A pharmacokinetic and pharmacodynamic study of pioglitazone in a model of induced insulin resistance in normal horses

Jamie Macquarie Gollan Wearn

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

Master of Science
In
Biomedical and Veterinary Sciences

Mark V. Crisman
(Committee Chair)
L. Jill McCutcheon
David R Hodgson
R. Scott Pleasant

May 28th 2010
Blacksburg, Virginia, USA

Keywords: Horse, Pioglitazone, Thiazolidinedione, Insulin Resistance, Equine Metabolic Syndrome

A pharmacokinetic and pharmacodynamic study of pioglitazone in a model of induced insulin resistance in normal horses

Jamie Macquarie Gollan Wearn

ABSTRACT

Equine Metabolic Syndrome (EMS) is a unique condition of horses characterized by adiposity, insulin resistance, and an increased risk of laminitis. Reducing insulin resistance may decrease the incidence of laminitis in horses with EMS. Pioglitazone, a thiazolidinedione class of anti-diabetic drug, has proven efficacy in humans with type 2 diabetes, a syndrome of insulin resistance sharing some similarities with EMS. The ability of pioglitazone to influence insulin sensitivity in an endotoxin-infusion model of induced insulin resistance was investigated. Our hypothesis was that piogltiazone would preserve insulin sensitivity in a model of induced insulin resistance. The specific aims were to investigate the pharmacokinetics and pharmacodynamics of pioglitazone in an endotoxin infusion model of insulin resistance.

16 normal adult horses were enrolled. Pioglitazone was administered to 8 horses (1 mg/kg, PO, q24h) for 14 days, and 8 horses served as their controls. Liquid chromatography with tandem mass spectroscopy was used to quantitate plasma concentration of pioglitazone. A frequently sampled intravenous glucose tolerance test with minimum model analysis was used to compare indices of glucose and insulin dynamics prior to, and following, endotoxin infusion in horses treated with pioglitazone and their controls. Parameters of clinical examination and lipid metabolism were compared prior to, and following, endotoxin administration.

Pioglitazone administered orally at 1 mg/kg q 24 h resulted in plasma concentrations lower, and more variable, compared to those considered therapeutic in humans. No significant effect of drug treatment was detected on clinical parameters or indices of insulin dynamics or lipid homeostasis following endotoxin challenge.

GRANT INFORMATION

Source: Center for Equine Research in Virginia
Title: Evaluation of insulin sensitizing agents for management of Equine Metabolic Syndrome
Amount: $ 74,217

DEDICATION

“Dream, Believe, Create, Succeed” is the motto of my life. I would never have been able to achieve my dreams without the amazing support I have been so privileged to have received from family and friends. My parents Michele and David and grandmother June are the most important people in my life and any success I achieve is testament to their boundless support. Jenny Raffetto’s unconditional belief in me is my foundation, without which I would be nothing and nobody. I am truly thankful to all my amazing friends who support and encourage me in all my endeavors.

ACKNOWLEDGEMENTS

The group of people I have been so fortunate to work with at Virginia Tech have provided me with so many opportunities for which I am immensely grateful. The publication of this thesis would never have been possible without their dedication, support, guidance, expertise, knowledge and patience. I would particularly like to acknowledge my graduate committee: Drs Mark Crisman (chair), Jill McCutcheon, David Hodgson, and Scott Pleasant; Dr Jennifer Davis for extensive assistance with pharmacokinetic analysis and Jessica Suagee for assistance with sample collection, data analysis, manuscript preparation and presentations.

I gratefully acknowledge the technical assistance provided by Elaine Meilahn, Tracy Smith, Robert Drake, Maureen Perry and Rebecca Wade.

Chapter 1 :

Literature Review

Chapter 2 :

Pharmacokinetics of pioglitazone after multiple oral dose administration in horses.

Chapter 3 :

Effects of pioglitazone on clinical parameters and minimal model parameters of the frequently sampled intravenous glucose tolerance test following intravenous endotoxin infusion, as a model of induced insulin resistance.

Chapter 4 : Conclusions

Chapter 5 : Appendices

List Of Figures

List Of Tables

ATTRIBUTION

Dr Jennifer L. Davis DVM PhD DACVIM DACVCP is an internist and clinical pharmacologist with over 5 years experience in equine clinical pharmacology research. Dr Davis performed the pharmacokinetic analysis and assisted with manuscript preparation. Assisted with all chapters.

Dr. Raymond J. Geor DVM MVSc PhD DACVIM. Formerly the Professor and Paul Mellon Distinguished Chair, Director of Research, Middleburg Agricultural Research and Extension (MARE) Center, College of Agriculture and Life Sciences. Currently a Professor and the Chairperson; Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University. Dr. Geor is a veterinary internist and equine nutritionist. He has over 20 years experience in equine research. Dr. Geor assisted with development of the study and preparation of the proposal. Dr. Geor assisted in each of experimental protocols, and assisted with data interpretation and manuscript preparation for all results of these studies. His laboratory performed blood biochemical and hormonal analyses. Assisted with chapters 2, 3 and 5.

Jessica K. Suagee MS is a PhD candidate in the Department of Animal and Poultry Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Ms Suagee assisted in each of experimental protocols, assisted with data interpretation and manuscript preparation for all results of these studies. Ms Suagee worked in the laboratory of Dr Geor and performed blood biochemical and hormonal analyses. Assisted with all chapters.

Dr Mehdi Ashraf-Khorassani PhD. Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia is an expert in his field of liquid chromatography and mass spectrometry. Dr Ashraf-Khorassani performed the chromatorgraphic and spectrometric analysis and assisted with manuscript preparation. Assisted with chapter 2.

Dr Steven Werre PhD is an expert in his field of epidemiology and statistics. Dr Were performed extensive statistical analysis of the data. Assisted with all chapters.

Chapter 1

Literature Review

Introduction

Equine metabolic syndrome (EMS) is a term recently coined to describe a collection of metabolic dysfunctions centered around obesity in adult horses and ponies. These dysfunctions result in a heightened risk of developing laminitis. The significance of EMS is twofold. First the impact on the horse's health and welfare that resultant laminitis imposes, and second, the size of the 'at risk' population for developing EMS is large and likely expanding. Laminitis is a painful and potentially life threatening condition, that often eventually requires euthanasia.[1] Similar to obesity in humans, obesity in horses appears to be an emerging and expanding problem. In an owner completed survey of the horse population of the USA (1999), assessment of the body condition of the horse or horses under the care of each person, 5% of the horse population was reported to be overweight.[1] This study included horses in 28 of the 48 continental US states. A recent 2006 cross-sectional prevalence study[2] performed at the Virginia Maryland Regional College of Veterinary Medicine of horses in Southwestern Virginia, assessment of body condition score (BCS) by two independent veterinarians identified 19% prevalence of obese horses. Obesity defined as a body condition score >7.5 out of 9, and 51% cumulative prevalence of horses over-conditioned or obese (BCS > 6.5 out of 9).

The first known description of the features of acute laminitis was made by Aristotle in 350BC, when he described an affliction of horses he called 'barley disease' and associated its occurrence with the over consumption of grain. Despite being recognized for over two thousand years, the pathophysiology of laminitis remains incompletely understood due to its extreme complexity. Commonly reported causes of acute laminitis in horses include acute gastrointestinal diseases, for example overconsumption of carbohydrates, particularly grain, and inflammatory diseases, for example endometritis. The term pasture associated laminitis (PAL) is given to horses that develop laminitis whilst grazing pasture without any other known concurrent disease, to distinguish these cases from those that develop following acute

disease. Laminitis developing whilst horses were grazing pasture accounted for an estimated 54% of cases in one investigation, highlighting the significance of this form of the disease.[1] A collection of associated metabolic dysfunctions affecting adult horses with a specific, defined, phenotype is accepted as a predisposing factor to horses developing PAL. Equine metabolic syndrome is the name given to this collection of metabolic dysfunctions.

Equine Metabolic Syndrome

The term equine metabolic syndrome was first proposed by Johnson in 2002[3]. The describd syndrome in horses involving a metabolic disorder, characterized by insulin resistance (IR) and obesity, that puts the horse at a higher risk of developing pasture associated laminitis. Pathophysiologic similarities between obese, laminitic, horses and obese, type-2 diabetic humans, was the basis for the application of the term metabolic syndrome in horses. In humans, the term metabolic syndrome is used as a collection of risk factors to predict the occurrence of non-insulin dependent (type 2) diabetes mellitus (NIDDM) or coronary artery disease.[4] The extrapolation of this term initially prompted controversy due to lack of definitive evidence of the existence of IR in affected horses. Also there were pathophysiologic differences between the human and equine diseases.[5] Other terms that have been used to describe this condition include; peripheral cushingoid syndrome[6], prelaminitic metabolic syndrome[7] and pasture associated laminitis syndrome[7]. However a recent consensus statement published by the American College of Veterinary Internal Medicine, supported the use of the term equine metabolic syndrome for the description of this clinical syndrome unique to equids.[8] Although EMS likely involves different pathophysiology than NIDDM of humans, IR is a key feature of both NIDDM and EMS.[9]

Clinical features of horses with equine metabolic syndrome

Horses with EMS are characterized by a distinctive phenotype. Typically horses are 5-18 years of age with no gender predilection having been described. Welsh, Dartmoor, and Shetland ponies and Morgan Horse, Paso Fino, Arabian, Saddlebred, domesticated Spanish Mustang, and Warmblood breeds appear to be overrepresented and hence potentially more susceptible to EMS. Increased deposits of adipose tissue can either be generalized (obesity)

or localized to specific locations, with expansion of subcutaneous adipose tissue surrounding the nuchal ligament (cresty neck), tail head, behind the shoulder, or in geldings, in the sheath, and mares, in the udder region. Abundant intra-abdominal omental fat is also observed. Generalized obesity is not present in all affected horses, and some horses have a leaner overall body condition with regional adiposity, and others have no detectable abnormalities of body fat distribution.[8]

Horses affected with EMS are reported by owners to be difficult to induce weight reduction by dietary restriction, being commonly referred to as “easy keepers”.[8,10] This trait potentially evolved as an evolutionary adaptation to survival on poor quality natural pastures, whereby ability to extract energy from the diet and store it as adipose tissue would provide a survival benefit.[7,11] The term “thrifty genes” has been applied to the genetic traits to facilitate survival via this mechanism. To develop insulin resistance (IR) in a time when dietary simple carbohydrates are scarce would benefit survival by sparing glucose for supply to essential tissues (nervous tissue and erythrocytes). This is achieved by IR reducing the utilization of glucose in non-essential tissues, which are reliant on insulin for glucose uptake, forcing these tissues that are able to produce energy from lipids derived from adipose tissue mobilization to do so. This conserves glucose for essential tissues, which require constant glucose supply and are not reliant on insulin for glucose uptake. Adipose stores increase during fall for use throughout winter and adipogenesis and appetite are stimulated during the fall. However, modern equine diets of improved pastures and supplemental concentrate feeding, may result in the loss of the annual cyclicity of the development and regression of adipose stores, which could result in disruption of normal metabolic homeostasis.

Laminitis in EMS-affected horses is often chronic, recurrent and develops whilst the horse is predominantly grazing pasture without any other recognized inciting event, such as excessive grain consumption, gastrointestinal disease or retained placenta.[8] Laminitis can be clinical, with associated lameness, or subclinical, with evidence of chronic abnormalities of the hoof structures without currently detectable lameness. Classically described hoof abnormalities include widening of the white line and diverging rings on the hoof wall.

Other reported features of EMS include altered reproductive cycling in mares[12], hypertriglyceridemia or dyslipidemia (increased very low density lipoproteins, non-esterified fatty acids and high-density lipoprotein cholesterol)[7,10], seasonal alterations in blood pressure[13], hyperleptinemia with possible leptin resistance[14], and elevated markers of systemic inflammation associated with obesity[15].[8] A familial predisposition to the development of EMS has been identified through genealogic analysis.[7]

A cresty neck (CN) score has been developed as a standardized, objective, assessment of the degree of adipose tissue expansion in the neck around the nuchal ligament.[16] The CN scoring system was designed to be used in conjunction with the BCS, as originally described by Henneke et al.[17]. The Henneke BCS score was designed to assess generalized subcutaneous fat deposits, whereas the CN score was specifically designed to assess regional adiposity in the neck region. A cresty neck was described as greater than or equal to 3 (out of 5). A grade 3 is described as the crest being: enlarged and thickened, so fat is deposited more heavily in the middle of the neck than toward poll and withers, giving a mounded appearance.

To assess for association of the CN score with the pathophysiology of EMS, an association between CN score and evidence of IR was investigated in a group of horses (n=34) and ponies(n=75).[16] Elevated baseline insulin (hyperinsulinemia) was used as an indicator of IR being present in that horse. The CN score was strongly associated with hyperinsulinemia in ponies (as assessed by a single baseline assessment of insulin). Ponies with a CN score of > 3 versus those with a normal neck score (CN score < 3) were 18.9 times more likely to be hyperinsulinemic. In the same study, this association of CN score and insulin concentration was not present in studied horses. However, the horses in this study, when compared to ponies, had a significantly lower CN score, BCS and leptin concentrations compared to ponies, indicating they were less likely to have been affected by EMS and therefore have insulin resistance. As such an association between CN score and evidence of IR could not be definitively assessed.

Frank et al., used a different assessment of neck adiposity, using an average neck circumference measured at 3 sites between the poll and withers.[10] Enlarged neck circumference was found to be significantly correlated with indicators of the EMS phenotype and pathophysiology (resting hyperinsulinemia, glucose to insulin ratio and leptin concentration).

Pathophysiology of equine metabolic syndrome.

As described by Johnson[3,6] and recently reviewed[8], EMS is a metabolic disorder, with concurrent endocrinologic abnormalities, that may explain the association between obesity and PAL in adult horses and ponies. The core elements defining EMS are IR, regional adiposity or generalized obesity and increased susceptibility to laminitis.

An association between obesity and an increased risk of developing laminitis in mature horses grazing pasture has been long recognized by equine veterinarians.[6,18] However the pathophysiologic link has only been recently investigated. Insulin resistance is accepted as the most likely link between obesity and a predisposition to PAL in horses and ponies.[18-22] Obesity has been demonstrated to result in IR [15,23,24], and obese ponies with IR have been demonstrated to be at a higher risk of developing laminitis[7,13,20]. This association of IR and laminitis offers a potential explanation for the cases of laminitis without an identifiable inciting event. The first known reference to an investigation of the relationship between insulin dynamics as the core link between obesity, breed and laminitis was performed by Jeffcott and collegues.[25] This investigation identified that ponies of normal body condition were insulin resistant compared to horses, and obese ponies and those with previous laminitis were insulin resistant when compared to ponies without a history of laminitis.

The phenotypic features of EMS were previously attributed to a disorder of thyroid metabolism, as low concentrations of thyroid hormones are often detected in horses with this phenotype.[3,26] A number of pieces of evidence do not support this association of causality. First, thyroid dysfunction is an uncommon endocrine abnormality of horses, unlike EMS which is a prevalent condition.[27] Furthermore, hypothyroidism induced experimentally in normal horses via propothyrouracil administration[28] or thyroidectomy[29,30] , was not associated with

the development of obesity or laminitis. The clinical signs identified following experimental thyroidectomy included retarded growth, increased sensitivity to cold, delayed shedding of the hair coat, edema of the hind legs, a coarse coat, reduced feed consumption and decrease in body weight gain. A number of factors which could potentially account for the decreased thyroid hormone concentrations in horses with EMS phenotype have been demonstrated to result in decreased circulating thyroid hormones in euthyroid horses, including; phenylbutazone administration, high energy diets, high protein diets, diets rich in zinc and copper, glucocorticoid administration, food deprivation and ingestion of endophyte-infected fescue grass.[27]

Although horses with the EMS phenotype share features of equine pituitary pars intermedia dysfunction (PPID), these conditions are separate disease entities.[8] It is possible for a horse to have both disorders concurrently.[8] Regional adiposity, IR, hyperinsulinemia and laminitis occur in horses with both PPID and EMS. However hirsutism, which is an integral clinical sign and pathologic feature of PPID, is not a feature of EMS.[31] The specificity of hirsutism as a diagnostic test for the presence of a pituitary gland abnormalityis reported as between 95 and 100%, indicating that almost all horses with hirsutism have abnormal pituitary function.[31,32] Horses and ponies with EMS have been demonstrated to have normal results for appropriate diagnostic testing of pituitary gland function and to have normal pituitary glands when examined histopathologically at necropsy.[8,33]

Insulin Resistance.

Metabolic effects of insulin.

The physiologic roles of insulin include modulation of carbohydrate, lipid and protein metabolism. In carbohydrate metabolism, insulin functions to regulate glucose homeostasis and promote glucose utilization by its actions on trans-membrane glucose transporters. Glucose utilization by cells requires transportation from the blood plasma across cell membranes into the cytosol by facilitative glucose-transporters.[34] Five isoforms of these glucose transporters have been identified, and are referred to as GLUT – 1 through – 5.[35,36] Different isoforms have different tissue distribution and biochemical properties. These

facilitative transporters are functionally and structurally different compared to the sodium-glucose co-transporter expressed by absorptive epithelial cells of the small intestine and renal epithelial cells.

- GLUT-1 facilitative transporter: predominantly expressed on erythrocytes in adults and is responsible for basal or constitutive glucose uptake. Responsible for non-insulin dependent transmembrane glucose transport.
- GLUT-2 facilitative transporter: mediates the bidirectional transport of glucose by hepatocytes. Responsible, at least in part, for the movement of glucose out of absorptive epithelial cells into the circulation in the small intestine and renal tubules. Comprise part of the glucose sensing mechanism of the insulin producing pancreatic islet of Langerhans β cells.
- GLUT-3 facilitative transporter: similar function to GLUT-1 however is primarily located on neural tissue.
- GLUT-4 facilitative transporter: the insulin responsive transmembrane glucose transporter. Responsible for most of the insulin-stimulated uptake of glucose that occurs in muscle and adipose tissue, as well as other body tissues.
- GLUT-5 facilitative transporter: located on the small intestine and is responsible for transcellular transport of glucose by absorptive epithelial cells.[35,36]

GLUT 1 and 4 have been identified in equine muscle, fat and liver.[37,38] The presence of other glucose transporters in equine tissues is a speculative extrapolation from human and rodent studies.

Insulin is released from the pancreatic β cells located in the islets of Langerhans in response to elevated blood glucose concentration. Glucose enters pancreatic β cells through the GLUT-2 glucose transporters and is metabolized such that ATP is produced, which increases the ATP/ADP ratio. This in turn closes ATP-sensitive K (K_{atp}) channels. This depolarizes the membrane, which opens L-type Ca^{2+} channels and promotes Ca^{2+} influx. The resulting increase in the cytosolic Ca^{2+} concentration evokes exocytosis of insulin granules. This model is described as the consensus model for glucose-stimulated insulin secretion from

pancreatic β cells.[39] The release of insulin from pancreatic β cells is pulsatile, with both fast (tens of seconds) and slow (4–6 minute) oscillations of insulin secretion.[39-41] In humans, the slow phase of oscillation has been demonstrated to have an important physiological role in insulin action and is lost in people with type-2 diabetes.[40] It is currently unknown if the oscillatory nature of insulin secretion is similarly affected in horses with EMS.

Following insulin binding to the insulin receptor, two different intracellular signaling pathways are stimulated;

1. The mitogen-activated protein kinase (MAPK) pathway, and
2. The phosphatidylinositol-3-kinase (PI3K) pathway.[42,43]

These pathways transduce the signal of insulin binding to the insulin receptor to initiate a variety of intracellular effects through a number of tyrosine kinase intermediaries.

An effect of insulin binding to the insulin receptor is to initiate two major metabolic functions relating to carbohydrate metabolism. First, insulin triggers a cascade of intracellular events that culminates in the translocation of glucose transport (GLUT-4) proteins to the cell membrane.[18] The PI3K pathway regulates the translocation of GLUT-4, however the exact steps in the pathway whereby insulin binding to the insulin receptor ultimately results in translocation of GLUT-4 has not yet been fully elucidated.[42] Skeletal muscle and adipose tissue represent the major site of insulin mediated glucose uptake, however the liver is also insulin responsive. Second, insulin has a number of other intracellular glucose metabolism. Functions of insulin in carbohydrate metabolism include stimulation of glycogen synthesis in skeletal muscle and liver, and inhibition of hepatic gluconeogenesis.[34]

Insulin has many other actions beyond the regulation of carbohydrate metabolism, including actions on lipid and protein metabolism during states of abundant energy supply. During this physiological state, insulin stimulates lipogenesis and inhibits lipolysis in liver and adipose tissue and stimulates protein anabolism and cell growth in all body tissues. Insulin also has critical regulatory functions on vascular endothelium function and vascular tone regulation.[21,34]

Diabetes mellitus.

In human medicine, disorders of insulin secretion, action or both that result in resting hyperglycemia are referred to as diabetes mellitus (DM).[4] Two types of DM are described. Type 1 DM, also referred to as insulin dependent DM, is due to a primary deficiency of pancreatic islet of Langerhans β cells to secrete insulin in response to elevated blood glucose concentrations, resulting in hyperglycemia and glucosuria.[34,44] This develops due to a primary disease process of the pancreas resulting in β cell destruction.[4] In cases of pure type 1 DM, there is no reduction in tissue insulin sensitivity. Pancreatic disease resulting in β cell dysfunction and type 1 DM is rare in horses.[45]

Type 2 DM, also referred to as non-insulin dependent DM (NIDDM), is due to decreased target tissue sensitivity to the metabolic effects of insulin.[34] In humans, progressive reduction in pancreatic β cell production of insulin and IR are two central, interrelated, defects in the pathophysiology of type 2 diabetes.[4,46] β cell changes in patients with type 2 DM include defects in insulin secretion, proinsulin conversion to insulin, and amyloid deposition in islets.[46] The majority of horses with EMS have decreased insulin sensitivity and β cell damage, as exists with humans, has not been reported to be present in horses with EMS. Compensation for reduced insulin sensitivity by increased pancreatic β cell production of insulin is considered the normal situation in horses, and less frequently recognized in humans.

Horses with EMS appear to display some of the features of human type 2 DM. As such a descriptive term of a “pre-diabetic state” has been proposed to describe the features of DM occurring in horses with EMS, rather than describing them as being affected with type 2 DM as the majority lack the β cell damage.

In humans a sequela to type 2 DM induced hyperinsulinemia is β cell exhaustion, resulting in hypoinsulinemia in the face of hyperglycemia and IR. This situation was previously considered rare in horses, but is however now being recognized more frequently in equine patients and is being referred to as end-stage type 2 DM.[47,48]

Type 1 DM has traditionally been considered rare in the equine population, with one report of hyperglycemia and hypoinsulinema in a domesticated Spanish mustang.[49] Whether this case represents type 1 DM or decompensated type 2 DM was not determined, as measures of insulin sensitivity were not pursued. Treiber et al.[50] report a laminitic pony with evidence of reduced capacity for insulin secretion from the pancreatic β cell and resting hyperglycemia, consistent with end stage type 2 DM. A more recent publication in which three confirmed cases of low insulin sensitivity, low pancreatic β cell response to exogenous glucose and concurrent hyperglycemia was reported suggests that end stage type 2 DM is not as rare as initially believed.[47] No histopathology is reported in these horses to identify that pathophysiology occurring in the pancreatic β cells of these affected horses. As such, there are 4 described cases of end stage type 2 DM in horses, which still is vastly less prevalent than the pre-diabetic state in the equine population.

Pathophysiology of insulin resistance.

Insulin resistance is defined as a state where a normal concentration of insulin fails to elicit its normal level of biological action.[51] Insulin resistance represents a shift to the right of an insulin dose-response curve.[51,52] Focusing on the role of insulin on glucose metabolism, IR represents a reduction in insulin mediated glucose transport into the cell for the same concentration of plasma insulin.[52] The PI3K pathway is believed likely to be dysfunctional in animals with IR as it is the PI3K pathway that is involved in the regulation of GLUT4 expression and therefore insulin responsive glucose transport.[21,43] In insulin resistant states, insulin mediated activation of the PI3K pathway is selectively impaired, whilst the MAPK pathway is spared.[43]

Insulin resistance is able to be compensated for by a higher circulating concentration of insulin, as supraphysiologic concentrations of insulin are able to achieve the maximal response.[52] The terms insulin resistance and reduced insulin sensitivity are used interchangeably in the equine literature.

Insulin responsiveness or effectiveness represents the maximal effect of insulin. Conversely, the term insulin has been used to describe a reduction in the maximal biological

response to insulin.[52] This represents a failure of insulin facilitated intracellular glucose metabolism.[52]

The exact mechanism resulting in IR in horses with EMS is yet to be fully elucidated, however the pathophysiology is likely multifactorial. Possible mechanisms that may result in the development of IR include: [34,44,53]

1. Reduced presentation of β cell-produced insulin to the insulin receptor
2. Increased hepatic degradation or antibody destruction of circulating insulin
3. Reduced insulin receptor density on the surface of insulin responsive cells due to down-regulation of receptor expression
4. Malfunction of the insulin receptor binding or signal transduction
5. Defective internal signaling cascade, or
6. Interference with the translocation or function of the GLUT-4 proteins.

Effects of inflammation in adipose tissue and the liver as well as the accumulation of by-products of nutritional overload (eg. diacylglycerol) in insulin sensitive tissues, have been suggested as possibly contributing to the etiology of the development of IR.[18,54]

Relationship of obesity and insulin resistance.

A number of factors contribute to the development of obesity in horses including environmental (eg. diet, level of physical activity, season, precipitation, pasture management) and intrinsic (eg. genetic) factors. Horses and ponies with EMS appear to have enhanced metabolic efficiency with respect to the extraction and utilization of dietary energy, when compared to normal horses not affected by this affliction.[8]

Jeffcott et al. were the first to make the association between obesity and IR.[25] In this study, when compared to normal ponies and horses, obese ponies or those that had previously suffered laminitis were found to have lower insulin sensitivity and increased plasma insulin concentrations after glucose loading, an estimate of the acute insulin response to glucose. A subsequent study by Hoffman et al. of horses that were maintained at their existing body weight utilized quantitative testing of insulin and glucose dynamics utilizing the frequently sampled intravenous glucose tolerance (FSIGT) test with minimal model

analysis.[24] Results confirmed the findings of the Jeffcott study that, in horses ranging from normal to obese body condition, obesity was associated with approximately 80% lower insulin sensitivity when compared to non-obese horses. Glucose effectiveness was also elevated in obese when compared to non-obese horses, suggesting obese horses rely on glucose mediated glucose disposal, and existence of elevated pancreatic insulin production. Similar findings of an inverse correlation between insulin sensitivity, as measured by the hyperinsulinemic, euglycemic clamp technique, and percentage body fat and body condition score, was identified by Vick et al.[15]

A study in which weight gain was induced by dietary modification, provided conclusive evidence for the association of obesity and IR.[55] Insulin homeostasis was monitored using the FSIGT test and minimal model analysis. Dietary modification resulted in a 20% increase in body weight and an increase in body condition score[17] from a mean of 6 ± 1 (out of 9) to 8 ± 1 (out of 9). Insulin sensitivity decreased by 71 ± 28% and acute insulin response to glucose increased by 408 ± 201 %. This is consistent with effective compensation of reduced insulin sensitivity by increased pancreatic production of insulin.

Insulin resistance, obesity and equine metabolic syndrome.

The first cohort study investigating whether an EMS phenotype and glucose-insulin homeostasis dysregulation was related to the incidence of pasture associated laminitis was performed by Treiber et al..[7] A herd of 160 Dartmoor and Welsh ponies managed as a semi-feral herd under natural, unregulated grazing conditions was investigated. The ponies were analyzed as three groups, those with no history of laminitis (NL; n=106), those previously laminitic (PL; n=54), as determined by hoof abnormalities and confirmed with farm records, and those that developed clinical laminitis during the spring months (CL; n=13). As these ponies received no access to grain feeds and experienced no observed episodes of gastrointestinal disease, the laminitis observed was assessed to be PAL. Proxies of glucose and insulin dynamics, body condition scores and pedigree were investigated at two time points; March, at the onset of spring prior to lush spring pasture growth, and May, at the end of spring at the peak of spring pasture growth was occurring.

At the onset of the study, all horses had been exposed to the same environmental conditions of natural pasture, however ponies that had previously experienced an episode of laminitis had a higher body condition score when compared to the ponies that had never had an episode of laminitis. An 80% decline in plasma nonesterified fatty acid concentrations between the two sampling periods occurred. This is consistent with mobilization of fat stores for energy due to reduced pasture energy supply during March and a shift away from lipolysis in May as pasture supply increased. Although not able to be analyzed statistically, the horses with historical laminitis (PL and CL horses) had regional adiposity, often having accumulations of fat along the dorsal crest of the neck, shoulder and tail head regions. This supports the theory of propensity of horses with the EMS phenotype to develop and maintain larger stores of adipose tissue as well as frequently having regional adiposity. Furthermore, the body condition score in the previously laminitic ponies did not change significantly during the spring. In contrast BCS increased in the NL group, suggestive that PL ponies did not lose body condition during the period of poorer pasture supply over winter, compared to the NL group whose body condition score increased with the spring increase in pasture supply.

Pedigree analysis within this herd identified the mode of inheritance of a previous diagnosis of laminitis to be consistent with dominant major gene or genes with reduced penetrance attributed to sex mediated factors, age of onset and further epigenetic factors.

When defined by reduced insulin sensitivity and increased pancreatic β cell insulin response, ponies with a previous history of pasture-associated laminitis were insulin resistant compared to those without evidence of previous laminitis.

Finally, the existence of a link between the suspected metabolic dysfunction of EMS as a predisposing factor for the development of pasture-associated laminitis was confirmed. In this study, a prelaminitic metabolic profile was defined as the presence of any three of the following metabolic derangements (now accepted as the metabolic profile of horses with EMS): Hypertryglyceridaemia, insulin insensitivity (as measured by basal insulin proxy), compensatory increased β cell response (as measured by basal insulin and glucose ratio proxy) and obesity (BCS > 6 out of 8) with regional adiposity. The presence of the prelaminitic

metabolic profile was associated with a 10 times higher risk of the development of PAL (odds ratio 10.0; 90% confidence interval 2.4-34.1), than horses lacking the prelaminitic metabolic profile.

This study was the first investigation confirming the existence of a causal link between evidence of IR, the central core of the prelaminitic metabolic dysfunction profile, and the development of PAL in ponies. An increase in pasture starch content was also identified and was proposed as a potential contributing factor to the development of IR and laminitis in these ponies, as high simple carbohydrate diets have previously been demonstrated to exacerbate IR.[56]

The same research group investigated the diagnostic accuracy of a number of variables for predicting the development of laminitis in a cohort of ponies.[20] The predictive power of body condition score, cresty neck score, girth and neck circumference to withers height, blood pressure, hoof surface temperature and plasma insulin, glucose, triglyceride, leptin, cortisol, adrenocortiotropic hormone (ACTH), uric acid and tumor necrosis factor-α (TNFα) were analyzed. Proxies of insulin sensitivity and pancreatic β cell insulin production were calculated and analyzed. Variables confirmed to have diagnostic accuracy for the prediction of laminitis developing were:

- Plasma insulin and leptin concentration,
- Proxy estimation of insulin sensitivity (reciprocal inverse square of the insulin; RISQI),
- Body condition score,
- Cresty neck score, and
- Neck circumference to withers height ratio.

This confirmed that the phenotypic features of EMS (regional or generalized obesity with a cresty neck) as well as disrupted insulin homeostasis (IR with compensatory hyperinsulinemia) and hyperleptinemia were associated with an increased risk of developing PAL.

Diagnostic tests for insulin resistance

The two primary variables involved in glucose homeostasis are disrupted during glucose dysregulation in horses with EMS and therefore represent the primary variables to be analyzed by diagnostic testing are[44] :

1. The insulin secretory response of pancreatic β cells of the islets of Langerhans to increased blood glucose concentration, and
2. The sensitivity of skeletal muscle and adipose tissue (insulin responsive tissues with the most metabolic mass) to circulating insulin concentrations.

A number of different methods have been described to assess insulin sensitivity in horses, including quantitative methods and non-specific indicators (see table 1). Quantitative indices include clamp techniques and dynamic modeling. Nonspecific indices include single sample measurement of basal blood insulin and glucose and mathematical proxies of quantitative techniques based upon these single samples.

Table 1. Quantitative and Semiquantitiative proxy methods for assessing insulin and glucose homeostasis.

Semiquantitiative proxy techniques	Formulae	Reference
Basal glucose and insulin measurement		
Tolerance testing		
○ Oral glucose tolerance test		
○ Intravenous glucose tolerance test		
○ Insulin tolerance testing		
○ Intravenous glucose insulin tolerance testing		
Surrogates – proxies		
Insulin sensitivity		
○ **RISQI** : Reciprocal inverse square of the insulin	$1 \div \text{insulin}^{-0.5}$	Treiber et al. 2005
○ **FGIR** : fasting glucose insulin ratio	Fasting glucose ÷ fasting insulin	Uwaifo et al. 2002
○ **QUICKI** : Quantitative insulin sensitivity check index	1 ÷ [log fasting insulin + log fasting glucose]	Katz et al. 2000
Insulin resistance		
○ **HOMA – IR** : Homeostasis model assessment for IR	[fasting insulin x fasting glucose] ÷ 22.5	Matthews et al. 1985
β cell function		
○ **I:G ratio** : Fasting insulin to glucose ratio	Fasting insulin ÷ fasting glucose	Uwaifo et al. 2002
○ **MIRG** : Modified insulin to glucose ratio	[800 - 0.3 x (insulin - 50)2] ÷ [glucose - 30]	Treiber et al. 2005
○ **HOMA-β%** : Homeostasis model assessment of % β cell function	[20 x fasting insulin] ÷ [fasting glucose - 3.5]	Matthews et al. 1985
Quantitative techniques		
Insulin Suppression test		
Clamping tests		
○ Hyperglycaemic clamp		Andres et al. 1966
○ Euglycaemic, hyperinsulinaemic clamp		Ringen et al. 2003
Dynamic Modelling		
○ Frequently sampled intravenous glucose tolerance (FSIGT) test with minimum model analysis		Hoffman et al. 2003
○ Modified FSIGT test with minimum model analysis		Toth et al. 2009
○ Combined glucose-insulin test		Eiler et al. 2005

Quantitative techniques.

Quantitative methods offer the most reliable, repeatable, accurate and thorough testing and are thus considered to be the best methods to assess insulin homeostasis, particularly for research studies. However, these tests are often not practical to be performed on clinical cases due to the prolonged time to perform the tests (many hours), intensive requirements for frequent samples, technical complexity, cost and less than ideal repeatability. The two most commonly used quantitative techniques are the euglycemic hyperinsulinemic clamp (EHC) and FSIGT test, which is analyzed using the minimal model technique. An adaptation of the FSIGT and minimal model, the combined glucose-insulin tolerance (CGIT) test, has recently been reported for the use in horses.[57]

Clamping techniques attempt to override endogenous insulin and glucose concentrations to allow more accurate assessment of glucose and insulin homeostasis. Originally a hyperglycaemic clamp was described by Andres et al. [58] and subsequently a euglycemic, hyperinsulinemic clamp technique was developed, and more recently applied to horses[59]. The EHC involves a 3 hour intravenous (IV) infusion of regular insulin at 3μg/kg/min. This represents a supraphysiologic, steady state of insulin to override any endogenous insulin secretion. An IV infusion of 50% dextrose is adjusted to maintain blood glucose at a physiologic concentration (5mmol/L or 90mg/dL). The actions of insulin on glucose uptake by tissues is quantitated by the rate of glucose infusion over the last 90 minutes of the study, once a plateau period of insulin infusion rate is achieved, indexed per unit mean plasma insulin. The disadvantages of the EHC include

1. Technical difficulties involved with undertaking the procedure,
2. Insulin concentrations are non-physiologic, set at an arbitrary supra-physiologic concentration, and
3. Other endogenous hormones, eg. cortisol, may influence the metabolic effects of insulin, thus compromising the results.

4. The EHC being only able to assess the insulin sensitivity, and no other aspects of insulin homeostasis.

Notwithstanding these limitations, the EHC has been demonstrated to be the most repeatable method of measuring insulin sensitivity.[60]

The FSIGT test protocol used in horses is based upon that described originally in humans[61] and modified for application in horses[24,59]. Briefly, 10 and 5 minutes prior to dextrose infusion, blood is collected for baseline blood glucose and insulin assessment. A bolus dose of 300mg glucose/kg body weight (50% [wt/v] dextrose solution) is administered rapidly IV. Time 0 is set at the completion of the dextrose infusion and blood samples are collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 19 minutes. At 20 minutes regular insulin (30mU/kg body) is administered IV. Blood samples are subsequently collected at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes.

To analyze the data of the FSIGT test, a non-linear mathematical model was developed to assess how glucose and insulin together control the production and disposal of glucose in the body. This model is called the minimal model and was originally designed for use in humans by Bergman et al.[61,62], and the methods modified slightly for application to horses[24,59], and then automated through the development of computer software by Boston et al.[63]. The model is used to calculate values for insulin sensitivity (SI; $x10^{-4}$ $L \cdot min^{-1} \cdot mU^{-1}$); acute insulin response to glucose (AIRg; $mU \cdot L \cdot min^{-1}$); glucose effectiveness (Sg; $x10^{-2}$ min^{-1}) and disposition index (DI; $x10^{-2}$ dimensionless).

Interpretation of each of the four indices measured by the FSIGT and minimal model analysis is as follows.[24] Glucose effectiveness (Sg) estimates the capacity of glucose to mediate it's own disposal independent of a change in plasma insulin. Glucose disposal is presumed to be mediated via the non-insulin dependent cell membrane glucose transporter (GLUT-1). This is calculated over the first 10 minutes of the FSIGT test from the rate of change in blood glucose concentration. Insulin sensitivity (SI) represents the capacity of insulin to promote glucose disposal or the rate of glucose clearance from the plasma ($L \cdot min^{-1}$) per unit of plasma insulin (mU). This is assessed as the acceleration of glucose disposal

associated with increased concentration of blood insulin (induced by exogenous insulin administration) over basal concentration of blood insulin. Acute insulin response to glucose (AIRg) quantifies the endogenous insulin secretion in response to the exogenously administered glucose dose. This assesses the pancreatic β-cell response of insulin production to increased blood glucose. Finally the disposition index (DI) describes β-cell responsiveness and accounts for the influences of endogenous insulin secretion and insulin sensitivity, allowing for assessment of pancreatic response without interference of changes in insulin sensitivity. A number of studies have investigated the minimal model parameters of the FSIGT test in horses of various breeds and body conditions (Tables 2-4).

Table 2. Values reported for minimal model parameters in thoroughbred geldings at existing levels of body condition.[24]

	Non-obese horses n=4	**Moderately obese horses** n=3	**Obese horses** n=3
Sg $x10^{-2}$ min^{-1}	1.43 ± 0.16	1.59 ± 0.19	3.02 ± 0.22
SI x 10^{-4} $L•mU^{-1}•min^{-1}$	1.94 ± 0.19	1.47 ± 0.23	0.37 ± 0.27
AIRg $mU•min•L^{-1}$	211 ± 34.7	221 ± 40.1	408 ± 49.1
DI x 10^{-2}	1.22 ± 0.64	2.68 ± 0.77	0.50 ± 0.90

Sg: glucose effectiveness; SI: insulin sensitivity; AIRg: acute insulin response to glucose; DI: disposition index (DI)

Table 3. Values reported for minimal model parameters derived from examination of 46 normal adult horses of mixed breeds.[50] Mean; median (95% CI for median)

Sg $x10^{-2}$ min^{-1}	1.0 0.95 (0.12-2.95)
SI x 10^{-4} $L•mU^{-1}•min^{-1}$	2.09 1.89 (0.16-58)
AIRg $mU•min•L^{-1}$	270 218 (67-805)
DI x 10^{-2}	0.476 0.381 (0.0393-1.675)

Sg: glucose effectiveness; SI: insulin sensitivity; AIRg: acute insulin response to glucose; DI: disposition index (DI)

Table 4. Effects of weight gain on values of minimal model parameters in adult geldings. Mean ± SD (range).[55]

	Prior to weight gain; BCS 6±1	**When obese BCS : 8±1**
Sg $x10^{-2}$ min^{-1}	1.07 ± 0.52 (0.24 – 2.05)	1.17 ± 0.73 (0.35 – 2.74)
SI $x\ 10^{-4}$ $L•mU^{-1}•min^{-1}$	2.07 ± 1.76 (0.50 – 6.65)	0.39 ± 0.27 (0.11-1.0)
AIRg $mU•min•L^{-1}$	206 ± 88 (89-403)	973 ± 393 (487 – 1864)
DI $x\ 10^{-2}$	3.70 ± 2.38 (0.91 - 8.41)	3.63 ± 2.60 (0.87 – 9.47)

Sg: glucose effectiveness; SI: insulin sensitivity; AIRg: acute insulin response to glucose; DI: disposition index (DI)

An investigation of the repeatability of measures of glucose and insulin homeostasis, identified significant aberrations from repeatability of both EHC and FSIGT measures of insulin sensitivity.[60] The EHC was identified to have greater repeatability than the FSIGT test. Results of the minimal model analysis of the FSIGT correlate well with measures of insulin sensitivity obtained by the use of clamping techniques.[60] Notwithstanding this inferior repeatability, FSIGT has a significant advantage in that it provides estimates of four different phases of insulin and glucose homeostasis, that are not measured by other testing techniques.[64]

A theoretical criticism of the minimal model is that glucose mediated glucose disposal is explained by a single rate constant. Discrepancies in the FSIGT and minimal model analysis occur due to the variability in response to glucose and insulin administration that naturally occurs within a horse, affecting repeatability.

A concern regarding the physiologic basis of the FSIGT test is that blood glucose concentrations during the first 20 minutes of the test (mean ± SD; 360 ± 51 mg/dL in our investigation of n=24 FSIGT tests) exceed the renal glucose reabsorptive threshold for glucose (estimated at 160-180mg/dL[65]), resulting in an undetermined quantity of glucose being lost in the renal tubular filtrate. As urinary loss of glucose is not accounted for in the

model, this may confound the results of the minimal model analysis. The most likely variable to be affected is the glucose effectiveness, which is calculated from blood glucose concentrations from between 8 and 20 minutes. If urinary spillage continues after 20 minutes, estimation of insulin sensitivity could be affected.[66]

A recent publication[66] addresses this concern of urinary glucose loss compromising the integrity of the FSIGT test, reports the investigation of a modification to the FSIGT test using different glucose and insulin doses. The doses of insulin and glucose in the initial report of the application of the FSIGT test to horses were a direct, empiric extrapolation of doses used in humans[61], without being critically evaluated.[48] Tóth et al.[66] investigated 6 insulin doses (5-30μU/kg) and 6 glucose doses (50-300mg/kg) and compared results for minimal model parameters of insulin and glucose homeostasis. The modified FSIGT test protocol utilized in this publication is a glucose dose of 100mg/kg followed by insulin dose of 20mU/kg at 20 minutes. Modification to these doses provided sufficient data for minimal model analysis and minimized urinary glucose loss by maintaining peak blood glucose below 200mg/dL. Using this modified protocol there was no statistically significant difference in minimal model calculated parameters ($P>0.133$). Furthermore urinary glucose loss was investigated in 2 horses, and although not totally abolished, was significantly reduced from 9 and 11.2% using the established FSIGT protocol, to 3.1 and 2.3% using the modified protocol in the 2 horses, and occurred for a shorter duration.

Semi-quantitative techniques.

The application of specific quantitative methods of analyzing insulin and glucose homeostasis to clinical patients is limited by their technical complexity and expense.[5,50] These limitations stimulated the generation of semi-quantitative, non-specific, single sample, surrogate indicators for application in clinical and research situations in humans for assessment of DM, and many such proxies have been described.[67] Semi-quantitaive indicators are technically simpler to perform and require less time as they are based upon a single sample analysis of insulin and glucose concentrations. Due to the single sample protocol, they also allow for sampling from larger populations.

Several factors regarding the accuracy and repeatability of these single sample proxies include:

1. Insulin and glucose concentrations can fluctuate significantly in an individual animal over a short period as a result of diurnal variation, feeding, stress and other factors. Fluctuation and the reliance of on a single sample is the basis for questioning of the accuracy of these measurements.[44,68]

2. The pulsatile and oscillatory release of insulin from the pancreatic β cells may also contribute to the variability in minute-to-minute plasma glucose and insulin concentrations.[39]

Notwithstanding these potential sources of variability, proxies developed for use in humans have been investigated, and subsequently validated, as acceptable surrogate indicators of the state of insulin and glucose metabolism in horses when compared to quantitative techniques in a research setting.[50] Proxy methods however have not been validated for use in clinical cases of IR in horses.[48] In a study by Treiber et al.[50], a number of single sample predictors of indices of insulin homeostasis used in humans were calculated. These were based upon reported mathematical equations using basal insulin and glucose concentrations and compared to the results of a quantitative method (FSIGT with minimal model analysis) in 46 normal horses of mixed breeds. The study was designed to compare a number of proxies for their accuracy in predicting the two primary parameters of glucose and insulin homeostasis as assessed by the minimal model quantitative technique; namely tissue SI, and pancreatic islet β cell responsiveness as determined by the AIRg.

Based upon the results of testing reference quintiles were developed for parameters of insulin homeostasis to allow diagnosis and characterization of the state of glucose and insulin homeostasis in clinical cases. Results of this study indentified that the reciprocal inverse square of basal insulin (RISQI) was the proxy with the highest correlation coefficient for prediction of the SI (r=0.774), and the modified insulin to glucose ratio (MIRG) of the AIRg (r=0.754) (Table 5).

Although the proxies are less accurate than specific quantitative parameters they predict, they represent useful tools as a screening assessment of insulin homeostasis in horses and are superior to simple measurements of basal insulin and glucose.[50]

Table 5: Non-specific proxy estimations with best predictive capability of minimal model parameters of insulin and glucose homeostasis.[50]

Homeostatic property	Minimum model parameter	Proxies
Tissue insulin sensitivity	Insulin Sensitivity (SI)	Reciprocal inverse square of basal insulin (RISQI)
Pancreatic islet β cell response	Acute insulin response to glucose (AIRg)	Modified Insulin:Glucose Ratio (MIRG)

Eiler and colleagues, recognizing the importance of quantitative testing of insulin and glucose homeostasis and the limitations of the currently available tests in application to clinical cases, developed a new diagnostic testing method.[57] They developed the combined intravenous glucose and insulin test (CGIT)[57], which was designed with the goal of providing information of similar quality to the FSIGT and EHC without the technical demands, computer modelling and frequent prolonged sampling. This test, however, falls in the non-quantitative measurement as it only provides non-specific indications of IR. This test involves a rapid IV glucose bolus administration (50% dextrose solution 150 mg/kg), immediately followed IV by regular insulin (0.1 U/kg) with blood glucose measurement at 0 (baseline prior to injection of dextrose) and then subsequently a 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, and 150 minutes. Accordingly the duration of the test was 2 and one half hours in duration, and required only 14 blood samples to be collected for glucose measurement. The glucose dose was chosen to result in approximately 100% increase in blood glucose at 5 minutes and without exceeding the renal threshold or transport maximum for glucose.

The CGIT results in the generation of a 2-phase curve of blood glucose clearance, a positive (hyperglycemic) phase above baseline glucose concentration, and negative (hypoglycemic) phase below the baseline. Analysis of the slope of the curve during each

portion of each phase is performed. An estimation of the rate of glucose clearance is generated by the gradient of the curve during the descending portion of the positive phase, as an indicator of insulin sensitivity. Time to blood glucose return to baseline is also analyzed and in this relatively small population of horses without clinically detectably abnormalities, the mean time to glucose return to baseline was 30 ± 6 minutes.[57]

Repeatability of the test was assessed by analysis of the coefficient of variation for glucose clearance rate estimation during the initial portion of the slope of the descending portion of the positive phase, which ranged from 2.3-59.3% (14.9± 6.4% mean ± SD). [57]

In a subsequent study by the same research group, the results of a CGIT test were compared between obese and non-obese horses, and correlated with measurement of body condition scores, neck circumference, resting glucose and insulin concentrations and circulating lipid analyses.[10] During this study, a modification of the CGIT was undertaken, with insulin being analyzed at each time point, in addition to blood glucose. Horses in the obese group were post-hoc assessed to fulfill their definition of insulin resistant, as having prolonged time to return to basal blood glucose (> 45 minutes) during the CGIT. The obese horses being hyperinsulinemic and having significantly higher basal insulin than the non-obese horses, supports this definition. During the CGIT test, the obese horses had prolonged time to return to basal blood glucose (> 45 minutes), whilst the non-obese horses returned to baseline within 25 minutes. To objectively analyze the CGIT, area beneath the curve (AUC) analysis was performed for both plasma insulin (AUC_i) and glucose (AUC_g). Obese horses had significantly larger AUC insulin and glucose compared to non-obese horses.

A test that is clearly ideal in terms of practicality and accuracy is yet to be established[44], however the CGIT test is suggested to be the accepted standard for clinical testing.[8,57] A delayed return of blood glucose to baseline (> 45 minutes) is suggested as an indicator of the possible presence of IR, because blood glucose should rapidly return to normal after exogenous insulin administration. The CGIT test procedure was subsequently modified to include quantitation of insulin concentration, allowing values of area under the curve of glucose and insulin to be generated. The AUC_i and AUC_g described as objective

measures of insulin-glucose dynamics during the CGIT[10] requires computer modeling, however offers no described benefit over the FSIGT and minimal model analysis. The FSIGT and minimal model analysis have proven diagnostic power as an assessment of insulin homeostasis in horses. No clear benefit of co-administering the glucose and insulin, as is done in the CGIT, has been described over the FSIGT, where the insulin is administered 20 minutes after glucose administration. An insulin concentration >100μU/mL at 45 minutes following CGIT commencement is described as evidence of increased insulin secretion and/or reduced rate of insulin clearance. These findings are recommended to be interpreted as evidence of IR, however, it is the opinion of this author that there is a lack of definitive experimental evidence to support these conclusions.[8,10,57,66]

In a report comparing the CGIT, the FSIGT and a modified FSIGT protocol, significant differences ($P<0.003$) were detected between area under the insulin and glucose curves between these two testing protocols.[66] As such, it is the opinion of the author that the CGIT test requires further investigation to be able to definitively provide evidence of glucose and insulin homeostasis in horses. The correlation of the CGIT with a quantitative parameter of insulin homeostasis would provide evidence to support the use of the CGIT as a diagnostic test of IR.

The role of inflammation in EMS

Relationship of obesity, inflammation and insulin resistance.

Investigations in humans provide evidence that elevations in circulating concentrations of inflammatory cytokines play direct roles in the development of obesity associated IR.[15,69] Specifically, interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF-α) are elevated in obesity and contribute to IR.[70,71]

The relationship between obesity, inflammatory cytokines and insulin sensitivity were evaluated in a study by Vick et al.[15] In this investigation, insulin sensitivity was inversely proportional to body condition score and percentage body fat. Furthermore insulin sensitivity

was inversely and obesity positively correlated with circulating concentration of TNFα protein and IL-1.

Adipose tissue as an endocrine gland.

There has been a recent paradigm shift in the understanding of adipose tissue, from believing it to be a benign storage site of lipid to an endocrinologically active tissue, producing a large number of biologically active mediators. These mediators possess both inflammatory actions and direct effects (stimulation or inhibition) on insulin sensitivity.[72] Inflammatory mediators derived from adipose tissue are termed adipokines, and include cytokines and other soluble bioactive mediators. Currently known and described adipokines include leptin, apelin, TNF-α, IL-1β, IL-8, IL-18, plasminogen activator inhibitor (PAI) -1, macrophage chemoattractant protein 1, adiponectin, 11-β- hydroxysteroid dehydrogenase 1 (11β HSD1) and visfatin.[8,72-74]

Human obesity and NIDDM are recognized as pro-inflammatory states and elevated inflammatory cytokines have been demonstrated to play a direct role in the development of IR associated with these conditions.[69] Obesity in horses is similarly associated with elevated concentrations of circulating inflammatory cytokines,[15,75] Visceral adipose tissue deposits in particular are highly endocrinologically active, when compared to subcutaneous adipose deposits.[44] In humans, mesenteric and omental adipose deposits are thought to play a more important role in the development of type 2 DM than other adipose deposits, not only because of their greater endocrinologic activity, but also because of their vascular proximity to the liver, having a more profound effect on hepatic metabolism and insulin clearance.[8,76]

The glucocorticoid effects of cortisol are believed to contribute to obesity associated IR.[44] 11-β- hydroxysteroid dehydrogenase 1 is an enzyme that converts the inactive precursor cortisone in plasma to active cortisol that possesses glucocorticoid properties. Omental adipocytes express 11βHSD1 to a greater degree than subcutaneous adipocytes. This enzyme is associated with the development of IR in transgenic 11βHSD1 over-expression mice.[77]

Leptin is potentially one of the most significant adipokines in horses with EMS.[8] Leptin is produced by adipocytes and signals to the hypothalamus that a state of energy excess exists within the adipose tissue and therefore results in a reduction in feed intake.[11] As such, leptin is referred to as a satiety factor.

Circulating leptin concentration in horses has been demonstrated to be positively correlated with body condition score, with horses with higher BCS having higher leptin concentrations.[78,79] Hyperleptinemia has also been associated with obese horses with evidence of IR, as indicated by greater insulin response to intravenous glucose challenge.[78] A number of studies have identified leptin to be in significantly higher concentrations in obese horses with evidence suggestive of IR[10], and hyperleptinemia is a significant predictor of the development of PAL in ponies with the EMS phenotype.[20] In humans, obesity has been suggested to occur as a result of relative or absolute insensitivity to leptin at its site of action.[11] The demonstration of hyperleptinemia in horses with EMS, is suggested to have similar pathophysiologic basis.[8]

Etiology of laminitis in EMS

Obesity, insulin resistance and inflammation : Potential links to laminitis in equine metabolic syndrome.

Despite a large amount of observational evidence linking this cluster of risk factors that form the phenotype of EMS with the development of laminitis, the underlying pathophysiologic mechanism of this increased susceptibility is yet to be proven.

Glucose starvation theory was originally proposed as a link between IR and laminitis whereby IR reduced glucose uptake into metabolically active lamellar tissue. This theory was disproven with the absence of insulin mediated glucose transporter (GLUT-4) mRNA expression, and lack of effect of insulin stimulation on glucose uptake, in hoof lamellar tissue explants.[80]

Hyperinsulinemia develops as a physiological compensatory mechanism for reduced tissue insulin sensitivity in horses[24,55] as is described in humans[81,82]. Hyperinsulinemia,

through a multitude of metabolic and vascular effects, has been proposed as the link between IR and the development of laminitis.[21,22,80] Support of this theory is provided by the demonstration of induction of laminitis in healthy ponies with no previous history of laminitis by prolonged (>72 hour) administration of insulin resulting in supraphysiologic hyperinsulinemia.[80] Although the degree of hyperinsulinemia in this experimental model is far above that recorded in horses with EMS, it provides strong evidence of a causal link between hyperinsulinemia and laminitis.

Insulin has many vasoactive roles with hemodynamic effects, some of which have been investigated in horses, but many extrapolated from human medicine.[21] The physiologic vasoactive actions of insulin serve to couple regulation of metabolic and hemodynamic homeostasis.[43] Insulin has been demonstrated in rats and humans to act as a slow vasodilator of high resistance and terminal arterioles, resulting in increased blood flow to muscles during periods of ample glucose supply.[83-85] The mechanism of this vasodilation is mediated through the PI3K pathway induced increased endothelial cell derived nitric oxide (NO).[43,86] However, insulin also has vasoconstrictive actions via promotion of endothelin-1 (ET-1) synthesis via the MAPK pathway and activation of the sympathetic nervous system.[21,43] The intracellular balance of activation of the MAPK and PI3K signal transduction pathways likely influences the vascular effects of insulin.

In an insulin resistant state, selective impairment of the PI3K pathway would result in reduced insulin mediated stimulation of endothelial NO production and hence vasodilatory actions, whilst the MAPK pathway remains active, resulting in endothelial ET-1 production and induced vasoconstriction.[43] In a carbohydrate overload model of laminitis induction in healthy horses, increased plasma ET-1 concentrations have been detected in digital blood 12 hours after carbohydrate administration.[87] This effect would be compounded by the hyperinsulinemic state in horses with EMS, increasing MAPK pathway stimulation, establishing a generalized vasoconstrictive state. The resultant effect would be a generalized vasoconstrictive state, potentially contributing to digital ischemia and laminitis.

Endotoxin and vasoactive amines trigger vasoconstriction and endothelial NO synthesis is the counteractive homeostatic protective vasodilatory mechanism. Insulin resistance in humans has also been shown to result in reduction in endothelial NO synthesis.[86] If this vasoconstriction was to occur in the digit it may compromise lamellar perfusion and therefore result in laminitis. If the same reduction in NO synthesis was to occur in horses with EMS induced IR, horses with EMS may have a reduced ability to withstand the vasoconstriction triggered by endotoxin or vasoactive amines contributing to IR induced laminitis.[21]

Obesity and a predisposition to laminitis have been independently associated with a systemic pro-inflammatory state, as evidenced by elevated inflammatory cytokines identified in the circulation of laminitis prone ponies.[9,75] This inflammatory process is postulated to contribute to vascular dysfunction and thus heightened susceptibility to laminitis.[18]

The future of diagnostics for equine metabolic syndrome.

Possible future directions for the diagnosis of EMS include the investigation of adipokines, leptin, adiponectin and resistin, fructosamine and measures of systemic inflammation (eg TNFα, IL-1, IL-6, serum amyloid A and plasminogen activating factor).[8]

An assay for connecting peptide (C-peptide) has recently been validated for use in equine serum.[88] Quantitation of C-peptide is a potential modality for the investigation of pancreatic insulin secretion.[8] C-peptide is released from the pancreas in amounts equimolar to insulin, but is not subjected to the same first pass hepatic extraction as is the case for insulin.[88] Decrease in the insulin-to-C-peptide ratio might be useful to characterize reduced insulin clearance, a suspected contributor to hyperinsulinemia in horses with EMS.[8]

Models of insulin resistance

Endotoxin (lipopolysaccharide).

Lipopolysaccharide (LPS) is an integral membrane component of the outer membrane of the gram negative bacterial cell wall. Administration of exogenous LPS has been investigated as a model of inducible IR, without causing mortality or lasting morbidity (eg.

inducing laminitis) in horses. Tóth et al. reported that administration of Escherichia coli O55:B5 LPS at a dose of 20 ng/kg administered as an infusion over 30 minutes, induced IR in 13 of 16 horses.[64] Clinical signs of mild fever, tachycardia, mild signs of colic and leucopenia were also observed. Furthermore, significant reduction in insulin sensitivity was identified at 24 and 48 hours following LPS administration, with the maximal aberration at 24 hours. Clinical and insulin homeostatic non-response to administration of LPS, as observed in 3 horses, was attributed to either circulating anti-LPS antibodies or genetic modification of intracellular pathways that result in insulin resistance following exposure to LPS. Similar effects of LPS infusion on insulin dynamics have been reported in other investigations.[89]

The mechanism whereby LPS administration results in IR was hypothesized to be related to induced elevations in inflammatory cytokines. Elevated TNF-α and IL-6 have been reported following LPS administration to horses,[89] and these cytokines are known to induce IR in humans.[70,71] As elevations in these cytokines have also been identified in obese, insulin resistant horses[15], this provides support for the use of a LPS infusion model for experimental reproduction of insulin resistance of similar pathophysiologic basis to that occurring in horses with EMS.

Dexamethasone.

The effects of dexamethasone (0.08mg/kg), administered intravenously at 48 hourly intervals for 21 days to normal horses, on insulin homeostasis were investigated and reported by Tiley et al.[90]. Administration of dexamethasone resulted in a significant (70%) decrease in insulin sensitivity as measured by a hyperinsulinemic, euglycemic clamp. The means by which glucocorticoids induce this insulin resistance in horses is not known.[90] Corticosteroids are associated with anti-inflammatory properties through their genomic and non-genomic actions. As such the insulin resistance induced by the administration of dexamethasone is likely induced via different mechanisms that occurs in horses with obesity associated insulin resistance, as obesity in horses is associated with a systemic pro-inflammatory state as discussed previously. For this reason it is this author's opinion, that dexamethasone induced insulin resistance is an inferior model for the study of obesity induced insulin resistance in horses when compared to LPS induced insulin resistance.

Current Treatments For Equine Metabolic Syndrome.

The primary therapeutic target of medical management in horses with EMS is improved insulin sensitivity to mitigate hyperinsulinemia, thus lowering the risk of laminitis. Effective countermeasures ameliorating the IR and pro-inflammatory state in EMS affected horses would have significant benefits to horse health and welfare. Weight loss, exercise, and dietary modification have been proven to be pivotal therapeutic interventions to improve insulin sensitivity in both insulin resistant horses[25,91] and humans[92,93]. In humans, lifestyle changes (modest, appropriate, weight loss and physical activity) have proven more effective than pharmacological interventions in controlling IR.[94] Pharmacologic intervention has an important role in specific circumstances, such as active laminitis where daily exercise is not possible, however does not replace the need for management modifications. The goal of pharmacologic intervention is to affect a more rapid decrease in hyperinsulinemia in order to alleviate the risks of adverse sequelae developing. In the initial period after diagnosis of EMS, prior to physiologic responses to instigated management changes, the risk for laminitis is highest, and represents a period of indication for pharmacologic intervention. In some horses, insulin resistance is reported to persist after the horse has reached its ideal body weight, representing a further indication for targeted drug therapy.[21] Furthermore, horses with active laminitis are often unable to exercise, and pharmacologic intervention is the only method of breaking the obesity - IR cycle. Various pharmacologic agents have been investigated in experimental models and clinical trials of horses with EMS without definitive evidence of an ideal treatment.

Weight loss, diet and exercise.

Weight loss, exercise, and dietary modification are central to effective therapeutic intervention to improve insulin sensitivity in both insulin resistant horses and humans.[25,91-93]

Dietary management of horses with EMS includes a feeding regimen to induce weight loss and the formulation of a diet low in nonstructural carbohydrates in order to minimize the glycemic and insulinemic response to meals.[21] The feeding of diets rich in starch and sugar has been proven to induce reduced insulin sensitivity when compared to feeding diets rich in fat and fiber.[24]

To reduce body weight, digestible energy (DE) intake must be reduced. Pasture can be a significant contributor to dietary DE, and daily pasture intake is unable to be accurately quantified. Furthermore carbohydrate content of the diet needs to be addressed. Simple sugars, starches and fructans are non-structural carbohydrates, whereas cellulose, hemicelluloses, and lignins are structural carbohydrates. [95] Fructans, although unable to be digested by mammalian enzymes, may contribute to the glycemic response following feeding by microbial and acid hydrolysis in the large colon. Evidence for this is provided by the insulin response to dietary fructans.[19] Maintaining total dietary non-structural carbohydrates to less than 10% dry matter is the current recommendation.[8]

Levothyroxine sodium (Thyro-L®).

Sommardahl and colleagues[96] investigated the administration of exogenous levothyroxine (LT4) to normal horses. Using an incremental dose study of 24,48,72, 96mg orally at 24 hour intervals for 2 week periods at each dose, they investigated the effects on thyroid gland hormones, weight and behavior. In euthyroid horses, treatment with exogenous LT4 resulted in:

- Increased total thyroxine (tT4) free thyroxine (fT4), total triiodothyronine (tT3) and free triiodothyronine (fT3)
- Decreased thyroid stimulating hormone (TSH)
- Increased reverse triiodothyronine (rT3), an isomer that is devoid of hormonal activity but acts as a competitive inhibitor of thyroid hormone receptor and
- Blunted TSH response to thyrotropin-releasing hormone (TRH) stimulation test, when compared to non-treated controls.

Concentration of TSH varied widely in treated and control horses, indicating that clinical assessment based upon a single sample is unreliable. Abnormal behavior was identified at the 96mg dose, however this is significantly above the clinically used dose of 24 or 48 mg.

During this study feed was restricted to maintenance requirements. Significant findings included exogenous LT4 induced 4 % body weight reduction (median weight loss 19 kg with

median original weight of 470 kg) during the 8 week study period. The study protocol of restriction of feed to maintenance was a significant feature of the study design, as rats increase feed intake by 47% when administered triiodothyroxine intraperitoneally for 14 days.[97] This was explained by increased metabolic rate, thermogenesis and consumption of body fat stores as triglycerides are hydrolyzed to supply fatty acids for energy in these rats.

In a companion study, the effects of levothyroxine on indices of insulin homeostasis was reported by Frank et al..[98] A CGIT was used to assess insulin sensitivity in the treated and control horses over the 8 week dosing period. A statistically significant difference in insulin sensitivity (increase) was detected in the treated horses, however no such statistical difference was identified in control horses. Notwithstanding the statistical difference being detected only in the treated horses, insulin sensitivity increased in the control as well as treatment horses. The magnitude of the increase in insulin sensitivity after treatment was almost identical to the change in control horses, and the magnitude of the average insulin sensitivity increase was larger in the control horses compared to the treatment horses. One possible explanation for the lack of detection of a statistical difference in the control horses relates to the variability in baseline insulin sensitivity in the control horse population, or potentially an outlier skewing the data. In the control horses baseline insulin sensitivity had a range of 0.09-12.33 $x10^{-4}$ L/mU/min whereas in the treated horse the range was 0.5-2.36 $x10^{-4}$ L/mU/min. Following treatment the range of insulin sensitivity in the control horses was markedly smaller (2.05-2.50), which was similar to the treated horses (0.85-4.40). As such the horse with the 12.33 $x10^{-4}$ L/mU/min insulin sensitivity in the baseline, non-treated group, was possibly an outlier, or alternatively the control population was not an accurate representation of the general horse population. Furthermore, no comparison was made between the treated and control horses in terms of change in insulin sensitivity resulting from the treatment, detracting from the strength of the conclusion that the increase in insulin sensitivity was caused by the treatment with LT4.

In a companion study, the effects of 8 weeks of LT4 on lipid metabolism and glucose homeostasis were investigated.[98] Levothyroxine was demonstrated to decrease plasma

triglyceride and very low density lipoprotein (VLDL) concentrations with no effect on NEFAs or VLDL composition.[98]

To determine whether the effects on insulin dynamics detected in an 8 week trial would persist over a longer duration, 6 euthyroid horses were recruited into a 1 year study and were administered 48mg LT4 in feed q24 hours.[99] Treatment with LT4 was determined to induce and maintain persistent weight loss, despite access to pasture for 12 hours per day and thus uncontrolled dietary intake. Unfortunately testing constraints prohibited the inclusion of a control group in the 48 week trial, and thus effects of exogenous factors were not able to be definitively excluded and were acknowledged to potentially confound results. Seasonal effects, changes in feed composition and pasture availability would be potentially influential factors on body weight and glucose-insulin dynamics. The weight loss induced by LT4 was attributed to increased metabolic rate and reduced fat stores.

Effects of long term administration of LT4 in healthy, euthyroid horses on serum thyroid hormone concentrations, clinicopathologic variables, and echocardiographic measurements were assessed in a companion study.[100] Persistent increase in tT4, increased tT3 at 32 weeks and blunted tT3 and tT4 response to TRH stimulation tests were identified, indicating higher circulating thyroid hormone concentrations and reduced thyroid gland responsiveness. A transient decrease in cardiac fractional shortening (20-15% at 16 and 32 weeks) without any effect on indicators of myocardial injury (CKMB or cardiac troponin I) was identified. Mild changes in clinical pathological variables (increased anion gap and decreased blood urea nitrogen) attributed to subtle effects on metabolism or protein metabolism. All of these changes were considered unlikely to be clinically relevant.

The effects of LT4 in clinical cases of EMS have yet to be reported, however anecdotal reports yield favorable results. The consistent effect on reduction in body weight identified in the experimental studies on normal horses, are observed in clinical cases, however dietary modification is crucial.

Metformin.

Metformin is an oral biguanide anti-diabetic agent, and is the most widely prescribed insulin sensitizing drug in humans.[101] Although a recent investigation in horses[102] identified an effect of treatment with metformin at 15mg/kg orally administered at 12-hour intervals on increased insulin sensitivity in an uncontrolled study, these results have not been able to be replicated. Using a euglycemic, hyperinsulinemic clamp technique in a placebo controlled study of normal horses, no effect of metformin treatment (15mg/kg orally at 8-hour intervals for 15 days) was identified.[103] Similar lack of significant effects have been reported in other publications.[12,47] A pharmacokinetic investigation of metformin identified that bioavailability was low (4-7%) after oral administration and clearance (Cl) rapid (>10 times the rate in humans), precluding achievement of concentrations considered therapeutic in humans at current dosing regimens in horses.[104] These findings may have accounted for the lack of statistically significant effect of drug treatment at the dose rate administered in previous studies.

Pioglitazone : pharmacology and pharmacodynamics.

Pioglitazone represents a novel therapeutic agent for horses with EMS. Yet to be investigated in horses, it possesses a mechanism of action targeted against the currently proposed pathological lesion of EMS. Pioglitazone ([(±)-5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4-] thiazolidinedione monohydrochloride) is a member of the thiazolidinedione (TZD) class of oral anti-diabetic agents used in human medicine to treat NIDDM by restoring insulin sensitivity.[105-107]

Thiazolidinediones are synthetic activators of the γ isoform of the peroxisome proliferator-activated receptor-γ (PPARγ).[108] Peroxisome proliferator-activated receptors are nuclear receptor transcription factors.[109] Three isoforms of PPARs are recognized, -α, -β, and –γ and these isoforms vary in their tissue distribution within the body. PPAR-γ is predominantly expressed in adipose tissue, and to a lesser degree in vascular endothelium, vascular smooth muscle and macrophages.[109] Activation of PPARs by its specific ligand, results in the formation of a heterodimer with retinoid-X receptors (RXR).[109] The PPAR-RXR dimer then binds to peroxisome proliferator-response elements of target genes, and can act

as a promoter or inhibitor of transcription.[109] By this mechanism PPARγ has regulatory action on more than 100 genes involved in glucose and lipid metabolism, and inflammation.[108-113] Genes known to be regulated by PPARγ include adiponectin[74], tumor necrosis factor-α[114], resistin[115], and 11β-hydroxysteroid dehydrogenase 1[116]. By modulating expression of these genes, TZDs restore insulin sensitivity and reduce hyperglycemia without effecting pancreatic β cell insulin secretion, therefore minimizing the risk of inducing hypoglycemia.[117,118] Endogenous ligands for PPARγ include fatty acids and fatty acid derived eicosanoids.[109,119,120]

Pharmacokinetics of pioglitazone have been investigated in humans that are healthy, elderly, with type 2 diabetes, hepatic insufficiency[117,121], and those with impaired renal function[122]. Pioglitazone is an enantomeric drug, administered as a racemate. The ratio of + to - enantomer was 1:1 in humans and 2:3 rats. The drug is extensively protein bound in human and animal plasma, the free fraction representing < 3% of total plasma concentration. The drug undergoes significant hepatic metabolism via hydroxylation and oxidation resulting in a number of known metabolites (M_1-M_6). M_3 and M_4 demonstrate pharmacological activity in diabetic animal models, retaining 40-60% of the hypoglycemic potential (ED50) of the pioglitazone parent compound. M2 has twice the potency for lowering triglycerides as the parent compound, while the potency of M3 and M4 was slightly less than the parent compound.

Human and animal studies have shown drug kinetics to follow one compartment pharmacokinetic model with first order absorption. Bioavailability was 83% (95% CI: 74-93) in healthy humans. Mean time to maximal plasma concentration (T_{max}) following oral administration was 1.5 hours (range 0.5-3.0hrs). Food delayed the rate of absorption (T_{max} 3hrs compared to 2hrs), but did not alter extent of absorption.[117]

Pioglitazone has a low volume of distribution in humans (0.199-0.299 L/kg) attributed to extensive binding to plasma protein of parent drug and active metabolites. The drug is eliminated slowly despite the low volume of distribution. This is attributed to the extensive protein binding resulting in low hepatic extraction ratio

In humans with NIDDM there was no significant change in maximal plasma concentration (C_{max}) or the area under the concentration versus time curve for 24 hours (AUC_{0-24}). Similar concentrations of metabolites were identified and overall the pharmacokinetic profile was similar in patients with NIDDM to healthy volunteers. In patients with renal insufficiency a lower C_{max} and AUC_{0-24} was identified and explained by reduced protein binding, common in patients with renal impairment, resulting in increased free fraction of pioglitazone affecting intrinsic hepatic clearance. Pioglitazone does not inhibit nor induce metabolism of commonly co-administered drugs in humans.

Reported adverse effects of pioglitazone therapy in humans include weight gain, vascular volume expansion, edema and increased lipoprotein(a) concentrations.[113,120] Troglitazone, the first clinically available TZD, was associated with life threatening hepatotoxicity in humans and for this reason was withdrawn from the market. Pioglitazone has been proven to not share this hepatotoxic profile in humans.[122,123]

Pioglitazone has been investigated in a variety of experimental animals with proven effects of enhancing insulin action on peripheral tissues. In genetically obese and insulin resistant mice[124] and rats[124-126], pioglitazone decreased hyperglycemia, hypertriglyceridemia, hyperinsulinemia and IR. Similar effects on blood glucose and triglyceride concentrations were achieved in obese dogs with moderate IR, however plasma insulin concentrations were not affected.[124]

Adipose tissue is rich in PPARγ receptors and is the primary origin of TZD induced increased insulin sensitivity in mice[120] and humans.[113,127,128] This was demonstrated in genetically modified mice lacking adipose tissue which were refractory to the insulin sensitizing actions of TZDs.[113] Adipose tissue however, only accounts for a small fraction of insulin-dependent glucose clearance and therefore does not explain the effect of improved systemic insulin sensitivity.[113] Skeletal muscle and liver represent the major site of insulin mediated glucose disposal and PPARγ receptors have been identified in skeletal muscle and liver, however at much lower numbers than in adipose tissue.[109,127] No known studies have investigated PPARγ expression in equine tissue.

Effects on free fatty acid (FFA) metabolism are proposed as one link between TZD action in adipose tissue and the improved insulin mediated glucose disposal in muscle and liver. TZDs promote free fatty acid uptake and storage in subcutaneous adipose tissue, thereby reducing circulating concentrations of FFAs.[113,128] By this mechanism, TZDs spare other insulin-sensitive tissues, such as the liver and skeletal muscle, from the deleterious effects of high concentrations of FFAs, which interference with intracellular insulin signaling.[120]

Another reported mechanism by which PPARγ agonists increase insulin sensitivity is by regulating adipokine synthesis and secretion of adipocytes and adipose tissue associated macrophages, thereby modulating the endocrine activity of adipose tissue.[113,120] PPARγ agonists increase adiponectin, an adipokine that potentiates hepatic insulin sensitivity, and reduces resistin and proinflammatory adipokines (TNF- α, PAI 1, and IL-6) released from macrophages in adipose tissue.[113,128] PPARγ agonists down-regulate the activity of the enzyme 11βHSD1, representing another mechanism whereby TZDs as PPARγ agonists improve insulin sensitivity.[113]

Summary

Equine metabolic syndrome is a collection of metabolic and endocrine abnormalities that result in the affected horse being at higher risk of developing laminitis. The pathophysiology of this disorder is currently incompletely understood. Whether this syndrome is a primary metabolic disorder with endocrine consequences, a primary endocrine disorder with metabolic consequences, concurrent endocrine and metabolic abnormalities or a genetic predisposition to obesity with resultant metabolic and endocrinologic effects is as yet unknown. Diagnostic testing for this disorder is in its infancy of development. An ideal therapeutic intervention for all affected horses has yet to be identified.

References

1. USDA. Lameness and Laminitis in U.S. Horses #N318.0400. *USDA: APHIS: VS, CEAH, National Animal Health Monitoring System Fort Collins, CO* 2000.
2. Thatcher CD, Pleasant RS, Geor RJ, et al. Prevalence of obesity in mature horses: an equine body condition study. *The American Academy of Veterinary Nutrition 7th Annual Clinical Nutrition and Research Symposium* 2007;6 (abstract).
3. Johnson PJ. The equine metabolic syndrome peripheral Cushing's syndrome. *Vet Clin North Am Equine Pract* 2002;18:271-293.
4. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998;15:539-553.
5. Kronfeld DS, Treiber KH, Geor RJ. Comparison of nonspecific indications and quantitative methods for the assessment of insulin resistance in horses and ponies. *J Am Vet Med Assoc* 2005;226:712-719.
6. Johnson PJ, Ganjam VK. Laminitis, "hypothyroidism", and obesity : a peripheral cushingoid syndrome in horses? American College of Veterinary Internal Medicine 1999;192-194.
7. Treiber KH, Kronfeld DS, Hess TM, et al. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. *J Am Vet Med Assoc* 2006;228:1538-1545.
8. Frank N, Geor RJ, Bailey SR, et al. Equine Metabolic Syndrome. *J Vet Intern Med* 2010;24:467-475.
9. Geor RJ. Metabolic predispositions to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. *Journal of Equine Veterinary Science* 2008;28:753-759.
10. Frank N, Elliott SB, Brandt LE, et al. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. *J Am Vet Med Assoc* 2006;228:1383-1390.
11. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998;395:763-770.
12. Vick MM, Sessions DR, Murphy BA, et al. Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. *Reprod Fertil Dev* 2006;18:609-617.
13. Bailey SR, Habershon-Butcher JL, Ransom KJ, et al. Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. *Am J Vet Res* 2008;69:122-129.
14. Carter RA, Treiber KH, Geor RJ, et al. Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies. *Equine Veterinary Journal* 2009;41:171-178.
15. Vick MM, Adams AA, Murphy BA, et al. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J Anim Sci* 2007;85:1144-1155.
16. Carter RA, Geor RJ, Burton Staniar W, et al. Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. *Vet J* 2009;179:204-210.

17. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* 1983;15:371-372.
18. Geor RJ. Metabolic Predispositions to Laminitis in Horses and Ponies: Obesity, Insulin Resistance and Metabolic Syndromes. *J Equine Vet Sci* 2008;28:753-759.
19. Bailey SR, Menzies-Gow NJ, Harris PA, et al. Effect of dietary fructans and dexamethasone administration on the insulin response of ponies predisposed to laminitis. *J Am Vet Med Assoc* 2007;231:1365-1373.
20. Carter RA, Treiber KH, Geor RJ, et al. Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies. *Equine Vet J* 2009;41:171-178.
21. Frank N. Equine metabolic syndrome. *Journal of Equine Veterinary Science* 2009;29:259-267.
22. Geor R, Frank N. Metabolic syndrome-From human organ disease to laminar failure in equids. *Vet Immunol Immunopathol* 2009;129:151-154.
23. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.
24. Hoffman RM, Boston RC, Stefanovski D, et al. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci* 2003;81:2333-2342.
25. Jeffcott LB, Field JR, McLean JG, et al. Glucose tolerance and insulin sensitivity in ponies and Standardbred horses. *Equine Vet J* 1986;18:97-101.
26. Frank N, Sojka J, Messer NT. Equine thyroid dysfunction. *Vet Clin North Am Equine Pract* 2002;18:305-319.
27. Messer NT. Thyroid disease (Dysfunction) In: Robinson NE, ed. *Current therapy in equine medicine 4*. Philadelphia, PA: W.B. Saunders, 1997;502-503.
28. Breuhaus BA. Thyroid stimulating hormone in euthyroid and hypothyroid horses. *Proceedings of the 17th American College of Veterinary Internal Medicine Forum, Chicago* 1999:71.
29. Lowe JE, Baldwin BH, Foote RH, et al. Equine hypothyroidism: the long term effects of thyroidectomy on metabolism and growth in mares and stallions. *Cornell Vet* 1974;64:276-295.
30. Frank N, Sojka JE, Latour MA, et al. Effect of hypothyroidism on blood lipid concentrations in horses. *Am J Vet Res* 1999;60:730-733.
31. Miller MA, Pardo ID, Jackson LP, et al. Correlation of pituitary histomorphometry with adrenocorticotrophic hormone response to domperidone administration in the diagnosis of equine pituitary pars intermedia dysfunction. *Vet Pathol* 2008;45:26-38.
32. Frank N, Andrews FM, Sommardahl CS, et al. Evaluation of the combined dexamethasone suppression/ thyrotropin-releasing hormone stimulation test for detection of pars intermedia pituitary adenomas in horses. *J Vet Intern Med* 2006;20:987-993.
33. Donaldson MT, McDonnell SM, Schanbacher BJ, et al. Variation in plasma adrenocorticotropic hormone concentration and dexamethasone suppression test results with season, age, and sex in healthy ponies and horses. *J Vet Intern Med* 2005;19:217-222.
34. Guyton AC, Hall JE. Insulin, Glucagon and Diabetes Mellitus In: Guyton AC,Hall JE, eds. *Textbook of medical physiology*. 11th. ed. Philadelphia.: Elsevier Saunders, 2006;961-978.

35. Mueckler MM. The molecular biology of mammalian glucose transporters. *Curr Opin Nephrol Hypertens* 1992;1:12-20.
36. Bell GI, Kayano T, Buse JB, et al. Molecular biology of mammalian glucose transporters. *Diabetes Care* 1990;13:198-208.
37. Annandale EJ, Valberg SJ, Mickelson JR, et al. Insulin sensitivity and skeletal muscle glucose transport in horses with equine polysaccharide storage myopathy. *Neuromuscul Disord* 2004;14:666-674.
38. McCutcheon LJ, Geor RJ, Hinchcliff KW. Changes in skeletal muscle GLUT4 content and muscle membrane glucose transport following 6 weeks of exercise training. *Equine Vet J Suppl* 2002:199-204.
39. Bertram R, Sherman A, Satin LS. Metabolic and electrical oscillations: partners in controlling pulsatile insulin secretion. *Am J Physiol Endocrinol Metab* 2007;293:E890-900.
40. Matthews DR, Lang DA, Burnett MA, et al. Control of pulsatile insulin secretion in man. *Diabetologia* 1983;24:231-237.
41. Porksen N. The in vivo regulation of pulsatile insulin secretion. *Diabetologia* 2002;45:3-20.
42. Lizcano JM, Alessi DR. The insulin signalling pathway. *Curr Biol* 2002;12:R236-238.
43. Schulman IH, Zhou MS. Vascular insulin resistance: a potential link between cardiovascular and metabolic diseases. *Curr Hypertens Rep* 2009;11:48-55.
44. Firshman AM, Valberg SJ. Factors affecting clinical assessment of insulin sensitivity in horses. *Equine Vet J* 2007;39:567-575.
45. Jeffrey JR. Diabetes mellitus secondary to chronic pancreatitis in a pony. *J Am Vet Med Assoc* 1968;153:1168-1175.
46. Kahn SE. The importance of the beta-cell in the pathogenesis of type 2 diabetes mellitus. *Am J Med* 2000;108 Suppl 6a:2S-8S.
47. Durham AE, Hughes KJ, Cottle HJ, et al. Type 2 diabetes mellitus with pancreatic 13 cell dysfunction in 3 horses confirmed with minimal model analysis. *Equine Vet J* 2009;41:924-929.
48. Menzies-Gow N. Diabetes in the horse: a condition of increasing clinical awareness for differential diagnosis and interpretation of tests. *Equine Vet J* 2009;41:841-843.
49. Johnson PJ, Scotty NC, Wiedmeyer C, et al. Diabetes mellitus in a domesticated Spanish mustang. *J Am Vet Med Assoc* 2005;226:584-588, 542.
50. Treiber KH, Kronfeld DS, Hess TM, et al. Use of proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic beta-cell responsiveness in horses. *Am J Vet Res* 2005;66:2114-2121.
51. Kahn CR. Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. *Metabolism* 1978;27:1893-1902.
52. Kronfeld DS, Treiber KH, Hess TM, et al. Insulin resistance in the horse: Definition, detection, and dietetics. *J Anim Sci* 2005;83:E22-31.
53. Frank N. Diagnosis of Equine Metabolic Syndrome. American College of Veterinary Internal Medicine 2007.
54. Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 2008;9:193-205.

55. Carter RA, McCutcheon LJ, George LA, et al. Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. *Am J Vet Res* 2009;70:1250-1258.
56. Treiber KH, Boston RC, Kronfeld DS, et al. Insulin resistance and compensation in Thoroughbred weanlings adapted to high-glycemic meals. *J Anim Sci* 2005;83:2357-2364.
57. Eiler H, Frank N, Andrews FM, et al. Physiologic assessment of blood glucose homeostasis via combined intravenous glucose and insulin testing in horses. *Am J Vet Res* 2005;66:1598-1604.
58. Andres R, Swerdloff R, Pozefsky T. Manual feedback techniques for the control of blood glucose concentrations. In: Skeggs LTJ, ed. *Automation in analytical chemistry*. New York: Mediad, 1966;486-491.
59. Rijnen KE, van der Kolk JH. Determination of reference range values indicative of glucose metabolism and insulin resistance by use of glucose clamp techniques in horses and ponies. *Am J Vet Res* 2003;64:1260-1264.
60. Pratt SE, Geor RJ, McCutcheon LJ. Repeatability of 2 methods for assessment of insulin sensitivity and glucose dynamics in horses. *J Vet Intern Med* 2005;19:883-888.
61. Bergman RN, Phillips LS, Cobelli C. Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and beta-cell glucose sensitivity from the response to intravenous glucose. *J Clin Invest* 1981;68:1456-1467.
62. Bergman RN, Ider YZ, Bowden CR, et al. Quantitative estimation of insulin sensitivity. *Am J Physiol* 1979;236:E667-677.
63. Boston RC, Stefanovski D, Moate PJ, et al. MINMOD Millennium: a computer program to calculate glucose effectiveness and insulin sensitivity from the frequently sampled intravenous glucose tolerance test. *Diabetes Technol Ther* 2003;5:1003-1015.
64. Toth F, Frank N, Elliott SB, et al. Effects of an intravenous endotoxin challenge on glucose and insulin dynamics in horses. *Am J Vet Res* 2008;69:82-88.
65. Carlson GP. Clinical chemistry tests. In: Smith BP, ed. *Large Animal Internal Medicine*. 4th ed. St Louis.: Mosby, 2002;p396.
66. Toth F, Frank N, Elliott SB, et al. Optimisation of the frequently sampled intravenous glucose tolerance test to reduce urinary glucose spilling in horses. *Equine Vet J* 2009;41:844-851.
67. Fukushima M, Taniguchi A, Sakai M, et al. Homeostasis model assessment as a clinical index of insulin resistance. Comparison with the minimal model analysis. *Diabetes Care* 1999;22:1911-1912.
68. Treiber KH, Kronfeld DS, Geor RJ. Insulin resistance in equids: possible role in laminitis. *J Nutr* 2006;136:2094S-2098S.
69. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 2004;25:4-7.
70. He J, Usui I, Ishizuka K, et al. Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. *Mol Endocrinol* 2006;20:114-124.
71. Vozarova B, Weyer C, Hanson K, et al. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. *Obes Res* 2001;9:414-417.
72. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 2006;6:772-783.

73. Chaldakov GN, Stankulov IS, Hristova M, et al. Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 2003;9:1023-1031.
74. Matsuda M, Shimomura I, Sata M, et al. Role of adiponectin in preventing vascular stenosis. The missing link of adipo-vascular axis. *J Biol Chem* 2002;277:37487-37491.
75. Treiber K, Carter R, Gay L, et al. Inflammatory and redox status of ponies with a history of pasture-associated laminitis. *Vet Immunol Immunopathol* 2009;129:216-220.
76. Santosa S, Jensen MD. Why are we shaped differently, and why does it matter? *Am J Physiol Endocrinol Metab* 2008;295:E531-535.
77. Masuzaki H, Paterson J, Shinyama H, et al. A transgenic model of visceral obesity and the metabolic syndrome. *Science* 2001;294:2166-2170.
78. Cartmill JA, Thompson DL, Jr., Storer WA, et al. Endocrine responses in mares and geldings with high body condition scores grouped by high vs. low resting leptin concentrations. *J Anim Sci* 2003;81:2311-2321.
79. Buff PR, Dodds AC, Morrison CD, et al. Leptin in horses: tissue localization and relationship between peripheral concentrations of leptin and body condition. *J Anim Sci* 2002;80:2942-2948.
80. Asplin KE, Sillence MN, Pollitt CC, et al. Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *Vet J* 2007;174:530-535.
81. Reaven GM. Compensatory hyperinsulinemia and the development of an atherogenic lipoprotein profile: the price paid to maintain glucose homeostasis in insulin-resistant individuals. *Endocrinol Metab Clin North Am* 2005;34:49-62.
82. Reaven GM. Insulin resistance/compensatory hyperinsulinemia, essential hypertension, and cardiovascular disease. *J Clin Endocrinol Metab* 2003;88:2399-2403.
83. Vincent MA, Dawson D, Clark AD, et al. Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes* 2002;51:42-48.
84. Zhang L, Vincent MA, Richards SM, et al. Insulin sensitivity of muscle capillary recruitment in vivo. *Diabetes* 2004;53:447-453.
85. Vincent MA, Clerk LH, Lindner JR, et al. Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab* 2006;290:E1191-1197.
86. Muniyappa R, Montagnani M, Koh KK, et al. Cardiovascular actions of insulin. *Endocr Rev* 2007;28:463-491.
87. Eades SC, Stokes AM, Johnson PJ, et al. Serial alterations in digital hemodynamics and endothelin-1 immunoreactivity, platelet-neutrophil aggregation, and concentrations of nitric oxide, insulin, and glucose in blood obtained from horses following carbohydrate overload. *Am J Vet Res* 2007;68:87-94.
88. Toth F, Frank N, Martin-Jimenez T, et al. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. *Equine Vet J* 2010;42:149-155.
89. Vick MM, Murphy BA, Sessions DR, et al. Effects of systemic inflammation on insulin sensitivity in horses and inflammatory cytokine expression in adipose tissue. *Am J Vet Res* 2008;69:130-139.
90. Tiley HA, Geor RJ, McCutcheon LJ. Effects of dexamethasone on glucose dynamics and insulin sensitivity in healthy horses. *Am J Vet Res* 2007;68:753-759.

91. Freestone JF, Beadle R, Shoemaker K, et al. Improved insulin sensitivity in hyperinsulinaemic ponies through physical conditioning and controlled feed intake. *Equine Vet J* 1992;24:187-190.
92. Goodpaster BH, Kelley DE, Wing RR, et al. Effects of weight loss on regional fat distribution and insulin sensitivity in obesity. *Diabetes* 1999;48:839-847.
93. Ryan AS, Nicklas BJ. Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women. *Diabetes Care* 2004;27:1699-1705.
94. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 2002;346:393-403.
95. Longland AC, Byrd BM. Pasture nonstructural carbohydrates and equine laminitis. *J Nutr* 2006;136:2099S-2102S.
96. Sommardahl CS, Frank N, Elliott SB, et al. Effects of oral administration of levothyroxine sodium on serum concentrations of thyroid gland hormones and responses to injections of thyrotropin-releasing hormone in healthy adult mares. *Am J Vet Res* 2005;66:1025-1031.
97. Oppenheimer JH, Schwartz HL, Lane JT, et al. Functional relationship of thyroid hormone-induced lipogenesis, lipolysis, and thermogenesis in the rat. *J Clin Invest* 1991;87:125-132.
98. Frank N, Sommardahl CS, Eiler H, et al. Effects of oral administration of levothyroxine sodium on concentrations of plasma lipids, concentration and composition of very-low-density lipoproteins, and glucose dynamics in healthy adult mares. *Am J Vet Res* 2005;66:1032-1038.
99. Frank N, Elliott SB, Boston RC. Effects of long-term oral administration of levothyroxine sodium on glucose dynamics in healthy adult horses. *Am J Vet Res* 2008;69:76-81.
100. Frank N, Buchanan BR, Elliott SB. Effects of long-term oral administration of levothyroxine sodium on serum thyroid hormone concentrations, clinicopathologic variables, and echocardiographic measurements in healthy adult horses. *Am J Vet Res* 2008;69:68-75.
101. Nathan DM, Buse JB, Davidson MB, et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2009;32:193-203.
102. Durham AE, Rendle DI, Newton JE. The effect of metformin on measurements of insulin sensitivity and beta cell response in 18 horses and ponies with insulin resistance. *Equine Vet J* 2008;40:493-500.
103. Firshman AM, Hustace JL, Peterson K, et al. The effect of metformin on insulin sensitivity in horses. American College of Veterinary Internal Medicine 2009.
104. Hustace JL, Firshman AM, Mata JE. Pharmacokinetics and bioavailability of metformin in horses. *Am J Vet Res* 2009;70:665-668.
105. Miyazaki Y, Mahankali A, Matsuda M, et al. Improved glycemic control and enhanced insulin sensitivity in type 2 diabetic subjects treated with pioglitazone. *Diabetes Care* 2001;24:710-719.

106. Rosenblatt S, Miskin B, Glazer NB, et al. The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. *Coron Artery Dis* 2001;12:413-423.
107. Yamasaki Y, Kawamori R, Wasada T, et al. Pioglitazone (AD-4833) ameliorates insulin resistance in patients with NIDDM. AD-4833 Glucose Clamp Study Group, Japan. *Tohoku J Exp Med* 1997;183:173-183.
108. Willson TM, Lambert MH, Kliewer SA. Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem* 2001;70:341-367.
109. Staels B, Fruchart JC. Therapeutic roles of peroxisome proliferator-activated receptor agonists. *Diabetes* 2005;54:2460-2470.
110. Kramer D, Shapiro R, Adler A, et al. Insulin-sensitizing effect of rosiglitazone (BRL-49653) by regulation of glucose transporters in muscle and fat of Zucker rats. *Metabolism* 2001;50:1294-1300.
111. Cabrero A, Laguna JC, Vazquez M. Peroxisome proliferator-activated receptors and the control of inflammation. *Curr Drug Targets Inflamm Allergy* 2002;1:243-248.
112. Berger JP, Petro AE, Macnaul KL, et al. Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. *Mol Endocrinol* 2003;17:662-676.
113. Rangwala SM, Lazar MA. Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. *Trends Pharmacol Sci* 2004;25:331-336.
114. Peraldi P, Spiegelman B. TNF-alpha and insulin resistance: summary and future prospects. *Mol Cell Biochem* 1998;182:169-175.
115. Steppan CM, Bailey ST, Bhat S, et al. The hormone resistin links obesity to diabetes. *Nature* 2001;409:307-312.
116. Berger J, Tanen M, Elbrecht A, et al. Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11beta -hydroxysteroid dehydrogenase type 1 expression and activity. *J Biol Chem* 2001;276:12629-12635.
117. Eckland D, Danhof M. Clinical pharmacokinetics of pioglitazone. *Exp Clin Endocrinol Diabetes* 2000;108:S234-242.
118. Aronoff S, Rosenblatt S, Braithwaite S, et al. Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. *Diabetes Care* 2000;23:1605-1611.
119. Szeles L, Torocsik D, Nagy L. PPARgamma in immunity and inflammation: cell types and diseases. *Biochim Biophys Acta* 2007;1771:1014-1030.
120. Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004;351:1106-1118.
121. Hanefeld M. Pharmacokinetics and clinical efficacy of pioglitazone. *Int J Clin Pract Suppl* 2001:19-25.
122. Budde K, Neumayer HH, Fritsche L, et al. The pharmacokinetics of pioglitazone in patients with impaired renal function. *Br J Clin Pharmacol* 2003;55:368-374.
123. Scheen AJ. Thiazolidinediones and liver toxicity. *Diabetes Metab* 2001;27:305-313.
124. Ikeda H, Taketomi S, Sugiyama Y, et al. Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. *Arzneimittelforschung* 1990;40:156-162.
125. Sugiyama Y, Shimura Y, Ikeda H. Effects of pioglitazone on hepatic and peripheral insulin resistance in Wistar fatty rats. *Arzneimittelforschung* 1990;40:436-440.

126. Sugiyama Y, Taketomi S, Shimura Y, et al. Effects of pioglitazone on glucose and lipid metabolism in Wistar fatty rats. *Arzneimittelforschung* 1990;40:263-267.
127. Vidal-Puig AJ, Considine RV, Jimenez-Linan M, et al. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest* 1997;99:2416-2422.
128. Berger JP, Akiyama TE, Meinke PT. PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci* 2005;26:244-251.

Chapter 2

Pharmacokinetics of pioglitazone after multiple oral dose administration in horses.

J. M. G. Wearn[1], M. V. Crisman[1], J. L. Davis[2], R. J. Geor[3], D. R. Hodgson[1], J. K. Suagee[3], M. Ashraf-Khorassani[4] and L. J. McCutcheon[5].

1. Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
2. Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.
3. Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
4. Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
5. Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Running title: pioglitazone pharmacokinetics in horses

Keywords: pioglitazone, insulin resistance, horses, equine metabolic syndrome, thiazolidinedione

1. Abstract

Pioglitazone is a thiazolidinedione class of anti-diabetic agent with proven efficacy in increasing insulin sensitivity in humans with non-insulin dependent diabetes mellitus, a syndrome of insulin resistance sharing similarities with equine metabolic syndrome. The purpose of this study was to determine the pharmacokinetics of pioglitazone in adult horses following multiple oral dose administration. Pioglitazone hydrochloride (1 mg/kg) was administered orally for 11 doses at 24 h intervals and plasma samples were collected. Initially, a pilot study was performed using one horse; and thereafter the drug was administered to six horses. Samples were analyzed by liquid chromatography with tandem mass spectrometry and pharmacokinetic parameters were calculated using non-compartmental modeling. The maximum plasma concentration was 509.1 ± 413.5 ng/mL achieved at 1.88 ± 1.39 h following oral administration of the first dose, and 448.1 ± 303.5 ng/mL achieved at 2.83 ± 1.81 h (mean ±SD) following the eleventh dose. Apparent elimination half-life was 9.94 ± 4.57 and 9.63 ± 5.33 h after the first and eleventh dose respectively. This study showed that in healthy horses pioglitazone administered at a daily oral dose of 1 mg/kg results in plasma concentrations and total drug exposure approximating, but slightly below, those considered therapeutic in humans.

2. Introduction

Pioglitazone ([(±)-5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4-] thiazolidinedione monohydrochloride) is a member of the thiazolidinedione (TZD) class of oral anti-diabetic agents used in human medicine to treat non-insulin dependent (type 2) diabetes mellitus (NIDDM) by improving insulin sensitivity (Yamasaki et al., 1997; Miyazaki et al., 2001; Rosenblatt et al., 2001). In human medicine, pioglitazone is administered orally at 15 or 45 mg once daily dosing, and pharmacokinetic studies have been published (Eckland & Danhof, 2000; Kalliokoski et al., 2008). Thiazolidinediones (TZDs) are synthetic activators of the peroxisome proliferator-activated receptor-γ (PPARγ), a class of nuclear receptor (Willson et al., 2001). Pioglitazone binds to these receptors and acts as a transcription factor with regulatory action on more than 100 genes associated with glucose and lipid metabolism, and inflammation (Berger et al., 2003; Rangwala & Lazar, 2004). By modulating expression of these genes, TZDs improve insulin sensitivity and reduce hyperglycemia without affecting pancreatic β cell insulin secretion, therefore minimizing the risk of inducing hypoglycemia (Aronoff et al., 2000; Eckland & Danhof, 2000).

Equine metabolic syndrome (EMS) is a disorder of adult horses characterized by insulin resistance (IR), regional or generalized obesity and increased susceptibility to laminitis (Johnson, 2002). Although EMS likely involves different pathophysiology than NIDDM of humans, insulin resistance is an integral component of both NIDDM and EMS (Geor, 2008). Hyperinsulinemia develops as a physiological compensatory mechanism for reduced tissue insulin sensitivity in horses with EMS (Hoffman et al., 2003). Hyperinsulinemia, through a multitude of metabolic and vascular effects, has been proposed as the link between insulin resistance and the development of laminitis (Asplin et al., 2007; Frank, 2009; Geor & Frank, 2009). Further, obesity and predisposition to laminitis have been independently associated with a systemic pro-inflammatory state, as evidenced by elevated inflammatory cytokines identified in the circulation of obese and laminitis prone ponies (Geor, 2008; Treiber et al., 2009). This inflammatory process

is postulated to contribute to vascular dysfunction and thus heightened susceptibility to laminitis (Geor, 2008).

The TZD class represents a potential therapeutic option for horses with EMS specifically targeting the molecular origin of the metabolic dysfunction of this disorder. To the authors' knowledge, the TZD class has not yet been investigated in horses. Safety and efficacy of pioglitazone have been investigated in animal and human experimental and clinical trials of NIDDM (Ikeda et al., 1990; Sugiyama et al., 1990; Sugiyama et al., 1990; Aronoff et al., 2000; Eckland & Danhof, 2000; Budde et al., 2003; Zhou et al., 2007; Lu et al., 2008). With the drug soon to be available as a generic preparation, pioglitazone may become an affordable therapeutic option targeting the reduction of insulin resistance and hyperinsulinemia in horses with EMS. The lack of pharmacokinetic data currently precludes the use of this insulin sensitizing agent in horses. A recent study investigating the pharmacokinetics of metformin, an orally administered antihyperglycemic agent widely used in humans with NIDDM, suggested the drug failed to reach therapeutic concentrations in horses (Hustace et al., 2009). The objective of the present study was to determine the pharmacokinetic disposition of pioglitazone after oral administration to normal adult horses at a dose of 1 mg/kg at 24 h intervals for 11 doses and to investigate for any significant differences in pharmacokinetic parameters that develop over 11 days of administration. Because concentrations have been reported in other species to vary over the dosing period(Budde et al., 2003), plasma concentrations and pharmacokinetics were determined following the first and eleventh doses, and statistical comparisons were made between single and multiple oral dose administration.

3. Materials And Methods

3.1 Pilot Study

A pilot study was performed to establish the safety of orally administered pioglitazone and to determine whether plasma concentrations would approximate human therapeutic concentrations when administered to horses. One horse (6 year old, Quarter Horse, mare) was administered pioglitazone hydrochloride (Actos®, 45 mg tablets, Takeda Pharmaceuticals North America, Inc., Deerfield, IL) at a dose of 1 mg/kg at 24 h intervals for 5 doses. The dose rate of 1 mg/kg administered enterally at 24 h intervals was empirically extrapolated from reports in other species (laboratory rodents (Ikeda et al., 1990; Sugiyama et al., 1990; Sugiyama et al., 1990), pigs (Lu et al., 2008), monkeys (Zhou et al., 2007), and humans (Eckland & Danhof, 2000; Budde et al., 2003; Kalliokoski et al., 2008)). The tablets were crushed, suspended in 500 mL water, and administered intragastrically via a nasogastric tube; the tube was then flushed with 1.5 L of water to clear drug residue from the tube. The horse was maintained on a diet of timothy hay and feed was withheld for a period of 4 hours before and after administration of the drug. Water and an iodized salt block were available at all times. Serial blood samples were collected via an indwelling jugular catheter at 0 (pre-treatment), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 7, 12, 16 and 24 h on days 1 and 5. Additionally, peak and trough (2 and 24 h respectively) concentrations were determined on days 2, 3 and 4, and samples were collected 48 and 72 hours after the fifth dose to ensure adequate characterization of the elimination phase. Blood was centrifuged within 5 min at 3000 x g for 10 min. Plasma was stored in 1.5 mL aliquots and was frozen at -80^{o}C until assayed. Physical examinations were performed twice daily, complete blood count was performed on day -1 and biochemical monitoring including plasma testing for AST, GGT, SDH, bile acids, and ammonia concentrations were performed on days -1, 3, 5 and 8 of the drug administration period. The mare was euthanized at the completion of the study due to chronic lameness. A necropsy examination was performed.

3.2 Animals

Six healthy, non-pregnant, Thoroughbred mares from a maintained herd of research animals with age and body weight of 14.6 ± 4.1 years and 602 ± 38 kg (mean ± SD) respectively, were enrolled. Physical examination of each horse prior to commencement of the study identified no abnormal findings. The horses were weighed on the day prior to commencement of the study and on day 10. The horses were housed in a dry lot for 1 month prior to the commencement of the study and throughout the study except during days 0 – 5 and 11, when the horses were housed individually in 3.6 m^2 box stalls. The horses were acclimated to all procedures and locations prior to commencement of the study. Throughout the study period, including days of drug administration, horses were not fasted so as to mimic natural conditions of this drug's clinical application, and had *ad libitum* access to mixed alfalfa/grass hay, water, and iodized salt. The horses were provided 2.2 kg of a forage balancer concentrate once daily.

The Virginia Polytechnic Institute and State University, Institutional Animal Care and Use Committee approved the use of animals for this study and all study protocols. The study was undertaken at the Middleburg Agricultural Research and Extension Center, in Middleburg, VA.

3.3 Drug Administration

Pioglitazone hydrochloride (Actos®, 45 mg tablets, Takeda Pharmaceuticals North America, Inc., Deerfield, IL) tablets were crushed, and then administered orally via oral dose syringe at 1 mg/kg (range 0.94-1.13 mg/kg) at 24 h intervals for a total of 11 doses, in an inert carrier (unflavoured gelatin) mixed immediately prior to delivery.

3.4 Blood Collection

A 14 gauge, 5 ¼ inch, polyurethane catheter (Milacath®, MILA International, Inc., Erlanger, KY) was placed in the left jugular vein using sterile technique the day prior to pharmacokinetic analysis on days 1 and 11 and was maintained in situ for 48 hours. Blood samples were collected via the indwelling jugular catheter at 0 (pre-treatment),

0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16 and 24 hours following the first and eleventh doses administered. Blood was placed into sterile tubes containing lithium heparin (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ), immediately stored at 4°C and centrifuged within 15 minutes at 3000 x g and 4°C for 10 min. Plasma was stored in 1.5 mL aliquots and was frozen at -80°C until assayed by liquid chromatography with tandem mass spectrometry/mass spectrometry (LC-MS/MS).

Based upon results of the pilot study, peak plasma concentration was expected at approximately 2 h post dose administration. Thus, plasma was collected to determine peak and trough (2 and 24 h post administration, respectively) concentrations of pioglitazone following doses 2, 4 and 5 to assess for variations in drug absorption or accumulation. Direct jugular venipuncture was performed for collection of blood samples when a catheter was not in place.

3.5 Drug Analysis

Plasma concentrations of pioglitazone were determined by LC-MS/MS after liquid-liquid extraction according to a previously established and validated technique (Shen et al., 2003; Christensen et al., 2005). Rosiglitazone maleate ((±)-5-[[4-[2-(methyl-2-pyridinylamino)ethoxy]phenyl]methyl]-2,4-thiazolidinedione, (Z)-2-butenedioate) was used as the internal standard (IS). Pioglitazone hydrochloride reference standard and rosiglitazone maleate internal standard was purchased from Molcan Corp. Toronto, Ontario, Canada. Samples were prepared as previously reported (Wang & Miksa, 2007). Briefly, reference and internal standard, were prepared in 60:40 (v:v) of acetonitrile (ACN):H_2O. Samples were prepared by combining 100 µL of plasma, 100 µL internal standard (1 ng/mL) and 300 µL of ACN in 1.8 mL microcentrifuge tubes (Eppendorf Flex-Tubes® Eppendorf North America, Westbury, NY). Each sample was then vortexed for 10 s before being centrifuged (Eppendorf Microcentrifuge Model 5415c, Eppendorf North America, Westbury, NY) at 12,000 x g for 8 min. The supernatant solution was transferred into a clean 2 mL vial and the solvent was evaporated to dryness under a stream of compressed nitrogen at 45°C. The residue

was reconstituted in 1 mL of 60:40 (v:v) ACN:H_2O and loaded into the LC-MS/MS system.

Sample extracts were subjected to chromatographic separation performed with an Agilent 1100 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) with a C18 column (Luna®, 150 mm length, 2.1 mm diameter, 4 μm dp; Phenomenex, Torrance, CA). Twenty μL of sample was injected onto the column using an autosampler (Thermo Survey, San Jose, CA) maintained at 10°C. Mobile phase A consisted of 1% (v:v) aqueous formic acid, and mobile phase B consisted of 1% (vol/vol) formic acid in ACN. The mobile phase was delivered to the HPLC column at a flow rate of 0.2 mL/min. The gradient elution program was as follows: time 0: 70/30% A/B; time 4 min:40/60% A/B; time 7 min: 90/10% A/B; time 9 min: 90/10% A/B; time 11 min: 70/30% A/B; time 17min: 70/30% A/B. The HPLC column effluent was pumped directly without any split into a triple quadrapole mass spectrometer (Thermo Instrument TSQ Finnigan, San Jose, CA) equipped with electrospray ionization source, which was used in positive ion mode using multiple reaction monitoring (MRM). Commercial software (Thermo Electron Xcalibur® version 1.4, Thermo Electron Corporation San Jose Products, Somerset, NJ), was used to analyze the data. The instrument was calibrated with a solution of polytyrosin according to the manufacturer's recommendation. Tuning was performed on each analyte by direct infusion of standard solution (1 ng/μL) at a rate of 10 μL/min. Table 6 shows the tuning parameters for each analyte. Also, Table 6 shows the parent ion and product ion transition for each analyte when operated with MRM mode. Mass spectrometry parameters use for detection of pioglitazone were: Spray Voltage: 4100 V; Sheath Gas Pressure: 49 arbitrary units; Auxillary Gas Pressure :13 arbitrary units; and capillary temperature : 300°C.

Calibration curves, with a range of 1-200 ng/mL, were run before and after every 35 samples for quality control purposes. A linear calibration curve was constructed using regression analysis (Excel 2008, Microsoft corporation, Redmond, WA) to determine pioglitazone concentration in samples based on the sample/IS ratio.

Pioglitazone concentrations above the range of the calibration curve were diluted 1:10 with ACN so that their concentration fell within the limits of the linear portion of the calibration curve. The coefficient of determination (R^2) for all curves was >0.99, and all standard values were within ± 12% of the expected range. The system had a limit of detection (LOD) of 0.4 ng/mL, as determined by the signal to noise ratio, and the limit of quantification (LOQ), determined by the lowest concentration on a linear regression line of the calibration curve, was 1 ng/mL. Average extraction efficiency of the drug in plasma, assessed by spiking equine plasma with known concentrations of reference standards, was 95.6 ± 6.2% (mean ± SD).

3.6 Pharmacokinetic Analysis

Noncompartmental pharmacokinetic analysis was performed using commercially available software (WinNonlin, version 4.0, Pharsight corp., Mountain View, CA). Pharmacokinetic parameters determined included time to maximum plasma concentration (T_{max}), maximum plasma concentration (C_{max}), slope of the terminal phase (λ), half-life of the terminal phase ($t_{1/2\lambda}$), area under the concentration-time curve from 0-24 h (AUC_{0-24}), area under the first moment-time curve (AUMC), and mean residence time (MRT).

Pharmacokinetic parameters calculated for day 1 and day 11 sampling were compared using commercial statistical software (JMP 7.0, SAS Analytical, Cary, NC) using a paired *t*-test, except for AUMC. Values of AUMC varied significantly from the normal distribution and were compared using Wilcoxon signed-rank test. Chi-square test was used to evaluate if peak plasma concentrations achieved by individual horses were different on days 1, 2, 4, 5 and 11 of dosing. Hypothesis tests were assessed with 2 tailed analysis, and results for all analyses were considered statistically significance if $P < 0.05$.

4. Results

4.1 Pilot Study

No clinically detectable adverse effects were observed and the horse remained euglycemic throughout drug administration. Hematological and biochemical monitoring identified no values outside the laboratory reference intervals throughout the course of drug administration. The concentration-versus-time curves for pioglitazone after oral administration were determined for days 1 and 5 of administration, and are represented in Figure 1. The relevant pharmacokinetic parameters are summarized in Table 7. Maximum plasma concentrations of pioglitazone achieved were 102 ng/mL, occurring at 1.5 h after oral administration of the first dose, and 123 ng/mL occurring at 3 h following the fifth dose. Following the fifth dose, drug was still detectable at 48 h (0.9 ng/mL), but not at 72 h post administration (Appendix A, Table 23). Necropsy examination identified no pathological renal or hepatic abnormalities.

4.2 Pharmacokinetics

No clinically detectable adverse effects were identified in any of the experimental horses during the 2 week study period. Pioglitazone was rapidly absorbed with detectable pioglitazone concentrations present at 15 min following oral administration of the first dose in all six horses. Drug was still detectable in 5 of the 6 horses for 24 h following administration of the first and eleventh doses (Appendix B, Table 24). The relevant pharmacokinetic parameters are summarized in Table 8. Values of T_{max} (P =0.179), C_{max} (P =0.436), λ (P =0.677), $t_{1/2\lambda}$ (P =0.913), AUC_{0-24} (P =0.190), AUMC (P =0.438), or MRT (P =0.894) for individual horses were not different on day 1 and 11. The maximum plasma concentration of pioglitazone was 509.1 ± 413.5 and 448.1 ± 303.5 ng/mL (mean ± SD) achieved at 1.88 ± 1.39 and 2.83 ± 1.81 h (mean ± SD) following oral administration of the first and eleventh doses, respectively. Apparent elimination half life ($t_{1/2}$) was 9.94 ± 4.57 and 9.63 ± 5.33 h (mean ± SD) after the first and eleventh doses, respectively. The concentration-versus-time curves for pioglitazone after oral administration of the first and eleventh doses are represented in Figure 2 and

for each horse on days 1 and 11 of dosing in Appendix B, Figures12 and 13, respectively.

Peak concentrations achieved by each horse varied between the sampling days (P <0.001). The difference in maximum and minimum peak plasma concentration achieved by each horse compared to mean C_{max} of each horse was 62% ± 48% during the main study and 42% ±0.9% (mean ± SD) during the pilot study.

5. Discussion

The results of this study indicate that pioglitazone is absorbed following oral administration to horses and that multiple doses did not cause clinically detectable adverse effects. As such, the authors propose that pioglitazone should be investigated as a potentially useful treatment for insulin resistance in horses with EMS. To the authors' knowledge, this is the first report of administration of pioglitazone to horses. As such, a pilot study was performed on one horse to determine whether the drug was detectable in plasma after oral administration and whether the drug would induce any adverse effects during the treatment period. Reported adverse effects of pioglitazone therapy in humans include weight gain, vascular volume expansion and edema (Rangwala & Lazar, 2004; Yki-Jarvinen, 2004). Troglitazone, the first clinically available TZD, was associated with life threatening hepatotoxicity in humans and for this reason was withdrawn from the market. Pioglitazone has been proven to not share this hepatotoxic profile in humans (Scheen, 2001; Budde et al., 2003). However, to assess for any hepatotoxicity, in the pilot horse, hepatocellular and hepatobilliary specific biochemical indices were monitored and a necropsy examination was performed. No evidence of hepatic pathology was detected on biochemical analysis or histopathology, however the duration of drug administration was relatively brief (5 days). While no adverse effects as seen in humans were noted in this study, the small number of animals investigated in this study precludes categorically concluding that the drug will be safe in all horses.

Results of this study indicated that pioglitazone peak plasma concentrations are lower compared to reported values in humans, with an extrapolated dose comparison. Table 9 summarizes reported human pharmacokinetic parameters of doses considered therapeutically effective. Human doses range from 15-45 mg orally once daily, approximated to be 0.2-0.65 mg/kg assuming the average 70 kg human. Oral administration of 1 mg/kg to horses achieved similar mean peak concentrations on day one compared to humans receiving a single dose of 0.2 mg/kg (509 and 617 ng/mL, respectively). However the peak plasma concentrations achieved in this study after oral administration varied widely (range 103.8-1164 ng/mL) and only 2 of 6 horses achieved maximum plasma concentrations greater than the mean C_{max} reported for humans at 0.2 mg/kg. Comparison of the AUC_{0-24} for horses receiving multiple doses of 1 mg/kg with the AUC_{0-24} in humans receiving multiple doses of 0.65 mg/kg shows a lower mean AUC in horses (2282 versus 14565 h•ng/mL, respectively) and greater variability with the range in horses in the present study being 352-5792 h•ng/mL and versus 9333-18395 h•ng/mL in humans. The lower AUC suggests a lower bioavailability, larger volume of distribution or more rapid metabolism or elimination in horses compared to humans (Kalliokoski et al., 2008). The variability in pharmacokinetic parameters noted in this study is not an uncommon finding with oral drug administration to horses (Davis et al., 2006). Numerous factors influence peak plasma concentration, including bioavailability, rate of absorption and drug distribution. Feeding is associated with differences in the absorption of a number of drugs administered orally to horses. During the pilot study, the drug was administered via a nasogastric tube to ensure the entire dose was administered and feed was withheld for 4 hours before and after drug administration. This was not continued for the remainder of the study and the drug was administered orally via dose syringe as the clinical application of the drug necessitates oral administration. Feed was also not withheld during the remainder of the study to mimic the drug's clinical application. Feeding did not appear to adversely affect the extent of, or variability in, drug absorption as higher plasma pioglitazone concentrations were achieved when feed was not withheld, when compared to when feed was withheld during the pilot study. The reason for this difference is not known. Values of T_{max} and

$t_{1/2\lambda}$ in studied horses (Table 8) were similar to reported pharmacokinetic studies in humans (Table 9) (Eckland & Danhof, 2000; Kalliokoski et al., 2008).

In this study, no significant differences in the pharmacokinetic parameters were detected between values determined after the first and eleventh doses. This is a similar finding to studies in humans of repeat dosing, where a slight decrease in AUC_{0-24} was the only difference in a 10 day dosing study (Budde et al., 2003). The small sample size in our study combined with the large individual horse variability, however, may have resulted in an insufficient statistical power to detect significant differences in pharmacokinetic variables, if such differences existed.

Pioglitazone is extensively (>97%) bound to plasma proteins in rodents and humans (Eckland & Danhof, 2000). Due to this extensive protein binding, the volume of distribution is low at 0.253L/kg in healthy humans (Eckland & Danhof, 2000). Oral bioavailability in humans is reported to be 83% and administration with food slightly delayed the rate of absorption, but did not significantly affect the extent of absorption (Eckland & Danhof, 2000). Lack of an available intravenous formulation of the medication precluded bioavailability analysis, as well as determination of a true volume of distribution and clearance, in this study.

The drug undergoes significant hepatic metabolism via hydroxylation and oxidation resulting in a number of known metabolites (M1-M6). M3 and M4 demonstrate pharmacological activity in diabetic animal models and retain 40-60% of pioglitazone parent compound hypoglycemic potential (ED50). M2 has twice the potency for lowering triglycerides as the parent compound, while the potency of M3 and M4 was slightly less than the parent compound (Eckland & Danhof, 2000). Standards for these metabolites could not be sourced, and therefore could not be analyzed.

In summary, the results of this study suggest that pioglitazone administered orally to horses at 1 mg/kg at 24 h intervals for 11 doses resulted in no observed adverse effects. This study provides evidence of attainment of plasma concentrations in horses similar to, although lower, than those considered therapeutic in humans. However

further investigation of the pharmacodynamic effects of pioglitazone in horses is required investigating whether this orally administered dose achieves therapeutic efficacy in horses. The pharmacokinetic profile was similar to reported values in humans with proven therapeutic efficacy of increased insulin sensitivity in insulin resistant individuals. A higher dose rate may be appropriate for future studies, as the peak plasma concentrations achieved and systemic drug exposure, as estimated by $AUC_{0\text{-}24}$, were at the lower end of the reported plasma values considered therapeutic in humans. Pioglitazone offers a novel therapeutic option for the management of EMS targeted at the suspected molecular origin of the disorder. Management strategies, specifically exercise and weight loss through dietary modification remain the cornerstone of treatment for insulin resistance in horses with EMS. Additionally, pharmacologic intervention has a role in the treatment of insulin resistance in horses with EMS and humans with NIDDM. The authors suggest that further investigation of the therapeutic efficacy of pioglitazone in horses with EMS is warranted; however further safety investigation of a specific dose and for longer durations is required before clinical use of this agent will be considered safe in this species.

6. Acknowledgements

This research was supported by a grant from the Center for Equine Research in Virginia. The authors would like to thank Elaine Meilahn, Tracy Smith, Robert Drake, Maureen Perry and Becky Wade for technical assistance.

7. References

Aronoff, S., Rosenblatt, S., Braithwaite, S., Egan, J.W., Mathisen, A.L. & Schneider, R.L. (2000). Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. *Diabetes Care*, **23**(11), 1605-1611.

Asplin, K.E., Sillence, M.N., Pollitt, C.C. & McGowan, C.M. (2007). Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *Vet J*, **174**(3), 530-535.

Berger, J.P., Petro, A.E., Macnaul, K.L., Kelly, L.J., Zhang, B.B., Richards, K., Elbrecht, A., Johnson, B.A., Zhou, G., Doebber, T.W., Biswas, C., Parikh, M., Sharma, N., Tanen, M.R., Thompson, G.M., Ventre, J., Adams, A.D., Mosley, R., Surwit, R.S. & Moller, D.E. (2003). Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. *Mol Endocrinol*, **17**(4), 662-676.

Budde, K., Neumayer, H.H., Fritsche, L., Sulowicz, W., Stompor, T. & Eckland, D. (2003). The pharmacokinetics of pioglitazone in patients with impaired renal function. *Br J Clin Pharmacol*, **55**(4), 368-374.

Christensen, M.L., Meibohm, B., Capparelli, E.V., Velasquez-Mieyer, P., Burghen, G.A. & Tamborlane, W.V. (2005). Single- and multiple-dose pharmacokinetics of pioglitazone in adolescents with type 2 diabetes. *J Clin Pharmacol*, **45**(10), 1137-1144.

Davis, J.L., Little, D., Blikslager, A.T. & Papich, M.G. (2006). Mucosal permeability of water-soluble drugs in the equine jejunum: a preliminary investigation. *J Vet Pharmacol Ther*, **29**(5), 379-385.

Eckland, D. & Danhof, M. (2000). Clinical pharmacokinetics of pioglitazone. *Exp Clin Endocrinol Diabetes*, **108**(Supp/2), S234-242.

Frank, N. (2009). Equine metabolic syndrome. *Journal of Equine Veterinary Science*, **29**(5), 259-267.

Geor, R. & Frank, N. (2009). Metabolic syndrome-From human organ disease to laminar failure in equids. *Vet Immunol Immunopathol*, **129**(3-4), 151-154.

Geor, R.J. (2008). Metabolic Predispositions to Laminitis in Horses and Ponies: Obesity, Insulin Resistance and Metabolic Syndromes. *J. Equine Vet Sci*, **28**(12), 753-759.

Hoffman, R.M., Boston, R.C., Stefanovski, D., Kronfeld, D.S. & Harris, P.A. (2003). Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci*, **81**(9), 2333-2342.

Hustace, J.L., Firshman, A.M. & Mata, J.E. (2009). Pharmacokinetics and bioavailability of metformin in horses. *Am J Vet Res*, **70**(5), 665-668.

Ikeda, H., Taketomi, S., Sugiyama, Y., Shimura, Y., Sohda, T., Meguro, K. & Fujita, T. (1990). Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. *Arzneimittelforschung*, **40**(2 Pt 1), 156-162.

Johnson, P.J. (2002). The equine metabolic syndrome peripheral Cushing's syndrome. *Vet Clin North Am Equine Pract*, **18**(2), 271-293.

Kalliokoski, A., Neuvonen, M., Neuvonen, P.J. & Niemi, M. (2008). No significant effect of SLCO1B1 polymorphism on the pharmacokinetics of rosiglitazone and pioglitazone. *Br J Clin Pharmacol*, **65**(1), 78-86.

Lu, L., Reiter, M.J., Xu, Y., Chicco, A., Greyson, C.R. & Schwartz, G.G. (2008). Thiazolidinedione drugs block cardiac KATP channels and may increase propensity for ischaemic ventricular fibrillation in pigs. *Diabetologia*, **51**(4), 675-685.

Miyazaki, Y., Mahankali, A., Matsuda, M., Glass, L., Mahankali, S., Ferrannini, E., Cusi, K., Mandarino, L.J. & DeFronzo, R.A. (2001). Improved glycemic control and enhanced insulin sensitivity in type 2 diabetic subjects treated with pioglitazone. *Diabetes Care*, **24**(4), 710-719.

Rangwala, S.M. & Lazar, M.A. (2004). Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. *Trends Pharmacol Sci*, **25**(6), 331-336.

Rosenblatt, S., Miskin, B., Glazer, N.B., Prince, M.J. & Robertson, K.E. (2001). The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. *Coron Artery Dis*, **12**(5), 413-423.

Scheen, A.J. (2001). Thiazolidinediones and liver toxicity. *Diabetes Metab*, **27**(3), 305-313.

Shen, Z., Reed, J.R., Creighton, M., Liu, D.Q., Tang, Y.S., Hora, D.F., Feeney, W., Szewczyk, J., Bakhtiar, R., Franklin, R.B. & Vincent, S.H. (2003). Identification of novel metabolites of pioglitazone in rat and dog. *Xenobiotica*, **33**(5), 499-509.

Sugiyama, Y., Shimura, Y. & Ikeda, H. (1990). Effects of pioglitazone on hepatic and peripheral insulin resistance in Wistar fatty rats. *Arzneimittelforschung*, **40**(4), 436-440.

Sugiyama, Y., Taketomi, S., Shimura, Y., Ikeda, H. & Fujita, T. (1990). Effects of pioglitazone on glucose and lipid metabolism in Wistar fatty rats. *Arzneimittelforschung*, **40**(3), 263-267.

Treiber, K., Carter, R., Gay, L., Williams, C. & Geor, R. (2009). Inflammatory and redox status of ponies with a history of pasture-associated laminitis. *Vet Immunol Immunopathol*, **129**(3-4), 216-220.

Wang, M. & Miksa, I.R. (2007). Multi-component plasma quantitation of anti-hyperglycemic pharmaceutical compounds using liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, **856**(1-2), 318-327.

Willson, T.M., Lambert, M.H. & Kliewer, S.A. (2001). Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem*, **70**, 341-367.

Yamasaki, Y., Kawamori, R., Wasada, T., Sato, A., Omori, Y., Eguchi, H., Tominaga, M., Sasaki, H., Ikeda, M., Kubota, M., Ishida, Y., Hozumi, T., Baba, S., Uehara, M., Shichiri, M. & Kaneko, T. (1997). Pioglitazone (AD-4833) ameliorates insulin resistance in patients with NIDDM. AD-4833 Glucose Clamp Study Group, Japan. *Tohoku J Exp Med*, **183**(3), 173-183.

Yki-Jarvinen, H. (2004). Thiazolidinediones. *N Engl J Med*, **351**(11), 1106-1118.

Zhou, R., Bruns, C.M., Bird, I.M., Kemnitz, J.W., Goodfriend, T.L., Dumesic, D.A. & Abbott, D.H. (2007). Pioglitazone improves insulin action and normalizes menstrual

cycles in a majority of prenatally androgenized female rhesus monkeys. *Reprod Toxicol*, **23**(3), 438-448.

8. Tables and Figures

Table 6. Parent ion and product ion transition for each analyte when operated with MRM mode.

Analyte	Parent Ion (amu)	Product Ion (amu)	Collision Energy (eV)	Q2 Collision gas Pressure
Pioglitazone	357.00	134.00	27	1.2
Rosiglitazone	358.00	135.00	21	1.2

Table 7. Noncompartmental pharmacokinetic parameters for pioglitazone after intragastric administration of 1 mg/kg at 24 h intervals to 1 normal horse for 5 doses.

Pharmacokinetic variable	**Dose 1**	**Dose 5**
T_{max} (h)	3	1.5
C_{max} (ng/mL)	102.1	123.5
λ (h^{-1})	0.06	0.09
$t_{1/2\lambda}$ (h)	11.16	8.07
AUC_{0-24} (h•ng/mL)	1332.82	1268.78
AUMC (h•h•ng/mL)	13221.54	10813.85
MRT (h)	17.53	12.13

T_{max} = time to maximum plasma concentration; C_{max} = maximum plasma concentration; λ = slope of the terminal phase; $t_{1/2\lambda}$ = half-life of terminal phase; AUC_{0-24} = area under the concentration-time curve from 0-24 hours; AUMC = area under the first moment-time curve; MRT = mean residence time.

Table 8 Noncompartmental pharmacokinetic parameters (mean ± SD) for pioglitazone after single and multiple oral dose administration of 1 mg/kg at 24 h intervals to 6 normal horses.

Pharmacokinetic variable	Dose 1	Dose 11
T_{max} (h)	1.88 ± 1.39	2.83 ± 1.81
C_{max} (ng/mL)	509.1 ± 413.5	448.07 ± 303.48
λ (h^{-1})	0.08 ± 0.03	0.09 ± 0.04
$t_{1/2\lambda}$ (h)	9.94 ± 4.57	9.63 ± 5.33
AUC_{0-24} (h•ng/mL)	2756.52 ± 2315.54	2882.09 ± 2443.06
AUMC (h•h•ng/mL)	18636.77 ± 15845.38	20932.49 ± 18986.34
MRT (h)	11.56 ± 3.30	11.98 ± 6.82

T_{max} = time to maximum plasma concentration; C_{max} = maximum plasma concentration; λ = slope of the terminal phase; $t_{1/2\lambda}$ = half-life of terminal phase; AUC_{0-24} = area under the concentration-time curve from 0-24 hours; AUMC = area under the first moment-time curve; MRT = mean residence time.

Table 9. Reported human pharmacokinetic data for orally administered pioglitazone.

Reference	Budde et al. 2003	Budde et al. 2003	Eckland & Danhof, 2000	Kalliokoski et al., 2008
Oral dose	45 mg single dose	45 mg multiple dose	45 mg single dose	15 mg single dose
T_{max} (h) median (range)	2 (1-4)	3.5 (1-4)	3 (2-5)	1.8 (1-4)
C_{max} (ng/mL) mean ± SD	1392 ± 667	1587 ± 225	1482 ± 500	617 ±129
$t_{1/2}$ (h) mean ± SD	13.7± 5.7	11.1± 5.5	9.2 ± 7.5	7.9 ± 2.5
AUC_{0-24} (h•ng/mL) mean (range) or mean ± SD	17387 (11161-27034)	14565 (9333-18395)	13854 ± 4996	
AUC_{0-72} (h•ng/mL) mean ± SD				6228 ± 1892

Figure 1. Plasma concentration vs time curve for pioglitazone hydrochloride (1mg/kg) administered intragastrically for 5 doses. Data represent one horse.

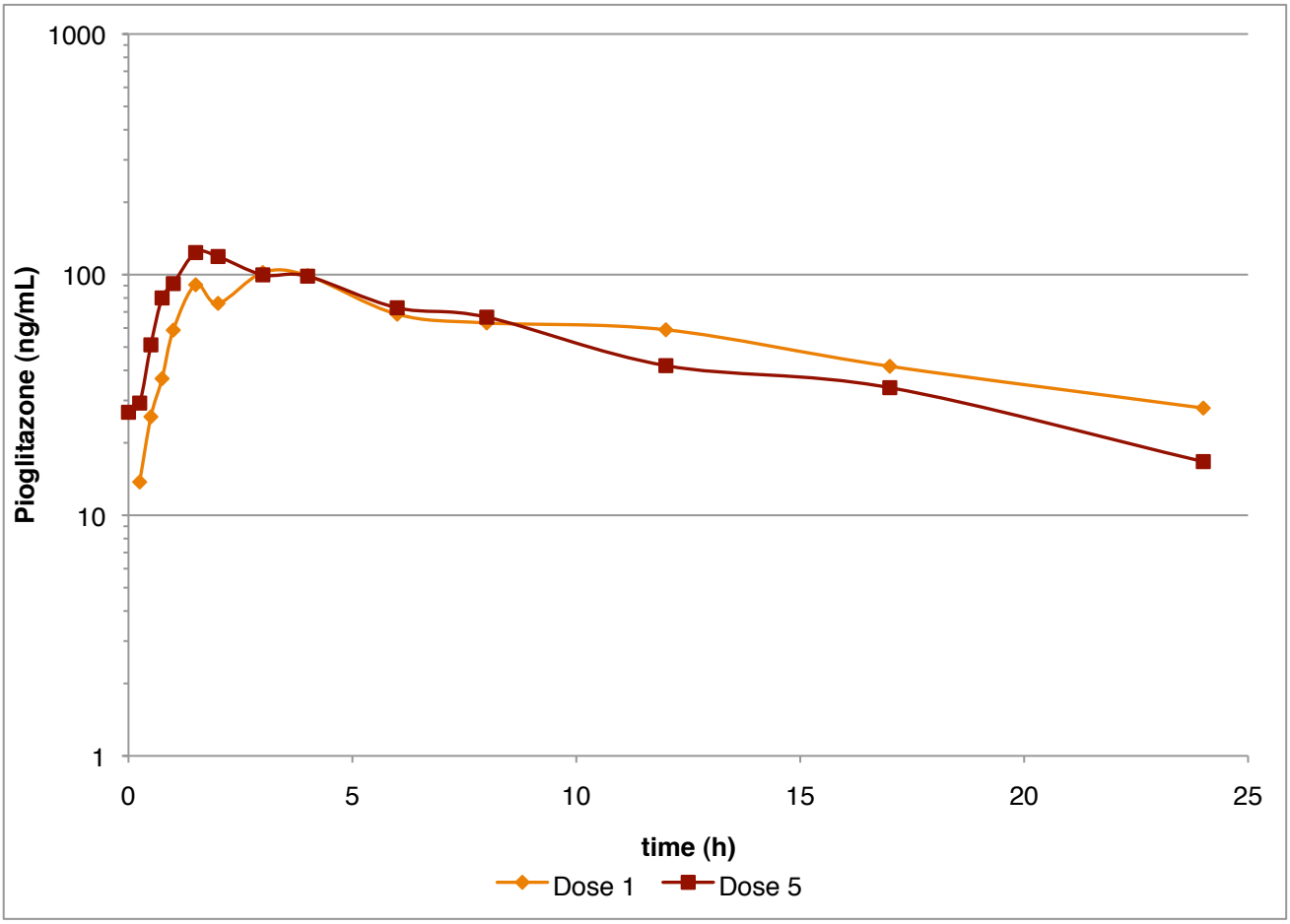
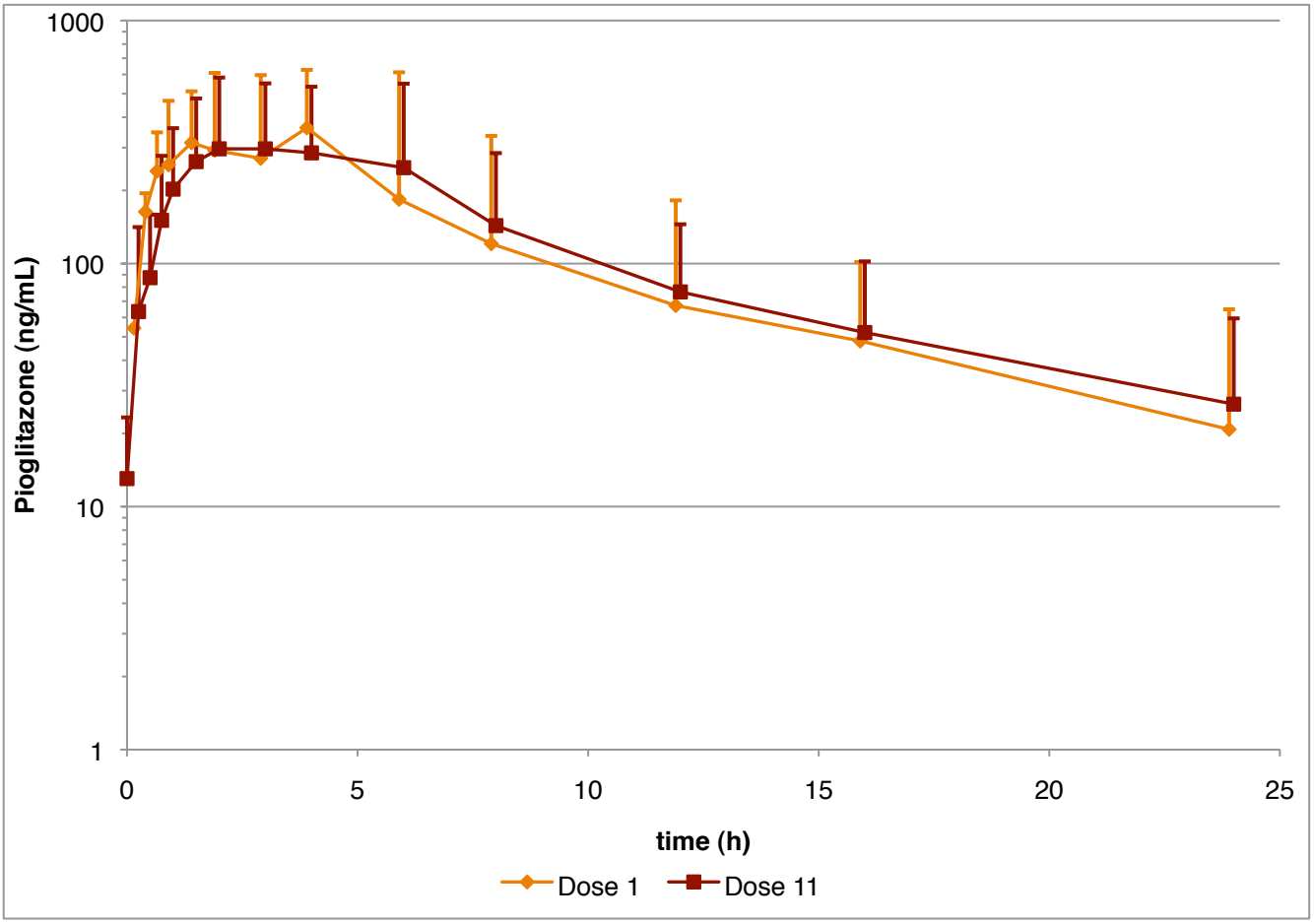


Figure 2. Plasma concentration vs time curve for pioglitazone hydrochloride (1mg/kg) administered orally. Data represent mean + SD for six horses.



Chapter 3

Effects of pioglitazone on clinical parameters and minimal model parameters of the frequently sampled intravenous glucose tolerance test following intravenous endotoxin infusion, as a model of induced insulin resistance.

J. M. G. Wearn[1], M. V. Crisman[1], J. L. Davis[2], R. J. Geor[3], D. R. Hodgson[1], J. K. Suagee[3], S. Were[4] and L. J. McCutcheon[4].

1. Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
2. Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.
3. Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
4. Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Running title: pioglitazone pharmacodynamics in horses

Keywords: pioglitazone, insulin resistance, horses, equine metabolic syndrome, thiazolidinedione

1. Study Objectives

The objectives of this study were to evaluate the effects of orally administered pioglitazone on clinical examination variables, indices of glucose and insulin dynamics, and plasma lipids following an endotoxin infusion administered IV; an experimental model of induced insulin resistance. We hypothesized that pioglitazone would (1) preserve indices of glucose-insulin homeostasis, as measured by frequently-sampled intravenous glucose tolerance (FSIGT) test with minimal model (minmod) analysis, (2) minimize aberrations in clinical examination and hematological variables and (3) mitigate alterations in plasma lipids (non-esterified fatty acids (NEFA) and triglycerides), following an endotoxin challenge model of induced insulin resistance. Minimal model analysis of a FSIGT test was selected to evaluate this hypothesis. This test was selected as it allows not only assessment of insulin sensitivity, but in addition, three further indices of glucose-insulin homeostasis not available with other methods. Endotoxin infusion model was selected as a proven, repeatable, minimally invasive, low risk method of induction of insulin resistance, able to be standardized between horses. The development of antibodies against endotoxin after IV administration in some horses, precluded a cross over study design.

2. Material and methods

2.1 Animals

Sixteen, healthy, non-pregnant, Thoroughbred mares from a maintained herd of research animals with age and body weight range of 8-21 years and 528 – 672 kg respectively, were enrolled. Horses were weighed prior to and on days 10 and 12 of the study. No abnormalities were detected on physical examination in any of the horses prior to commencement of the study. Body condition score was estimated by two independent observers, using the scale of 1-9 as described by Henneke et al.[1] Horses were housed in a dry lot for one month prior to the study and throughout the study

except during days 0 – 5 and 11-15, when the horses were housed individually, in 3.6 m^2 box stalls. Horses were acclimated to all procedures and locations prior to commencement of the study. Throughout the study period, horses had *ad libitum* access to mixed alfalfa/grass hay, water, and iodized salt. Horses were provided 2.2 kg of a forage balancer concentrate once daily.

The Virginia Polytechnic Institute and State University, Institutional Animal Care and Use Committee approved study protocols. The study was undertaken at the Middleburg Agricultural Research and Extension Center, in Middleburg, VA, USA.

2.2 Experimental Design

Eight horses (group 1) were randomly allocated to receive pioglitazone hydrochloride[a] (1 mg/kg, orally, q 24 h for 14 days), and 8 horses were allocated to serve as controls (group 2) (Table 3). An infusion of endotoxin (35ng/kg) was administered on day 13 as an insulin resistance induction challenge (Appendix C, Table 25). The horses were also randomly allocated into 4 groups (A-D) (n=4) that were staggered by 4-7 days to facilitate testing constraints. A FSIGT test was performed three times on each horse, day 0 (time 1, FSIGT 1), day 12 (time 2, FSIGT 2) and day 14 (time 3, FSIGT 3) (Figure 3). The FSIGT was performed on day 0 (FSIGT 1) as a baseline for each horse for comparison throughout the study, and to ensure homogeneity within the groups. Changes between FSIGT 1 and FSIGT 2 represented any effect of pioglitazone on normal horses, and were compared to the control group. The change in parameters of the FSIGT test performed before, (time 2), and after, (time 3), endotoxin challenge was assessed. The results were analyzed to evaluate the effects of pioglitazone to mitigate against the development of insulin resistance following endotoxin challenge. On days 0, 12 and 14 prior to the commencement of the FSIGT test, analysis of plasma NEFA and triglyceride concentrations were performed.

2.3. Drug Administration

Pioglitazone hydrochloride[a] was administered orally at 0700 h via oral dose syringe at 1 mg/kg q 24 hours for 14 doses in an inert carrier (unflavoured gelatin) mixed immediately prior to delivery.

2.4 Frequently sampled intravenous glucose tolerance test procedure

The horses were moved into individual box stalls on the day prior to FSIGT test procedure. A 14 gauge 5 ¼ inch, polyurethane catheter[b] was placed in the left jugular vein using sterile technique on days 0 and 11 and was maintained for a maximal period of 4 days. To maintain patency of the catheter, 1.5 mL sodium heparin[c] (1000 iU/mL) was injected into the catheter every 12 hours whilst not in use. On each occasion this was aspirated prior to lavaging the catheter with heparinized saline. An extension set, injection cap, three way stopcock were attached to the indwelling jugular catheter.

During the experiment horses were provided with mixed grass/alfalfa hay and water *ad libitum*. Each horse was weighed on the day prior to each test. All FSIGT tests commenced between 0730 and 0830 h. FSIGT test protocol was based upon that described originally in humans[2] and modified for application in horses[3]. Briefly ten and five minutes prior to dextrose infusion blood was collected via the catheter for baseline blood glucose and insulin assessment. A bolus dose of 300mg glucose/kg body weight (50% [wt/vol] dextrose solution[d]) was administered as rapidly as possible (duration of infusion time <2 minutes). The extension set and catheter were then flushed with 30mL heparianized saline (0.9% NaCl[d] + 10IU/mL heparin sodium[c]) to clear any residual glucose from the line. Time 0 was set at the completion of the dextrose infusion. Blood samples were then collected via the catheter and extension set at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 19 minutes. At 20 minutes regular insulin[e] (30mU/kg body weight diluted to 10mL with 0.9% sodium chloride) was rapidly infused (duration of infusion < 30 seconds), immediately followed by 30mL heparianized saline to clear any residual insulin from the line. Blood samples were subsequently collected at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes. At each time point 12mL

of blood was withdrawn from the line (3x volume of the catheter and extensions set) and discarded. A 10mL blood sample was then collected, followed by infusion of 15mL of heparinized saline to clear blood from the extension set and catheter. Blood was placed into glass tubes containing sodium heparin[c] that were immediately cooled in an ice bath at 4°C. Blood samples were centrifuged at 1000x g and 4°C within 15 minutes of collection. Plasma was withdrawn and stored in 1.5mL aliquots at -80°C until analysis (within 1 month).

2.5 Lipopolysaccharide administration

Escherichia coli O55:B5 lipopolysaccharide (LPS) was supplied as 1mg/mL and was diluted with sterile saline solution to create a stock solution of 10,000ng/mL (Appendix H). The dose of 35ng/kg was diluted into 500mL 0.9% sterile saline and was administered as an infusion over 30 minutes on day 13. Time zero was set at commencement of infusion. After the completion of the infusion, a 500mL bolus of 0.9% saline was administered to ensure administration of residual LPS in the infusion line. Horses were observed for signs of colic (colic score **:** 0: normal ; 1: signs of mild depression, flehming, washing in water bucket, yawning; 2: agitation, pawing, flank watching; 3 : rolling) and physical examination variables (heart rate, respiratory rate, rectal temperature, intestinal motility, mucous membrane color, and capillary refill time) were recorded every 30 minutes for 4 hours.

2.6 White blood cell count analysis

Blood was collected from the indwelling jugular catheter and placed into tubes containing EDTA immediately before commencing the LPS infusion (commenced at time = 0) and then at 60, 120, and 180 minutes. Samples were cooled to 4°C, transported to the clinical pathology laboratory and analyzed for total leukocyte count (WBC) within 4 hours.

2.7 Plasma NEFA and Triglycerides

Blood was collected from the indwelling catheter and placed into tubes containing sodium heparin on days 0, 12 and 14 prior to the commencement of the FSIGT test.

Triglycerides and NEFA concentrations were assayed in duplicate using an enzymatic spectrophotometric[g, h, i] method and an automated analyzerr[j].

2.8 Plasma glucose and insulin concentrations

Plasma glucose, triglycerides and NEFA concentrations were assayed in duplicate using an enzymatic spectrophotometric[k, l, m] method and an automated analyzer[n]. Plasma insulin concentrations were determined using a solid phase radioimmunoassay[o] previously validated for determination of insulin in equine plasma.[4,5]

2.9 Interpretation of FSIGT test data via minimal model analysis

Glucose and insulin dynamics was assessed in accordance with the minimal model[2] designed for use in humans using commercially available software[p] and previously described methods for application in horses[3,6]. The model is used to calculate values for the insulin sensitivity (Si;$x10^{-4}$ L•min^{-1}•mU^{-1}); acute insulin response to glucose (AIRg; mU•L•min^{-1}); Glucose effectiveness (Sg; $x10^{-2}$ min^{-1}) and disposition index (DI; $x10^{-2}$ dimensionless).

3. Statistical analysis

Animals were randomly allocated to groups using a random number generator (JMP 7.0, SAS Analytical, Cary, NC). Age and body weight at commencement of the study, prior to, and following LPS infusion were compared between study groups and treatments using two way analysis of variance (ANOVA) using commercial statistical software (SAS v. 9.2, SAS Institute Inc. Cary, NC). Body condition score data was skewed from normality and lacked variance in certain groups, as such the Wilcoxon 2-sample test was used to compare between treatments and the Kruskal-Wallis test was used to compare between groups. Repeated measures ANOVA was used to compare body weight at times 1, 2 and 3.

For minmod parameters repeated measures, mixed model ANOVA was used to compare 4 indices (AIRg, SI, DI, Sg) between treatment groups at baseline and to

determine if an individual's values differed between FSIGT 1 compared to FSIGT 2 for each of the 4 variables. Then, using FSIGT 1 (baseline) as a covariate repeated measures-mixed model analysis of covariance was used to compare treatment and control horses between FSIGT 2 and 3. For data of the $\Delta_{2\text{-}3}$ minmod parameters ($\Delta_{2\text{-}3}$ AIRg, $\Delta_{2\text{-}3}$ Si, $\Delta_{2\text{-}3}$ Sg, $\Delta_{2\text{-}3}$ DI), a mixed model ANOVA using Tukey-Kramer adjustment for multiple comparisons, least squares means for change in each of the variables between FSIGT 2 and FSIGT 3 ($\Delta_{2\text{-}3}$) was compared between treatments.

For clinical data (heart rate, respiratory rate and rectal temperature), repeated measures, mixed model ANOVA was used to evaluate the effect of time following endotoxin infusion and the effect of treatment. Data of respiratory rate were natural logarithm transformed to achieve normality. For categorical data of colic score, Friedman's chi square test was used to determine if scores differ over time within horses. Results of Δ_{max} clinical data was analyzed using mixed model ANOVA to compare maximal change in clinical parameters (Δ_{max} white blood cell count (WBC), Δ_{max} rectal temperature (Temp), Δ_{max} heart rate(HR)) between treatments.

Natural logarithmic transformation of the NEFA and triglyceride data was performed to achieve normality. NEFA and triglyceride data was compared using repeated measures mixed model analysis of variance (ANOVA) to evaluate for difference between treatments at baseline. Repeated measures mixed model analysis of covariance (ANCOVA) using FSIGT 1 as a covariate was used to evaluate for a difference prior to, and following, LPS administration and for differences between treatments at these time points. The change in NEFA and triglyceride concentration between FSIGT 2 and FSIGT 3 (sample taken at baseline) was calculated and presented as the $\Delta_{2\text{-}3}$ NEFA and $\Delta_{2\text{-}3}$ triglyceride, respectively. $\Delta_{2\text{-}3}$ NEFA and $\Delta_{2\text{-}3}$ triglyceride data were compared using mixed model ANOVA using Tukey-Kramer adjustment for multiple comparisons to compare least square means for change in each of the variables between time point 2 and time point 3 ($\Delta_{2\text{-}3}$) NEFA and triglyceride different between treatment and controls for each of the 2 variables.

Hypothesis tests were assessed with 2 tailed analysis, and results for all analyses were considered statistically significanct if $P < 0.05$. Data presented as mean ± SD unless otherwise stated.

4. Results

4.1 Physical parameters of studied animals

Age of enrolled horses was 14.2 ± 3.2 years and 14.6 ± 4.0 years (Mean ± SD) in control and drug treated horses respectively (Appendix D, Table 26). There were no significant differences between groups A-D (P =0.321) nor between drug treated and control horses (p=0.836) for age of enrolled horses. Baseline body weight in control (576.5 ± 38.8 kg) and drug treated horses (602.4 ± 38.5 kg), was not significantly different between groups A-D (P =0.879) or between drug treated and control horses (P =0.246) (Appendix D Table 27 and 28). Nor at time points 2 and 3 did drug treated and control horses have different body weights, P =0.1204 and P =0.5610, respectively. There was no significant change in body weight over the course of the study in either control (P =0.156) or drug treated (P =0.137) horses. Body condition score was not different between the individual horses (n=16; P =0.999), between groups (P =0.4695) or between drug treated horses or their controls (P =0.711) (appendix D Table 29). Median (range) body condition score in drug treated and control horses was 5.75 (5-8) and 5.75 (5-7).

4.2 Doses administered

Pioglitazone was administered at 0.99 ± 0.08 mg/kg, 0.99 ± 0.05 mg/kg and 1.02 ± 0.07 mg/kg at time points 1, 2 and 3 respectively (Appendix D, Table 28). Doses administered to each horse were not significantly different from each other at any time point (P = 1.00). There was a significant effect of time on dose administered (P = 0.023) , however the magnitude of the difference was so small it was considered clinically insignificant (Appendix D, Table 30).

4.3 Physical examination data following LPS administration

Values of, and graphical representations of, each variable assessed during physical examinations following LPS infusion for each horse are presented in Appendix F (Tables 31-35 and Figures 14-18). Measurements of baseline physical examination variables and hematological analyses were compared between drug treated and control horses, with no significant differences identified (Table 10).

Baseline WBC were not different between drug treated and control groups (p=0.542). Within drug treated (P = <0.0001) and control (P = <0.0001) groups, white blood cell count differed over time. Significant differences (decrease) were recorded at 60 (P = < 0.0001, P = < 0.0001) and 90 (P = < 0.0001, P = 0.003) minutes following initiation of LPS infusion in both drug treated and control groups respectively, but was not significantly different from baseline at 180 minutes (P = 0.999, P = 0.424). No effect of treatment over time was detected for WBC (P = 0.8254). At no time point was the mean WBC for drug treated and control horses significantly different.

A significant effect of time (P < 0.001) and treatment over time (P =0.0013) was identified in analysis of heart rate measurements following LPS administration. Within control horses, heart rate differed significantly from baseline at 90 (P = 0.032) and 120 (P = 0.032) minutes following LPS administration. Within drug treated horses heart rate differed (increased) from baseline at 60(P = 0.0005), 90(P < 0.0001), and 120 (P = 0.021) minutes. At 60 (P = 0.030) and 90 (P = 0.004) minutes mean heart rate in drug treated horses was significantly different from control horses.

In drug treated horses mean respiratory rate changed (elevated) significantly from baseline over time (P = 0.0213). This change was only recorded at one time point, 210 minutes following LPS initiation, with the respiratory rate in drug treated horses significantly different from baseline (P = 0.017). No such significant change over time or at any individual time point occurred in control horses (P = 0.767). At no individual time point were respiratory rates between drug treated and control horses significantly

different, and no effect of treatment over time for respiratory rate was recorded (P = 0.749).

Within both drug treated (P = <0.0001) and control (P = <0.0001) groups, measured rectal temperatures increased over time, with significant differences (elevation) in temperature recorded at 90 minutes following initiation of LPS infusion in both drug treated and control groups (P = <0.0001). Rectal temperatures were significantly different (P = <0.05) from baseline (elevated) in both drug treated and control group was 90 – 240 minutes. At no time point are the mean temperatures for drug treated and control groups significantly different (P = >0.05), and there was no effect of treatment over time (P = 0.769).

Colic scores differed from baseline in both control (P = <0.0001) and drug treated (P = 0.0003) horses following LPS infusion. In drug treated horses, colic scores were significantly different from baseline at 90 – 210 minutes, and in controls from 60-210 minutes.

To assess the effect of 12 days of pioglitazone therapy in mitigating the effects of LPS infusion-induced aberrations in physical examination variables, the maximal change in clinical parameters detected in the 4 hours following LPS administration was compared between treatment and control horses. No significant differences were detected in the Δ_{max} values between drug treated horses and controls for any of the clinical variables assessed (Table 11, Figure 4).

4.4 Minimal model parameters of the frequently sampled intravenous glucose tolerance tests.

Minimal model (minmod) parameters of the FSIGT were calculated for drug treated horses and their controls prior to the commencement of pioglitazone administration. Natural *log* transformation of the values for AIRg, DI and Sg was performed to achieve normality. Values for each parameter at baseline are presented in Table 12. Values of, and graphical representations of, each parameter for each individual horse are presented in Appendix G (Tables 36-43 and Figures 19-26).

Values of each of the four minmod parameters was not significantly different between drug treated and control horses at baseline time point 1 (Figure 5). Values for each minmod parameter was compared between time point 1 and time point 2 to determine whether there was any effect of treatment with 12 days of pioglitazone, and no significant difference was detected in either drug treated or control horses (Table 13).

Using minmod parameters at time point 1 as the covariate, ANCOVA was used to determine whether a significant difference existed between FSIGT 2 and FSIGT 3 for each of the 4 variables measured in the minmod analysis in drug treated horses and their controls. A significant difference (decrease) in SI was detected in both groups, indicating that the LPS infusion induced insulin resistance as expected. Significant differences were also detected for DI in both drug treated and control horses, and Sg in drug treated horses (Table 14). Using minmod1 as the covariate, ANCOVA identified no significant difference between drug treated horses and their controls for each of the 4 minmod parameters at FSIGT 2 and 3 (Table 15)

To assess the effect of 12 days of pioglitazone therapy in mitigating the effects of LPS induced aberration of glucose – insulin homeostasis, the change in minmod parameters was compared between time points 2 and 3. No significant differences were detected in the $\Delta_{2\text{-}3}$ values between drug treated horses and their controls for any of the 4 minmod parameters (Tables 16 and 17 and Figure 6-8).

4.5 Nonesterified Fatty Acids (NEFA) and Triglycerides

No significant differences in concentrations of NEFA or triglycerides were present at baseline between drug treated horses and their controls (Table 18, Figure 10) allowing time point 1 to be used as a covariate for ANCOVA analysis as individual horse values varied widely.

To assess for effect of treatment with pioglitazone for 12 days without other exogenous influences, comparison between time points 1 and 2 were made. No such effect of drug treatment was identified as no significant difference was identified for concentrations of NEFA or triglycerides between time points 1 and 2 (Table 19).

Significant differences existed between time 2 and time 3 in both drug treated (P = 0.043) and control (P = 0.024) horses. At no time point was a significant difference in plasma concentration of NEFA or triglycerides identified between drug treated and control horses. Using baseline concentrations as a covariate, ANCOVA identified a significant change in NEFA and triglyceride concentrations from time point 2 to time point 3, prior to, and following the LPS infusion respectively, for both triglyceride and NEFA data, except for NEFA in the control horses (Table 20). However, no significant differences were identified between drug treated and control horses at any of the two time points (Table 21). Furthermore, comparing the change in plasma concentrations in NEFA and triglycerides between time points 2 and 3 ($\Delta_{2\text{-}3}$ NEFA and triglycerides), there was no significant effect of drug treatment (Table 22, Figure 11).

4.6 Outlier analysis

One horse (horse # 15; control, group B) did not respond clinically to the LPS administration. This horse was determined to be a statistical outlier for all minmod and clinical parameters at the time point 3. As such this horse was removed from the analysis of the minmod, blood, lipid and clinical parameters.

5. Discussion

A number of aberrations in clinical, hematological, and blood lipid parameters are reported to occur following LPS administration to horses, namely development of fever, tachycardia, signs of mild abdominal pain, leucopenia and elevations in NEFA and triglycerides.[7] Furthermore, disturbances in insulin and glucose homeostasis, primarily characterized by reduced insulin sensitivity and increased AIRg, develop following LPS administration and are maximally affected at approximately 24 hours post LPS administration.[7]

Certain horses do not respond with the expected clinical, hematological and insulin resistance aberrations as expected to endotoxin infusion, and are described as LPS non-responders. These animals are thought to either possess preexisting anti-

lipopolysaccharide antibodies or have different LPS receptors or genetic polymorphism within intracellular LPS signal transduction pathway.[7]

The physical parameters of the enrolled horses, including age, body weight, body condition score, was equally distributed across treatment and control horses. At the initiation of this study, there were no significant differences in any clinical, hematologic or blood lipid parameter between treatment and control horses, supporting the effective random allocation of horses in this investigation. There was no significant change in bodyweight during the study, supporting the diet being supplied to horses accurately provided their maintenance requirements. The desired dose of 1mg/kg was able to be maintained throughout the dosing period.

At baseline prior to endotoxin infusion, all horses had normal clinical physical examination variables, that did not differ between treatment and control animals. Expected clinical aberrations were observed following LPS administration, with significant reduction in white blood cell count, elevations in heart rate, respiratory rate, rectal temperature and colic score being recorded. Similarly, significant reduction in SI and DI were observed. No significant change in AIRg was observed following LPS. No explanation for this can be offered.

The purpose of this study was to investigate the homeostatic protective effects of pioglitazone hydrochloride in an insulin resistance induction model using an endotoxin infusion model. The clinical application of this study was to investigate a possible therapeutic intervention for horses with insulin resistance associated with obesity. Obesity in horses, similarly to humans, is also associated with a systemic pro-inflammatory state. This dual disturbance of insulin resistance and inflammation was the basis for the selection of an LPS infusion as the experimental model, as LPS induces a systemic inflammatory state and insulin resistance.

There was no effect of administration of pioglitazone orally at 1mg/kg for 12 days prior to endotoxin infusion for either mean value of, or magnitude of the change prior to, of following the LPS administration for any studied variable in drug treated horses when

compared to controls. The authors' propose several possible reasons for this lack of identification of a significant effect of administration of a drug (pioglitazone hydrochloride) with proven efficacy in the improvement of insulin sensitivity in humans with type 2, non-insulin dependent diabetes;

1. The model we selected for induction of insulin resistance did not accurately mimic the insulin resistance that develops with type 2 diabetes in humans where the beneficial effects of pioglitazone have been proven.

2. The magnitude of the insulin resistance was too great and overwhelmed any effects of pioglitazone.

3. The speed with which the insulin resistance developed was too rapid to allow for clinical effects of pioglitazone. Insulin resistance in obesity is chronic, developing gradually over months to years, whereas profound insulin resistance developed within 24 hours following LPS administration.

4. The duration of treatment with pioglitazone was insufficient to have an identifiable clinical effect. In humans 6-8 months of treatment is required before significant effects are noted.

5. The dose rate administered to horses in this study resulted in plasma concentrations and total drug exposure lower than those described in humans where clinical benefits have been reported.

6. The individual horse variability in repeatability of minmod parameters was of sufficient magnitude to reduce statistical power. Thus althougha significant difference existed between drug treated and control horses, this was not able to be identified statistically.[6]

a. Actos®, 45mg tablets, Takeda Pharmaceuticals North America, Inc., Deerfield, IL.
b. Milacath®, MILA International, Inc., Erlanger, KY.
c. Heparin sodium, APP Pharmaceuticals, Schaumburg, IL
d. Baxter, Deerfield, IL.
e. Novolin R, Novo Nordisk Inc., Princeton, NJ.
f. BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ
g. Glucose Procedure No. 16-UV, Sigma Diagnostics, St. Louis, MO.
h. Triglyceride GPO reagent, Beckman Coulter, Inc., Fullerton, CA.
i. HR Series NEFA-HR(2), Wako Chemicals, Richmond, VA.
j. Beckman SYNCHRON CX®5 Delta autoanalyzer, Beckman Coulter, Inc., Fullerton, CA.
k. Glucose Procedure No. 16-UV, Sigma Diagnostics, St. Louis, MO.
l. Triglyceride GPO reagent, Beckman Coulter, Inc., Fullerton, CA.
m. HR Series NEFA-HR(2), Wako Chemicals, Richmond, VA.
n. Beckman SYNCHRON CX®5 Delta autoanalyzer, Beckman Coulter, Inc., Fullerton, CA
o. Coat-A-Count Insulin, Diagnostic Products Corp., Los Angeles, CA.
p. MinMod Millenium version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, PA.

6. References.

1. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* 1983;15:371-372.
2. Bergman RN, Phillips LS, Cobelli C. Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and beta-cell glucose sensitivity from the response to intravenous glucose. *J Clin Invest* 1981;68:1456-1467.
3. Hoffman RM, Boston RC, Stefanovski D, et al. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci* 2003;81:2333-2342.
4. Reimers TJ, Cowan RG, McCann JP, et al. Validation of a rapid solid-phase radioimmunoassay for canine, bovine, and equine insulin. *Am J Vet Res* 1982;43:1274-1278.
5. Freestone JF, Wolfsheimer KJ, Kamerling SG, et al. Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. *Equine Vet J* 1991;23:219-223.
6. Boston RC, Stefanovski D, Moate PJ, et al. MINMOD Millennium: a computer program to calculate glucose effectiveness and insulin sensitivity from the frequently sampled intravenous glucose tolerance test. *Diabetes Technol Ther* 2003;5:1003-1015.
7. Toth F, Frank N, Elliott SB, et al. Effects of an intravenous endotoxin challenge on glucose and insulin dynamics in horses. *Am J Vet Res* 2008;69:82-88.

7. Tables and Figures

Figure 3. Pharmacodynamic study design.

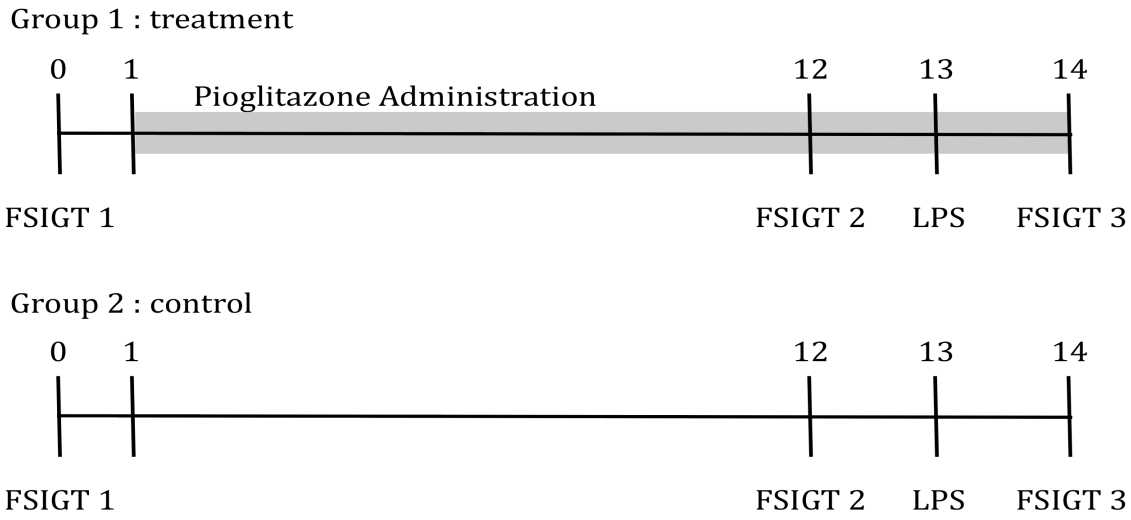


Table 10. Baseline physical examination parameters and statistical comparison

	Treatment		Control		*P* for comparison between drug treated and control horses
	Mean	SEM	Mean	SEM	
WBC cells/μL	6925.0	562.72	6508.59	594.24	0.542
HR beats per minute	41.75	1.7718	39.61	1.8747	0.358
***ln*RR** breaths per minute	2.485	0.187	2.617	0.194	0.454
Temperature °F	99.7	0.3052	99.8	0.316	0.862
Colic Score arbitrary units	0	0	0	0	1.000

Table 11. Statistical comparison of drug treated and control horses for values of Δ_{max} of clinical variables.

	Drug treated		Control		
	Mean	SEM	Mean	SEM	*P* (difference)
Δ_{max} WBC cells/μL	-2937.50	346.63	-2457.14	370.56	0.361
Δ_{max} Temperature °F	2.4250	0.339	2.257	0.357	0.669
Δ_{max} heart rate beats per minute	13.250	2.962	11.120	3.036	0.371

Figure 4. Comparison of Δ_{max} white blood cell count, temperature and heart rate between drug treated and control horses from time 2 to time 3, before and after LPS infusion.

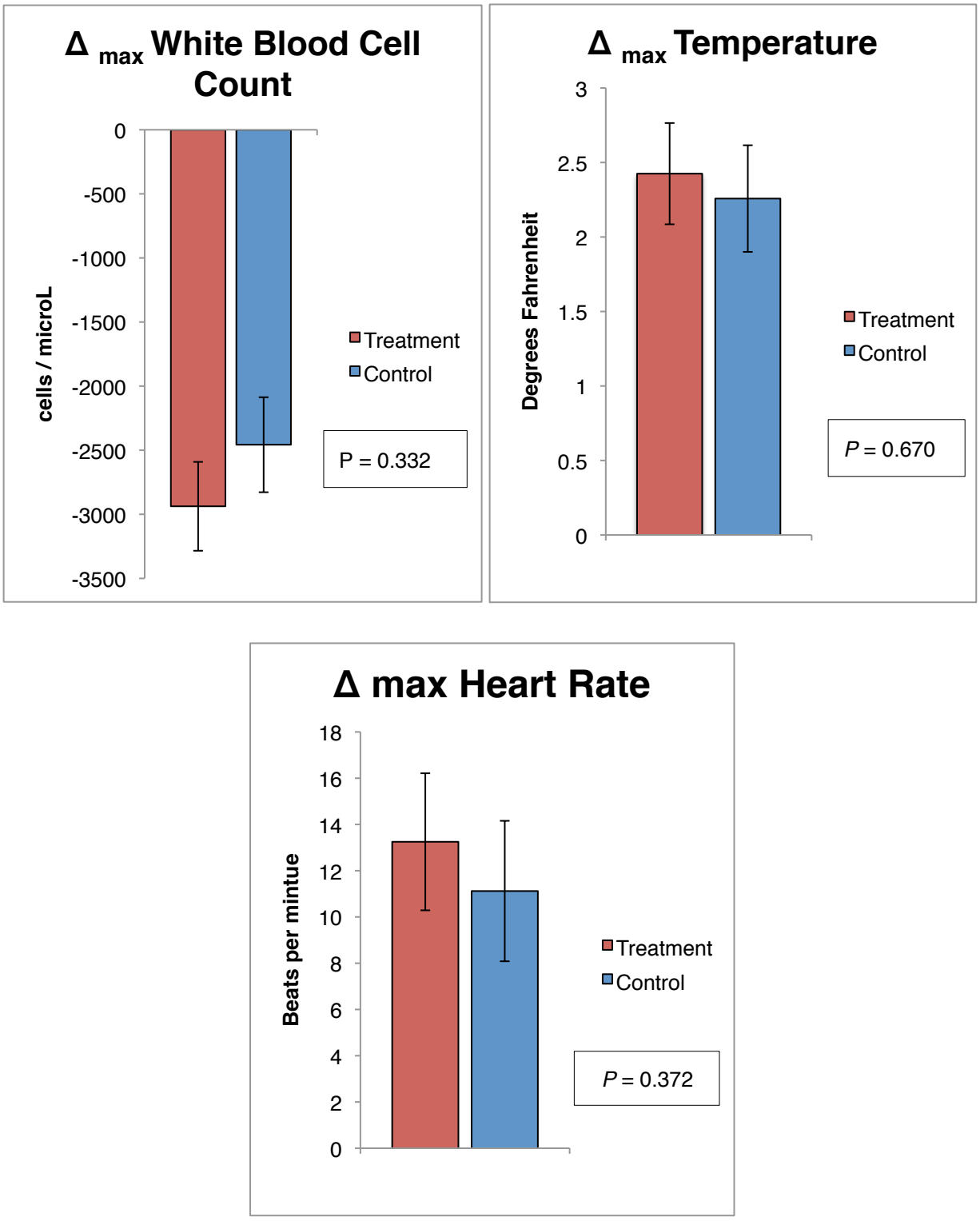


Table 12. Baseline (time1) minmod data : back transformed geometric least square mean ± 95% confidence interval (CI) of the geometric mean.

	Drug treated			Control			P(difference)
	Mean	*-ive 95% CI*	*+ive 95% CI*	Mean	*-ive 95% CI*	*+ive 95% CI*	
*ln*AIRg	6.35	5.81	6.88	6.59	6.01	7.16	0.525
*ln*DI	6.86	6.39	7.33	7.20	6.70	7.70	0.322
SI	2.53	1.65	3.41	2.29	1.35	3.23	0.717
*ln*Sg	-3.83	-4.26	-3.39	-3.90	-4.35	-3.46	0.666

Figure 5. Geometric least squares (LS) means of baseline minimal model parameters and repeated measures mixed model ANOVA to determine whether drug treated and control horses are different at baseline time point 1 for minmod parameters.

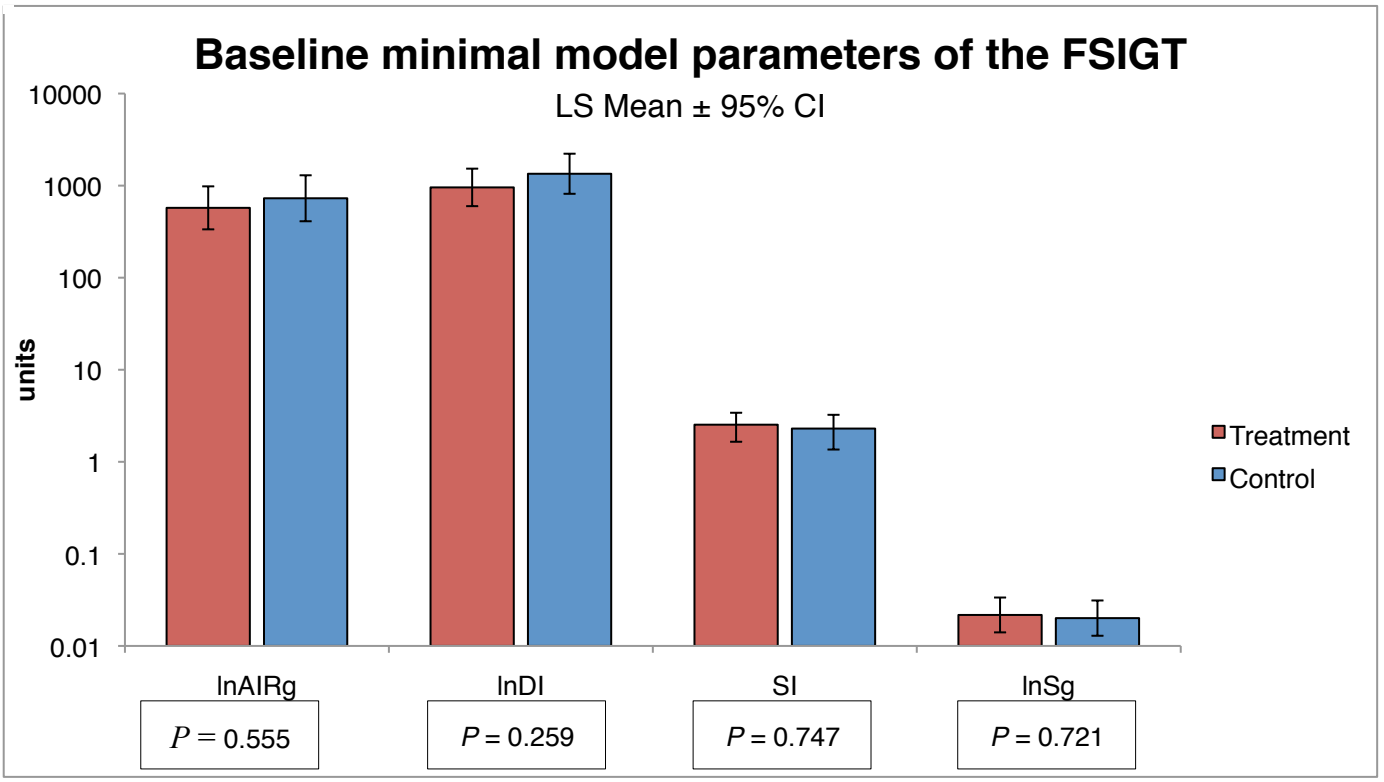


Table 13. Repeated measures mixed model ANOVA to determine whether minmod 1 is different from minmod 2 in drug treated and control horses

	P that minmod1 different from minmod 2 (time point 1 vs time point 2)			
	*ln*AIRg	*ln*DI	SI	*ln*Sg
Control	0.310	0.459	0.255	0.444
Drug treated	0.507	0.981	0.543	0.651

Table 14 : ANCOVA, using FSIGT 1 parameters as a covariate, to determine whether a significant difference exits between time points 2 and 3 for each of the minmod parameters.

	P that there was a change in value from time point 2 to time point 3			
	*ln*AIRg	*ln*DI	SI	*ln*Sg
Control	0.428	<0.0001	0.024	0.100
Drug treated	0.903	<0.0001	0.0005	0.016

Table 15. Using minmod1 as covariate, analysis of covariance was used to determine for each of the 4 variables measured with FSIGT and minmod analysis whether treatment and control horses differ at minmod 2 and 3.

	P that drug treated and control horses were different at time 2 and time 3			
	*ln*AIRg	*ln*DI	SI	*ln*Sg
FSIGT 2	0.529	0.777	0.174	0.888
FSIGT 3	0.978	0.764	0.887	0.384

Table 16. Least square mean and standard error of the mean of the $\Delta_{2\text{-}3}$ of the minmod parameters

	Drug treated		Control		
	Mean	SEM	Mean	SEM	P (difference)
$\Delta_{2\text{-}3}$ AIRg	-138.62	153.2	-45.82	154.69	0.236
$\Delta_{2\text{-}3}$ Si	-1.55	0.40	-1.022	0.42	0.240
$\Delta_{2\text{-}3}$ Sg	-0.01	0.0051	-0.01	0.0059	0.984
$\Delta_{2\text{-}3}$ DI	-979.42	230.87	-865.27	246.82	0.740

Table 17. Back-transformed geometric least square mean ± 95% CI of the geometric mean of the Δ_{2-3} of the minmod parameters

	Drug treated			Control			
	Mean	*-ive 95% CI*	*+ive 95% CI*	Mean	*-ive 95% CI*	*+ive 95% CI*	P (difference)
Δ_{2-3} AIRg	-138.62	-601.21	324.07	-45.82	-504.81	413.16	0.236
Δ_{2-3} Si	-1.55	-2.64	-0.46	-1.02	-2.11	0.071	0.240
Δ_{2-3} Sg	-0.01	-0.02	0.002	-0.01	-0.02	0.003	0.984
Δ_{2-3} DI	-979.42	-1478.2	-480.65	-865.27	-1398.48	-332.05	0.740

Figure 6. Geometric least square mean ± 95% CI of the geometric mean of the Δ_{2-3} of the minmod parameters and the repeared measures ANOVA probability that drug treated and control horses are significantly different.

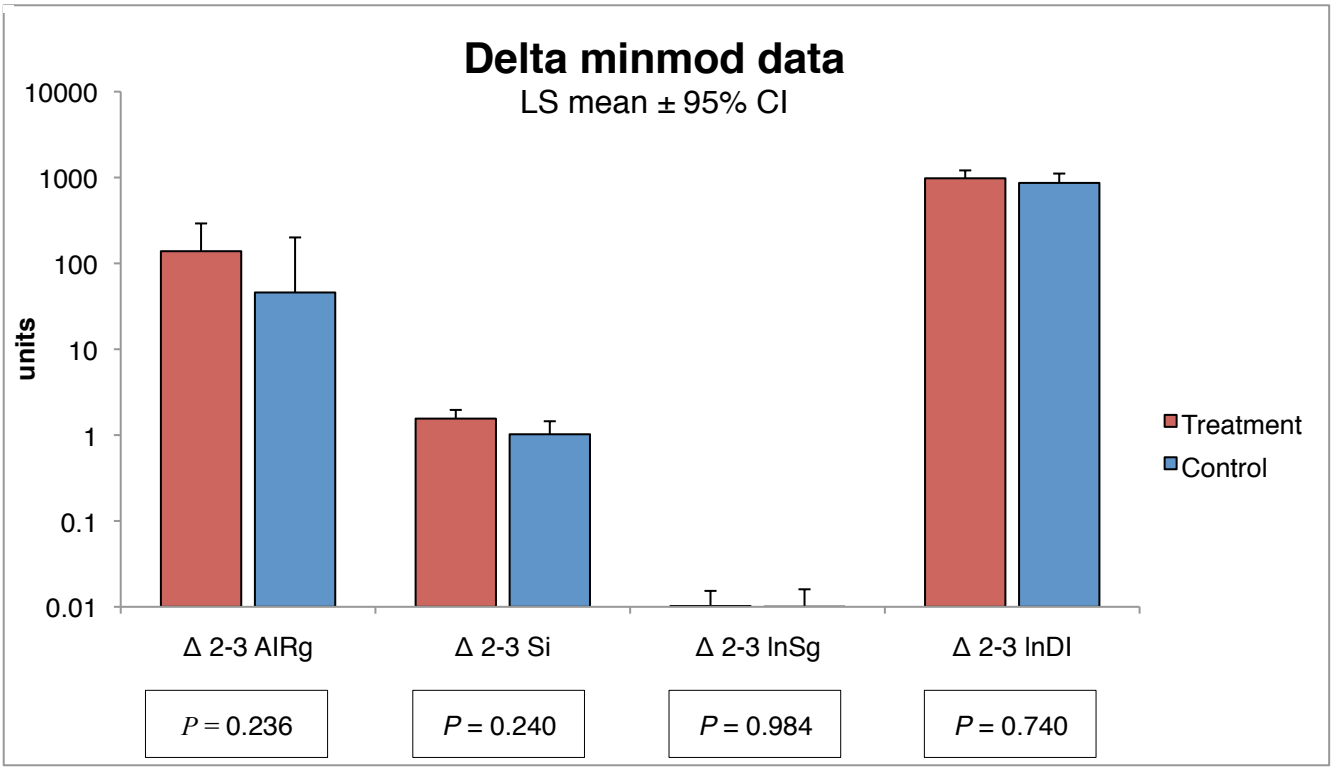


Figure 7. Comparison of $\Delta_{2\text{-}3}$ minimal model parameters (AIRg and DI) between drug treated and control horses from time 2 to time 3, before and after LPS infusion.

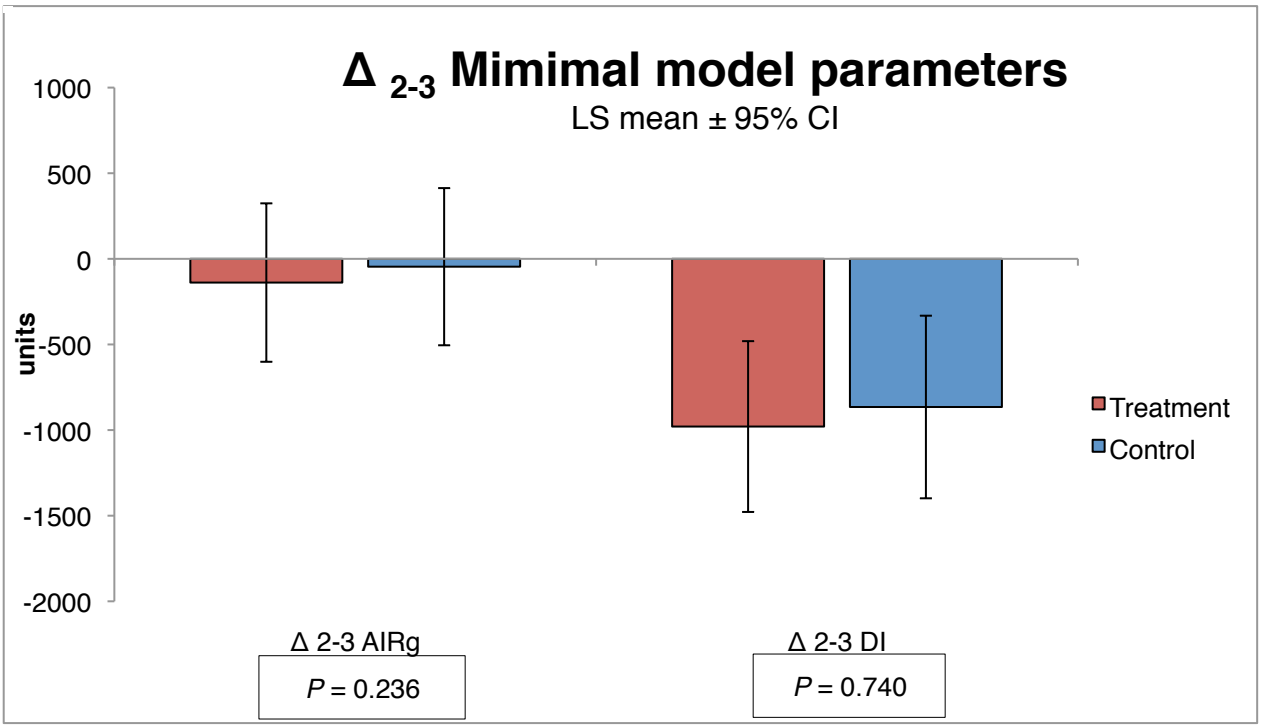


Figure 8. Comparison of $\Delta_{2\text{-}3}$ minimal model parameters (SI and Sg) between drug treated and control horses from time 2 to time 3, before and after LPS infusion.

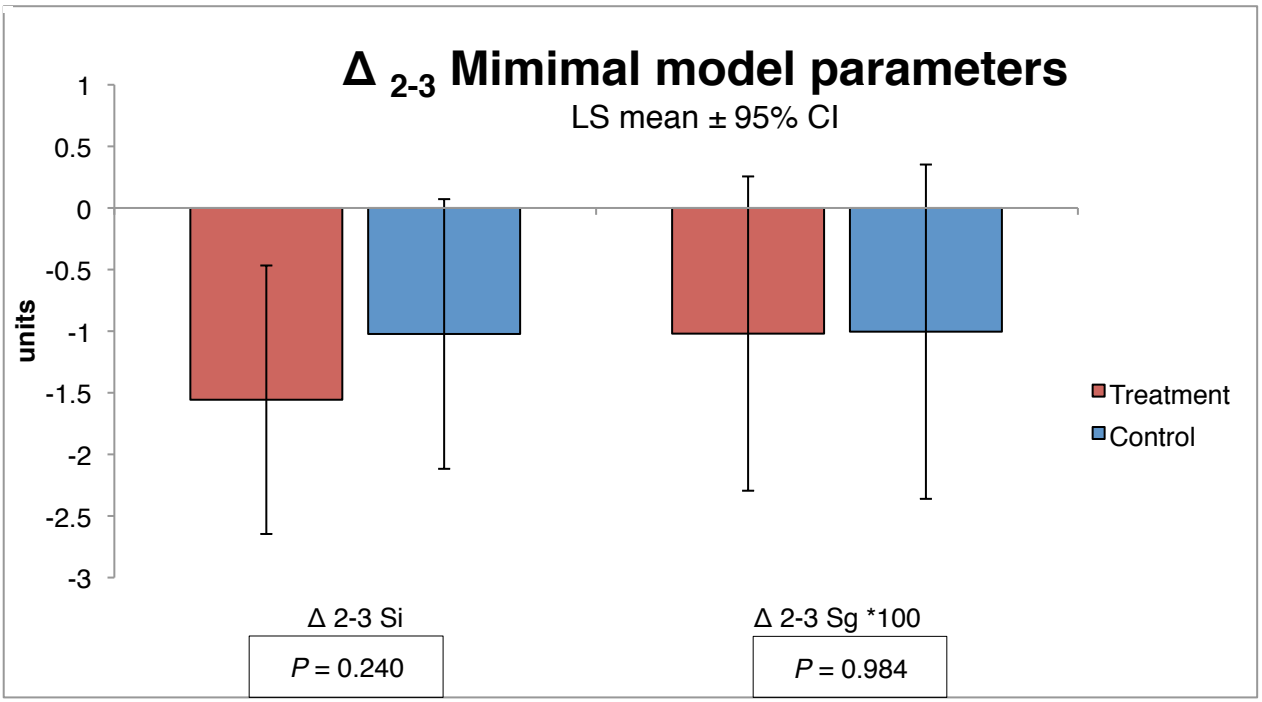


Table 18 : Statistical comparison of NEFA and triglyceride concentrations in pioglitazone treated horses and their controls at baseline.

	Drug treated		Control		*P* (difference)
	Mean	SEM	Mean	SEM	
*ln*NEFA	-2.45	0.19	-2.69	0.20	0.406
*ln*TRIG	*3.24*	*0.13*	*3.04*	*0.14*	0.332

Figure 9. Least squares means of baseline NEFT and triglyceride data in drug treated and control horses.

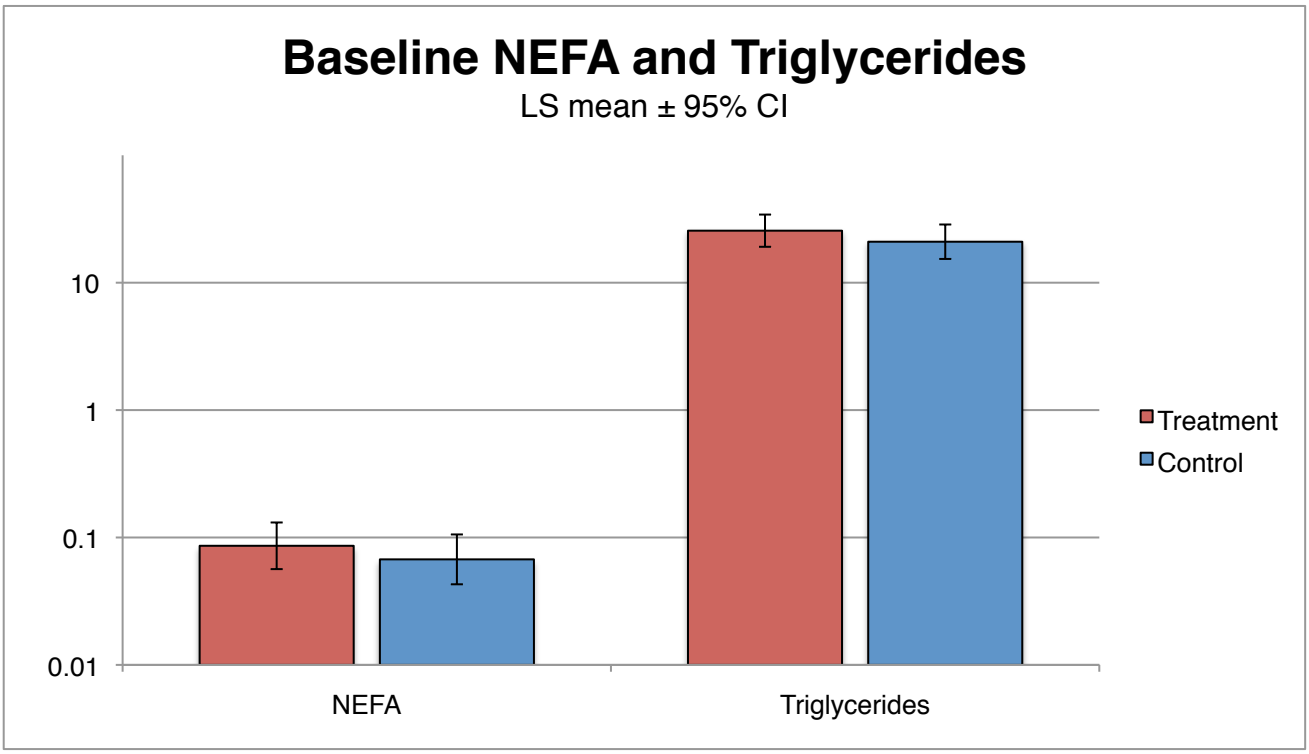


Table 19 : Comparison of NEFA and triglyceride concentration at time points 1 and 2

	P that time 1 significantly different from time 2	
	Drug treated	Control
*ln*NEFA	0.938	0.377
ln triglyceride	0.746	0.474

Table 20 : Repeated measures mixed model ANCOVA to identify change in NEFA and triglyceride concentrations between time points 2 and 3 in drug treated and control horses.

	P that there was a change in value from time point 2 to time point 3	
	***ln* NEFA**	***ln* triglyceride**
Control	0.209	0.008
Drug treated	0.030	0.016

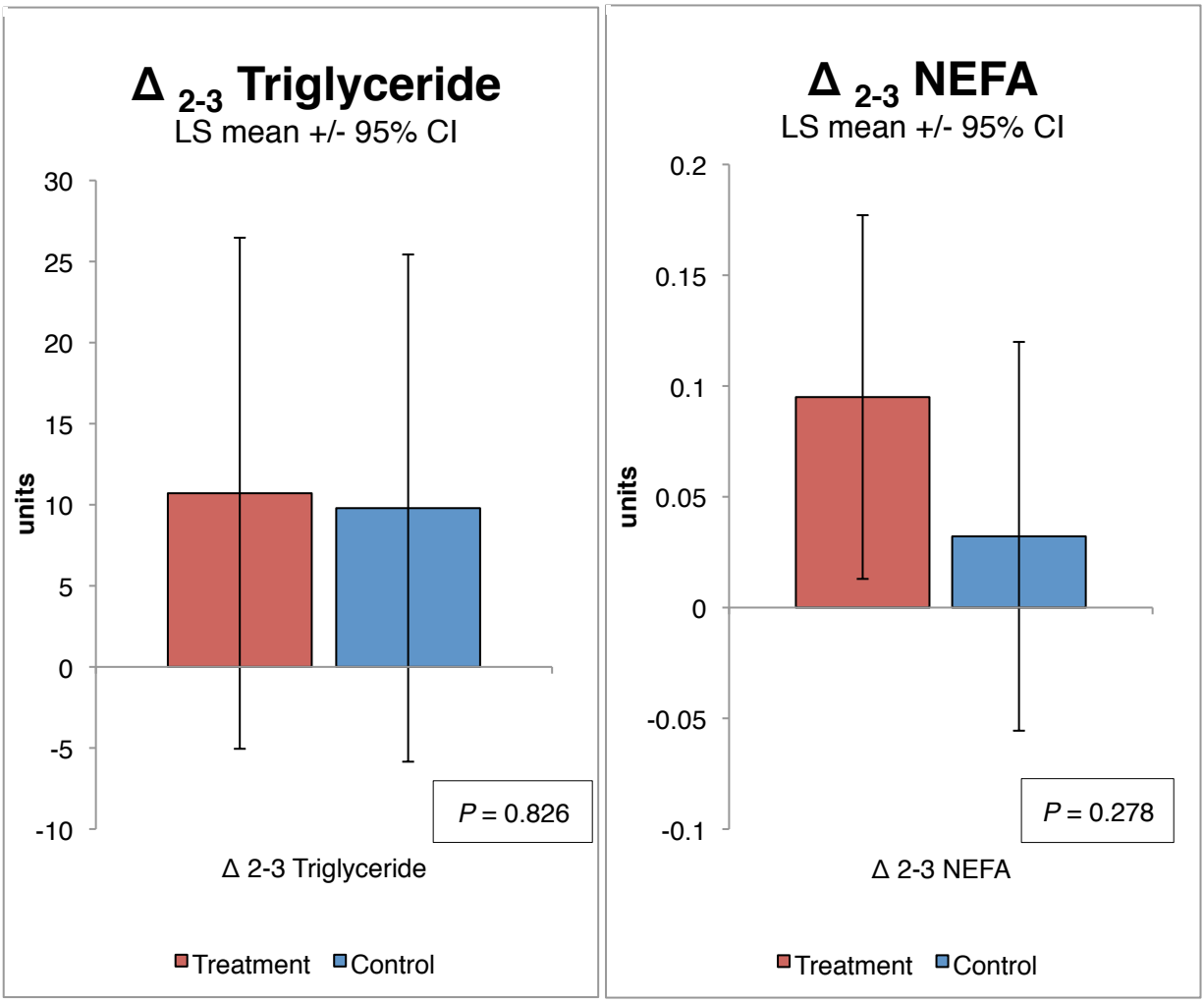
Table 21 : Repeated measures mixed model ANCOVA to identify for any difference in NEFA and triglyceride concentrations between time points 2 and 3 in drug treated and control horses.

	P that drug treated and control horses were different at time 2 and time 3	
	***ln* NEFA**	***ln* triglyceride**
FSIGT 2	0.766	0.964
FSIGT 3	0.837	0.578

Table 22. Comparison of $\Delta_{2\text{-}3}$ NEFA and triglycerides by mixed model ANOVA.

	Drug treated			Control			
	Mean	*-ive 95% CI*	*+ive 95% CI*	Mean	*-ive 95% CI*	*+ive 95% CI*	P (difference)
$\Delta_{2\text{-}3}$ NEFA	0.095	0.013	0.17	0.032	-0.055	0.11	0.278
$\Delta_{2\text{-}3}$ triglyceride	10.70	-5.05	26.46	9.79	-5.84	25.43	0.826

Figure 10. Comparison of Δ $_{2\text{-}3}$ NEFA and triglyceride by mixed model ANOVA.



Chapter 4

Conclusions

Equine metabolic syndrome involves a number of hormonal and metabolic disturbances that result in placing horses at increased risk of developing pasture associated laminitis. The pathophysiology of this condition is as yet incompletely understood. Obesity resulting in a pro-inflammatory and insulin resistant state are the core pathyophysiologic elements of this disorder. The exact mechanism whereby this results in an increased risk of developing laminitis is as yet incompletely understood.

The similarities of EMS with human type 2 diabetes, both having insulin resistance and obesity as core elements, is the basis upon which therapies with proven efficacy in humans are extrapolated to equine medicine for investigation. Pioglitazone has proven efficacy in the modulation of insulin resistance in humans with type 2 diabetes mellitus. This drug had yet to be investigated in horses.

The pharmacokinetics of pioglitazone in horses following oral administration of 1 mg/kg at 24 hour intervals, resulted in peak concentrations and total dose exposures that were lower than those described in humans. The marked physiologic differences in the equine compared to horses in intragastric pH profiles, dietary composition and transit times may have contributed to these differences in the pharmacokinetic profile. This lower total drug exposure may have contributed to the lack of significant differences in the pioglitazone treated horses and their controls in the pharmacodynamic phase of the study.

All but one of the 16 investigated horses developed expected clinical and endocrinologic responses to the LPS infusion. One horse appeared to be an LPS non-responder. No significant effect of drug therapy was able to be identified in the study model. Proposed explanations for the lack of significant findings include the model chosen to investigate insulin resistance, the lower drug concentrations and total drug

exposure measured, insufficient duration of drug administration, the less than ideal repeatability and individual horse variability in the FSIGT testing procedure and low number of investigated horses.

Further investigation of the effects of pioglitazone in horses with insulin resistance is indicated. Administration of the drug at a higher dose, more frequent dosing interval, for a longer duration and in horses with naturally occurring insulin resistance would be recommended for future investigations.

APPENDICES

Appendix A. Pilot Horse Data

A.1 Pharmacokinetic data

Table 23. Pilot horse plasma pioglitazone concentrations (ng/mL) as determined by LCMS-MS.

DOSE	TIME FROM DOSE (hrs)	TIME FROM DOSE (min)	Plasma piogltiazone concentration (ng/mL)
Dose 1	0	0	0.00
	0.25	15	13.75
	0.5	30	25.67
	0.75	45	36.97
	1	60	58.80
	1.5	90	90.76
	2	120	75.96
	3	180	102.07
	4	240	99.07
	6	360	68.48
	8	480	62.98
	12	720	59.00
	17	1020	41.60
	24	1440	27.89
Dose 2	2	120	77.83
	24	1440	26.83
Dose 3	2	120	89.49
	24	1440	22.09
Dose 4	2	120	156.77
	24	0	26.79
Dose 5	0.25	15	29.26
	0.5	30	51.07
	0.75	45	79.99
	1	60	91.78
	1.5	90	123.52
	2	120	118.95
	3	180	99.80
	4	240	98.56
	6	360	72.82
	8	480	66.57
	12	720	41.86
	17	1020	33.91
	24	1440	16.71
	48	2880	0.88
	72	4320	-0.52

Appendix B. Pharmacokinetic Data.

Table 24. Plasma pioglitazone concentrations (ng/mL) in individual animals as determined by LCMS-MS.

			Group A		Group B		Group C	
DOSE	TIME FROM DOSE (hrs)	TIME FROM DOSE (min)	95	123	35	125	3	51
Dose 1	0	0	7.8	7.8	0	0	0.5	0.5
	0.25	15	62.9	40.0	2.7	94.6	72.7	52.7
	0.5	30	172.7	141.0	28.4	199.7	342.4	96.0
	0.75	45			41.6	351.2	483.8	83.9
	1	60	147.9	193.0	77.8	533.5	473.2	104.5
	1.5	90	114.7	200.6	166.2	898.7	448.2	57.4
	2	120	46.1	170.2	201.6	904.2	389.2	44.1
	3	180	33.7	274.9	269.2	755.5	264.7	28.7
	4	240	23.9	182.8	1114.6	621.9	204.8	24.6
	6	360	21.1	46.1	551.4	326.4	139.5	18.3
	8	480	19.7	24.7	269.0	235.0	156.3	19.5
	12	720	20.4	22.2	95.1	123.2	126.3	16.0
	16	960	14.6	17.3	53.3	101.7	99.2	2.0
	24	1440	9.5	13.5	25.5	38.6	36.3	1.4
Dose 2	2	120			643.3	806.0	364.2	157.6
Dose 4	2	120	205.6	144.8	626.7	1163.8	527.5	609.6
	24	1440	8.6	15.3	16.1	34.8	110.6	0
Dose 5	2	120	272.1	259.3	475.4	854.4	474.9	240.5
Dose 10	24	0	9.7	8.3	19.7	19.7	24.4	-3.5
Dose 11	0.25	15	21.3	13.4	28.6	89.4	211.5	16.8
	0.5	30	40.8	24.7	48.7	126.3	214.7	69.8
	0.75	45	54.3	35.4	48.1	270.0	332.5	163.4
	1	60	64.7	79.6	99.5	460.0	320.9	189.9
	1.5	90	103.8	105.0	156.0	605.3		343.6
	2	120	101.8	224.5	205.5	838.4	358.8	50.6
	3	180	60.1	193.3	461.4	684.8	359.2	20.1
	4	240	32.2	168.4	506.5	607.6	378.2	19.9
	6	360	23.2	57.9	800.0	347.5	243.9	16.2
	8	480	23.1	40.0	349.6	262.9	172.8	11.5
	12	720	22.1	25.2	102.1	183.7	115.1	10.6
	16	960	21.7	14.5	57.3	136.9	77.8	4.0
	24	1440	14.7	10.3	16.7	90.6	29.1	-3.0
Dose 12	2	120			277.2	1062.6	397.1	46.5
	24	1440	16.5	21.2	72.0	99.0	21.2	-3.8
Dose 13	2	120	190.4	334.9	619.1	673.6	226.7	80.2
	24	1440	21.1	12.5	71.5	109.2	52.4	-3.2
Dose 14	2	120	91.7	259.2	203.5	764.7	557.7	41.8
	24	1440	16.9	24.5	83.6	97.9	28.9	-3.0

Figure 11. Plasma pioglitazone concentration vs time for individual horse following the first oral dose. Horses identified by number.

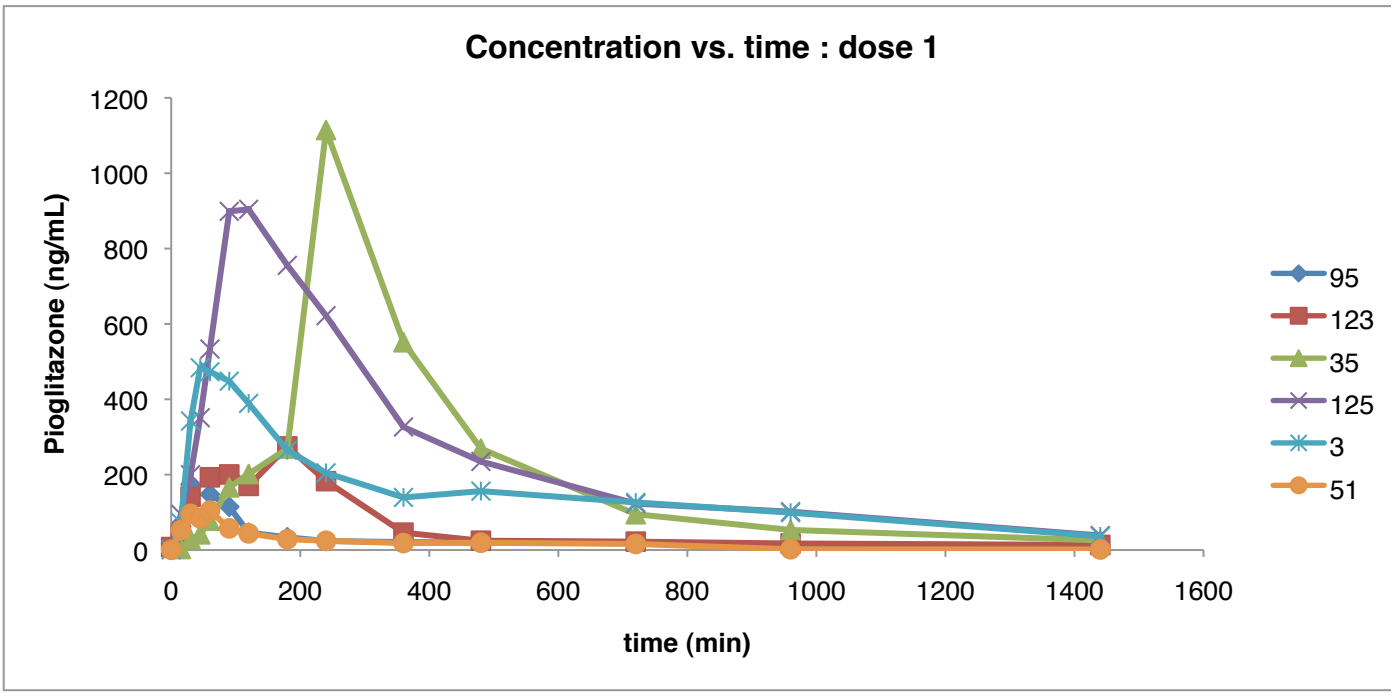
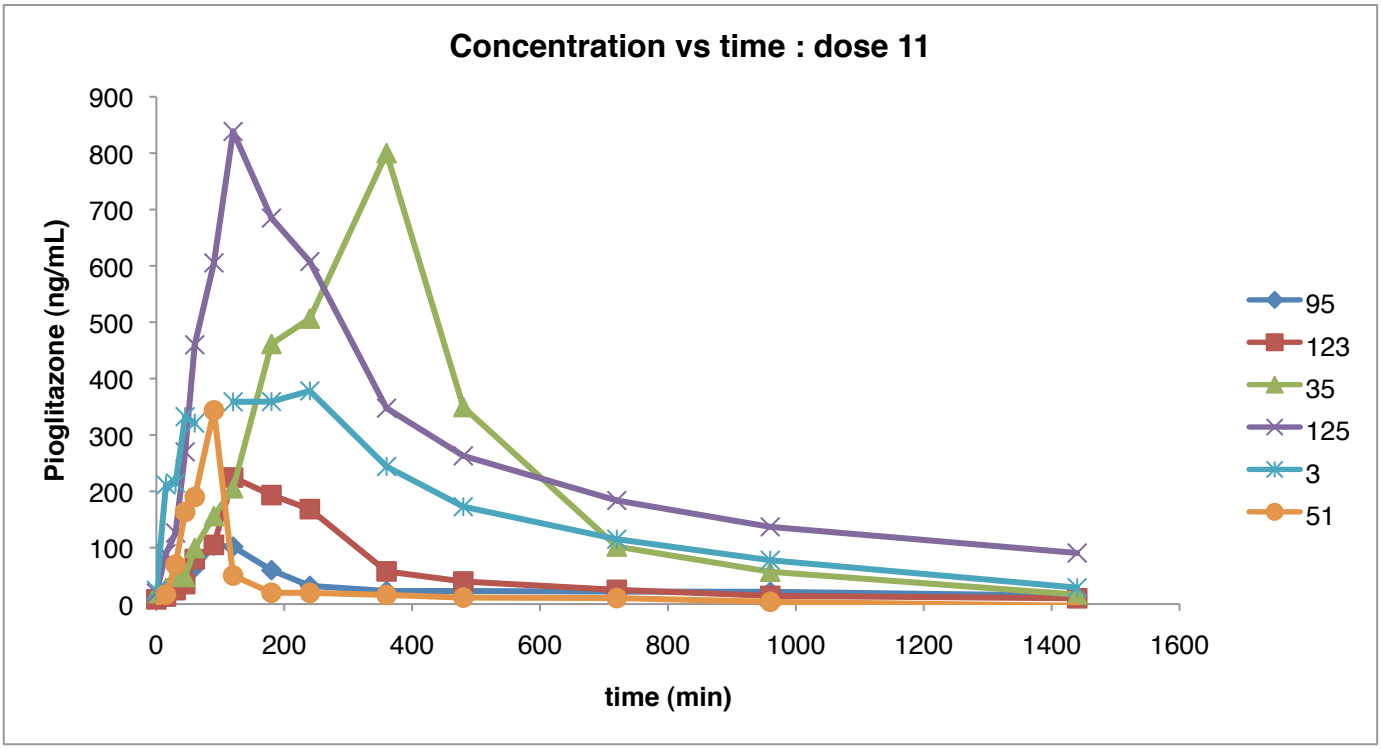


Figure 12. Plasma pioglitazone concentration vs time for individual horse following the eleventh oral dose. Horses identified by number.



Appendix C. Study Design.

Table 25. Study design.

Study Design					
DAY	**Treatments - 7am**	**8am**	**Biopsy**	**Catheters**	**Drug Tests**
-1				Place catheter	
0		FSIGT 1 : Pre-treatment	Biopsy 1		
1	Pioglitazone 1		Biopsy Care		PK 1 : 14 samples - Dose 1
2	Pioglitazone 2		Biopsy Care	Catheter out 9am	PK1 Trough 6:59am; Peak 9am
3	Pioglitazone 3		Biopsy Care		
4	Pioglitazone 4		Biopsy Care		Peak 9am
5	Pioglitazone 5		Biopsy Care		Trough 6:59am, Peak 9am
6	Pioglitazone 6				
7	Pioglitazone 7				
8	Pioglitazone 8				
9	Pioglitazone 9				
10	Pioglitazone 10				
11	Pioglitazone 11			Place catheter	PK 11 : 14 samples - Dose 11
12	Pioglitazone 12	FSIGT 2 : Treatment	Biopsy 2		PK 11 Trough 6:59am;
13	Pioglitazone 13	**LPS INFUSION**	Biopsy Care	WCC (x3) + TNFα (x7)	Trough 6:59am; Peak 9am
14	Pioglitazone 14	FSIGT 3 : Challenge	Biopsy 3		Trough 6:59am, Peak 9am
15			Biopsy Care	Catheter out	Trough 6:59am
16			Biopsy Care		
17			Biopsy Care		
18			Biopsy Care		

Appendix D : Horse Signalment And Physical Parameters.

Table 26. Horses' signalment.

Horse	Group	Treatment	Name	Age (y)
7	A	Control	Good Rockin'	13
135	A	Control	Castle Mellay	18
95	A	Drug	Seattle Slugger	10
123	A	Drug	Magic Dot	21
15	B	Control	Balalia	12
124	B	Control	Christmas Magic	15
35	B	Drug	My Lady's a Knockout	13
125	B	Drug	Cyclonic Girl	19
33	C	Control	Brooke's Sarah	15
87	C	Control	Our Lady Slew	17
44	C	Drug	Empress Kell	15
128	C	Drug	Tess Trull	17
3	D	Control	Herb Garden	11
37	D	Control	Uniteski	16
51	D	Drug	Lynn's Bluffin'	11
21	D	Drug	Amyscharming way	8

Table 27. Body weight of control group horses.

Horse	Group	Control	Time	BW (kg)
7	A	Control	1	575
7	A	Control	2	575
7	A	Control	3	584
135	A	Control	1	643
135	A	Control	2	643
135	A	Control	3	661
15	B	Control	1	588
15	B	Control	2	585
15	B	Control	3	585
124	B	Control	1	590
124	B	Control	2	596
124	B	Control	3	596
33	C	Control	1	528
33	C	Control	2	531
33	C	Control	3	539
87	C	Control	1	606
87	C	Control	2	514
87	C	Control	3	603
21	D	Control	1	536
21	D	Control	2	547
21	D	Control	3	535
37	D	Control	1	546
37	D	Control	2	567
37	D	Control	3	551

Table 28. Body weight & drug dose of treatment group. Incorrect dose for horses 95 and 123 for first pharmacokinetic study displayed in red.

Horse	Group	Treatment	Date	BW (kg)	Tablets per horse	mg/tab	mg/horse	mg/kg
95	A	Drug	1	572	**12**	**45**	**540**	**0.944**
95	A	Drug	2	572	13	45	585	1.023
95	A	Drug	3	574	13	45	585	1.019
123	A	Drug	1	623	**12**	**45**	**540**	**0.867**
123	A	Drug	2	623	13	45	585	0.939
123	A	Drug	3	620	13	45	585	0.944
35	B	Drug	1	617	13	45	585	0.948
35	B	Drug	2	615	13	45	585	0.951
35	B	Drug	3	613	13	45	585	0.954
125	B	Drug	1	555	13	45	585	1.054
125	B	Drug	2	554	13	45	585	1.056
125	B	Drug	3	527	13	45	585	1.110
3	D	Drug	1	564	14	45	630	1.117
3	D	Drug	2	579	14	45	630	1.088
3	D	Drug	3	560	14	45	630	1.125
51	D	Drug	1	672	15	45	675	1.004
51	D	Drug	2	691	15	45	675	0.977
51	D	Drug	3	672	15	45	675	1.005

Excluded first two doses in this analysis

Use Minimal model 1 weight for PK 1

Use Minimal model 2 weight for PK 11

Mean	1.020
Median	1.012
Max	1.125
Min	0.939
SD	0.066

Table 29. Body condition score (out of 9) of enrolled horses.

Horse	Group	Treatment	Body Condition Score (out of 9)
7	A	Control	6
15	B	Control	5.5
21	D	Control	6
33	C	Control	5
37	D	Control	5
87	C	Control	6.5
124	B	Control	5.5
135	A	Control	7
3	D	Control	5
35	B	Drug	5.5
44	C	Drug	6.5
51	D	Drug	8
95	A	Drug	5.5
123	A	Drug	6.5
125	B	Drug	5.5
128	C	Drug	6

Appendix E. Dose Differences Between Time Points.

Table 30. Differences (mg/kg) in dose of pioglitazone administered to each individual horse between time points.

Group	Time point 1	Time point 2	Dose difference (mg/kg)
a	1	2	-0.07545
	1	3	-0.07549
	2	3	-0.00049
b	1	2	-0.00249
	1	3	-0.03109
	2	3	-0.0286
c	1	2	0.01697
	1	3	-0.00494
	2	3	-0.02191
d	1	2	0.02828
	1	3	-0.00436
	2	3	-0.03264
Maximal positive dose difference			0.02828
Maximal negative dose difference			-0.07549

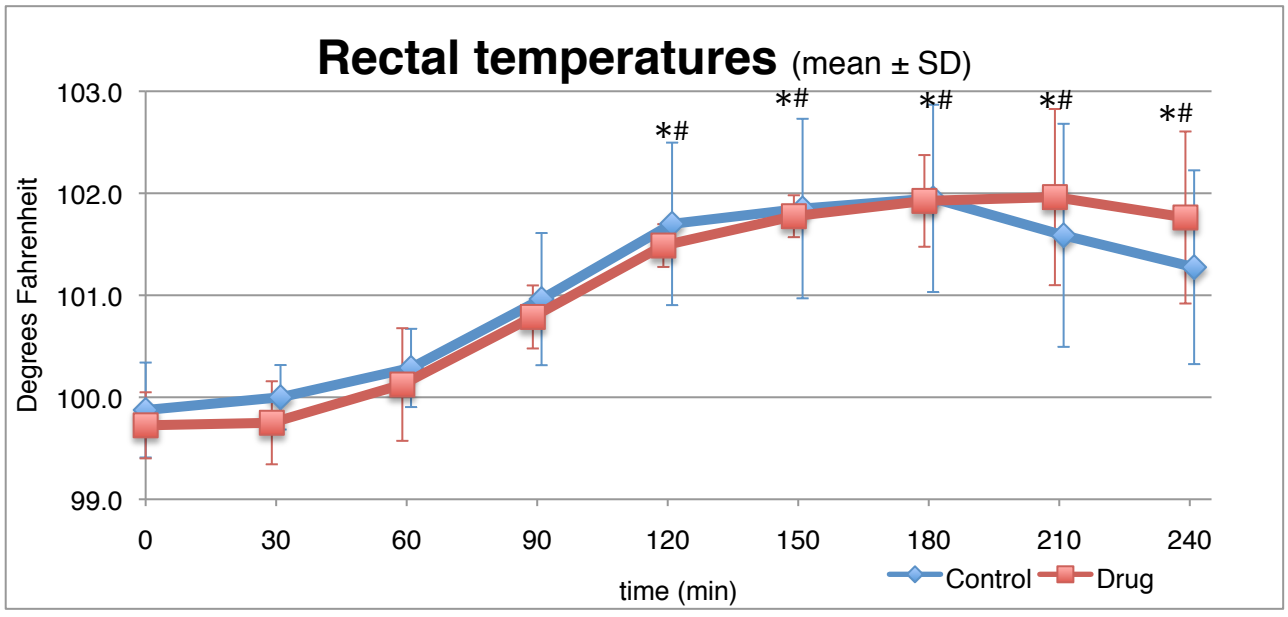
Appendix F: Physical Examination Following Lipopolysaccharide Infusion Data.

F.1 Rectal Temperature

Table 31. Rectal temperature (°F) following endotoxin infusion.

Group	Treatment	Horse	0	30	60	90	120	150	180	210	240
A	Drug	95	100.3	100.2	99.4	100.8	101.5	101.5	101.6	101.2	101.0
A	Drug	123	99.7	99.3	99.2	100.4	101.4	101.8	101.8	101.6	101.1
B	Drug	35	99.8	99.8	100.4	100.9	101.6	101.6	101.5	101.5	101.6
B	Drug	125	99.8	99.8	100.4	100.9	101.6	101.6	101.5	101.5	101.6
C	Drug	44	99.3	99.4	100.7	100.8	101.8	102.1	101.8	101.6	101.2
C	Drug	128	99.3	99.3	100.0	100.5	101.2	101.8	102.2	101.8	101.6
D	Drug	3	99.7	99.8	100.3	100.6	101.2	101.8	102.8	103.8	103.5
D	Drug	51	99.9	100.4	100.6	101.4	101.6	102.0	102.2	102.7	102.5
		Mean	**99.7**	**99.8**	**100.1**	**100.8**	**101.5**	**101.8**	**101.9**	**102.0**	**101.8**
		SD	**0.3**	**0.4**	**0.6**	**0.3**	**0.2**	**0.2**	**0.4**	**0.9**	**0.8**
A	Control	7	100.2	100.1	100.5	101.1	101.9	102.4	102.4	102.3	102.0
A	Control	135	99.9	99.8	100.2	100.8	101.4	101.7	101.9	101.4	101.3
B	Control	124	99.5	99.5	99.9	101.2	102.1	101.6	101.2	100.2	100.1
C	Control	33	98.9	99.7	99.8	100.2	101	101.4	101.4	101.3	100.6
C	Control	87	99.9	100.1	100.1	100.4	100.8	100.9	100.7	100.9	100.6
D	Control	37	100.2	100.1	100.5	101.1	101.9	102.4	102.4	102.3	102.0
D	Control	21	100.2	100.5	101.0	102.3	103.3	103.5	103.7	103.6	102.9
		Mean	**99.8**	**100.0**	**100.3**	**101.0**	**101.8**	**102.0**	**102.0**	**101.7**	**101.4**
		SD	**0.5**	**0.3**	**0.4**	**0.7**	**0.8**	**0.9**	**1.0**	**1.1**	**1.0**

Figure 13. Rectal temperature (°F; mean ± SD) following IV LPS infusion.



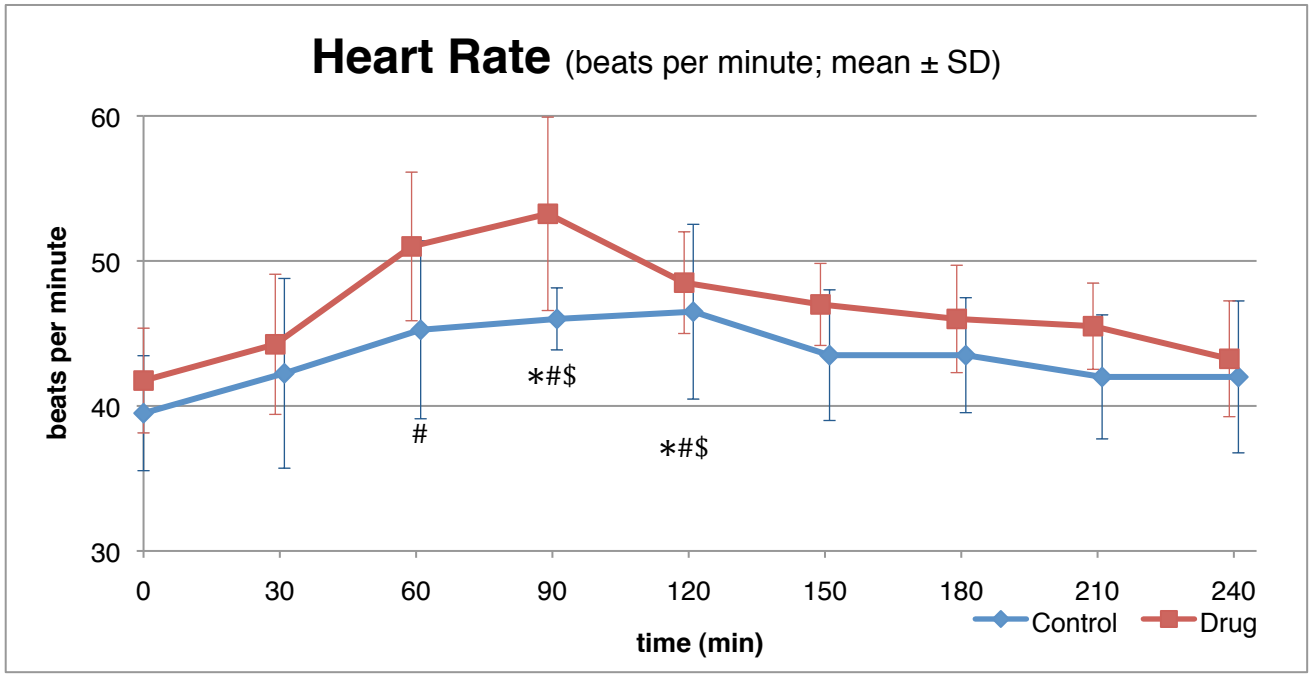
*** Significant difference from baseline in control horses; # significant difference from baseline in drug treated horses**. At no time point does a significant difference exist between treatment and control horses.

F.2 Heart rate

Table 32 : Heart rate (beats per minute) following endotoxin infusion.

GROUP	TREATMENT	HORSE	0	30	60	90	120	150	180	210	240
A	Drug	95	44	40	52	52	54	48	44	44	40
A	Drug	123	40	40	44	44	44	40	40	40	36
B	Drug	35	36	44	52	48	48	48	48	48	44
B	Drug	125	40	54	56	60	44	48	44	48	48
C	Drug	44	44	44	48	64	48	48	48	44	44
C	Drug	128	42	44	60	56	52	48	44	44	42
D	Drug	3	40	40	48	54	50	48	48	48	44
D	Drug	51	48	48	48	48	48	48	52	48	48
		Mean	**41.8**	**44.3**	**51.0**	**53.3**	**48.5**	**47.0**	**46.0**	**45.5**	**43.3**
		SD	4	5	5	7	4	3	4	3	4
A	Control	7	40	40	44	48	44	40	44	40	44
A	Control	135	40	44	44	44	44	44	40	40	44
B	Control	124	44	40	60	44	60	44	44	44	44
C	Control	33	32	54	44	48	44	40	40	40	36
C	Control	87	36	32	40	44	40	40	40	36	32
D	Control	37	40	40	44	48	44	40	44	40	44
D	Control	21	44	48	44	48	48	52	52	48	48
		Mean	**39.4**	**42.6**	**45.7**	**46.3**	**46.3**	**42.9**	**43.4**	**41.1**	**41.7**
		SD	4	7	6	2	6	4	4	4	6

Figure 14. Heart rate (beats per minute; mean ± SD) following IV LPS infusion.



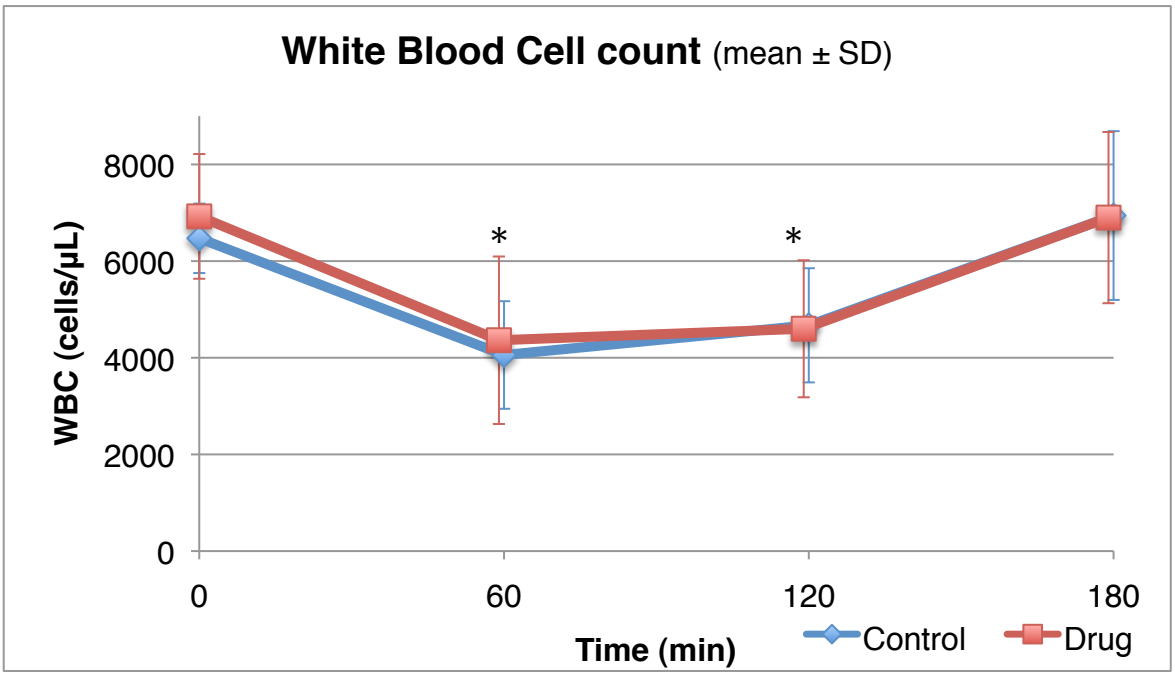
* Significant difference from baseline in control horses; # significant difference from baseline in drug treated horses; $ significant difference between drug treatment and control horses

F.3 White blood cell count

Table 33 : White blood cell count (cells/μL) following endotoxin infusion.

GROUP	TREATMENT	HORSE	0	60	120	180
A	Drug	95	6900	3800	3300	4800
A	Drug	123	6100	5600	3900	6700
B	Drug	35	8400	4900	6000	7400
B	Drug	125	6200	5200	5400	7900
C	Drug	44	7600	3900	5700	8900
C	Drug	128	9000	7200	6400	9300
D	Drug	3	5600	2400	2900	5000
D	Drug	51	5600	1900	3200	5200
		Mean	**6925**	**4362.5**	**4600**	**6900**
		SD	1290	1733	1418	1771
A	Control	7	7000	3700	3800	6800
A	Control	135	6200	2000	2800	3700
B	Control	124	6600	5000	4700	7100
C	Control	33	6400	3900	6200	8500
C	Control	87	5800	4600	4600	6800
D	Control	37	5600	3800	4600	6500
D	Control	21	7700	5400	6000	9200
		Mean	**6471**	**4057**	**4671**	**6943**
		SD	718	1113	1181	1746

Figure 15. White blood cell count (cells/μL; mean ± SD) following IV LPS infusion.



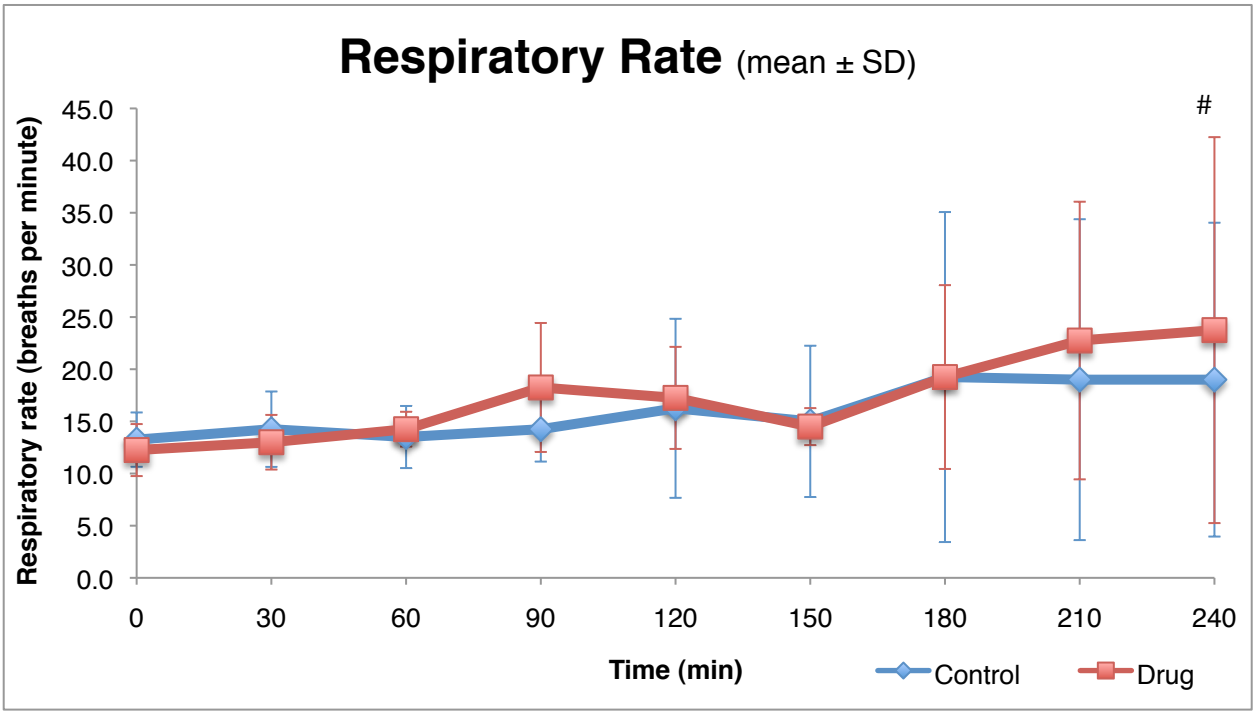
* represents time points where mean significantly (P<0.05) different from baseline. At no time point does a significant difference exist between treatment and control horses.

F.4 Respiratory rate

Table 34 : Respiratory Rate (breaths per minute) following endotoxin infusion.

GROUP	TREATMENT	HORSE	0	30	60	90	120	150	180	210	240
A	Drug	95	12	16	12	12	14	14	36	30	20
A	Drug	123	14	12	14	12	12	16	16	16	22
B	Drug	35	10	12	12	16	16	14	16	12	12
B	Drug	125	12	12	16	30	16	12	12	12	12
C	Drug	44	14	12	16	16	12	12	12	12	10
C	Drug	128	8	10	14	16	24	16	16	16	10
D	Drug	3	12	12	14	20	20	16	16	40	60
D	Drug	51	16	18	16	24	24	16	30	44	44
		Mean	**12.3**	**13.0**	**14.3**	**18.3**	**17.3**	**14.5**	**19.3**	**22.8**	**23.8**
		SD	2.5	2.6	1.7	6.2	4.9	1.8	8.8	13.3	18.5
A	Control	7	16	20	16	16	16	16	12	16	22
A	Control	135	18	20	20	20	20	16	32	28	28
B	Control	124	12	12	12	10	10	10	10	10	10
C	Control	33	12	12	12	12	12	12	12	10	10
C	Control	87	12	12	12	14	12	12	12	12	10
D	Control	37	12	12	12	14	12	12	12	12	10
D	Control	21	14	14	12	16	36	32	54	54	52
		Mean	**13.7**	**14.6**	**13.7**	**14.6**	**16.9**	**15.7**	**20.6**	**20.3**	**20.3**
		SD	2.4	3.8	3.1	3.2	9.1	7.5	16.6	16.1	15.8

Figure 16. Respiratory rate (breaths per minute; mean ± SD) following IV LPS infusion.



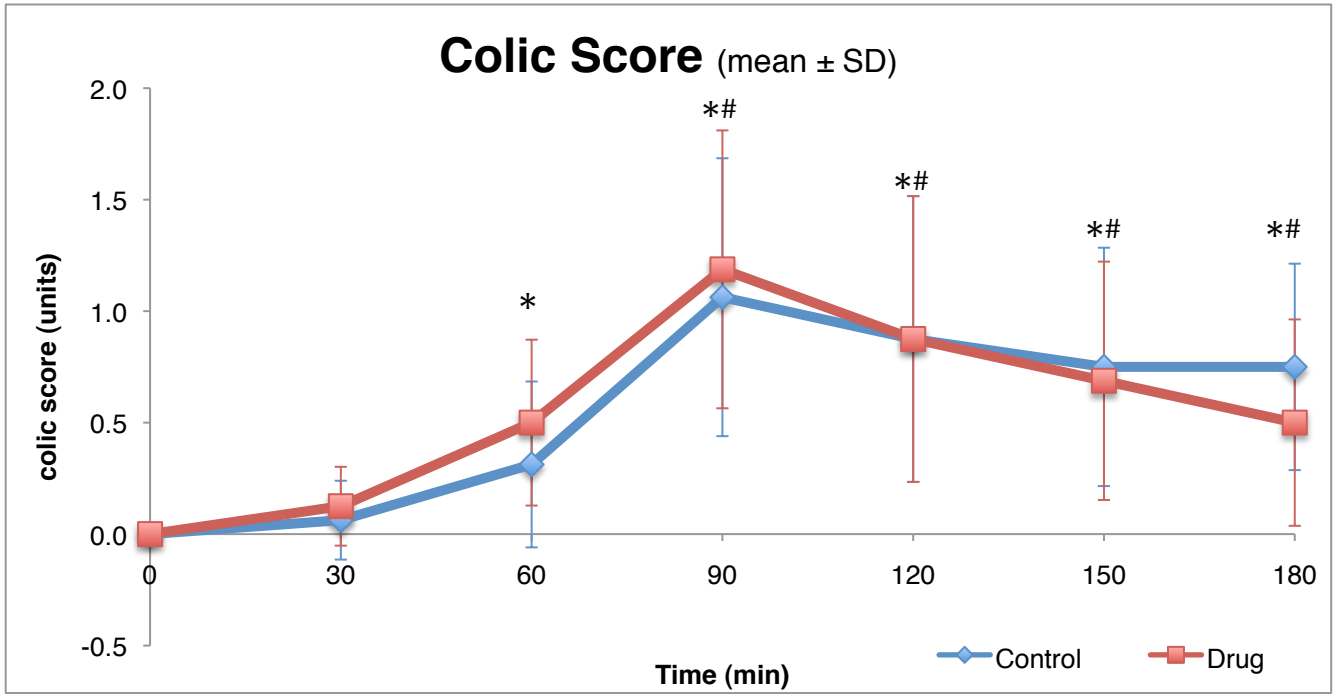
significant difference from baseline in drug treated horses. At no time point does a significant difference exist between treatment and control horses.

F.5 Colic Score

Table 35. Colic Score (arbitrary units) following endotoxin infusion.

GROUP	TREATMENT	HORSE	0	30	60	90	120	150	180	210	240
A	Drug	95	0	0	0	0	0	0	0.5	0.5	0
A	Drug	123	0	0	0	1	1	1	0.5	0	0
B	Drug	35	0	0	0	1.5	1	1	1	0.5	0
B	Drug	125	0	1	0	1	0	0	0	0	0
C	Drug	44	0	0	1	1.5	1	0.5	0	0	0
C	Drug	128	0	0	2	1.5	1	1	0	0	0
D	Drug	3	0	0	1	2	2	1	1	1	1
D	Drug	51	0	0	0	1	1	1	1	1	0
		Mean	**0.0**	**0.1**	**0.5**	**1.2**	**0.9**	**0.7**	**0.5**	**0.4**	**0.1**
		SD	0.0	0.4	0.8	0.6	0.6	0.5	0.5	0.4	0.4
A	Control	7	0	0	0.5	1	1	1	1	0.5	0
A	Control	135	0	0	0	1.5	0	0.5	1	0	0
B	Control	124	0	0	0	0.5	1	0	0	0	0
C	Control	33	0	0.5	1	1.5	1	1.5	1	0	0
C	Control	87	0	0	0.5	1	1	1	1	0.5	0
D	Control	37	0	0	0	0	0	0	0	0	0
D	Control	21	0	0	0.5	1	1	1	1	0	0
		Mean	0	0	0	2	2	1	1	1	1
		SD	**0.0**	**0.1**	**0.3**	**1.1**	**0.9**	**0.8**	**0.8**	**0.3**	**0.1**

Figure 17. Colic score (arbitrary units; mean ± SD) following IV LPS infusion.



* Significant difference from baseline in control horses; # significant difference from baseline in drug treated horses. At no time point does a significant difference exist between treatment and control horses.

Appendix G: Minimal Model Analysis Of The Frequently Sampled Intravenous Glucose Tolerance Test Data For Individual Animals.

G.1 Individual horse glucose effectiveness

Table 36. Glucose Effectiveness (Sg x10^{-2}; min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	0.0354	0.0153	0.0273	0.0245	0.0202	0.0068	0.0258	0.0384
FSIGT 2	0.0304	0.0309	0.0182	0.0363	0.0128	0.0184	0.0351	0.0339
FSIGT 3	0.0203	0.0154	0.0284	0.0121	0.0134	0.0133	0.0195	0.0119
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	0.0167	0.0313	0.0348	0.0162	0.0097	0.0194	0.020354	
FSIGT 2	0.0189	0.0333	0.0259	0.0243	0.0116	0.0220	0.070548	
FSIGT 3	0.0210	0.0142	0.0275	0.0111	0.0158	0.0220	0.023736	

Table 37. Glucose Effectiveness (Sg x10^{-2}; min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline Sg.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	1	1	1	1	1	1	1	1
FSIGT 2	0.86	2.02	0.67	1.48	0.63	2.71	1.36	0.88
FSIGT 3	1.01	0.57	1.04	0.50	0.66	1.95	0.76	0.31
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	1	1	1	1	1	1	1	
FSIGT 2	1.14	1.06	0.74	1.50	1.20	1.13	3.47	
FSIGT 3	1.26	0.45	0.79	0.68	1.64	1.13	1.17	

Figure 18. Glucose Effectiveness (Sg x10^{-2}; min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

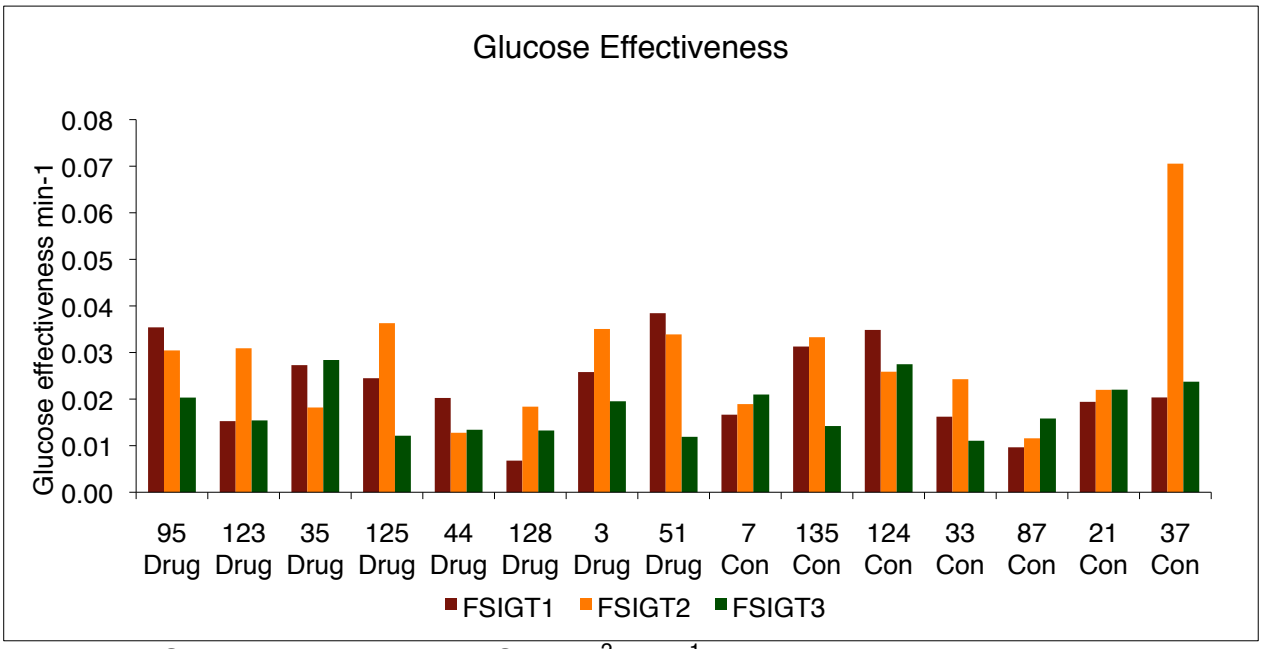
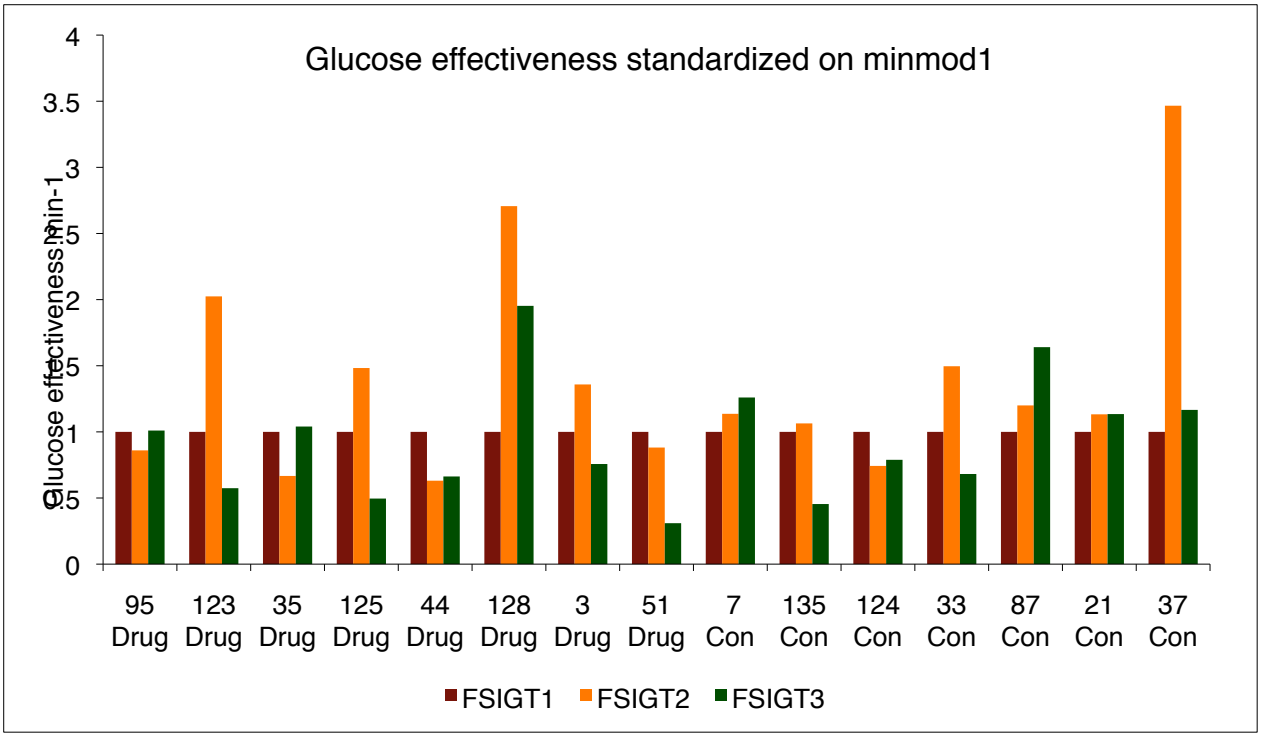


Figure 19. Glucose Effectiveness (Sg x10^{-2}; min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1.



G.2 Individual horse insulin sensitivity

Table 38. Insulin sensitivity (SI x10^{-4}; L•min^{-1}•mU^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	5.02	0.48	3.98	2.16	3.04	0.17	3.96	1.44
FSIGT 2	2.02	2.16	4.22	3.03	1.95	0.21	0.85	1.15
FSIGT 3	0.35	0.09	0.38	0.83	1.05	0.05	0.09	0.29
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	1.10	0.74	1.66	5.76	1.23	3.29	2.32	
FSIGT 2	0.78	0.13	1.60	2.42	0.92	0.75	2.59	
FSIGT 3	0.39	0.04	0.07	0.98	0.29	0.16	0.79	

Table 39. Insulin sensitivity (SI x10^{-4}; L•min^{-1}•mU^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline SI.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	1	1	1	1	1	1	1	1
FSIGT 2	0.40	4.51	1.06	1.40	0.64	1.25	0.21	0.80
FSIGT 3	0.07	0.20	0.09	0.39	0.35	0.29	0.02	0.20
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	1	1	1	1	1	1	1	
FSIGT 2	0.71	0.17	0.96	0.42	0.75	0.23	1.12	
FSIGT 3	0.35	0.06	0.04	0.17	0.24	0.05	0.34	

Figure 20. Insulin sensitivity (SI x10^{-4}; L•min^{-1}•mU^{-1})values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

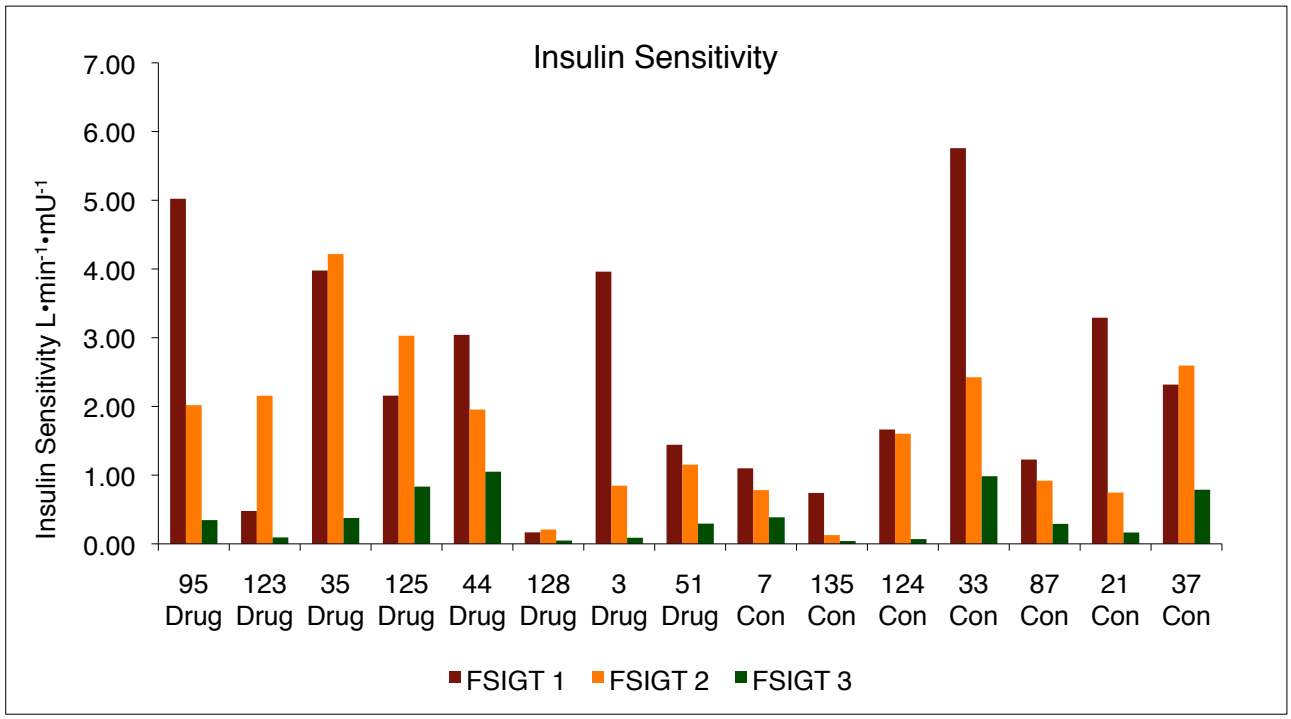
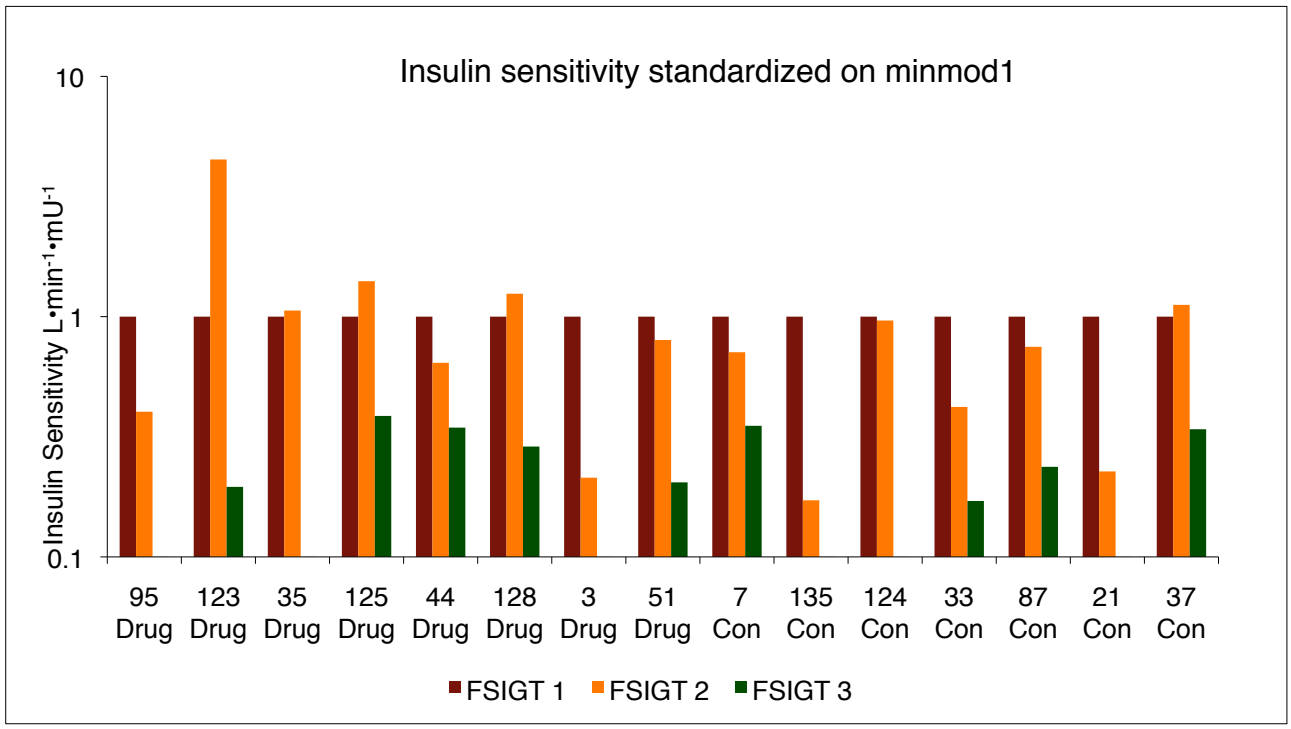


Figure 21. Insulin sensitivity (SI x10^{-4}; L•min^{-1}•mU^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline SI.



G.3 Individual horse acute insulin response to glucose

Table 40. Acute insulin response to glucose (AIRg; mU•L•min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	400.95	670.65	239.65	526.50	309.00	2878.90	479.00	811.00
FSIGT 2	260.30	1031.40	155.85	516.80	603.20	2638.20	733.30	1956.30
FSIGT 3	504.30	1064.10	308.00	610.50	230.20	1994.70	624.75	1449.80

	Control						
Group	A	A	B	C	C	D	D
Horse	7	135	124	33	87	21	37
FSIGT 1	812.05	2251.10	650.65	396.20	707.60	576.20	569.15
FSIGT 2	876.20	2856.10	623.50	980.80	657.60	1182.80	765.80
FSIGT 3	1142.50	2945.60	819.35	498.35	442.30	972.60	565.05

Table 41. Acute insulin response to glucose (AIRg; mU•L•min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline AIRg.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	1	1	1	1	1	1	1	1
FSIGT 2	0.65	1.54	0.65	0.98	1.95	0.92	1.53	2.41
FSIGT 3	1.26	1.59	1.29	1.16	0.74	0.69	1.30	1.79

	Control						
Group	A	A	B	C	C	D	D
Horse	7	135	124	33	87	21	37
FSIGT 1	1	1	1	1	1	1	1
FSIGT 2	0.65	1.54	0.65	0.98	1.95	0.92	1.53
FSIGT 3	1.26	1.59	1.29	1.16	0.74	0.69	1.30

Figure 22. Acute insulin response to glucose (AIRg; mU•L•min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

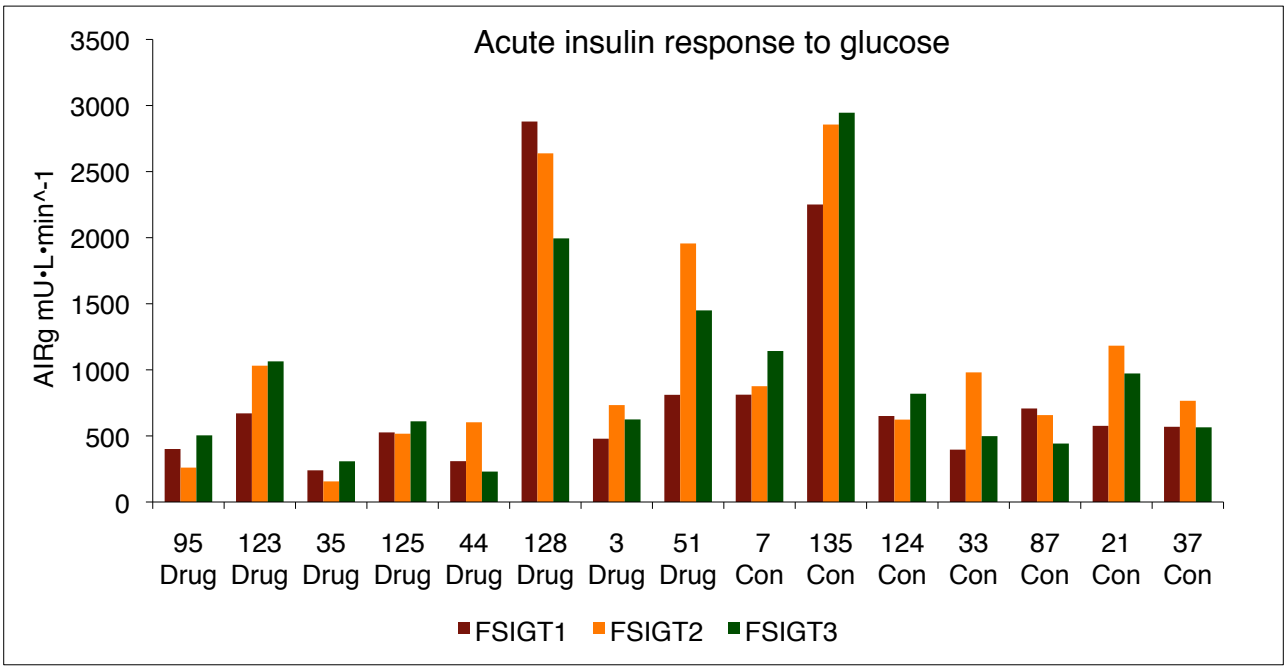
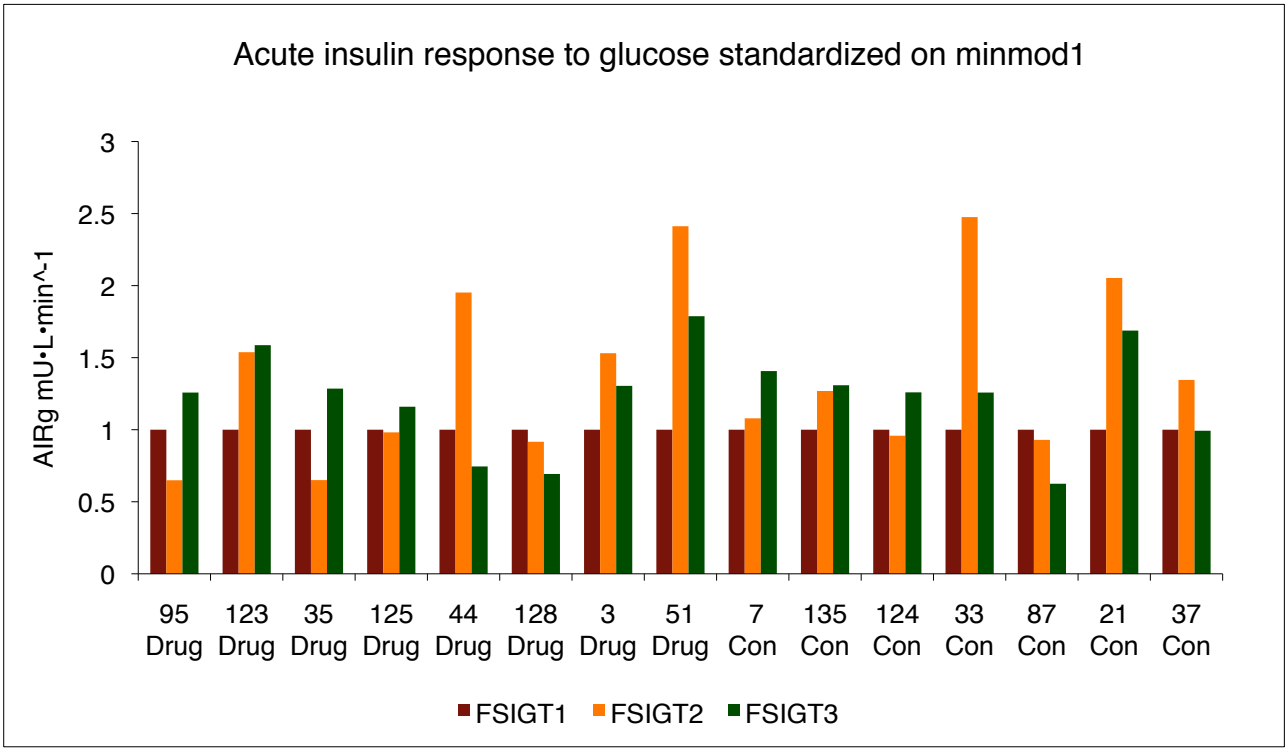


Figure 23. Acute insulin response to glucose (AIRg; mU•L•min^{-1}) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline AIRg.



G.4 Individual horse disposition index

Table 42. Disposition index (DI; $x10^{-2}$ dimensionless) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	2013.40	320.58	953.25	1135.50	939.82	481.40	1897.40	1169.20
FSIGT 2	525.55	2223.20	657.20	1565.20	1178.90	549.45	620.58	2255.10
FSIGT 3	174.28	99.53	115.72	508.40	241.00	96.05	77.86	426.95
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	892.77	1667.60	1082.60	2281.50	868.18	1896.10	1319.10	
FSIGT 2	684.94	363.91	999.54	2377.40	604.26	882.98	1986.80	
FSIGT 3	441.20	120.33	56.94	490.39	128.65	160.30	445.16	

Table 43. Disposition index (DI; $x10^{-2}$ dimensionless) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline DI.

	Treatment							
Group	A	A	B	B	C	C	D	D
Horse	95	123	35	125	44	128	3	51
FSIGT 1	1	1	1	1	1	1	1	1
FSIGT 2	0.26	6.93	0.69	1.38	1.25	1.14	0.33	1.93
FSIGT 3	0.31	0.09	0.12	0.45	0.26	0.20	0.04	0.37
	Control							
Group	A	A	B	C	C	D	D	
Horse	7	135	124	33	87	21	37	
FSIGT 1	1	1	1	1	1	1	1	
FSIGT 2	0.77	0.22	0.92	1.04	0.70	0.47	1.51	
FSIGT 3	0.49	0.07	0.05	0.21	0.15	0.08	0.34	

Figure 24. Disposition index (DI; x10^{-2} dimensionless) values calculated via minimal model analysis of each FSIGT for individual horses at each time point.

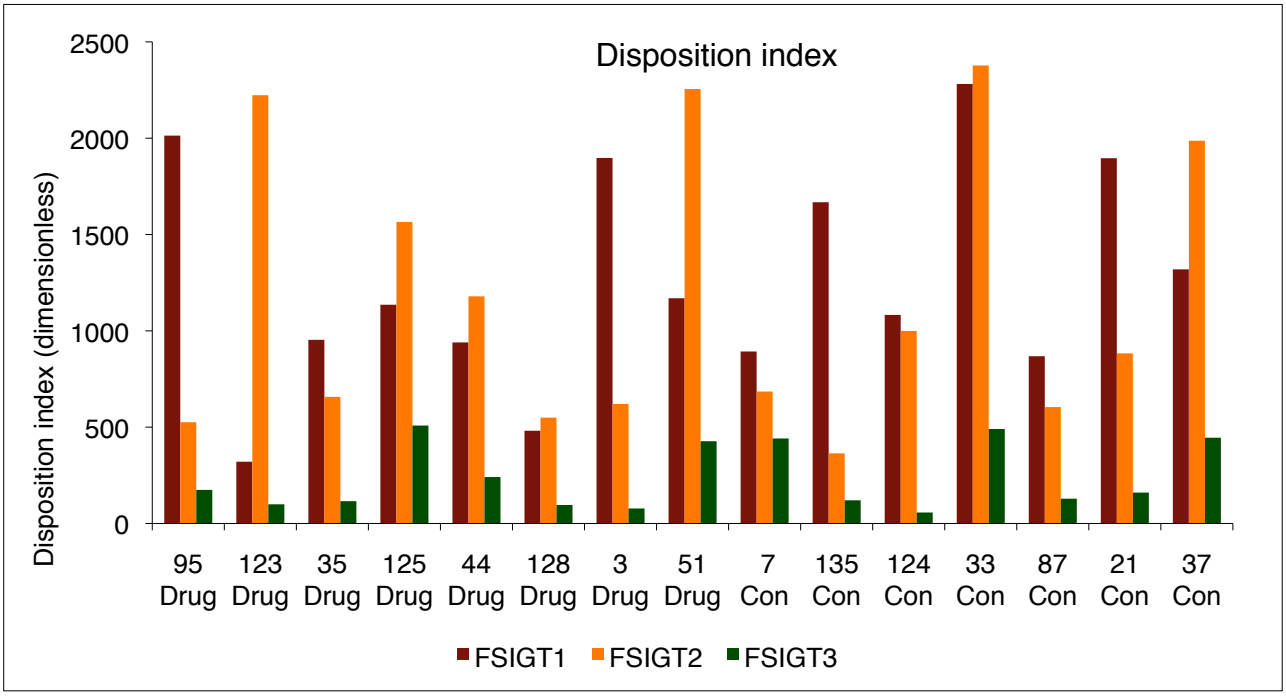
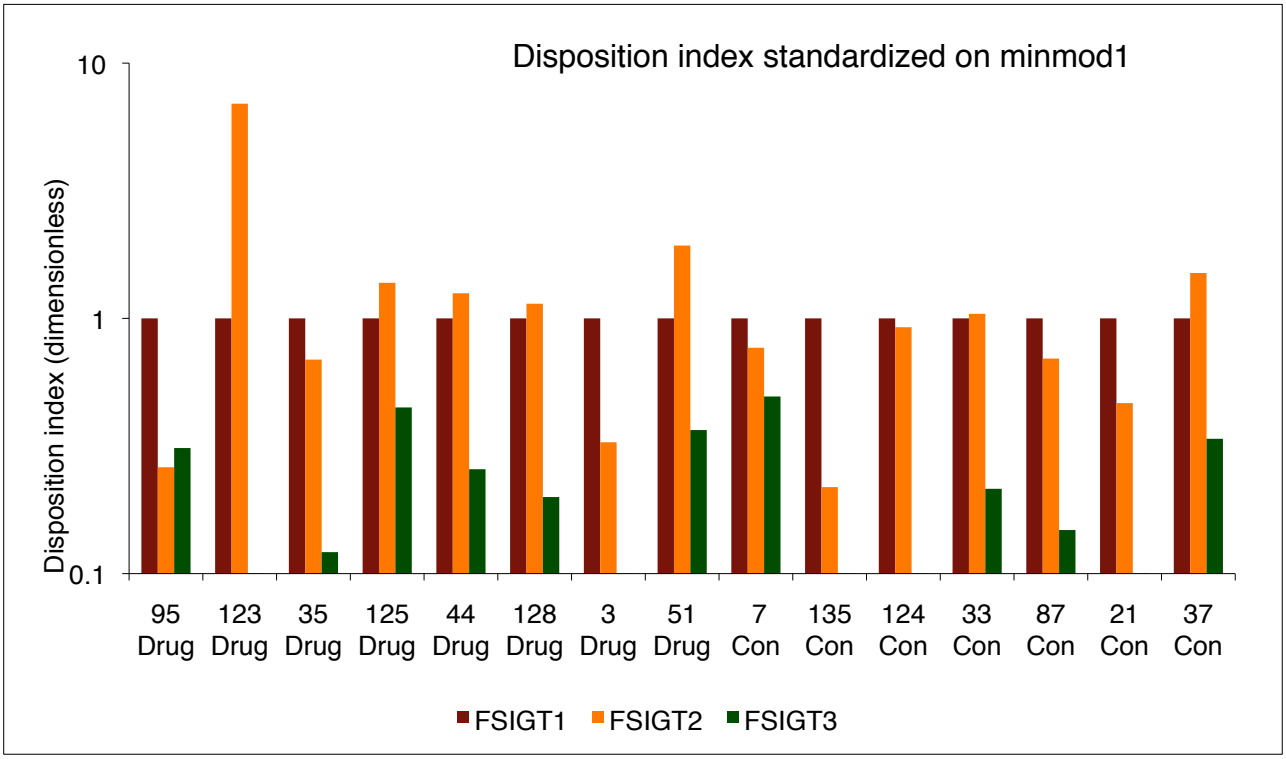


Figure 25. Disposition index (DI; x10^{-2} dimensionless) values calculated via minimal model analysis of each FSIGT for individual horses at each time point standardized on FSIGT 1 to account for individual horse variability in baseline DI.



Appendix H : LPS Stock Solution formulation

- Supplied as 1mg/mL 2mL vial
- Dilute 1mL in **99mL** Saline → 100mL Stock solution 10,000ng/mL
- Take stock solution and dilute up to 500mL with 0.9% saline for administration.
- Then 500mL saline flush after LPS bolus to ensure line clearance.

1g = 1,000,000,000 ng (10^{-9})

= 1,000,000 μg

= 1,000 mg

1ng = 0.000,000,001 g

1mg/mL	=	1,000	μg/mL
	=	1,000,000	ng/mL

1mL in 100mL	= 1,000,000	ng/100mL
	= 10,000	ng/mL