29-MTX cell line. *Clin Otolaryngol* **28**, 39-42.

Gelb, A.F., Taylor, C.F., Nussbaum, E., Gutierrez, C., Schein, A., Shinar, C.M., Schein, M.J., Epstein, J.D. and Zamel, N. (2004) Alveolar and airway sites of nitric oxide inflammation in treated asthma. *Am J Respir Crit Care Med* **170**, 737-741.

Gerber, H. (1968) [On the treatment of chronic respiratory diseases in horses]. *Schweiz Arch Tierheilkd* **110**, 139-153.

Gerber, H. (1969) [Clinical features and therapy of chronic pulmonary diseases in the horse]. *Dtsch Tierarztl Wochenschr* **76**, 234-239.

Gerber, V. and Bailey, E. (1995) Genetics and disease in the horse. *Equine Vet J* **27**, 400-401.

Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J. and Franchini, M. (2002) Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet Immunol Immunopathol* **85**, 147-158.

Groneberg, D.A., Eynott, P.R., Lim, S., Oates, T., Wu, R., Carlstedt, I., Roberts, P., McCann, B., Nicholson, A.G., Harrison, B.D. and Chung, K.F. (2002) Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology* **40**, 367-373.

Guelfi, J.F. and Kraouchi, D.E. (1989) [The effect of a single dose of dexamethasone on the production of superoxide anion and on the chemotaxis of blood polynuclear neutrophils in the horse]. *Comp Immunol Microbiol Infect Dis* **12**, 105-113.

Hammond, R.A., Hannon, R., Frean, S.P., Armstrong, S.J., Flower, R.J. and Bryant, C.E. (1999) Endotoxin induction of nitric oxide synthase and cyclooxygenase-2 in equine alveolar macrophages. *Am J Vet Res* **60**, 426-431.

Hart, L., Lim, S., Adcock, I., Barnes, P.J. and Chung, K.F. (2000) Effects of inhaled corticosteroid therapy on expression and DNA-binding activity of nuclear factor kappaB in asthma. *Am J Respir Crit Care Med* **161**, 224-231.

Hoshino, M., Takahashi, M., Takai, Y. and Sim, J. (1999) Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* **104**, 356-363.

Jackson, C.A., Berney, C., Jefcoat, A.M. and Robinson, N.E. (2000) Environment and prednisone interactions in the treatment of recurrent airway obstruction (heaves). *Equine Vet J* **32**, 432-438.

Jackson, K.A., Stott, J.L., Horohov, D.W. and Watson, J.L. (2004) IL-4 induced CD23 (FcepsilonRII) up-regulation in equine peripheral blood mononuclear cells and pulmonary alveolar macrophages. *Vet Immunol Immunopathol* **101**, 243-250.

Kirschvink, N., Di Silvestro, F., Sbai, I., Vandenput, S., Art, T., Roberts, C. and Lekeux, P. (2002) The use of cardboard bedding material as part of an environmental control regime for heaves-affected horses: in vitro assessment of airborne dust and aeroallergen concentration and in vivo effects on lung function. *Vet J* **163**, 319-325.

Kravis, L.P. and Lecks, H.I. (1966) Inhalational vs oral steroid therapy in childhood asthma. *Am J Dis Child* **111**, 343-352.

Lapointe, J.M., Lavoie, J.P. and Vrins, A.A. (1993) Effects of triamcinolone acetonide on pulmonary function and bronchoalveolar lavage cytologic features in horses with chronic obstructive pulmonary disease. *Am J Vet Res* **54**, 1310-1316.

Lee, Y.M., Park, J.S., Hwang, J.H., Park, S.W., Uh, S.T., Kim, Y.H. and Park, C.S. (2004) High-resolution CT findings in patients with near-fatal asthma: comparison of patients with mild-to-severe asthma and normal control subjects and changes in airway abnormalities following steroid treatment. *Chest* **126**, 1840-1848.

Lehtimaki, L., Kankaanranta, H., Saarelainen, S., Hahtola, P., Jarvenpaa, R., Koivula, T., Turjanmaa, V. and Moilanen, E. (2001) Extended exhaled NO measurement differentiates between alveolar and bronchial inflammation. *Am J Respir Crit Care Med* **163**, 1557-1561.

Leung, T.F., Wong, G.W., Ko, F.W., Lam, C.W. and Fok, T.F. (2004) Increased macrophage-derived chemokine in exhaled breath condensate and plasma from children with asthma. *Clin Exp Allergy* **34**, 786-791.

Li, K.X.Z., Obeyeserkere, V.R., Krozowski, Z.S. and Ferrari, P. (1997) Oxoreductase and dehydrogenase activities of the human and rat 11-beta-hydroxysteroid dehydrogenase type II enzyme. *Endocrinology* **138**, 2948-2952.

Liden, J., Ek, A., Palmberg, L., Okret, S. and Larsson, K. (2003) Organic dust activates NF-kappaB in lung epithelial cells. *Respir Med* **97**, 882-892.

Liles, W.C., Dale, D.C. and Klebanoff, S.J. (1995) Glucocorticoids inhibit apoptosis of human neutrophils. *Blood* **86**, 3181-3188.

Lindberg, A., Nasman-Glaser, B., Lindgren, J.A. and Robinson, N.E. (2002) Evaluation of leukotriene biosynthetic capacity in lung tissues from horses with recurrent airway obstruction. *Am J Vet Res* **63**, 794-798.

Lindberg, A., Robinson, N.E., Nasman-Glaser, B., Jensen-Waern, M. and Lindgren, J.A. (2004) Assessment of leukotriene B4 production in leukocytes from horses with recurrent airway obstruction. *Am J Vet Res* **65**, 289-295.

Littenberg, B. and Gluck, E.H. (1986) A controlled trial of methylprednisolone in the emergency treatment of acute asthma. *N Engl J Med* **314**, 150-152.

Lu, W., Lillehoj, E.P. and Kim, K.C. (2005) Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells. *Am J Physiol Lung Cell Mol Physiol* **288**, L52-60.

MacKay, R.J., King, R.R., Dankert, J.R., Reis, K.J. and Skelley, L.A. (1991) Cytotoxic tumor necrosis factor activity produced by equine alveolar macrophages: preliminary characterization. *Vet Immunol Immunopathol* **29**, 15-30.

Mair, T.S. (1996) Obstructive pulmonary disease in 18 horses at summer pasture. *Vet Rec* **138**, 89-91.

Mak, J.C., Nishikawa, M. and Barnes, P.J. (1995a) Glucocorticosteroids increase beta 2-adrenergic receptor transcription in human lung. *Am J Physiol* **268**, L41-46.

Mak, J.C., Nishikawa, M., Shirasaki, H., Miyayasu, K. and Barnes, P.J. (1995b) Protective effects of a glucocorticoid on downregulation of pulmonary beta 2-adrenergic receptors in vivo. *J Clin Invest* **96**, 99-106.

Marti, E., Gerber, H., Essich, G., Oulehla, J. and Lazary, S. (1991) The genetic basis of equine allergic diseases. 1. Chronic hypersensitivity bronchitis. *Equine Vet J* **23**, 457-460.

Marti, E., Horohov, D.W., Antzak, D.F., Lazary, S. and Paul Lunn, D. (2003) Advances in equine immunology: Havemeyer workshop reports from Santa Fe, New Mexico, and Hortobagy, Hungary. *Vet Immunol Immunopathol* **91**, 233-243.

Mazan, M.R., Hoffman, A.M., Kuehn, H. and Deveney, E.F. (2003) Effect of aerosolized albuterol sulfate on resting energy expenditure determined by use of open-flow indirect calorimetry in horses with recurrent airway obstruction. *Am J Vet Res* **64**, 235-242.

Morris, D.D., Strzemienski, P.J., Gaulin, G. and Spencer, P. (1988) The effect of corticosteroid administration on migration, phagocytosis and bacterial capacity of equine neutrophils. *Cornell Vet* **78**, 243-252.

Muylle, E. and Oyaert, W. (1973) Lung function tests in obstructive pulmonary disease in horses. *Equine Vet J* **5**, 37-44.

Nevalainen, M., Raulo, S.M., Brazil, T.J., Pirie, R.S., Sorsa, T., McGorum, B.C. and Maisi, P. (2002) Inhalation of organic dusts and lipopolysaccharide increases gelatinolytic matrix metalloproteinases (MMPs) in the lungs of heaves horses. *Equine Vet J* **34**, 150-155.

Newton, R., Hart, L.A., Stevens, D.A., Bergmann, M., Donnelly, L.E., Adcock, I.M. and Barnes, P.J. (1998) Effect of dexamethasone on interleukin-1beta-(IL-1beta)-induced nuclear factor-kappaB (NF-kappaB) and kappaB-dependent transcription in epithelial cells. *Eur J Biochem* **254**, 81-89.

Nishikawa, M., Mak, J.C. and Barnes, P.J. (1996) Effect of short- and long-acting beta 2-adrenoceptor agonists on pulmonary beta 2-adrenoceptor expression in human lung. *Eur J Pharmacol* **318**, 123-129.

Nittoh, T., Fujimori, H., Kozumi, Y., Ishihara, K., Mue, S. and Ohuchi, K. (1998) Effects of glucocorticoids on apoptosis of inflitrated eosinophils and neutrophils in rats. *Eur J Pharmacol* **354**, 73-81.

Pelaia, G., Cuda, G., Vatrella, A., Fratto, D., Grembiale, R.D., Tagliaferri, P., Maselli, R., Costanzo, F.S. and Marsico, S.A. (2003) Effects of transforming growth factor-[beta] and budesonide on mitogen-activated protein kinase activation and apoptosis in airway epithelial cells. *Am J Respir Cell Mol Biol* **29**, 12-18.

Peroni, D.L., Stanley, S., Kollias-Baker, C. and Robinson, N.E. (2002) Prednisone per os is likely to have limited efficacy in horses. *Equine Vet J* **34**, 283-287.

Picandet, V., Leguillette, R. and Lavoie, J.P. (2003) Comparison of efficacy and tolerability of isoflupredone and dexamethasone in the treatment of horses affected with recurrent airway obstruction ('heaves'). *Equine Vet J* **35**, 419-424.

Pirie, R.S., Collie, D.D., Dixon, P.M. and McGorum, B.C. (2003a) Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy* **33**, 676-683.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2003b) Endotoxin contamination contributes to the pulmonary inflammatory and functional response to Aspergillus fumigatus extract inhalation in heaves horses. *Clin Exp Allergy* **33**, 1289-1296.

Pirie, R.S., McLachlan, G. and McGorum, B.C. (2002) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 1: Preparation and composition of HDS. *Equine Vet J* **34**, 332-336.

Raulo, S.M., Sorsa, T.A. and Maisi, P.S. (2000) Concentrations of elastinolytic metalloproteinases in respiratory tract secretions of healthy horses and horses with chronic obstructive pulmonary disease. *Am J Vet Res* **61**, 1067-1073.

Robinson, N.E., Berney, C., Eberhart, S., deFeijter-Rupp, H.L., Jefcoat, A.M., Cornelisse, C.J., Gerber, V.M. and Derksen, F.J. (2003) Coughing, mucus accumulation, airway obstruction, and airway inflammation in control horses and horses affected with recurrent airway obstruction. *Am J Vet Res* **64**, 550-557.

Robinson, N.E., Derksen, F.J., Jackson, C., Peroni, D. and Gerber, V. (2001) Mangement of heaves. *Equine Vet Educ* **13**, 247-259.

Robinson, N.E., Derksen, F.J., Olszewski, M.A. and Buechner-Maxwell, V.A. (1996) The pathogenesis of chronic obstructive pulmonary disease of horses. *Br Vet J* **152**, 283-306.

Robinson, N.E., Jackson, C., Jefcoat, A., Berney, C., Peroni, D. and Derksen, F.J. (2002) Efficacy of three corticosteroids for the treatment of heaves. *Equine Vet J* **34**, 17-22.

Robinson, N.E., Olszewski, M.A., Boehler, D., Berney, C., Hakala, J., Matson, C. and Derksen, F.J. (2000) Relationship between clinical signs and lung function in horses with recurrent airway obstruction (heaves) during a bronchodilator trial. *Equine Vet J* **32**, 393-400.

Rohdewald, P., Mollmann, H.W. and Hochhaus, G. (1985) Affinities of glucocorticoids for glucocorticoid receptors in human lung. *Agents and Actions* **17**, 290-291.

Roumestan, C., Henriquet, C., Bousquet, J. and Mathieu, M. (2003) Fluticasone propionate and mometasone furoate have equivalent transcriptional potencies. *Clin Exp Allergy* **33**, 895-901.

Rush, B.R., Flaminio, M.J., Matson, C.J., Hakala, J.E. and Shuman, W. (1998a) Cytologic evaluation of bronchoalveolar lavage fluid from horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **59**, 1033-1038.

Rush, B.R., Hoskinson, J.J., Davis, E.G., Matson, C.J. and Hakala, J.E. (1999) Pulmonary distribution of aerosolized technetium Tc 99m pentetate after administration of a single dose of aerosolized albuterol sulfate in horses with recurrent airway obstruction. *Am J Vet Res* **60**, 764-769.

Rush, B.R., Raub, E.S., Rhoads, W.S., Flaminio, M.J., Matson, C.J., Hakala, J.E. and Gillespie, J.R. (1998b) Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **59**, 1039-1043.

Rush, B.R., Worster, A.A., Flaminio, M.J., Matson, C.J. and Hakala, J.E. (1998c) Alteration in adrenocortical function in horses with recurrent airway obstruction

after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **59**, 1044-1047.

Sandersen, C., Bureau, F., Turlej, R., Fievez, L., Dogne, S., Kirschvink, N. and Lekeux, P. (2001) p65 Homodimer activity in distal airway cells determines lung dysfunction in equine heaves. *Vet Immunol Immunopathol* **80**, 315-326.

Scarfone, R.J., Fuchs, S.M., Nager, A.L. and Shane, S.A. (1993) Controlled trial of oral prednisone in the emergency department treatment of children with acute asthma. *Pediatrics* **92**, 513-518.

Storr, J., Barrell, E., Barry, W., Lenney, W. and Hatcher, G. (1987) Effect of a single oral dose of prednisolone in acute childhood asthma. *Lancet* **1**, 879-882.

Tesarowski, D.B., Viel, L., McDonell, W.N. and Newhouse, M.T. (1994) The rapid and effective administration of a beta 2-agonist to horses with heaves using a compact inhalation device and metered-dose inhalers. *Can Vet J* **35**, 170-173.

Thomson, J.R. and McPherson, E.A. (1983) Chronic obstructive pulmonary disease in the horse. 2: Therapy. *Equine Vet J* **15**, 207-210.

Thomson, J.R. and McPherson, E.A. (1984) Effects of environmental control on pulmonary function of horses affected with chronic obstructive pulmonary disease. *Equine Vet J* **16**, 35-38.

Torneke, K., Ingvast Larsson, C. and Appelgren, L.E. (1998) A comparison between clenbuterol, salbutamol and terbutaline in relation to receptor binding and in vitro relaxation of equine tracheal muscle. *J Vet Pharmacol Ther* **21**, 388-392.

Traub-Dargatz, J.L., McKinnon, A.O., Thrall, M.A., Jones, R.L., Bruyninckx, W., Blancquaert, A.M. and Dargatz, D.A. (1992) Evaluation of clinical signs of disease, bronchoalveolar and tracheal wash analysis, and arterial blood gas tensions in 13 horses with chronic obstructive pulmonary disease treated with prednisone, methyl sulfonmethane, and clenbuterol hydrochloride. *Am J Vet Res* **53**, 1908-1916.

Trigg, C.J., Manolitsas, N.D., Wang, J., Calderon, M.A., McAulay, A., Jordan, S.E., Herdman, M.J., Jhalli, N., Duddle, J.M., Hamilton, S.A. and et al. (1994) Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. *Am J Respir Crit Care Med* **150**, 17-22.

Turgut, K. and Sasse, H.H. (1989) Influence of clenbuterol on mucociliary transport in healthy horses and horses with chronic obstructive pulmonary disease. *Vet Rec* **125**, 526-530.

Turiaf, J., Blanchon, P., Sauvan, R. and Georges, R. (1956) [Chronic pulmonary heart disease in asthma and therapy with adrenal cortex hormones (ACTH, cortisone and derivatives).]. *Bull Mem Soc Med Hop Paris* **72**, 188-216.

Umland, S.P., Schleimer, R.P. and Johnston, S.L. (2002) Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. *Pulm Pharmacol Ther* **15**, 35-50.

Vandenput, S., Duvivier, D.H., Votion, D., Art, T. and Lekeux, P. (1998) Environmental control to maintain stabled COPD horses in clinical remission: effects on pulmonary function. *Equine Vet J* **30**, 93-96.

Walsh, G.M., Sexton, D.W. and Blaylock, M.G. (2003) Mechanisms of steroid action and resistance in inflammation. *J Endocrinol* **178**, 37-43.

Woods, P.S., Robinson, N.E., Swanson, M.C., Reed, C.E., Broadstone, R.V. and Derksen, F.J. (1993) Airborne dust and aeroallergen concentration in a horse stable under two different management systems. *Equine Vet J* **25**, 208-213.

Yuan, Y., Wang, Z. and Dong, B. (2003) [Modulation of matrix metalloproteinase by dexamethasone in airway remodeling in asthmatic rats]. *Sichuan Da Xue Xue Bao Yi Xue Ban* **34**, 680-683.

Zhang, N., Moilanen, E. and Kankaanranta, H. (2001) Beclomethasone, budesonide and fluticasone proprionate inhibit human neutrophil apoptosis. *Eur J Pharmacol* **431**, 365-371.

Zhang, X., Moilanen, E., Adcock, I.M. and Lindsay, M.A.K., H. (2002) Divergent effect of momethasone on human eosinophils and neutrophil apoptosis. *Life Sciences* **71**, 1523-1534.

References chapter 3: Efficacy of oral and intravenous dexamethasone in horses with recurrent airway obstruction

Ammann, V.J., Vrins, A.A. and Lavoie, J.P. (1998) Effects of inhaled beclomethasone dipropionate on respiratory function in horses with chronic obstructive pulmonary disease (COPD). *Equine Vet. J.* **30**, 152-157.

Cunningham, F.E., Rogers, S., Fischer, J.H. and Jensen, R.C. (1996) The pharmacokinetics of dexamethasone in the thoroughbred racehorse. *J. Vet. Pharmacol. Ther.* **19**, 68-71.

Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J. and Franchini, M. (2002) Cytokine induction in pulmonary airways of horses with heaves and effect of

therapy with inhaled fluticasone propionate. *Vet. Immunol. Immunopathol.* **85**, 147-158.

Hoshino, M., Takahashi, M., Takai, Y., Sim, J. (1999) Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in asthma. *J. Allergy Clin. Immunol.* **104**, 356-363.

Jaffuel, D., deMoly, P., Gougat, C., Balaguer, P., Mautino, G., Godard, P., Bousquet, J., Mathieu, M. (2000) Transcriptional potencies of inhaled glucocorticoids. *Am. J. Respir. Crit. Care Med.* **162**, 57-63.

Lapointe, J.M., Lavoie, J.P. and Vrins, A.A. (1993) Effects of triamcinolone acetonide on pulmonary function and bronchoalveolar lavage cytologic features in horses with chronic obstructive pulmonary disease. *Am. J. Vet. Res.* **54**, 1310-1316.

Littenberg, B., Gluck, E.H. (1986) A controlled trial of methylprednisolone in the emergency treatment of acute asthma. *N. Engl. J. Med.* **314**, 150-152.

Peroni, D.L., Stanley, S., Kollias-Baker, C. and Robinson, N.E. (2002) Prednisone per os is likely to have limited efficacy in horses. *Equine Vet. J.* **34**, 283-287.

Petsche, V.M., Derksen, F.J. and Robinson, N.E. (1994) Tidal breathing flow-volume loops in horses with recurrent airway obstruction (heaves). *Am. J. Vet. Res.* **55**, 885-891.

Robinson, N.E., Jackson, C., Jefcoat, A., Berney, C., Peroni, D. and Derksen, F.J. (2002) Efficacy of three corticosteroids for the treatment of heaves. *Equine Vet. J.* **34**, 17-22.

Robinson, N.E., Olszewski, M.A., Boehler, D., Berney, C., Hakala, J., Matson, C. and Derksen, F.J. (2000) Relationship between clinical signs and lung function in horses with recurrent airway obstruction (heaves) during a bronchodilator trial. *Equine Vet. J.* **32**, 393-400.

Robinson, N.E., Berney, C., Defeijter-Rupp, H., Jefcoat, A.M., Cornelisse, C.J., Gerber, V. and Derksen, F.J. (2003) Cough, mucus, airway obstruction, and inflammation in RAO-affected and control horses. *Am. J. Vet. Res.* **64**, 550-557.

Rush, B.R., Raub, E.S., Rhoads, W.S., Flaminio, M.J., Matson, C.J., Hakala, J.E. and Gillespie J.R. (1998) Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am. J. Vet. Res.* **59**: 1039-1043.

Rush, B.R., Hoskinson, J.J., Davis, E.G., Matson, C.J. and Hakala, J.E. (1999) Pulmonary distribution of aerosolized technetium Tc 99m pentetate after administration of a

single dose of aerosolized albuterol sulfate in horses with recurrent airway obstruction. *Am. J. Vet. Res.* **60**, 764-769.

Sandersen, C., Bureau, F., Turlej, R., Fievez, L., Dogne, S., Kirschvink, N., Lekeux, P. (2001) p65 Homodimer activity in distal airway cells determines lung dysfunction in equine heaves. *Vet. Immunol. Immunopathol.* **80**, 315-326.

Schimmer, B.P., Parker,K.L. (1996) Chapter 59: Adrenocorticotrophic hormone; Adrenocortical steroids and their synthetic analogs;inhibitors of the synthesis and actions of adrenocortical hormones. In Goodman & Gilman's The pharmacological basis of therapeutics 9th edition. Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W. (Eds.). McGraw-Hill, New York. pp 1459-1485.

Scarfone, R.J., Fuchs, S.M., Nager, A.L., Shane, S.A. (1993) Controlled trial of oral prednisone in the emergency department treatment of children with acute asthma. *Pediatrics* **92**, 513-518.

Storr, J., Barrell, E., Barry, W., Lenney, W. and Hatcher, G. (1987) Effect of a single oral dose of prednisolone in acute childhood asthma. *Lancet* **1**, 879-882.

Trigg, C.J., Manolitsas, N.D., Wang, J., Calderon, M.A., McAulay, A., Jordan, S.E., Herdman, M.J., Jhalli, N., Duddle, J.M., Hamilton, S.A., Devalia, J.L., Davies, R.J. (1994) Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. *Am. J. Respir. Crit. Care Med.* **150**, 17-22.

References **chapter 4: Glucocorticoid therapy and equine laminitis: Fact or fiction?**

Adair, H. S., 3rd, Goble, D. O., Schmidhammer, J. L. and Shires, G. M. (2000). Laminar microvascular flow, measured by means of laser Doppler flowmetry, during the prodromal stages of black walnut-induced laminitis in horses. *Am. J. vet. Res.* **61,** 862-8.

Baxter, G. M. (1995). Alterations of endothelium-dependent digital vascular responses in horses given low dose endotoxin. *Vet. Surg.* **24,** 87-96.

Best, R., Nelson, S. M. and Walker, B. R. (1997). Dexamethasone and 11-dehydrodexamethasone as tools to investigate the isozymes of 11 beta-hydroxysteroid dehydrogenase in vitro and in vivo. *J. Endocrinol.* **153,** 41-8.

Cardillo, C., Nambi, S. S., Kilcoyne, C. M., Choucair, W. K., Katz, A., Quon, M. J. and Panza, J. A. (1999). Insulin stimulates both endothelin and nitric oxide activity in the human forearm. *Circulation* **100,** 820-5.

Chen, C. L., Sailor, J. A., Collier, J. and Wiegand, J. (1992). Synovial and serum levels of triamcinolone following intra-articular administration of triamcinolone acetonide in the horse. *J. vet. Pharmacol. Ther.* **15,** 240-6.

Cogswell, A. M., Johnson, P. J. and Adams, H. R. (1995). Evidence for endothelium-derived relaxing factor/nitric oxide in equine digital arteries. *Am. J. vet. Res.* **56,** 1637-41.

Cripps, P. J. and Eustace, R. A. (1999). Radiological measurements from the feet of normal horses with relevance to laminitis. *Equine vet. J.* **31,** 427-32.

Cunningham, F. E., Rogers, S., Fischer, J. H. and Jensen, R. C. (1996). The pharmacokinetics of dexamethasone in the thoroughbred racehorse. *J. vet. Pharmacol. Ther.* **19,** 68-71.

Diederich, S., Hanke, B., Burkhardt, P., Muller, M., Schoneshofer, M., Bahr, V. and Oelkers, W. (1998). Metabolism of synthetic corticosteroids by 11 beta-hydroxysteroid-dehydrogenases in man. *Steroids* **63,** 271-7.

Dybdal, N. O., Hargreaves, K. M., Madigan, J. E., Gribble, D. H., Kennedy, P. C. and Stabenfeldt, G. H. (1994). Diagnostic testing for pituitary pars intermedia dysfunction in horses. *J. Am. vet. med. Ass.* **204,** 627-32.

Eyre, P., Elmes, P. J. and Strickland, S. (1979). Corticosteroid-potentiated vascular responses of the equine digit: a possible pharmacologic basis for laminitis. *Am. J. vet. Med.* **40,** 135-8.

FDA (2002). Adverse Drug Experience Summary. **2002.**
Frederick, D. M. and Kehl, M. Case Report: back from the brink. Equus. **272:** 34-41.

French, K., Pollitt, C. C. and Pass, M. A. (2000). Pharmacokinetics and metabolic effects of triamcinolone acetonide and their possible relationships to glucocorticoid-induced laminitis in horses. *J. vet. Pharmacol. Ther.* **23,** 287-92.

Frisbie, D. D., Kawcak, C. E., Trotter, G. W., Powers, B. E., Walton, R. M. and McIlwraith, C. W. (1997). Effects of triamcinolone acetonide on an in vivo equine osteochondral fragment exercise model. *Equine vet. J.* **29,** 349-59.

Gerber, H. (1970). Nebenwirkungen von kortikosteroid-behandlungen beim grosstier. *Schweiz. Arch. Tierheilkd.* **112,** 51-57.

Grosenbaugh, D. A., Hood, D. M., Amoss, M. S., Jr. and Williams, J. D. (1991). Characterisation and distribution of epidermal growth factor receptors in equine hoof wall laminar tissue: comparison of normal horses and horses affected with chronic laminitis. *Equine vet. J.* **23,** 201-6.

Haapasaari, K. M., Risteli, J. and Oikarinen, A. (1996). Recovery of human skin collagen synthesis after short-term topical corticosteroid treatment and comparison between young and old subjects. *Brit. J. Dermatol.* **135,** 65-9.

Houdeshell, J. W. (1969). Field trials of a new long-acting corticosteroid in the treatment of equine arthropathies. *Vet. Med. small Anim. Clin.* **64,** 782-4.

Hunt, R. J. (1993). A retrospective evaluation of laminitis in horses. *Equine vet. J.* **25,** 61-64.

Jaffuel, D., Roumestan, C., Balaguer, P., Henriquet, C., Gougat, C., Bousquet, J., Demoly, P. and Mathieu, M. (2001). Correlation between different gene expression assays designed to measure trans-activation potencies of systemic glucocorticoids. *Steroids* **66,** 597-604.

Johnson, P. J. (1999). Laminitis, "hypothyroidism", and obesity: A peripheral cushingoid syndrome in horses? *17th Annual Veterinary Medical Forum*, Chicago, IL.

Katwa, L. C., Johnson, P. J., Ganjam, V. K., Kreeger, J. M. and Messer, N. T. (1999). Expression of endothelin in equine laminitis. *Equine vet. J.* **31,** 243-7.

Korting, H. C., Unholzer, A., Schafer-Korting, M., Tausch, I., Gassmueller, J. and Nietsch, K. H. (2002). Different skin thinning potential of equipotent medium-strength glucocorticoids. *Skin Pharmacol. appl Skin Physiol.* **15,** 85-91.

Koupai-Abyazani, M. R., Yu, N., Esaw, B. and Laviolette, B. (1995). Determination of triamcinolone acetonide in equine serum and urine by liquid chromatography-atmospheric pressure ionization mass spectrometry. *J Anal Toxicol* **19,** 182-6.

Kylmaniemi, M., Oikarinen, A., Oikarinen, K. and Salo, T. (1996). Effects of dexamethasone and cell proliferation on the expression of matrix metalloproteinases in human mucosal normal and malignant cells. *J. dent. Res.* **75,** 919-26.

LaPointe, J.-M., Lavoie, J.-P. and Vrins, A. A. (1993). Effects of triamcinolone acetonide on pulmonary function and bronchoalveolar lavage cytologic features in horses with chronic obstructive pulmonary disease. *Am. J. vet. Res.* **54,** 1310-1316.

Lepage, O. M., Laverty, S., Marcoux, M. and Dumas, G. (1993). Serum osteocalcin concentration in horses treated with triamcinolone acetonide. *Am. J. vet. Res.* **54,** 1209-12.

Lose, M. P. (1980). Drug-induced laminitis in a colt. *Mod. vet. Pract.* **61,** 608-10.

Maddison, J. E. (1994). Adverse drug reactions: report of the Australian Veterinary Association Adverse Drug Reaction Subcommittee, 1993. *Aust. vet. J.* **71,** 53-7.

Maddison, J. E. (1996). Adverse drug reactions: report of the Australian Veterinary Association Adverse Drug Reaction Subcommittee, 1994. *Aust. vet. J.* **73,** 132-6.

Madlener, M., Parks, W. C. and Werner, S. (1998). Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. *Exp. Cell. Res.* **242,** 201-10.

Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R. and Flier, J. S. (2001). A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294,** 2166-70.

Miller, A. W., Tulbert, C., Puskar, M. and Busija, D. W. (2002). Enhanced endothelin activity prevents vasodilation to insulin in insulin resistance. *Hypertension* **40,** 78-82.

Mungall, B. A., Kyaw-Tanner, M. and Pollitt, C. C. (2001). In vitro evidence for a bacterial pathogenesis of equine laminitis. *Vet. Microbiol.* **79,** 209-23.

Muylle, E. and Oyaert, W. (1973). Lung function tests in obstructive pulmonary disease in horses. *Equine vet. J.* **5,** 37-44.

Oikarinen, A., Haapasaari, K. M., Sutinen, M. and Tasanen, K. (1998). The molecular basis of glucocorticoid-induced skin atrophy: topical glucocorticoid apparently decreases both collagen synthesis and the corresponding collagen mRNA level in human skin in vivo. *Brit. J. Dermatol.* **139,** 1106-10.

Paulmyer-Lacroix, O., Boullu, S., Oliver, C., Alessi, M. C. and Grino, M. (2002). Expression of the mRNA coding for 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. *J. clin. Endocrinol. Metab.* **87,** 2701-5.

Pollitt, C. C. and Daradka, M. (1998). Equine laminitis basement membrane pathology: loss of type IV collagen, type VII collagen and laminin immunostaining. *Equine vet. J. Suppl.*, 139-44.

Robinson, N. E., Jackson, C., Jefcoat, A., Berney, C., Peroni, D. and Derksen, F. J. (2002). Efficacy of three corticosteroids for the treatment of heaves. *Equine vet. J.* **34,** 17-22.

Rohdewald, P., Mollman, H. W. and Hochhaus, G. (1986). Affinities of glucocorticoids for glucocorticoid receptors in the human lung. *Agents Actions* **17,** 290-1.

Rush, B. R., Raub, E. S., Rhoads, W. S., Matson, C. J., Hakala, J. E. and Gillespie, J. R.

(1998). Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am. J. vet. Res.* **59,** 1039-1043.

Schimmer, B. P. and Parker, K. L. (1996). Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. *Goodman and Gilman's The phamacological basis of therapeutics*. J. G. Hardman and L. E. Limbird. New York, McGraw-Hill: 1459-1486.

Schneider, D. A., Parks, A. H., Eades, S. C. and Tackett, R. L. (1999). Palmar digital vessel relaxation in healthy horses and in horses given carbohydrate. *Am. J. vet. Res.* **60,** 233-9.

Severino, C., Brizzi, P., Solinas, A., Secchi, G., Maioli, M. and Tonolo, G. (2002). Low-dose dexamethasone in the rat: a model to study insulin resistance. *Am J Physiol Endocrinol Metab* **283,** E367-73.

Slater, M. R., Hood, D. M. and Carter, G. K. (1995). Descriptive epidemiological study of equine laminitis. *Equine vet. J.* **27,** 364-7.

Slone, D. E., Purohit, R. C., Ganjam, V. K. and Lowe, J. L. (1983). Sodium retention and cortisol (hydrocortisone) suppression caused by dexamethasone and triamcinolone in equids. *Am. J. vet. Res.* **44,** 280-3.

Steinberg, H. O. and Baron, A. D. (2002). Vascular function, insulin resistance and fatty acids. *Diabetalogia* **45,** 623-34.

Swanstrom, O. G. and Dawson, H. A. (1974). Intra-articular betasone and depomedrol: A comparative study. *Proceedings of the 20th Annual Convention of the American Association of Equine Practitioners*, Las vegas, Nevada.

Trovati, M. and Anfossi, G. (2002). Influence of insulin and of insulin resistance on platelet and vascular smooth muscle cell function. *J. Diabetes Complications* **16,** 35-40.

Tumas, D. B., Hines, M. T., Perryman, L. E., Davis, W. C. and McGuire, T. C. (1994). Corticosteroid immunosuppression and monoclonal antibody-mediated CD5+ T lymphocyte depletion in normal and equine infectious anaemia virus-carrier horses. *J. gen. Virol.* **75 (Pt 5),** 959-68.

Vernimb, G. D., Van Hoose, L. M. and Hennessey, P. W. (1977). Onset and duration of corticosteroid effect after injection of Betasone for treating equine arthropathies. Results of laboratory and clinical studies. *Vet. Med. small Anim. Clin.* **72,** 241-4.

Verschooten, F., Desmet, P., Peremans, K. and Picavet, T. Navicular disease in the horse:

The effect of controlled intrabursal corticoid injection. *J. eq. vet. Sci.* **10,** 316-320.

Wattle, O. S. (2001). Cytokeratins of the matrices of the chestnut (torus carpeus) and periople in horses with acute laminitis. *Am. J. vet. Res.* **62,** 425-32.

Weiss, D. J. (1997). Equine Laminitis: A Review of Recent Research. *Eq, Pract.* **19,** 16 - 20.

References **chapter 5: Effects and mechanisms of glucocorticoid actions on microcirculation**

Ahluwalia, A. and R. J. Flower (1993). "Investigations into the mechanism of vasoconstrictor action of the topical steroid betamethasone-17-valerate in the rat." Br J Pharmacol **108**(2): 544-8.

Andersen, P., K. Milioni, et al. (1993). "The cutaneous corticosteroid vasoconstriction assay: a reflectance spectroscopic and laser-Doppler flowmetric study." Br J Dermatol **128**(6): 660-5.

Barbera, M., V. Fierabracci, et al. (2001). "Dexamethasone-induced insulin resistance and pancreatic adaptive response in aging rats are not modified by oral vanadyl sulfate treatment." Eur J Endocrinol **145**(6): 799-806.

Benamou, A. E., D. J. Marlin, et al. (2003). "Spasmogenic action of endothelin-1 on isolated equine pulmonary artery and bronchus." Equine Vet J **35**(2): 190-6.

Benamou, A. E., D. J. Marlin, et al. (2001). "Equine pulmonary and systemic haemodynamic responses to endothelin-1 and a selective ET(A) receptor antagonist." Equine Vet J **33**(4): 337-44.

Binnert, C., S. Ruchat, et al. (2004). "Dexamethasone-induced insulin resistance shows no gender difference in healthy humans." Diabetes Metab **30**(4): 321-6.

Boron, W. F. and E. L. Boulpaep, Eds. (2003). Medical Physiology. Philadelphia, Saunders.

Brain, S. D., D. C. Crossman, et al. (1989). "Endothelin-1: demonstration of potent effects on the microcirculation of humans and other species." J Cardiovasc Pharmacol **13 Suppl 5**: S147-9; discussion S150.

Buus, N. H., U. Simonsen, et al. (2000). "Nitric oxide, prostanoid and non-NO, non-prostanoid involvement in acetylcholine relaxation of isolated human small arteries." Br J Pharmacol **129**(1): 184-92.

Cardillo, C., C. M. Kilcoyne, et al. (2000). "Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo." Hypertension **35**(6): 1237-41.

Chaturvedi, N., L. K. Stevens, et al. (2001). "Risk factors, ethnic differences and mortality associated with lower-extremity gangrene and amputation in diabetes. The WHO Multinational Study of Vascular Disease in Diabetes." Diabetologia **44 Suppl 2**: S65-71.

Clozel, M., B. M. Loffler, et al. (1993). "Downregulation of endothelin receptors by autocrine production of endothelin-1." Am J Physiol **265**(1 Pt 1): C188-92.

Crossman, D. C., S. D. Brain, et al. (1991). "Potent vasoactive properties of endothelin 1 in human skin." J Appl Physiol **70**(1): 260-6.

Dasarathy, Y., J. J. Lanzillo, et al. (1992). "Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors." Am J Physiol **263**(6 Pt 1): L645-9.

Di Filippo, C., F. Rossi, et al. (2004). "The distinct alterations produced in cardiovascular functions by prednisolone and nitro-prednisolone (NCX-1015) in the rat highlight a causal role for endothelin-1." J Pharmacol Exp Ther **310**(3): 1133-41.

Ducluzeau, P. H., L. M. Fletcher, et al. (2002). "Molecular mechanisms of insulin-stimulated glucose uptake in adipocytes." Diabetes Metab **28**(2): 85-92.

Eyre, P., P. J. Elmes, et al. (1979). "Corticosteroid-potentiated vascular responses of the equine digit: a possible pharmacologic basis for laminitis." Am J Vet Res **40**(1): 135-8.

Goldsmith, P., C. Bunker, et al. (1996). "The effect of topical steroid on the actions of vasoconstrictor and vasodilator peptides in human skin." Skin Pharmacol **9**(5): 289-97.

Haigh, R. M. and C. T. Jones (1990). "Effect of glucocorticoids on alpha 1-adrenergic receptor binding in rat vascular smooth muscle." J Mol Endocrinol **5**(1): 41-8.

Haynes, W. G., J. G. Clarke, et al. (1991). "Pharmacology of endothelin-1 in vivo in humans." J Cardiovasc Pharmacol **17**(S7): s284-286.

Hood, D. M., M. S. Amoss, et al. (1978). "Equine laminitis I:Radioisotopic analysis of the hemodynamics of the footduring the acute phase." J Equine Med Surg **2**: 439-444.

Hood, D. M., D. A. Grosenbaugh, et al. (1993). "The role of vascular mechanisms in the development of acute equine laminitis." J Vet Intern Med **7**(4): 228-34.

Ialenti, A., A. Calignano, et al. (1986). "Glucocorticoid induction of angiotensin converting enzyme." Agents Actions **17**(3-4): 294-5.

Kanse, S. M., K. Takahashi, et al. (1991). "Glucocorticoids induce endothelin release from vascular smooth muscle cells but not endothelial cells." Eur J Pharmacol **199**(1): 99-101.

Katakam, P. V., J. S. Pollock, et al. (2001). "Enhanced endothelin-1 response and receptor expression in small mesenteric arteries of insulin-resistant rats." Am J Physiol Heart Circ Physiol **280**(2): H522-7.

Katz, L. M., C. M. Marr, et al. (2003). "Characterization and comparison of the responses of equine digital arteries and veins to endothelin-1." Am J Vet Res **64**(11): 1438-43.

Kellogg, D. L., Jr., Y. Liu, et al. (2001). "Genome and Hormones: gender differences in physiology selected contribution: gender differences in the endothelin-B receptro contribution to basal cutaneous vascular tone in humans." J Appl Physiol **91**: 2407-2411.

Kellogg, D. L., Jr., J. L. Zhao, et al. (2005). "Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin." J Appl Physiol **98**(2): 629-32.

Kenyon, C. J., W. B. Brown, et al. (1990). "Effects of dexamethasone on body fluid and electrolyte composition of rats." Acta Endocrinol (Copenh) **122**(5): 599-604.

Koshino, Y., T. Hayashi, et al. (1998). "Dexamethasone modulates the expression of endothelin-1 and -A receptors in A7r5 vascular smooth muscle cells." J Cardiovasc Pharmacol **32**(4): 665-72.

Kunz, D., G. Walker, et al. (1996). "Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1 beta-stimulated mesangial cells: evidence for the involvement of transcriptional and posttranscriptional regulation." Proc Natl Acad Sci U S A **93**(1): 255-9.

Mak, J. C., M. Nishikawa, et al. (1995). "Glucocorticosteroids increase beta 2-adrenergic receptor transcription in human lung." Am J Physiol **268**(1 Pt 1): L41-6.

Miller, A. W., C. Tulbert, et al. (2002). "Enhanced endothelin activity prevents vasodilation to insulin in insulin resistance." Hypertension **40**(1): 78-82.

Morawietz, H., R. Talanow, et al. (2000). "Regulation of the endothelin system by shear stress in human endothelial cells." J Physiol **525 Pt 3**: 761-70.

Morin, C., C. Asselin, et al. (1998). "Transcriptional regulation of pre-pro-endothelin-1 gene by glucocorticoids in vascular smooth muscle cells." Biochem Biophys Res Commun **244**(2): 583-7.

Nambi, P., M. Pullen, et al. (1992). "Dexamethasone down-regulates the expression of endothelin receptors in vascular smooth muscle cells." J Biol Chem **267**(27): 19555-9.

Piatti, P. M., L. D. Monti, et al. (1996). "Hypertriglyceridemia and hyperinsulinemia are potent inducers of endothelin-1 release in humans." Diabetes **45**(3): 316-21.

Piatti, P. M., L. D. Monti, et al. (2000). "Relationship between endothelin-1 concentration and metabolic alterations typical of the insulin resistance syndrome." Metabolism **49**(6): 748-52.

Provencher, P. H., J. Saltis, et al. (1995). "Glucocorticoids but not mineralocorticoids modulate endothelin-1 and angiotensin II binding in SHR vascular smooth muscle cells." J Steroid Biochem Mol Biol **52**(3): 219-25.

Provencher, P. H., A. Villeneuve, et al. (1998). "Glucocorticoids increase preproendothelin-1 expression in rat aorta." Endocr Res **24**(3-4): 737-41.

Robinson, N. E. (1990). "Digital blood flow, arteriovenous anastomoses and laminitis." Equine Vet J **22**(6): 381-3.

Rogers, K. M., C. A. Bonar, et al. (2002). "Inhibitory effect of glucocorticoid on coronary artery endothelial function." Am J Physiol Heart Circ Physiol **283**(5): H1922-8.

Roubert, P., I. Viossat, et al. (1993). "Endothelin receptor regulation by endothelin synthesis in vascular smooth muscle cells: effects of dexamethasone and phosphoramidon." J Vasc Res **30**(3): 139-44.

Sato, A., H. Suzuki, et al. (1994). "Increased expression of vascular angiotensin II type 1A receptor gene in glucocorticoid-induced hypertension." J Hypertens **12**(5): 511-6.

Severino, C., P. Brizzi, et al. (2002). "Low-dose dexamethasone in the rat: a model to study insulin resistance." Am J Physiol Endocrinol Metab **283**(2): E367-73.

Sommer, A., J. Veraart, et al. (1998). "Evaluation of the vasoconstrictive effects of topical steroids by laser-Doppler-perfusion-imaging." Acta Derm Venereol **78**(1): 15-8.

Steinberg, H. O. and A. D. Baron (2002). "Vascular function, insulin resistance and fatty acids." Diabetologia **45**(5): 623-34.

Steinberg, H. O., G. Brechtel, et al. (1994). "Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release." J Clin Invest **94**(3): 1172-9.

Stepp, D. W. and J. C. Frisbee (2002). "Augmented adrenergic vasoconstriction in hypertensive diabetic obese Zucker rats." Am J Physiol Heart Circ Physiol **282**(3): H816-20.

Telemaque-Potts, S., R. E. Kuc, et al. (2002). "Elevated systemic levels of endothelin-1 and blood pressure correlate with blunted constrictor responses and downregulation of endothelin(A), but not endothelin(B), receptors in an animal model of hypertension." Clin Sci (Lond) **103 Suppl 48**: 357S-362S.

Tooke, J. (1999). "The association between insulin resistance and endotheliopathy." Diabetes Obes Metab **1 Suppl 1**: S17-22.

Ullian, M. E., L. G. Walsh, et al. (1996). "Potentiation of angiotensin II action by corticosteroids in vascular tissue." Cardiovasc Res **32**(2): 266-73.

Venugopal, C. S., E. P. Holmes, et al. (2001). "In vitro pharmacologic effect of two endothelin-1 antagonists on equine colonic arteries and veins." Am J Vet Res **62**(2): 154-9.

Wallerath, T., A. Godecke, et al. (2004). "Dexamethasone lacks effect on blood pressure in mice with a disrupted endothelial NO synthase gene." Nitric Oxide **10**(1): 36-41.

Wallerath, T., K. Witte, et al. (1999). "Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension." Proc Natl Acad Sci U S A **96**(23): 13357-62.

Warner, T. D., Ed. (2001). Endothelin and its inhibitors. Handbook of experimental pharmacology. Berlin, Springer-Verlag.

Webb, M. L., K. E. Dickinson, et al. (1992). "The endothelin receptor antagonist, BQ-123, inhibits angiotensin II-induced contractions in rabbit aorta." Biochem Biophys Res Commun **185**(3): 887-92.

Wenzel, R. R., J. Ruthemann, et al. (2001). "Endothelin-A receptor antagonist inhibits angiotensin II and noradrenaline in man." Br J Clin Pharmacol **52**(2): 151-7.

References chapter 6: A thermographic study of the *in vivo* modulation of vascular responses to phenylephrine and endothelin-1 by dexamethasone in the horse

Andersen, P., Milioni, K. and Maibach, H. (1993) The cutaneous corticosteroid vasoconstriction assay: a reflectance spectroscopic and laser-Doppler flowmetric study. *Br J Dermatol* **128**, 660-665.

Benamou, A.E., Marlin, D.J., Callingham, B.C., Hiley, R.C. and Lekeux, R. (2003) Spasmogenic action of endothelin-1 on isolated equine pulmonary artery and bronchus. *Equine Vet J* **35**, 190-196.

Bornmyr, S., Svensson, H., Lilja, B. and Sundkvist, G. (1997) Skin temperature changes and changes in skin blood flow monitored with laser Doppler flowmetry and imaging: a methodological study in normal humans. *Clin Physiol* **17**, 71-81.

Bowen, I.M., Marr, C.M., Chester, A.H., Wheeler-Jones, C.P. and Elliott, J. (2004) In-vitro contraction of the equine aortic valve. *J Heart Valve Dis* **13**, 593-599.

Brown, P.H., Teelucksingh, S., Matusiewicz, S.P., Greening, A.P., Crompton, G.K. and Edwards, C.R. (1991) Cutaneous vasoconstrictor response to glucocorticoids in asthma. *Lancet* **337**, 576-580.

Clark, S., Dunn, G., Moore, T., Jayson, M.t., King, T.A. and Herrick, A.L. (2003) Comparison of thermography and laser Doppler imaging in the assessment of Raynaud's phenomenon. *Microvasc Res* **66**, 73-76.

Clough, G.F. (1999) Role of nitric oxide in the regulation of microvascular perfusion in human skin in vivo. *J Physiol* **516 (Pt 2)**, 549-557.

Cornelisse, C.J. and Robinson, N.E. (2004) Glucocorticoid therapy and equine laminitis: fact or fiction ? *EVE* **16**, 90-93.

Di Filippo, C., Rossi, F., Ongini, E., Del Soldato, P., Perretti, M. and D'Amico, M. (2004) The distinct alterations produced in cardiovascular functions by prednisolone and nitro-prednisolone (NCX-1015) in the rat highlight a causal role for endothelin-1. *J Pharmacol Exp Ther* **310**, 1133-1141.

Elliott, J., Bryant, C.E. and Soydan, J. (1994) The role of nitric oxide in the responses of equine digital veins to vasodilator and vasoconstrictor agents. *Equine Vet J* **26**, 378-384.

Eyre, P., Elmes, P.J. and Strickland, S. (1979) Corticosteroid-potentiated vascular responses of the equine digit: a possible pharmacologic basis for laminitis. *Am J Vet Res* **40**, 135-138.

French, K., Pollitt, C.C. and Pass, M.A. (2000) Pharmacokinetics and metabolic effects of triamcinolone acetonide and their possible relationships to glucocorticoid-induced laminitis in horses. *J Vet Pharmacol Ther* **23**, 287-292.

Gerber, H. (1970) [Side effects of corticosteroid treatment in large animals]. *Schweiz Arch Tierheilkd* **112**, 51-58.

Haigh, R.M. and Jones, C.T. (1990) Effect of glucocorticoids on alpha 1-adrenergic receptor binding in rat vascular smooth muscle. *J Mol Endocrinol* **5**, 41-48.

Harrison, D.K., Abbot, N.C., Beck, J.S. and McCollum, P.T. (1994) Laser Doppler perfusion imaging compared with light-guide laser Doppler flowmetry, dynamic thermographic imaging and tissue spectrophotometry for investigating blood flow in human skin. *Adv Exp Med Biol* **345**, 853-859.

Hocher, B., Schwarz, A., Slowinski, T., Bachmann, S., Pfeilschifter, J., Neumayer, H.H. and Bauer, C. (2004) In-vivo interaction of nitric oxide and endothelin. *J Hypertens* **22**, 111-119.

Hood, D.M., Amoss, M.S. and Hightower, D. (1978) Equine laminitis I:Radioisotopic analysis of the hemodynamics of the footduring the acute phase. *J Equine Med Surg* **2**, 439-444.

Ialenti, A., Calignano, A., Carnuccio, R. and Di Rosa, M. (1986) Glucocorticoid induction of angiotensin converting enzyme. *Agents Actions* **17**, 294-295.

Johnson, P.J., Ganjam, V.K., Slight, S.H., Kreeger, J.M. and Messer, N.T. (2004) Tissue-specific dysregulation of cortisol metabolism in equine laminitis. *Equine Vet J* **36**, 41-45.

Kanse, S.M., Takahashi, K., Warren, J.B., Ghatei, M. and Bloom, S.R: (1991) Glucocorticoids induce endothelin release from vascular smooth muscle cells but not endothelial cells. *Eur J Pharmacol* **199**, 99-101.

Kato, H., Hayashi, T., Koshino, Y., Oida, K., Kutsumi, Y., Nakai, T. and Miyabo, S. (1995) Autocrine production of endothelin-1 participates in the glucocorticoid-induced Ca2+ influx into vascular smooth muscle cells. *Biochem Biophys Res Commun* **208**, 82-88.

Katugampola, R., Church, M.K. and Clough, G.F. (2000) The neurogenic vasodilator response to endothelin-1: a study in human skin in vivo. *Exp Physiol* **85**, 839-846.

Katz, L.M., Marr, C.M. and Elliott, J. (2003) Characterization and comparison of the responses of equine digital arteries and veins to endothelin-1. *Am J Vet Res* **64**, 1438-1443.

Koshino, Y., Hayashi, T., Matsukawa, S., Asazuma, K., Eguchi, K., Kato, H., Nakai, T. and Miyamori, I. (1998) Dexamethasone modulates the expression of endothelin-1 and -A receptors in A7r5 vascular smooth muscle cells. *J Cardiovasc Pharmacol* **32**, 665-672.

Lawrence, E. and Brain, S.D. (1994) Effect of BQ-123 and Ro 47-0203 (bosentan) on endothelin-induced vasoconstriction in the rat skin. *Eur J Pharmacol* **260**, 103-106.

Leslie, S.J., Rahman, M.Q., Denvir, M.A., Newby, D.E. and Webb, D.J. (2004) Endothelins and their inhibition in the human skin microcirculation: ET[1-31], a new vasoconstrictor peptide. *Br J Clin Pharmacol* **57**, 720-725.

Lipa, J.E., Neligan, P.C., Perreault, T.M., Baribeau, J., Levine, R.H., Knowlton, R.J. and Pang, C.Y. (1999) Vasoconstrictor effect of endothelin-1 in human skin: role of ETA and ETB receptors. *Am J Physiol* **276**, H359-367.

Liu, J., Haigh, R.M. and Jones, C.T. (1992) Enhancement of noradrenaline-induced inositol polyphosphate formation by glucocorticoids in rat vascular smooth muscle cells. *J Endocrinol* **133**, 405-411.

Mather, K.J., Lteif, A., Steinberg, H.O. and Baron, A.D. (2004) Interactions between endothelin and nitric oxide in the regulation of vascular tone in obesity and diabetes. *Diabetes* **53**, 2060-2066.

Morgan, K. (1998) Thermoneutral zone and critical temperatures of horses. *J Therm Biol* **23**, 59-61.

Morin, C., Asselin, C., Boudreau, F. and Provencher, P.H. (1998) Transcriptional regulation of pre-pro-endothelin-1 gene by glucocorticoids in vascular smooth muscle cells. *Biochem Biophys Res Commun* **244**, 583-587.

Nambi, P., Pullen, M., Wu, H.L., Nuthulaganti, P., Elshourbagy, N. and Kumar, C. (1992) Dexamethasone down-regulates the expression of endothelin receptors in vascular smooth muscle cells. *J Biol Chem* **267**, 19555-19559.

Noon, J.P., Evans, C.E., Haynes, W.G., Webb, D.J. and Walker, B.R. (1996) A comparison of techniques to assess skin blanching following the topical application of glucocorticoids. *Br J Dermatol* **134**, 837-842.

Pang, C.Y., Zhang, J., Xu, H., Lipa, J.E., Forrest, C.R. and Neligan, P.C. (1998) Role and mechanism of endothelin-B receptors in mediating ET-1-induced vasoconstriction in pig skin. *Am J Physiol* **275**, R1066-1074.

Pass, M.A., Pollitt, S. and Pollitt, C.C. (1998) Decreased glucose metabolism causes separation of hoof lamellae in vitro: a trigger for laminitis? *Equine Vet J Suppl*, 133-138.

Provencher, P.H., Saltis, J. and Funder, J.W. (1995) Glucocorticoids but not mineralocorticoids modulate endothelin-1 and angiotensin II binding in SHR vascular smooth muscle cells. *J Steroid Biochem Mol Biol* **52**, 219-225.

Robinson, N.E., Dabney, J.M., Weidner, W.J., Jones, G.A. and Scott, J.B. (1975) Vascular responses in the equine digit. *Am J Vet Res* **36**, 1249-1253.

Robinson, N.E., Derksen, F.J., Olszewski, M., Berney, C., Boehler, D., Matson, C. and Hakala, J. (1999) Determinants of the maximal change in pleural pressure during tidal breathing in COPD-affected horses. *Vet J* **157**, 160-165.

Robinson, N.E., Scott, J.B., Dabney, J.M. and Jones, G.A. (1976) Digital vascular responses and permeability in equine alimentary laminitis. *Am J Vet Res* **37**, 1171-1176.

Roubert, P., Viossat, I., Lonchampt, M.O., Chapelat, M., Schulz, J., Plas, P., Gillard-Roubert, V., Chabrier, P.E. and Braquet, P. (1993) Endothelin receptor regulation by endothelin synthesis in vascular smooth muscle cells: effects of dexamethasone and phosphoramidon. *J Vasc Res* **30**, 139-144.

Ryu, S.H., Kim, B.S., Lee, C.W., Yoon, J. and Lee, Y.L. (2004) Glucocorticoid-induced laminitis with hepatopathy in a Thoroughbred filly. *J Vet Sci* **5**, 271-274.

Sakaue, M. and Hoffman, B.B. (1991) Glucocorticoids induce transcription and expression of the alpha 1B adrenergic receptor gene in DTT1 MF-2 smooth muscle cells. *J Clin Invest* **88**, 385-389.

Severino, C., Brizzi, P., Solinas, A., Secchi, G., Maioli, M. and Tonolo, G. (2002) Low-dose dexamethasone in the rat: a model to study insulin resistance. *Am J Physiol Endocrinol Metab* **283**, E367-373.

Shastry, S., Minson, C.T., Wilson, S.A., Dietz, N.M. and Joyner, M.J. (2000) Effects of atropine and L-NAME on cutaneous blood flow during body heating in humans. *J Appl Physiol* **88**, 467-472.

Sommer, A., Veraart, J., Neumann, M. and Kessels, A. (1998) Evaluation of the vasoconstrictive effects of topical steroids by laser-Doppler-perfusion-imaging. *Acta Derm Venereol* **78**, 15-18.

Stepp, D.W. and Frisbee, J.C. (2002) Augmented adrenergic vasoconstriction in hypertensive diabetic obese Zucker rats. *Am J Physiol Heart Circ Physiol* **282**, H816-820.

Tooke, J. (1999) The association between insulin resistance and endotheliopathy. *Diabetes Obes Metab* **1 Suppl 1**, S17-22.

Tooke, J.E. and Goh, K.L. (1999) Vascular function in Type 2 diabetes mellitus and pre-diabetes: the case for intrinsic endotheiopathy. *Diabet Med* **16**, 710-715.

Venugopal, C.S., Holmes, E.P., Koch, C.E., Curtis, L.A., Holm, A.S. and Moore, R.M. (2001) In vitro pharmacologic effect of two endothelin-1 antagonists on equine colonic arteries and veins. *Am J Vet Res* **62**, 154-159.

Wallerath, T., Godecke, A., Molojavyi, A., Li, H., Schrader, J. and Forstermann, U. (2004) Dexamethasone lacks effect on blood pressure in mice with a disrupted endothelial NO synthase gene. *Nitric Oxide* **10**, 36-41.

Wallerath, T., Witte, K., Schafer, S.C., Schwarz, P.M., Prellwitz, W., Wohlfart, P., Kleinert, H., Lehr, H.A., Lemmer, B. and Forstermann, U. (1999) Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. *Proc Natl Acad Sci U S A* **96**, 13357-13362.

Wenzel, R.R., Ruthemann, J., Bruck, H., Schafers, R.F., Michel, M.C. and Philipp, T. (2001) Endothelin-A receptor antagonist inhibits angiotensin II and noradrenaline in man. *Br J Clin Pharmacol* **52**, 151-157.

Wilson, T.E., Monahan, K.D., Short, D.S. and Ray, C.A. (2004) Effect of age on cutaneous vasoconstrictor responses to norepinephrine in humans. *Am J Physiol Regul Integr Comp Physiol* **287**, R1230-1234.

Yosipovitch, G., Sackett-Lundeen, L., Goon, A., Yiong Huak, C., Leok Goh, C. and Haus, E. (2004) Circadian and ultradian (12 h) variations of skin blood flow and barrier function in non-irritated and irritated skin-effect of topical corticosteroids. *J Invest Dermatol* **122**, 824-829.

References **chapter 7: A thermographic study of the in vivo modulation of metacholine induced vasodilation by dexamethasone in the horse**

Cornelisse, C. J.Robinson, N. E. (2004) Glucocorticoid therapy and equine laminitis: fact or fiction ? EVE; 1690-93

Di Filippo, C., Rossi, F., Ongini, E., Del Soldato, P., Perretti, M.D'Amico, M. (2004) The distinct alterations produced in cardiovascular functions by prednisolone and nitro-prednisolone (NCX-1015) in the rat highlight a causal role for endothelin-1. J Pharmacol Exp Ther; 310; 1133-1141

Hiramatsu, K., Yamada, T.Katakura, M. (1984) Acute effects of cold on blood pressure, renin-angiotensin-aldosterone system, catecholamines and adrenal steroids in man. Clin Exp Pharmacol Physiol;11; 171-179

Hood, D. M., Amoss, M. S.Hightower, D. (1978) Equine laminitis I:Radioisotopic analysis of the hemodynamics of the footduring the acute phase. J Equine Med Surg; 2439-444

Jansky, L., Sramek, P., Savlikova, J., Ulicny, B., Janakova, H.Horky, K. (1996) Change in sympathetic activity, cardiovascular functions and plasma hormone concentrations due to cold water immersion in men. Eur J Appl Physiol Occup Physiol;74;148-152

Morin, C., Asselin, C., Boudreau, F.Provencher, P. H. (1998) Transcriptional regulation of pre-pro-endothelin-1 gene by glucocorticoids in vascular smooth muscle cells. Biochem Biophys Res Commun; 244; 583-587

Provencher, P. H., Villeneuve, A.Morin, C.1998Glucocorticoids increase preproendothelin-1 expression in rat aorta. Endocr Res 24; 737-741

Robinson, N. E., Derksen, F. J., Olszewski, M., Berney, C., Boehler, D., Matson, C.Hakala, J.1999Determinants of the maximal change in pleural pressure during tidal breathing in COPD-affected horses. Vet J;157;160-165

Robinson, N. E., Scott, J. B., Dabney, J. M.Jones, G. A.1976Digital vascular responses and permeability in equine alimentary laminitis. Am J Vet Res; 37; 1171-1176

Severino, C., Brizzi, P., Solinas, A., Secchi, G., Maioli, M.Tonolo, G.2002Low-dose dexamethasone in the rat: a model to study insulin resistance. Am J Physiol Endocrinol Metab; 283; E367-373

Wallerath, T., Witte, K., Schafer, S. C., Schwarz, P. M., Prellwitz, W., Wohlfart, P., Kleinert, H., Lehr, H. A., Lemmer, B.Forstermann, U.1999Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. Proc Natl Acad Sci U S A; 96; 13357-13362