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Insulin dose response curves and factors affecting insulin sensitivity in horses

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**INSULIN DOSE RESPONSE CURVES AND FACTORS AFFECTING
INSULIN SENSITIVITY IN HORSES**

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ABSTRACT

Three experiments were conducted to further study the use of intravenous insulin injection to measure insulin sensitivity in horses. In the first experiment, glucose responses to multiple doses of recombinant human insulin were compared in insulin sensitive and insensitive mares. Regression lines describing the responses in insensitive mares had less (P = 0.003) steep slopes and greater (P = 0.006) effective doses at 50% response (ED50) compared to sensitive mares. Curvilinear regression models provided a good fit ($R^2 = 0.95$) for the prediction of ED50 from the individual responses to 50 mU/kg BW insulin. The second experiment estimated the repeatability of the glucose responses to a fixed dose of insulin in 41 horses (mares and geldings) injected twice, between 7 and 14 days apart. Overall agreement between the responses was significant (P < 0.001) but not high enough to be predictive (R^2 value = 0.384). Intra-horse coefficients of variation (CV) ranged from 0 to 68.7%, and averaged 23%. Mares and geldings were similar in their repeatabilities. Horses in the bottom half of the sensitivity rankings had a greater (P = 0.03) average intra-horse CV (28.7 ± 4.6%) than horses in the top half (16.7 ± 2.2%). In the third experiment (single switch-back design), glucose responses to a fixed dose of insulin were compared after an overnight (about 12 hours) versus a 24-hour period of feed deprivation in geldings previously determined to be insulin sensitive (n = 4) versus insensitive (n = 4). The longer period of feed deprivation decreased (P = 0.06) the percentage decrease in blood glucose concentrations, indicating a lesser sensitivity relative to the 12-hour period of deprivation. Insulin sensitivity also affected (P = 0.0003) the glucose response, with sensitive geldings exhibiting the greater response. It was concluded that the response to a single dose of insulin is often a good predictor of a horse's insulin sensitivity, but that the lack of repeatability in some horses could lead

to errors in diagnosis. The decrease in glucose response seen after 24 hours of feed deprivation is likely due to the elevation of fatty acid concentrations in the blood.

INTRODUCTION

Caltabilota et al. (2010) first reported on the use of insulin injection to directly estimate insulin sensitivity in horses. In their experiments, three doses of recombinant human insulin were used to obtain the percentage decreases (% decreases) in blood glucose concentrations, and then linear regression analysis was used to estimate the dose that would result in a 50% reduction (effective dose 50, or ED50). Applying their technique to mares known to be hyperleptinemic versus those with normal leptin concentrations, Caltabilota et al. (2010) confirmed that hyperleptinemic mares were relatively insulin insensitive compared to mares with normal leptin concentrations. Earl et al. (2012) further applied the technique of Caltabilota et al. (2010) to show that feeding conditions prior to testing (overnight feed deprivation versus hay or pasture for ad libitum consumption) altered the estimates of insulin sensitivity, and that epinephrine administration immediately before testing totally obliterated the glucose response to injected insulin.

In the experiments reported by Caltabilota et al. (2010) and Earl et al. (2012), the incremental increases in % decreases produced by increasing doses of insulin seemed to differ between horses with low versus high insulin sensitivity. That is, not only was the ED50 value for insensitive horses shifted to the right (higher) on the dose-response curve, but the slope of the curve was altered as well. The first experiment described herein was designed to study these differences in more detail, i.e., to better describe the slopes and intercepts of the dose-response curves of horses predetermined to be insulin sensitive versus insensitive.

In an effort to simplify the method of assessing insulin sensitivity described by Caltabilota et al. (2010), a second experiment was designed to determine the repeatability of the glucose response to a single, fixed dose of insulin. Caltabilota et al. (2010) concluded, with a limited number of horses, that the glucose response to insulin at 50 mU/kg BW was correlated

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with the overall estimates of ED50 derived from 3 doses of insulin, but that the relationship seemed better for sensitive than insensitive horses. To be practical, such a simplified approach would need to be repeatable across a wide spectrum of sensitivities.

A final experiment was conducted to assess the effect of overnight versus 24 hours of feed deprivation on the glucose response in mares to a fixed dose of recombinant human insulin. Feed deprivation gradually leads to an elevation in the plasma concentration of nonesterified fatty acids (NEFA), also referred to as free fatty acids (FFA). In horses, Sticker et al. (1995) and Nadal et al. (1997) showed that NEFA concentrations were significantly elevated after 24 hours of feed deprivation. Elevated NEFA concentrations are reported to cause insulin insensitivity in mares (Sessions et al., 2004), thus it was hypothesized that extending the period of feed deprivation from overnight to 24 hours would further reduce the glucose response to a fixed dose of insulin.

CHAPTER I

REVIEW OF LITERATURE

Metabolic Syndrome in Humans

When first described, metabolic syndrome (METS) was considered a combination of hypertension, hyperglycemia, and gout (Kylin, 1923, as referenced by Janiszewski et al., 2008). The World Health Organization (WHO) has produced the most widely accepted definition established by the European Group for the Study of Insulin Resistance (Balkau and Charles, 1999; WHO, 1999). This definition constructed by WHO assumes insulin resistance as a major underlying contributor to METS. To be diagnosed with METS, the patient must also have 2 of the following symptoms, in addition to insulin resistance: hypertension, raised plasma triglycerides, low HDL cholesterol, or obesity (waist to hip ratio) at specific levels (WHO, 1999). Identification criteria for METS, according to the European Group for the Study of Insulin Resistance, is similar to that described by WHO, except measurements vary slightly for the above characteristics (Balkau and Charles, 1999).

To date, multiple symptoms have been associated with METS, including dyslipidemia, in which individuals have elevated triglyceride levels and low HDL-cholesterol concentrations (Carr, 2004). Elevated blood pressure is another symptom associated with obesity and commonly occurs in people with insulin resistance. Rises in C-reactive protein levels are positively correlated with adiposity and insulin resistance (Lemieux, 2001). The rise in C-reactive protein is promoted by inflammatory state caused by association with obesity. Adipocytes and macrophages release inflammatory cytokines causing aggravation throughout the body (Fresno et al., 2011).

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Equine Metabolic Syndrome

The term equine metabolic syndrome (EMS) was first described by Johnson in 2002. This syndrome represents a cluster of physiological alterations including obesity, insulin resistance, hypertriglyceridemia, regional adiposity, and hyperleptinemia. Furthermore, laminitis and altered reproductive functions have been described (Frank, 2009).

Horses that are affected by EMS are often called "easy keepers" due to the fact that they can maintain their body condition with no more than pasture for nutrient intake. Equine metabolic syndrome has been observed in various breeds, including ponies, Paso Finos, Morgans, Arabians, Quarter Horses, and Thoroughbreds. The best indicator of EMS is expansion of adipose tissue within the neck region. This "cresty neck" phenomenon has been associated with insulin resistance in horses and ponies (Frank et al., 2006 ; Carter et al., 2009). A scoring system for the assessment of adipose expansion around the crest of the neck has been defined by Carter et al. (2009).

Previous studies revealed that laminitis predisposition may be determined by the ability of glucose to be supplied to hoof tissues (Pass et al., 1998). Insulin receptors affect phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways. In insulin resistant animals, the PI3K pathway, which stimulates glucose uptake, is compromised. A PI3K pathway that is compromised cannot synthesize nitric oxide, which normally causes vasodilatation. Hyperinsulinemia also causes vasoconstriction by an increase in MAPK. Adhesion molecules are in greater abundance in the laminar vessels in horses with laminitis than those without (Loftus et al., 2007).

Obesity and Insulin Resistance

In humans, obesity represents an inflammatory state producing an increase of circulating inflammatory cytokines (Xu et al., 2003). Studies in some species show that obesity results in the

body secreting adipokines, endocrine signals produced by adipocytes, in stressful situations. There is an increased amount of adipokines associated with obesity and these signals can cause insulin resistance (Lyon et al., 2003).

Cortisol counteracts insulin's effect on regulating carbohydrate metabolism. An enzyme produced by omental adipocytes, 11-β hydroxysteroid dehydrogenase, is capable of converting cortisone, a relatively inactive glucocorticoid, to the highly active glucocorticoid, cortisol. Cortisol system causes impairment of peripheral tissue uptake of glucose and overproduction of hepatic glucose, which together leads to the development of insulin resistance (Masuzaki et al., 2001). Adipose tissues secrete more monocyte chemoattractant protein-1 when they reach storage capacity and are stressed (Vick et al., 2007). The number of macrophages in adipose tissue increase and these inflammatory cells secrete tumor necrosis factor alpha (TNFα; Kim et al., 2007). The more obese an animal is, the more TNFα is secreted, making an EMS horse more susceptible to laminitis (Vick, 2007). Leptin and adiponectin are adipokines, i.e., hormones produced by adipocytes, and are affected by obesity. High plasma leptin and low plasma adiponectin concentrations are associated with obesity in the horse (Kearns et al., 2006)

Pancreatic Insufficiency

In horses with chronic insulin resistance, elevated blood insulin concentrations are due to the pancreas over-secreting insulin to compensate for the reduction in action at the tissue level. Previous studies have described that horses with EMS for many years may develop a decrease in pancreatic insulin secretion and cause increased hyperglycemia. Pancreatic exhaustion or damage may develop in EMS as a result of insulin resistance (Kronfeld et al., 2006). In humans, this continual high output of insulin, common in type 2 diabetes, often leads to exhaustion of the pancreatic islets, which then stop producing and secreting insulin, resulting in frank, or type 1, diabetes (Guyton and Hall, 2006). Many reasons for decreased insulin secretion have been

described including pancreatic exhaustion, pancreatic failure, or pancreatic insufficiency (Treiber et al., 2005).

Insulin Resistance Characteristics

Insulin resistance has previously been described as a state in which a greater than normal amount of insulin is required to elicit a quantitatively normal response (Moller and Flier, 1991). Severe insulin resistance is measured by fasting insulin levels above 50 µU/mL or peak insulin levels above 350 µU/mL. Fasting insulin levels below 20 µU/mL and peak levels of insulin below 150 µU/mL are seen in normal individuals (Vidal-Puig and Moller, 1997). There are two important variables involved in glucose homeostasis: insulin secretion from beta cells in the pancreas in response to blood glucose, and the sensitivity of adipose tissues and skeletal muscle to serum insulin concentration (Kaneko,1989; Firshman and Valberg, 2007).

A decrease in normal tissue sensitivity is classified as insulin resistance and this problem can occur before insulin receptor binding, down regulation of the receptor, or because of disturbances of glucose uptake pathways after insulin binding (Kronfeld et al., 2005; Treiber et al., 2006).

Insulin resistance has been classified into distinct phenotypes, type A and type B syndrome. Type A was originally applied to lean adolescent females with severe insulin resistance, acanthrosis nigricans, severe ovarian hyperandrogenism, and decreased insulin binding to circulating leukocytes (Kahn et al., 1976). Type A is used to diagnose males and females (Mantzoros and Flier, 1995; Vidal-Puig and Moller, 1997). Type B syndrome has been described in middle-aged individuals with common symptoms of insulin resistance as well as autoimmunity (vitiligo and arthritis) and Hodgkin's disease (Kahn, 1976; Moller and Flier, 1991; Mantzoros and Flier, 1995; Vidal-Puig and Moller, 1997). The diagnosis hallmark of type B

patients is anti-insulin antibodies present in plasma (Kahn, 1976; Moller and Flier, 1991, Mantzoros and Flier, 1995).

Measuring Insulin Sensitivity in Horses

Over the years, insulin sensitivity in horses has been measured by one of two standard methods, both derived from extensive research in human medicine and applied to the horse. The hyperinsulinemic-euglycemic clamp involves the constant infusion of a fixed concentration of insulin and, simultaneously, sufficient glucose to maintain euglycemia (Kaske et al., 2001; Powell et al., 2002; Rijnen and van der Kolk, 2003). Insulin sensitivity is initially indicated by the glucose infusion rate once euglycemia is attained and held constant (sensitive horses require a higher infusion rate of glucose than insensitive horses). The other approach is the minimal modeling of the insulin-modified, frequently sampled intravenous glucose tolerance test (FSIGT; Bergman et al., 1987). The FSIGT involves an intravenous injection of a bolus of glucose at time 0, followed by an injection of insulin 20 min later (Hoffman et al., 2003; Treiber et al., 2005). Frequent samples are drawn before and after each injection, and glucose and insulin concentrations are measured; a computer program is then used to model the results and obtain estimates of insulin sensitivity, insulin-independent glucose clearance, and beta cell function (i.e., the degree of insulin response in the first 20 min).

Although the use of insulin per se as an injection had not been used to estimate insulin sensitivity, it had been used in various experimental settings with horses and mules (Silver et al., 1987; Alexander et al., 1997; Forhead and Dobson, 1997). One concern from a clinical standpoint was the possibility of insulin overdose, which might lead to hypoglycemic shock (Given et al., 1988), plus, there was also a chance of inducing laminitis with repeated injections (Asplin et al., 2007). However, doses of insulin up to 0.4 USP units (U) per kg BW were administered to feed-deprived donkeys without any reported serious side-effects (Forhead and

Dobson, 1997), and Gentry et al. (1999) noticed no detrimental effects in horses administered a single i.v. dose of insulin of 0.1 U/kg BW.

Based on these latter observations, Caltabilota et al. (2010) developed a method of assessing insulin sensitivity in horses based on the initial injection of human recombinant insulin at a fixed, low dose (50 mU/kg BW) followed by appropriate higher or lower doses, depending upon the horse's reaction to the initial injection. In that report (Caltabilota et al., 2010), it was also shown that the glucose response to the initial dose of 50 mU/kg BW, as well as the eventual estimate of insulin sensitivity (i.e., the dose of insulin predicted to cause a 50% decrease in blood glucose concentrations), were both highly repeatable when performed in 2 separate experiments. It was concluded that the intra-horse coefficient of variation (CV) was equivalent to the other, more established methods of assessing insulin sensitivity (the clamp and the minimal model). Subsequently, Earl et al. (2012) applied the insulin injection technique of Caltabilota et al. (2010) to show that two potential treatments for insulin resistance (cinnamon ingestion and fish oil ingestion) had no beneficial effect in mares.

Free Fatty Acids and Insulin Sensitivity

Insulin resistance is induced by free fatty acids (FFA) during starvation. The higher levels of FFA signal the body to reserve carbohydrates for fuel and glucose for the brain. In obese individuals, plasma FFA levels are elevated. Although lipolysis rates from fat cells appear normal (Reaven et al., 1988), larger amounts of FFA are released from the expanded adipose tissue in obese individuals (Gorden, 1960). When comparing healthy versus type 2 diabetic individuals, healthy patients had a twofold higher level of insulin stimulated glucose uptake versus type 2 diabetic patients (Boden and Chen, 1995). Infusion of triglycerides plus heparin, triglycerides without heparin, and a control group of saline infusion was used in his experiment to conclude that high levels of FFA inhibit glucose uptake by insulin (Boden, 1998). That

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experiment also revealed that depressing FFA beneath basal levels produce an increase in insulin stimulated glucose uptake.

Several mechanisms can account for FFA-induced defects in glucose uptake. Lab rat studies determined that fat-induced insulin resistance is linked to buildup of uridine diphosphate N-acetylglucosamine (UDP-N-acetyl glucosamine), the end product of hexosamine pathway. Accumulation of UDP-N-acetyl glucosamine hexosamine in skeletal muscle produces insulin resistance (Hawkins et al., 1997). In an experiment with men, those with a higher concentration of intramuscular triglycerides had a decreased response of whole body glucose uptake in reaction to insulin (Virkamaki et al., 2001), regardless of body weight or physical fitness. Sessions et al. (2004) found this consistent with their experimental results with mares; i.e., not all obese mares necessarily have high intramuscular triglyceride concentrations, and not all responded to elevation of plasma NEFA with an immediate reduction in insulin sensitivity (most did).

A number of long chain fatty acids have been found to decrease mRNA levels coding for the protein glucose transporter 4 (GLUT 4) by reducing Glut 4 gene transcription and disrupting its message. The Glut 4 gene expression in muscle tissue could be inhibited by insulin resistance due to FFA (Long et al., 1996).

Cell membrane fluidity can be changed by FFA. Research has shown that change in fatty acid content of membranes (insulin receptors are located in the lipid bilayer of plasma membrane) modifies insulin receptor accessibility, insulin binding, and insulin action. Polyunsaturated fatty acid content in the body increases binding of insulin and membrane fluidity, whereas decreasing their content has the opposite effect (Ginsberg et al., 1981, Grunfeld et al., 1981, Farias et al., 1987, Borkman et al., 1993).

Rationale for Present Experiments

The experiments described herein were conducted to further document the glucose response to insulin injection, as described by Caltabilota et al. (2010), and to determine the repeatability of the responses to individual injections versus series of injections as originally reported. Although the technical aspects of measuring insulin sensitivity via the Caltabilota approach are simple (insulin injection and glucose measurement by hand-held glucometer; Caltabilota et al., 2010), it is inevitable that a single-shot test will be desired for on-farm use. Also, factors affecting insulin sensitivity, such as duration of feed deprivation, need to be defined for future use of the technique to be applicable across a broad spectrum of conditions.

CHAPTER II

**GLUCOSE RESPONSE CURVES AFTER INSULIN INJECTION:
CHARACTERIZATION OF CURVES IN SENSITIVE VERSUS INSENSITIVE MARES**

Introduction

Caltabilota et al. (2010) reported that the percentage decrease in glucose concentrations in mares after injection of recombinant human insulin could be used to determine insulin sensitivity, with the limitation that sufficient data must be obtained between 20 and 60% decrease, at which the dose-response curve is basically linear. In the development of the technique described by Caltabilota et al. (2010), it was noticed that mares with very low sensitivities seemed to have a less steep dose-response curves. That is, the incremental increase in % decrease in insulin insensitive mares with each higher dose of insulin was not as much as that for insulin sensitive mares. Caltabilota et al. (2010) suggested that three doses of insulin produced the most reliable estimates of insulin sensitivity, even though the response to a single dose (50 mU/kg BW) did in fact provide a close approximation to the final sensitivity estimate in most insulin sensitive mares, but less so in mares eventually diagnosed as insensitive. The present experiment was conducted to better characterize the insulin-glucose dose-response curves in mares (a minimum of 5 points between 10 and 75% decrease) so that differences among horses of differing sensitivities could be assessed. Data from these curves were then used to estimate the predictability of single-injection results to those obtained with the multi-point curves.

Materials and Methods

Twelve mares, six of known insulin insensitivity and six known to be insulin sensitive (determined via the methods of Caltabilota et al., 2010), were used in this experiment. The mares were between 6 and 16 years of age, weighed between 450 and 650 kg, and were in good body

condition (>6; Henneke et al., 1983). They were routinely housed on native grass pasture, and were brought into an outdoor lot for the experimental procedures. The lot was devoid of feedstuff; however water was available for ad libitum consumption.

For the first day of the experiment, all mares were moved from pasture to the lot and deprived of feed from approximately 17:00 until 07:00 the next morning (September 13, 2010). Two samples of jugular blood were obtained 10 minutes apart from each mare via venipuncture with a 22 ga needle with attached syringe, and glucose concentration was estimated via a hand-held glucometer (Precision Xtra, Abbott Laboratories, www.abbottdiabetescare.com). Glucometer readings were typically not duplicated, except when an obtained value seemed unphysiologic or when the two samples at the 10-min interval differed by >10%.

Once the baseline glucose concentration was established, each mare was then administered intravenously recombinant human insulin (Sigma Chem. Vo., St. Louis, MO) in saline at 50 mU/kg BW. Injection volume was 0.01 mL/kg BW. Blood samples were drawn from the jugular vein for glucose concentration measurement at 40 and 60 min after insulin injection, and glucose measurements were again measured via the glucometer.

Percent decreases were calculated as described by Caltabilota et al. (2010). First, the glucose concentrations of the two pre-insulin injection samples were averaged to obtain a baseline mean. Then, the glucose concentrations for the 40- and 60-min samples were subtracted from this baseline and the differences expressed as a percentage of the baseline (% decrease). The greatest % decrease was used as the mare's response for that insulin injection.

Mares were returned to pasture after all samples had been drawn and assessed. The entire procedure was repeated on September 15, 17, 20, 22, and 24, with different doses of insulin (8, 12.6, 20, 32, 79, and 125 mU/kg BW). Based on the response to the 50 mU/kg BW dose on day 1, the second injection given was one dose higher (79 mU/kg BW; half the horses, primarily the

lowest responders) or lower (32 mU/kg BW; the rest). The goal was to get decreases in glucose concentrations between 10 and 75%, completing the curve in an alternating manner as much as possible (high then low, or low than high, etc.). The insensitive mares typically needed the highest dose (125 mU/kg BW) to get near a 50% decrease, thus one higher dose (198 mU/kg BW) was added for those mares as needed.

Data from each mare were analyzed by linear regression analysis (Steel et al., 1997) in which the x-values were the natural logs (ln) of the insulin doses and the y-values were the associated % decreases in blood glucose concentrations. The slopes and intercepts for each mare's responses were then analyzed in a one-way ANOVA in SAS (SAS Instit., Cary, NC) for comparison between the insulin sensitive and insensitive groups. Also, the ln of the ED50 values (lnED50) and the ED50 values were calculated from the individual regression equations as described by Caltabilota et al. (2010). Briefly, the lnED50 value was the calculated x-value associated with a y-value of 50%, and the ED50 value was the exponent of the lnED50 value. These data, as well as body weights, were analyzed in one-way ANOVA to test the effect of insulin sensitivity group.

Results

The regression lines of the log natural doses of insulin plotted versus the % decrease in glucose concentrations for the individual mares in the sensitive and insensitive groups in this experiment are shown in Figure 2.1. Analysis of those regression data revealed that mares considered insulin insensitive in previous trials had a higher y-intercept (-35.45 vs. -52.6%; $P = 0.066$; Figure 2.2). These insensitive mares also displayed a less steep slope (15.4 vs. 26.2% increase per ln dose unit; $P = 0.0003$) than mares of normal insulin sensitivity (Figure 2.2). Estimates of lnED50 ($P = 0.0002$) and ED50 ($P = 0.006$) derived from the full data sets for each
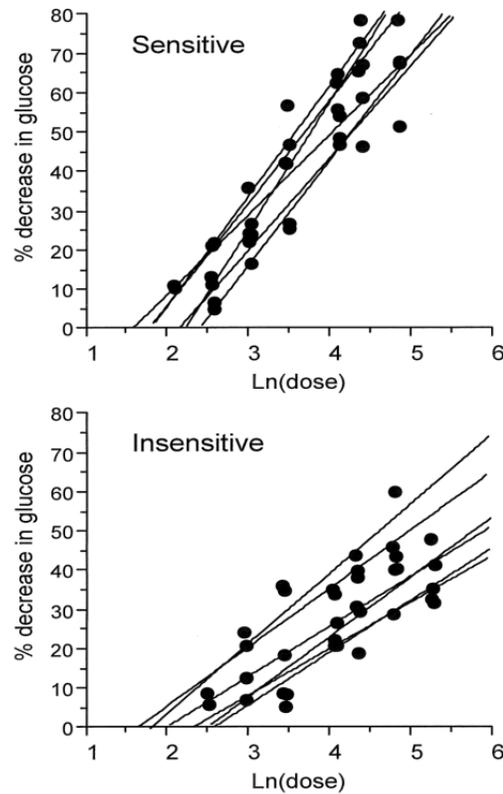
Figure 2.1. Combined plots of the decreases in blood glucose concentrations in response to various dose of recombinant human insulin in the 6 sensitive and 6 insensitive mares. Each line represents one mare, whereas the mares' individual data points are not differentiated. The degree of parallelism shows the relative agreement of the results within each group.

mare were also higher in the insulin insensitive mares than in the mares considered to be sensitive (Figure 2.2). Mean body weights, in contrast, did not differ ($P > 0.24$) between groups.

Estimates of lnED50 and ED50 derived from the full data sets for each mare were plotted against the raw percentage decreases for the 32, 50 and 79 mU/kg BW doses to determine how well the individual responses predicted the results from the more extensive analyses. Similar to reported by Caltabilota et al. (2010), the percentage decrease in glucose concentrations in response to the first dose used (50 mU/kg BW) was highly correlated to the overall estimates of both lnED50 and ED50 (Figure 2.3).

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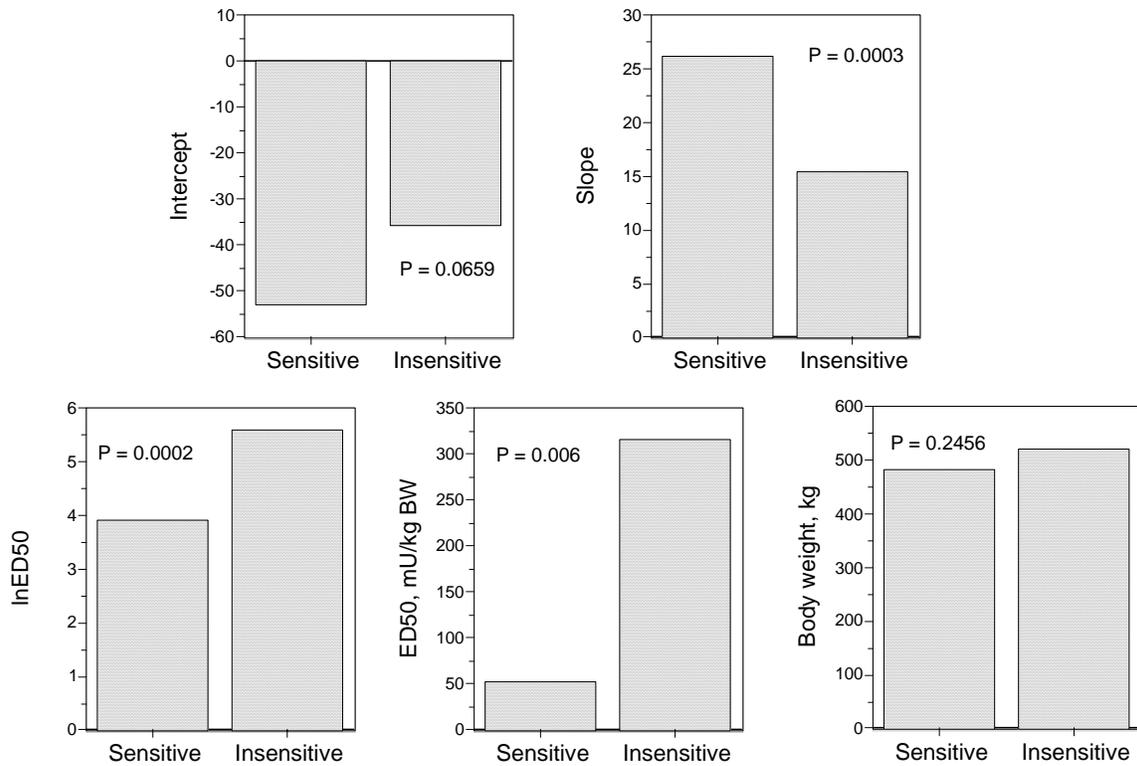
Figure 2.2. Mean slopes and intercepts, lnED50 and ED50 values, and body weights for sensitive and insensitive mares. The p-value for the differences between groups is indicated within each panel.

Discussion

Caltabilota et al. (2010) were the first to formalize the use of insulin injection to directly estimate insulin sensitivity in horses. The previous applications of the hyperinsulinemic-euglycemic clamp (Powell et al., 2002; Sessions et al., 2004) and the minimal modeling of the FSIGT (Hoffman et al., 2003; Treiber et al., 2005) were considered as the currently acceptable methods for estimating insulin sensitivity in horses, however neither approach was easy or convenient for on-farm use. Caltabilota et al. (2010) showed that their approach could easily be performed on the farm, and that at the doses of insulin used, no detrimental side effects of the
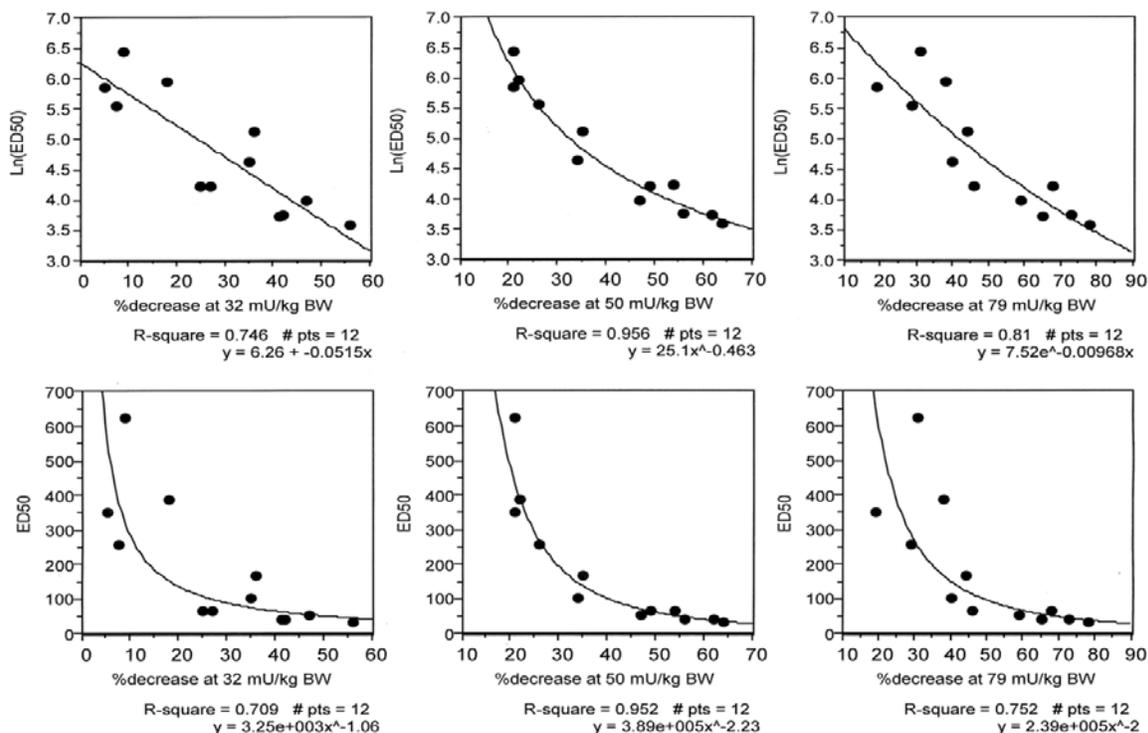
Figure 2.3 Relationships between the % decrease in glucose concentrations for the 32, 50 and 79 mU/kg BW insulin doses and the estimates of the lnED50 and ED50 derived from the complete data sets for each mare. The starting dose of 50 mU/kg BW provided the highest correlation coefficient (best fit of the data) in both cases.

injected insulin were found. Moreover, Caltabilota et al. (2010) applied the technique to mares previously categorized as hyperleptinemic, as described by Gentry et al. (2002) and Cartmill et al. (2003), to confirm that the hyperleptinemic state was indeed associated with a reduced insulin sensitivity. Subsequently, Earl et al. (2012) reported that a single injection of recombinant human insulin could be successfully used to assess the effects of epinephrine injection, prior feed intake, and cinnamon and fish oil supplementation in horses. However, just as Caltabilota et al. (2010) emphasized that dose of insulin is critical to the usefulness of the data collected, Earl et al. (2012) showed that the single-dose approach to studying the effects of various factors on insulin

sensitivity was best performed with a higher starting dose of insulin for horses known to be insensitive.

Given standard dose-response curve analysis, a shift in the curve towards the right (higher x-values) indicates an upward shift in amount of x required to produce a fixed response in y. Thus, it was not surprising that the dose-response curves for mares previously determined to be insulin insensitive were in fact shifted towards higher x-values (as seen in lnED50 values). In addition, the slopes of those regression lines for insensitive mares were also different, in that they were less steep than those for sensitive mares. This difference in slope indicates the inefficiency of insulin usage in insensitive mares; that is, for each fixed incremental increase in insulin dose, there's less of an increase in the % decrease in blood glucose concentrations relative to sensitive mares. Similar shifts in the response curve were reported for adipose cells cultured in vitro (Kronfeld et al., 2005). Where exactly this inefficiency occurs in the insulin-receptor-cellular response mechanism scheme is unknown. Insulin insensitivity in obesity in humans is thought to be due to high intracellular lipid content that leads to a series of intracellular changes resulting in fewer GLUT4 proteins incorporated into the plasma membrane in response to insulin (Pessin and Saltiel, 2000; Morino et al., 2006). However, Pessin and Saltiel (2000) emphasize that various forms of insulin insensitivity are likely caused by numerous factors or deficiencies in the insulin-response cascade, and that no one factor can account for all cases in humans.

The use of a single dose of insulin to predict lnED50 (and hence, ED50) would be a useful shortcut and a time-saver for estimating insulin sensitivity in horses, and in fact, the relationships of lnED50 and ED50 with the individual responses to the 50 mU/kg BW insulin dose were very good (Figure 2.3), albeit not linear in nature. Caltabilota et al. (2010) noted that the response to the 50 mU/kg BW dose was generally correlated to the estimates of ED50 derived from the responses to three insulin doses, but that the relationship seemed to be not as

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good for insensitive mares compared to sensitive mares. The lesser slopes of the insensitive mares, plus the fact that the 50 mU/kg BW dose generally produces a % decrease in blood glucose concentrations well below 50% in those mares, together probably contribute to this observation. Moreover, retrospective analysis of the individual coefficients of determination from the regression analyses for determination of lnED50 indicated that regression accounted for an average of 93.2% of the variation in the analysis of the sensitive mares' data, whereas the corresponding mean for the insensitive mares was 84.6% ($P = 0.053$). This likely confirms the implication by Caltabilota et al. (2010) that this technique for estimating insulin sensitivity is most reliable, and perhaps repeatable, in sensitive horses.

Humans with a higher concentration of intramuscular triglycerides, as in obesity, have a decreased response to glucose uptake by the whole body in reaction to insulin (Virkamaki et al., 2001). At high levels of exposure to exogenous fatty acids, muscle tissue becomes insulin resistant (referred to as the lipotoxicity theory; Holland et al., 2007; Savage et al., 2007). Similarly, the inflammation theory is based on the immune response creating an overproduction of inflammatory cytokines that disrupt normal metabolic function of glucose and insulin responses (Holland et al., 2011). Vick et al. (2007) confirmed that obese horses as well have increased inflammatory factors circulating in the blood and a reduced sensitivity to insulin, and perhaps the insensitive mares in the present experiment were more exposed to inflammatory cytokines and lipids in their muscle tissue. However, other factors are likely involved, because horses in the present experiment and in those of Caltabilota et al. (2010) and Earl et al. (2012) could be identified as insensitive, whereas horses of similar body weights and body condition scores were also identified that were insulin sensitive.

CHAPTER III

REPEATABILITY OF GLUCOSE RESPONSES TO A STANDARDIZED
DOSE OF INSULIN

Introduction

Previous research performed at the LSU Agricultural Center horse farm (Caltabilota et al., 2010; Earl et al., 2012) described the development of a standardized procedure to assess insulin sensitivity in horses by means of multiple doses of recombinant human insulin. In that procedure, an initial dose of 50 mU/kg BW was used, and subsequent doses, either higher or lower, were administered based on the response to the 50 mU/kg BW dose. Subsequent description by Caltabilota et al. (2010) of the dose-response curves between insulin dose and percentage decrease (% decrease) in blood glucose concentrations revealed that the calculated effective dose-50 (ED50), i.e., the dose that caused a 50% decrease in pre-injection glucose concentrations, was correlated to the original % decrease in blood glucose in response to the 50 mU/kg dose. Caltabilota et al. (2010) did point out that the correlation seemed better for sensitive mares than for insensitive mares. If a single injection of insulin is to be used reliably to estimate a horse's insulin sensitivity, it's imperative that the response to that single dose be repeatable itself. Thus, the purpose of the present experiment was to determine the repeatability of responses in blood glucose concentrations to the standard 50 mU/kg BW dose of insulin in horses across a wide range of insulin sensitivities.

Materials and Methods

Twenty-four mares and 17 geldings housed at the LSU Agricultural Center horse farm were used starting on August 28, 2011. They were all routinely maintained on native grass pasture, and were brought into smaller lots as needed for experimental procedures. They ranged

in age from 5 to 17 years, weighed between 450 and 650 kg, and had body condition scores between 5 and 8.

The horses were tested in subgroups (two gelding groups of approximately 8 to 9 per group, and three mare groups of 6 to 9 mares each) for their response to a single intravenous injection of recombinant human insulin (50 mU/kg BW; Sigma Chem. Co., St. Louis, MO) after an overnight (12-h minimum) period of feed deprivation. In general, the horses were brought in from pasture between 17:00 and 19:00 and were placed in a holding pen with no food but free access to water. The following morning (approximately 07:00), they were placed in an outdoor chute, or, in inclement weather, inside a covered, open-sided shed, for testing. Two samples of blood were drawn from the jugular vein by venipuncture 10 min apart and assessed for blood glucose concentration by means of one of two glucometers (both Precision Xtra).

The same glucometer was used for a given horse throughout the day. If the two readings agreed within 10%, insulin was injected at that time. If there was a large difference between pre-injection samples, a third was drawn; the two that agreed the most were used for the pre-injection average. Once insulin was injected, samples were drawn at 40 and 60 min thereafter for blood glucose assessment. The mean pre-injection glucose concentration and data from the 40- and 60-min samples were used to calculate % decrease as described in Chapter II.

Repeat injections of insulin at the same dose were given at least 7 days later, but no more than 14 days later. This provided a 7-day window in which to do the repeat injections. The procedures described above for the first injection were repeated as closely as possible for the second injection. Any noticeable differences in the horses, the environmental conditions, etc., between the day of first injection and the day of second injection were noted. Because excitement or stress can perturb the glucose response to insulin, horses were kept as quiet as possible and any physical exertion was avoided.

After all data were collected, regression analysis of first response (x axis) versus second

response (y axis) was run in SAS (proc reg; SAS Instit., Cary, NC) to obtain a slope, intercept,

and coefficient of determination (R^2; Steel et al., 1997). In a perfectly repeatable situation, the

respective values would be slope = 1, intercept = 0, and $R^2 = 1$.

Results

The plots of the paired % decreases for all horses are presented in Figure 3.1. The

resulting linear regression line was: % decrease to second injection = 0.59 x % decrease to first

injection + 14.4%. The P-values associated with the estimates of slope and intercept were P <

0.001 and P = 0.0057, respectively. The coefficient of determination (R^2) was 0.384.
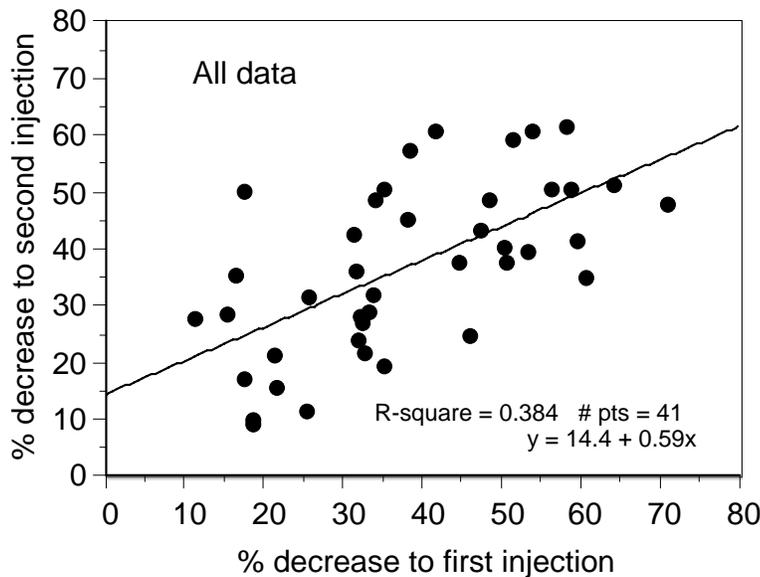
Figure 3.1. Scatterplot of the % decreases for the 24 mares and 17 geldings after the first versus
second injection of recombinant human insulin at 50 mU/kg BW. The resulting regression
equation and coefficient of determination are shown within the panel.

The data was further separated into that for mares versus geldings (Figure 3.2). The two resulting regression equations were similar to that for all data combined. The correlation coefficients were compared Using the Fisher r-to-z transformation (SAS) and were not different (P = 0.74).

The individual intra-horse coefficients of variation (CV), which are indices of the variation between the two estimates for the individual horses, were calculated. They ranged from 0 to 68.7%, and averaged 23% (S.D. was 17.4%). When horses were ranked by their mean % decrease, those below 40% (21 horses, or about the lowest half) had a mean intra-horse CV of $28.7 \pm 4.6\%$ (S.E.) and those in the highest half had a mean intra-horse CV of $16.7 \pm 2.2\%$ (P = 0.03)
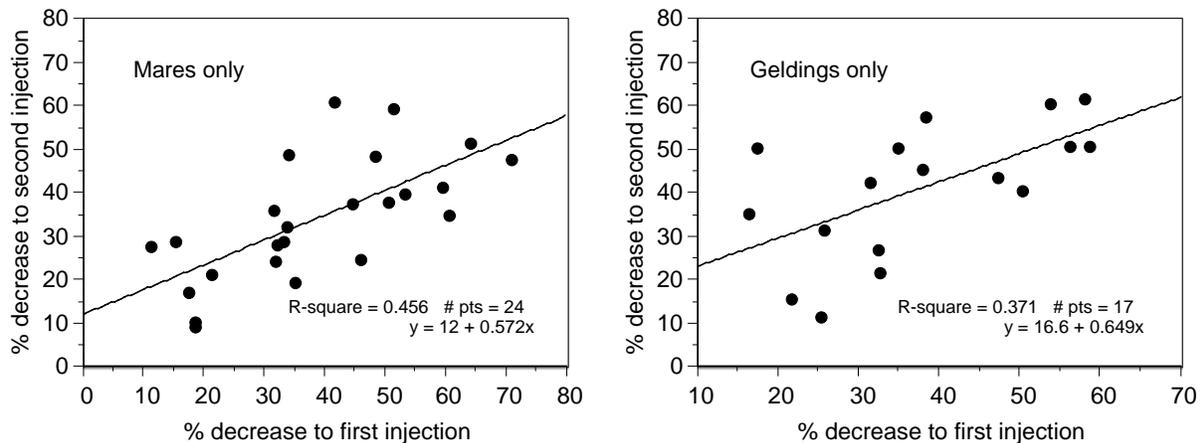
Figure 3.2. Scatterplot of the % decreases after the first versus second injection of recombinant human insulin at 50 mU/kg BW for the 24 mares (left panel) and 17 geldings (right panel) analyzed separately. The resulting regression equation and coefficient of determination are shown within the panel.

Using a % decrease of 50% as a demarcation of the sensitive (>50%) and insensitive (<50%) horses, 25 horses (61%) were categorized as insensitive in both cases (injections), 6 horses (15%) were categorized as sensitive in both cases, and 10 horses (24%) were categorized differently in the two cases.

Discussion

Caltabilota et al. (2010) reported good agreement between the % decreases in 12 mares treated with recombinant human insulin at 50 mU/kg BW in summer and again that following October (coefficient of determination = 0.85; decline in October = 0.72 × % decline in June and July + 6.98%). Application of this technique to a greater number of horses, and including both mares and geldings, in the present experiment did not result in as good an agreement between the two insulin challenges, and there was no difference between mares and geldings in the present experiment as a far as repeatability of their responses. Pratt et al. (2005) used the intra-horse CV of estimates of insulin sensitivity measured via the hyperinsulinemic-eugycemic clamp technique and the FSIGT as an estimate of their repeatabilities, and reported means (and ranges) of 14.1% (7 to 20%) and 23.7% (9 to 35%) for the two techniques, respectively. Caltabilota et al. (2010) applied the same calculation to the duplicate estimates of lnED50 mentioned above and reported a mean intra-horse CV of 8.9% (range 2.3 to 18.8%). The average intra-horse CV for the % decrease in blood glucose concentrations observed herein (23%) was similar to that reported for the FSIGT by Pratt et al. (2005), however the range was much greater (0 to 68.7%).

The fact that horses with the lowest % decreases had a higher mean intra-horse CV than those in the highest half somewhat confirms the concept discussed in Chapter II and implicated by Caltabilota et al. (2010) that this technique (insulin injection) for estimating insulin sensitivity seems most reliable, and perhaps most repeatable, in sensitive horses. However, based on the % decrease data collected in the present experiment, there does not appear to be distinct categories

of sensitive versus insensitive, but rather a continuum ranging from low to high. That is, the individual mean % decreases (averages of the two estimates per horse) in this experiment ranged from 13.9 to 59.9%, and breaking the data down in 10% increments (e.g., 10.1 to 20%, 20.1 to 30%, etc.) resulted in numbers of horses of 6, 8, 7, 11, and 9 in those respective increments. Given this continuous nature of the data, categorization of horses into sensitive and insensitive becomes complicated, not for those on the extremes (lowest end and highest end), but for those in the middle (e.g., in the 40 to 60% decrease range).

In general, horses with % decreases below 35% at the first injection also registered below 35% at the second injection (16 out of 20, or 80%). Thus, although a single injection response below 35% might predict an insensitive horse, there's always the possibility that it won't hold true for a repeat injection for any given horse. In addition to the inherent variation associated with most measurements in biological situations, short-term factors that might alter the estimate of % decrease include excitement of the horse before injection (Earl et al., 2012) and failure to inject the entire insulin dose into the vein. Precautions were taken in the present experiment to avoid any stress or excitement of the horses, thus that is not considered a factor here. The most variable two responses were from a gelding that registered a 17% decrease in blood glucose concentrations at the first injection and a 50% decrease at the second injection; a third injection would likely have agreed with one or the other. However, relying on either response individually would be misleading if the wrong one were chosen.

The glucometers used in this experiment (Precision Xtra) were originally used by Caltabilota et al. (2010) and Earl et al. (2012) because they were the brand used by Eiler et al. (2005) in their studies on blood glucose homeostasis. When tested in the laboratory with blood samples selected for low, medium, and high blood glucose concentrations, the agreement between measurements and between glucometers was very good (within a few %). However, in

the field (on the farm), replicate measurements on a given blood sample sometimes did not agree, and replicate measurements would be needed to ensure an accurate estimate. Temperature of the glucometer and strips, and of the blood itself, can alter the reading. Again, knowing this, precautions were taken to avoid having artifacts in the data. Blood glucose concentrations in horses deprived of feed overnight should be relatively constant, and the -10 and 0 samples generally reflected this. Whenever the two pre-injection estimates did not agree (greater than 10 percentage points different), a third sample was used to determine which of the two was the more correct estimate. Once insulin was injected, duplicate readings were performed only occasionally, or when a sample seemed truly outside of physiologic limits. One way of avoiding artifacts of poor estimates post-injection would be to add an addition sampling at 50 min after injection, or perhaps using duplicate readings at the 40 and 60 minute intervals to ensure accuracy.

In the procedure of Caltabilota et al. (2010), which relied on three doses of insulin to estimate ED50, minor fluctuations in the accuracy of a single response had little effect on the estimate of ED50 because the other two points also defined the slope. Given the results of the present experiment, it is concluded that Caltabilota's approach is likely the most reliable for estimating insulin sensitivity by direct insulin injection.

CHAPTER IV

GLUCOSE RESPONSE AFTER INSULIN INJECTION: EFFECT OF 24 HOURS VERSUS OVERNIGHT FEED DEPRIVATION

Introduction

Feed deprivation causes the body to spare glucose, primarily for brain function, and to switch to nonesterified fatty acid (NEFA) usage for energy for the body (Guyton and Hall, 1996). Feed deprivation of horses increases NEFA concentrations in plasma within 24 hours (Sticker et al., 1995, 1996; Christensen et al., 1997; McManus and Fitzgerald, 2000). Feed deprivation for 3 days, compared to overnight, resulted in a decreased glucose response to an intravenous insulin injection of 400 mU/kg BW in donkeys (Forhead and Dobson, 1997). The present experiment was designed to test the hypothesis that 24 hours of feed deprivation would result in a decreased glucose response in geldings to a fixed dose of insulin (50 mU/kg BW) relative to overnight feed deprivation.

Materials and Methods

Eight long-term geldings housed at the LSU Agricultural Center horse farm were used. They were routinely maintained on native grass pasture, and were brought into smaller lots as needed for experimental procedures. They ranged in age from 5 to 17 years, weighed between 450 and 650 kg, and had body condition scores between 5 and 8.

The experiment was conducted as a repeated (4 squares) 2 x 2 Latin square (commonly referred to as a single switchback design) with a 2 x 2 factorial arrangement of treatments (length of feed deprivation and insulin sensitivity status). There was 1 week between trials. The geldings in two of the squares were selected for low insulin sensitivity (the lowest in the results of Chapter 3) and the geldings in the remaining two squares were selected for normal insulin sensitivity (i.e., had the greatest glucose response to the 50 mU/kg BW dose of insulin).

In the first trial, the geldings were placed in stalls in a barn on the morning of February 7, 2012. Four of the geldings were thereafter feed deprived (water only), and the other 4 received grass hay and water for ad libitum consumption. Geldings of the two insulin sensitivity statuses were evenly distributed across groups. Between 17:00 and 19:00 that evening, all hay was removed from the latter group, resulting in a final feed deprivation time of 24 hours for the former group and overnight (12 to 14 hours) for the latter group. Overnight feed deprivation is the standard preparation in most studies of insulin sensitivity, and therefore served as the reference point for the experiment.

The next morning, two samples of blood were drawn from the jugular vein 10 min apart and assessed for blood glucose concentration by means of two glucometers (both Precision Xtras). The basic procedures for data collection and data reduction (to obtain % decreases) were the same as described for Chapter III. The insulin dose was 50 mU/kg BW. Once all data were collected, the geldings were returned to pasture. All procedures described for the first trial were repeated 1 week later, with the feed deprivation assignments reversed.

The percentage decreases were analyzed by ANOVA for a replicated Latin square design with a 2 x 2 factorial arrangement of treatments in SAS. The effects of insulin sensitivity status, duration of feed deprivation, and their interaction, as well as the effects of horse within sensitivity group and day, were tested with residual error. Means of the interaction were compared with the least-significant difference test (Steel et al., 1997).

Results

The mean % decreases for insulin sensitive and insensitive geldings feed deprived overnight or for 24 hours are presented in Figure 4.1. The % decreases for sensitive geldings were greater (P = 0.0003) than those for insensitive geldings, and the longer period of feed
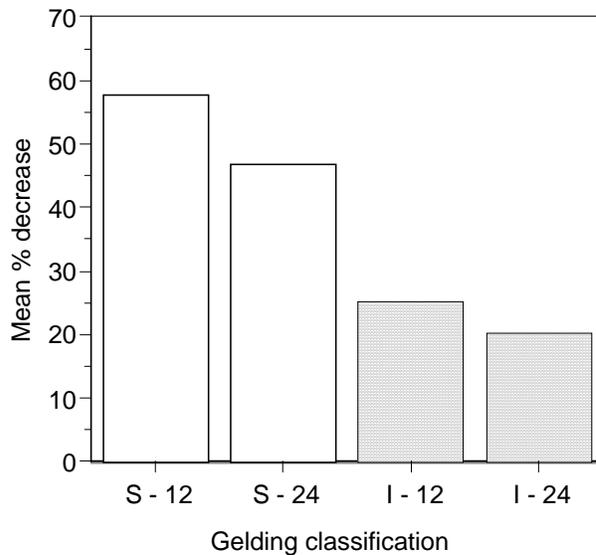
Figure 4.1. Mean % decreases for insulin sensitive (S) and insensitive (I) geldings feed deprived for 12 or 24 hours. There were main effects of insulin sensitivity classification (P = 0.0003) and duration of feed deprivation (P = 0.06) in the ANOVA. The pooled SEM was 3.2%.

deprivation resulted in a reduction (P = 0.06) in % decrease relative to the overnight period of feed deprivation. There was no interaction (P > 0.1) between sensitivity classification and length of feed deprivation.

Discussion

The standard procedure for assessing blood glucose concentrations in human patients involves an overnight fast, so that resting, or basal, glucose and insulin concentrations can be obtained. This same procedure (feed deprivation) is generally applied for animals in veterinary medicine as well, and is part of the hyperinsulinemic-euglycemic clamp protocol used by Powell et al. (2002) and Sessions et al. (2004). In contrast, those who have reported on the use of the FSIGT (Hoffman et al., 2003; Trieber et al., 2005) have provided their horses hay for ad libitum consumption overnight, based on the assertion that total feed deprivation reduces insulin sensitivity. In fact, Earl et al. (2012) confirmed that assertion, showing that the % decrease in

blood glucose concentrations to a fixed insulin dose in horses was 49.8% in horses deprived of feed overnight and 58.3% in horses provided grass hay overnight. As seen in the present experiment, extending the period of feed deprivation to 24 hours reduced the glucose response to insulin even further.

Boden (1998) reviewed the role of free fatty acids in insulin sensitivity and concluded that the elevated free fatty acid concentrations in obese individuals accounted for much of the insulin resistance seen in those individuals. Sessions et al. (2004) reported that elevating the plasma NEFA concentrations of horses resulted in an immediate reduction in insulin sensitivity, as measured by the hyperinsulinemic-euglycemic clamp technique. Feed deprivation elevates plasma NEFA in horses within about 24 hours (Sticker et al., 1995, 1996; Christensen et al., 1997; McManus and Fitzgerald, 2000), supposedly because of the extended period in which insulin secretion is low to nonexistent. Thus, it appears that mobilization of NEFA from the fat cells, in the absence of absorption of nutrients from the gut, provides both a source of energy for many tissues in the body as well as a signal for conservation of glucose. After an extended period of feed deprivation, when all feedstuffs are basically gone from the gut, blood glucose concentrations are maintained via liver glycogenolysis for the first 24 to 36 hours (Geor, 2003) and eventually gluconeogenesis (Geor, 2003). Resistance to any circulating insulin under these conditions would ensure that glucose is available for use by the central nervous system (brain) and red blood cells (Guyton and Hall, 1996).

The results of this experiment are consistent with the hypothesis that 24 hours of feed deprivation would result in a decreased glucose response in geldings to a fixed dose of insulin relative to overnight feed deprivation. It is assumed that an elevation of plasma NEFA concentrations contributed to this reduction in response. It is also possible that a rise in epinephrine output from the adrenal glands (Hadley and Levine, 2007) contributed to the reduced

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response, given that administration of epinephrine to horses totally obliterates the glucose

response to injected insulin (Earl et al., 2012).

SUMMARY AND CONCLUSIONS

Three experiments were conducted to further study the use of intravenous insulin injection to measure insulin sensitivity in horses. In the Chapter II, the glucose responses to multiple doses of recombinant human insulin were compared in insulin sensitive and insensitive mares. Regression lines describing the responses in insensitive mares had less steep slopes and greater ED50 values compared to sensitive mares. Curvilinear regression models provided a good fit ($R^2 = 0.95$) for the prediction of ED50 from the individual responses to 50 mU/kg BW insulin.

The second experiment estimated the repeatability of the glucose responses to a fixed dose of insulin in 41 horses (mares and geldings) injected twice, between 7 and 14 days apart. Overall agreement between the responses was significant but not high enough to be predictive (R^2 value = 0.384). Intra-horse CV ranged from 0 to 68.7%, and averaged 23%. Mares and geldings were similar in their repeatabilities. Horses in the bottom half of the sensitivity rankings had a greater average intra-horse CV ($28.7 \pm 4.6\%$) than horses in the top half ($16.7 \pm 2.2\%$).

In the third experiment (single switch-back design), glucose responses to a fixed dose of insulin were compared after an overnight (12 to 14 hours) versus a 24-hour period of feed deprivation in geldings previously determined to be insulin sensitive (n = 4) versus insensitive (n = 4). The longer period of feed deprivation decreased the percentage decrease in blood glucose concentrations, indicating a lesser sensitivity relative to the 12-hour period of deprivation. Insulin sensitivity also affected the glucose response, with sensitive geldings exhibiting the greater response.

Overall, it was concluded that the response to a single dose of insulin is often a good predictor of a horse's insulin sensitivity, but that the lack of repeatability in some horses could lead to errors in diagnosis. With on-farm use, the repeatability of glucometer readings and

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environmental and perhaps behavioral variations may affect the readings. The decrease in glucose response to a fixed insulin dose seen after 24 hours of feed deprivation is likely due to the elevation of fatty acid concentrations in the blood.

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VITA

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