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Cardiovascular markers during hyperinsulinemia in insulin resistant and insulin sensitive horses

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Cardiovascular markers during hyperinsulinemia in insulin resistant and insulin sensitive horses

Kardiovaskulära markörer under hyperinsulinemi hos insulinresistenta och insulinkänsliga hästar

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SUMMARY

Background: Insulin is an important metabolic hormone that also has cardiovascular effects. Insulin resistance is characterized by decreased tissue sensitivity to insulin, and is a part of two important endocrine diseases in horses, Equine Metabolic Syndrome and Pituitary Pars Intermedia Dysfunction. Both diseases carry an increased risk for laminitis.

Decreased insulin sensitivity in people has been shown to lead to a decrease of the vasodilating effect of insulin. Increased baseline blood pressure and heart rate have also been seen in insulin resistant dogs and rats. The effect of induced hyperinsulinemia on cardiovascular markers such as heart rate, blood pressure and peripheral blood flow has been studied in healthy as well as insulin resistant people and laboratory animals. Studies regarding the effect of insulin resistance and induced hyperinsulinemia on cardiovascular markers in horses are scarce. The purpose of this study was to compare heart rate, blood pressure and hoof wall surface temperature (HWST) in insulin resistant horses with controls and to study the effect of hyperinsulinemia.

Method: Ten insulin resistant (IR) horses and four healthy controls were included in the study. A 180 min euglycemic-hyperinsulinemic clamp (EHC) was performed in all horses. All horses were evaluated for body condition. Heart rate and systemic blood pressure was recorded before the start of the insulin infusion and at 45, 105 and 165 min of insulin infusion. Hoof wall surface temperature was recorded continuously during the 180 min EHC, starting 45 min before infusion start to obtain baseline temperatures. Blood samples for serum insulin and glucose were taken at 10 min intervals during the entire EHC, and insulin sensitivity was assessed from calculation of metabolized glucose per unit insulin (M/I ratio, with < 5 considered insulin resistant).

Results: Insulin resistant horses had higher baseline heart rates than control horses, and heart rate was positively associated with body condition score. There was no difference in systemic blood pressure or hoof wall surface temperatures between groups. Insulin infusion caused a decrease in systolic blood pressure in the control group, but not in the IR group. The induced hyperinsulinemia did not influence heart rate, mean arterial blood pressure, diastolic blood pressure or hoof wall surface temperature in either group.

Conclusion:
This study indicates that horses with insulin resistance have higher baseline heart rates compared to controls and that heart rate is associated with BCS. There are also indications that insulin resistant horses might have an altered cardiovascular response to hyperinsulinemia. Further studies are needed to validate this result, preferably with greater sample sizes and using different breeds of horses.

SAMMANFATTNING

Bakgrund: Insulin är ett viktigt metabolt hormon som även har kardiovaskulära effekter, dels genom kärlpåverkan men även genom stimulans av sympatiska nervsystemet. Insulinresistens är ett syndrom som innebär nedsatt känslighet för insulin och ingår i hästsjukdomarna Ekvint metabolt syndrom och Pituitary Pars Intermedia Dysfunction hos hästar, vilka båda medför ökad risk för fång.

På människa har nedsatt insulinkänslighet visat sig minska insulinets vasodilaterande effekt vilket leder till hypertension. På människa och försöksdjur har man studerat insulinets effekter på kardiovaskulära markörer såsom hjärtfrekvens, blodtryck och perifert blodflöde både på insulinresistenta individer och på individer med normal insulinkänslighet. Studier avseende effekten av insulinresistens och hyperinsulimemi på kardiovaskulära markörer på häst är få. Syftet med den här studien var att jämföra hjärtfrekvens, blodtryck och hovtemperatur hos insulinresistenta hästar med hästar med normal insulinkänslighet samt studera effekten av hyperinsulinemi.

Metod: Tio insulinresistenta hästar och fyra friska kontroller med normal insulinkänslighet genomgick en euglycemisk-hyperinsulinemisk clamp (EHC under 180 min under vilken kardiovaskulära parametrar mättes. Blodtryck och hjärtfrekvens mättes före och under infusionen och hovtemperatur (ett indirekt mått på blodflöde i hoven) mättes kontinuerligt med start 45 min före infusionens början. Seruminsulin och –glukos mättes under EHC, och insulinkänslighet bestämdes genom uträkning av metaboliserat glukos per enhet insulin (M/I-kvot, där ett värde <5 benämns som resistenta). Hästarna hullbedömdes enligt Hennekes skala för *body condition score* (BCS).

Resultat: Vilohjärtfrekvensen var högre hos insulinresistenta hästar jämfört med kontroller, och det fanns ett positivt samband mellan hjärtfrekvensen i vila och hög BCS. Ingen skillnad i blodtryck eller hovtemperatur sågs mellan friska och insulinresistenta hästar. Under insulininfusionen sjönk systoliska blodtrycket hos kontrollerna men var oförändrat hos de insulinresistenta hästarna. Ingen skillnad från ursprungsvärdena sågs i hjärtfrekvens, medelartärtryck, diastoliskt tryck eller hovtemperatur i någon av grupperna under infusionen.

Konklusion: Studien indikerar att insulinresistenta hästar har högre vilohjärtfrekvens än hästar med normal insulinkänslighet. Vilohjärtfrekvens var också associerad till grad av fetma, där feta hästar tenderade att ha högre hjärtfrekvens i vila. Det finns också indikationer på att insulinresistenta hästar har ett förändrad kardiovaskulärt svar på insulininfusion jämfört med friska insulinkänsliga kontroller. Fler studier på ett större material av hästar av olika raser är nödvändiga för att bekräfta detta samband.

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ABBREVATIONS

AUC	Area under the curve
BCS	Body condition score
BP	Blood pressure
CNS	Cresty neck score
DBP	Diastolic pressure
EHC	Euglycemic-hyperinsulinemic clamp
EMS	Equine Metabolic Syndrome
ET-1	Endothelin-1
GLUT	Glucose transporter
HR	Heart rate
HWST	Hoof wall surface temperature
IR	Insulin resistant / insulin resistance
IS	Insulin sensitive
MetS	Human Metabolic Syndrome
MAP	Mean arterial pressure
NO	Nitrous oxide
OGT	Oral Glucose Test
PBF	Peripheral blood flow
pEHC	prolonged Euglycemic-hyperinsulinemic clamp
PPID	Pituitary Pars Intermedia Dysfunction
RAAS	Renin-Angiontensin-Aldesteron-System
SBP	Systolic blood pressure

INTRODUCTION

Insulin resistance (IR) is a key feature of Equine Metabolic Syndrome (EMS) and Pituitary Pars Intermedia Dysfunction (PPID). Both diseases increase the risk of laminitis (Frank *et al.*, 2010). Laminitis is a detrimental disease of the hoof, causing pain, lameness and often leads to euthanasia (Johnson *et al.*, 2009). EMS has a counterpart in human medicine, human metabolic syndrome (MetS). The syndromes both include IR, obesity and dyslipidemia (Frank, 2011).

Insulin is a hormone produced by pancreatic β-cells, which besides its central metabolic function also has vasoactive effects (Kim *et al.*, 2006; Sjaastad, 2010). It acts primarily vasodilating, to increase postprandial glucose uptake in peripheral tissue, but can also cause vasoconstriction (Kim *et al.*, 2006). The physiological response to insulin is highly sophisticated, with vasoconstriction and vasodilation acting in balance through different mechanisms (Younk *et al.*, 2014). Hyperinsulinemia has been shown to increase peripheral blood flow (PBF), lower blood pressure (BP) and increase heart rate (HR) in studies on healthy people, dogs and rats, however conflicting results exists (Anderson *et al.*, 1991; Baron and Brechtel, 1993; Bourgoin *et al.*, 2008; Hall *et al.*, 1995, Santure *et al.*, 2002; van de Borne *et al.*, 1999).

Under IR conditions, the vasodilating effects of insulin are impaired through multifaceted changes in cellular signaling pathways (Kim *et al.*, 2006; Younk *et al.*, 2014). Elevated blood pressure is a vital part of human MetS, and higher baseline BP and/or HR has been shown in IR rats and dogs (Bourgoin *et al*., 2008; Hall *et al*., 1995). The acute hyperinsulinemic effect on HR, BP and PBF on IR subjects is controversial, with studies showing no difference between healthy or IR, or an impaired effect of insulin infusion (Baron and Brechtel, 1993; Bourgoin *et al.*, 2008; Hall *et al.*, 1995; Laakso *et al.*, 1990; Santure *et al.*, 2002).

Studies regarding the effect of insulin resistance and induced hyperinsulinemia on cardiovascular markers in horses are scarce. A study on previously laminitic ponies showed that they had higher baseline blood pressures during summer pasture than ponies with normal insulin sensitivity, and that this was associated with a decreased insulin sensitivity (Bailey *et al*., 2008). In healthy insulin sensitive horses, no effect of insulin infusion was shown on HR, but an increase in PBF was seen (with a decrease in variation in PBF compared to healthy controls) (de Laat *et al.*, 2010). Blood pressure has been shown to decrease during induced hyperinsulinemia in healthy horses (Nostell *et al.*, 2016).

The purpose of this study was to compare heart rate, blood pressure and hoof wall surface temperature (HWST) in insulin resistant horses with controls and to study the effect of hyperinsulinemia. The hypothesis, based on previous studies, is that baseline systemic blood pressure is higher in IR horses than in insulin sensitive horses. Another hypothesis was that hyperinsulinemia would decrease systemic blood pressure and increase HWST in both groups of horses, with changes seen to a lesser extent in IR horses.

In this paper, the review of the literature is focused on the physiological and pathological role of insulin in healthy and resistant individuals. The literature review will begin with a short

description of clinical relevance of IR in horses, and then discuss insulin and its physiological and pathological vascular effects. Finally, existent studies on cardiovascular markers during hyperinsulinemia (and the pathophysiology behind it) will be presented.

LITERATURE REVIEW

Insulin and insulin resistance – clinical relevance

Insulin resistance in horses is defined as "a reduction in the action of insulin on target tissues" (Frank, 2011). Insulin is an anabolic hormone that increases cellular glucose uptake, triglyceride and glycogen synthesis while inhibiting gluconeogenesis and lipolysis (Kim *et al.*, 2006). Besides the key role in metabolism, insulin has a wide array of different physiological functions including anti-inflammatory effects and regulation of the vascular tone (Geor, 2008). The metabolic and vasoactive effects of insulin are synchronized to optimize postprandial clearance of glucose from the blood stream (Younk *et al.*, 2014).

Diagnosis of insulin resistance

Diagnosis of IR in horses can be made using static/screening or dynamic tests (Frank, 2011; Johnson *et al.*, 2012). Static tests (i.e. resting serum insulin and glucose concentration) are less sensitive than dynamic. Dynamic tests measure the metabolic response in serum glucose and insulin to a glucose challenge. Many of the dynamic tests are labour-time-intensive and are primarily used for research purposes, i.e. Euglycemic-Hyperinsulineamic Clamp (EHC) and Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT). These two tests are considered as the Gold Standard for IR diagnosis (Johnson *et al.*, 2012). The Combined Glucose-Insulin Test (CGIT), the Oral Sugar Test (OST) and the Oral Glucose Test (OGT) are all examples of dynamic tests used in the clinical setting (Eiler *et al.*, 2005; Lindåse *et al.*, 2015; Schuver *et al.*, 2014). These tests are made by measuring plasma glucose and insulin after a single administration of oral or intravenous glucose (with or without insulin). Insulin resistance can be quantified using the M/I ratio, which is a method for approximating the amount of glucose metabolized per unit of insulin, which is lower in an IR subject (DeFronzo *et al*., 1979). The M/I ratio is calculated from serum insulin and glucose levels obtained during an EHC.

Insulin resistance and endocrine pathologies

Insulin resistance is a known characteristic in two endocrine diseases of the horse: Equine Metabolic Syndrome (EMS) and Pituitary Pars Intermedius Dysfunction (PPID). Laminitis is a key feature of both these diseases.

Insulin resistant horses have an increased risk of laminitis (Frank, 2011; Johnson *et al.*, 2010). Serum insulin levels have been found to be strongly correlated with laminitis score in both healthy and EMS/PPID horses (Walsh *et al.*, 2009). Laminitis associated with PPID or EMS is called endochrinopathic laminitis, the exact mechanism of which is still elusive. Different mechanisms have been proposed to cause laminitis such as inflammation, vascular/endothelial dysfunction, extracellular matrix degradation and glucose dysregulation (Johnson *et al.*, 2010; de Laat *et al.*, 2010).

The term EMS was first presented by Johnson in 2002, describing a disease in obese horses with glucose intolerance, insensitivity to insulin and a predisposition to laminitis (Johnson, 2002). The name of the disease was chosen from similarities with Human metabolic syndrome, as both conditions involve IR, obesity and dyslipidemia (Frank, 2011). Diagnosis of human MetS is based on the presence of three or more out of the following factors; abdominal obesity, elevation of serum triglycerides and glucose, elevated blood pressure and reduced levels of HDL cholesterol (Grundy, 2016).

Equine Metabolic Syndrome has later been defined by a consensus statement by the American College of Veterinary Internal Medicine in 2010. It was then established that the diagnosis of EMS should be based upon the presence of IR, (characterized by hyperinsulinemia or an abnormal response to glucose or insulin), increased adiposity or obesity, and a predisposition to laminitis (Frank *et al.*, 2010). Additional features are presence of dysplipidemia, hyperleptinemia, arterial hypertension and systemic inflammation (Frank *et al.*, 2010; Johnson *et al.*, 2012). Horses with EMS also have an increased risk of developing hyperlipidemia with secondary hepatic lipidosis (Johnson *et al.*, 2012). Unlike in people, horses with IR seldom develop type 2 Diabetes Mellitus (DM) (Johnson *et al.*, 2012). Management of EMS is focused on diet and increased activity, while pharmaceutical management needs further evaluation (Frank, 2011; Frank *et al.*, 2010; Menzies-Gow, 2015). For detection of obesity and increased adiposity in horses, two body scoring systems have been developed. Body condition score (BCS) is obtained by appreciating the amount of palpable fat cover present in six regions on the body of the horse (Henneke *et al*., 1983). Evaluation of subcutaneous fat in the neck region of horses can be made using the Cresty neck score (CNS) (Carter *et al*., 2009a). Both CNS and BCS have been shown to have a good correlation to serum insulin levels (Carter *et al*., 2009a).

Vascular effects of insulin - physiological

Insulin is a vasoactive substance which stimulates both vasoconstriction and vasodilatation. Vasodilatation is initiated by the release of nitric oxide (NO) from endothelium, which relaxes vascular smooth muscle cells, thereby increasing blood flow (Younk *et al*., 2014). Nitrous oxide also has anti-inflammatory and anti-platelet migration effects (Younk *et al.*, 2014). The release of NO as well as the metabolic effects of insulin is obtained through signaling in the PI-3 kinase-dependent transduction pathway in skeletal muscle and adipose cells (Kim *et al.*, 2006). Vasodilation acts biphasically; studies on people and rats have shown that capillary recruitment occurs after a few minutes and increased regional blood flow can be seen after 30 minutes (Baron *et al.*, 1996; Coggins *et al.*, 2001; Kim *et al.*, 2006; Vincent *et al.*, 2002). The physiological objective is to increase vascular access to peripheral cells for maximum blood glucose uptake.

During normal conditions, insulin regulates vascular tone by balancing vasodilation (by increased NO-release) and vasoconstriction. Vasoconstriction is stimulated through secretion of endothelin-1 (ET-1) from endothelium in a separate, MAP-kinase dependent pathway (Younk *et al.*, 2014). Insulin also has an effect on the sympathetic nervous system leading to increased blood pressure through the vasoconstrictive effects of catecholamines (Kim *et al.*, 2006). In an

insulin sensitive individual, acute hyperinsulinemia leads to a balanced effect of NO and ET-1 and sympathetic tone, and does not result in hypertension.

Vascular effects of insulin - pathological

Insulin resistance suppresses the vasodilating effects of insulin (Younk *et al.*, 2014). In two studies on rats (*ex* and *in vivo*) and people, IR has been shown to only interfere with the effect of insulin on the PI-3 kinase signaling pathway while the MAP-kinase signaling pathway is unaffected, thus leaving vasoconstriction as the only attainable effect of insulin (Cusi *et al.*, 2000; Jiang *et al.*, 1999). When IR conditions apply, acute hyperinsulinemia results in decreased NO production, shifting the balance towards ET-1 secretion and vasoconstriction (Younk *et al.*, 2014).

Exactly what causes tissue to become resistant to insulin is unknown. However, the mechanisms behind insulin resistance have been extensively studied in human medicine, and the theories centers around endothelial dysfunction and obesity-related inflammation (Kim *et al.*, 2006). Endothelial dysfunction has been proposed as an important part of human MetS as well as EMS in a review (Geor and Frank, 2009). Endothelial dysfunction is characterized by increased production of reactive oxygen agents (ROS) and cell adhesion products as well as a decreased ability for vascular relaxation (Geor, 2008). Glucotoxicity and lipotoxicity due to elevated free fatty acids (FFAs) in DM-patients can cause both IR and endothelial dysfunction (Kim *et al.*, 2006). Inflammation and pro-inflammatory conditions lead to an impairment of the PI-3 cell signaling pathway leading to IR. Human diseases causing endothelial dysfunction (e.g. atherosclerosis) leads to increased release of inflammatory proteins, which markedly decreases the endothelium's response to NO. In this way, inflammation, IR and endothelial dysfunction have a reciprocal relationship, where the causality is far from straightforward (Kim *et al.*, 2006).

Cardiovascular effects of hyperinsulinemia with or without insulin resistance

Physiological homeostasis of BP and HR is interconnected. Mean arterial pressure is the product of cardiac output (CO) and total peripheral resistance (TPR), and CO is in turn the product of heart rate and stroke volume, meaning an increase in blood volume leads to a rise in MAP (Sjaastad, 2010). Peripheral vasoconstriction leads to an increase in TPR and consequently in MAP. Total peripheral resistance is subject to neuro-hormonal regulation, with increased sympathetic activity and circulating epinephrine/norepinephrine leading to an increase in vasoconstriction and a rise in TPR. Arterial resistance is also subject to autoregulation by organs and the endothelium itself, independent of neuro-hormonal influence. In the long term, a major part of blood pressure regulation occurs through the renin-angiotensin-aldesterone system (RAAS), where a decrease of BP leads to increasing serum levels of Angiotensin II, Angiotensin II increases blood pressure by multiple mechanisms; through increased vasoconstriction as well as through an increase in blood volume; both due to increased renal resabsorption of fluids and an increase in thirst and thereby fluid intake (Sjaastad, 2010).

Heart rate is also subject to neuro-hormonal regulation. Increase of sympathetic tone as well as increasing circulating serum levels of norepinephrine and epinephrine leads to higher HR. In

contrast to BP, the parasympathetic system is also of importance in HR regulation. Changes in HR are generally mediated by concurring altered activity in both the sympathetic and the parasympathetic system (Sjaastad, 2010).

Blood pressure

Hypertension is a vital component of human MetS (Grundy, 2016). It has been proposed that several factors act together to elevate blood pressure; IR, enhanced renal absorption of sodium, increased intravascular volume, activation of renin-angiotensin-aldosterone-system (RAAS) and increased release of angiotensin from adipose tissue (Grundy, 2016). Also, as mentioned previously, IR contributes to endothelial dysfunction, with ROS leading to endothelium-dependent decreased vasodilation and thus hypertension (Kim *et al.*, 2006). Hyperinsulinemia under IR conditions has been proposed to lead to increased sympathetic tone as well as an unbalanced vasoactive activity regarding ET-1 and NO secretion (Younk *et al.*, 2014). Increased blood pressure has been reported in horses with history of laminitis (Bailey *et al*., 2008; Rugh *et al*., 1987). One study has shown that laminitis-prone ponies had a higher systemic blood pressure and lower insulin sensitivity during the summer months compared to healthy controls (Bailey *et al.*, 2008). No significant difference was detected in a similar study on a herd of ponies, where systemic blood pressure and serum insulin levels were compared between laminitic, previously laminitic and healthy ponies (Carter *et al.*, 2009b).

Hyperinsulinemia stimulates the sympathetic nervous system, but vasodilation occurring concurrently often cancels out a rise in blood pressure (Anderson *et al.*, 1991; Scherrer and Sartori, 1997). The effect of acute hyperinsulinemia on systemic blood pressure has been studied in different species with conflicting results. Systemic blood pressure decreased in horses after EHC, with a greater decrease seen in insulin sensitive horses compared to horses with IR (Nostell *et al.*, 2016). No effect was seen in healthy and obese dogs during hyperinsulinemia (Hall *et al.*, 1995; Villa *et al.*, 1998). In healthy and IR rats, systemic blood pressure has been shown to be unchanged during EHC (Bourgoin *et al.*, 2008; Santure *et al.*, 2002). Baseline systemic blood pressure was found to be higher in obese rats and dogs (Bourgoin *et al.*, 2008; Hall *et al.*, 1995). In people, results are equally conflicting, with no changes detected during insulin infusion in healthy people in one study, while another found significantly lower MAP and DBP, but not SBP (Anderson *et al.*, 1991; van de Borne *et al.*, 1999). MAP was found to decrease in lean but was unchanged in obese people (Baron and Brechtel, 1993).

Heart rate

Baseline heart rates have been shown to be higher in rats and dogs with induced IR and obesity (Bourgoin *et al*., 2008; Hall *et al*., 1995). No difference between baseline heart rates where shown between lean and obese IR people (Baron and Brechtel, 1993).

In people, insulin infusion has been shown to lead to increased sympathetic activation and increased serum levels of norepinephrine which contribute to higher heart rates (Anderson *et al.*, 1991; Sjaastad, 2010). No changes in heart rate were found in healthy horses with

hyperinsulinemia induced by glucose infusion or EHC (de Laat *et al.*, 2010; de Laat *et al.*, 2012). Heart rate has been shown to increase in healthy dogs and dogs with obesity-induced IR dogs during hyperinsulinemia (Hall *et al.*, 1995). No increase in heart rate was seen in healthy or IR rats (Bourgoin *et al.*, 2008; Santure *et al.*, 2002). Heart rate has been shown to increase in healthy people during EHC, but was reported to be unchanged in obese subjects (Anderson *et al.*, 1991; Baron and Brechtel, 1993; van de Borne *et al.*, 1999).

Digital blood flow

Peripheral blood flow in horses can be studied by measuring HWST, a method that has been shown to have good correlation with digital blood flow (Bailey *et al.*, 2004; Menzies-Gow *et al.*, 2008). In one study on healthy horses, horses receiving an EHC were shown to have increased HWST compared to horses receiving a saline infusion (de Laat *et al.*, 2010). In the same study, variation in HWST was smaller in horses receiving an insulin infusion compared to saline.

Hyperinsulinemia induced during an EHC has been shown to lead to higher PBF in several studies in insulin sensitive people and rats (Anderson *et al.*, 1991; Baron and Brechtel, 1993; Baron *et al.*, 1996; Baron *et al.*, 1995; Laakso *et al.*, 1990; van de Borne *et al.*, 1999; Vincent *et al.*, 2002). Lean subjects were found to have higher peripheral blood flow than obese insulin resistant individuals (Baron and Brechtel, 1993).

MATERIAL & METHODS

The study was approved by the Animal Ethics committee of Uppsala, Sweden (no C19/14), and is part of a PhD project on Equine Metabolic Syndrome.

Horses

The study consisted of two study groups; healthy controls and horses diagnosed with IR. The IR horses included in the study were privately owned Icelandic horses diagnosed with IR using an OGT-test (insulin responses over 1500 ng/L) performed by field active veterinarians. Horses showed no signs of pain or laminitis upon examination at arrival or during the stay at the clinic. Controls were recruited from the population at Wången National Center for Education in Trotting. Inclusion criteria for the control group were healthy Icelandic horses age 4-15 with a BCS<7 and no previous history of laminitis. An OGT and EHC were performed on all control candidates, and subjects with an M/I ratio<5 were excluded. Horses (in both groups) with ATCH-concentrations above the reference range (<11 pmol/L August-October or <7 pmol/L November-July) were excluded from the study.

Study protocol

Studies on IR horses were performed at the Equine clinic at the University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala. Studies on the control group were performed at Wången National Center for Education in Trotting. Studies on IR horses were performed from November 2014 throughout April 2015, and controls in December 2015.

Insulin resistant horses

Each horse was housed in a separate stable together with another horse in order to minimize stress. Boxes were bedded with wood or paper shavings and the horses were strictly fed the same food regimen as in their home environment and had access to water at all times during the day. Horses were exercised by hand walking several times per day.

All horses arrived at the clinic 3 or 4 days before the EHC (day 1). The day after arrival (day 2) an area over each jugular was clipped, and after antiseptic preparation and desensitizing with a topical local anesthetic (EMLA; Lidokain 25 mg/g + Pilokain 25 mg/g, Astra Zeneca AB), an indwelling intravenous catheter (Intranule 2.0 x 105 mm, Vygon Sweden AB) was introduced into one of the jugular veins. Catheters were flushed with sterile saline infusion (0,9% Sodium chloride, Fresenius Kabi). Horses were withheld feed overnight (12 h) but allowed free access to water. As this study was a part of a larger study regarding the reliability of the OGT-test, an additional OGT-test was performed in all horses on day 3. The results from this OGT test are not presented in this paper. After the OGT test the horses were allowed normal feed. The EHC was performed within 2 days after OGT. The day before EHC, an additional intravenous catheter was placed in the contralateral jugular in the same manner as described above, to provide one catheter for blood samples and one for insulin/glucose infusion. Horses were withheld feed for 12 hours before EHC but were allowed free access to water.

Controls

The EHC of the control horses were performed in the horses' home environment with normal feed and exercise routines followed, except for 12 hours before OGT and EHC, where feed, but not water, was withheld for 12 hours. Otherwise, procedure for EHC was identical to the IR horses.

Data collection

EHC protocol

The Euglycemic-hyperinsulinemic clamp (EHC) was performed as previously described (DeFronzo *et al.*, 1979; Powell *et al.*, 2002; Pratt *et al.*, 2005). During the 180 minutes long EHC, the horses were infused with a continuous infusion of human insulin at 3 $mU \cdot kg^{-1} \cdot min^{-1}$ (Humulin Regular, 100 IE/ml, Eli Lilly Sweden AB) and a 50% solution of glucose (500 mg/ml Glucose, Fresenius Kabi AB). The glucose infusion rate was adjusted to keep blood glucose at an euglycemic level (4.8-5.2 nmol/ml). Analysis of blood glucose was performed every five minutes using two hand held glucometers (Accu-Chek Aviva Plus Silver) for control of reliability of results. If measurements on the same sample differed more than 0.3 nmol/ml between the glucometers, samples were re-analyzed. Blood samples for subsequent analysis of plasma glucose and insulin were collected in lithium heparine tubes (Vacuette, Greiner Bio-One) before the start of the EHC, every 10 min during the EHC as well as immediately after the EHC. Blood samples were kept cold on ice until centrifuged (within 15 minutes after sampling) at 2770 x g for 10 min, and then stored at -80 °C until analysis.

Hoof wall superficial temperature measurements

Horses were fitted with a hoof temperature measuring equipment (Tinytag and PB-5003 unit, Intab) on the left front hoof. The method has been previously validated, and the equipment has been used in previous clinical studies on horses (de Laat *et al.*, 2012, Hood *et al.*, 2001). The measurement device was a probe that was attached to the dorsal hoof a few centimeters distally to the coronary band. Another measurement unit (Tinytag, Intab) was placed in the box to measure ambient temperatures. Measurements of room and hoof temperatures were started at least 45 min before the start of insulin infusion to allow the probe to adjust to room and hoof temperatures. Temperatures were registered automatically every two minutes during the entire EHC-clamp. Data were transferred to a computer program and compiled.

Blood pressure measurement

Systemic blood pressure and heart rate were measured using a non-invasive oscillometric technique via a sphygomanometer (Memo High Definition Oscillometry Monitor, horse model, S+B medVET GmbH) placed on the coccygeal artery. Both the method and device has been previously validated on horses (Muir *et al.*, 1983; Söder *et al.*, 2012; Tunsmeyer *et al.*, 2015). Parameters provided by the test were MAP, SBP, DBP and HR. The horses were fitted with an 8 cm cuff at the base of the tail, and measurements were made before, at 45 min, 105 min and 165 min after the start of the insulin infusion. Measurements of distance from the base of the tail and the overlap of the cuff's edges were noted to ensure consistent placement of the cuff. Six subsequent measurements of blood pressure were made at each time point. Several test measurements were made prior to data collection to allow the horses to get used to the testing procedure, and horses had also undergone BP measurements during OGT (not shown in this paper) on day 2. A first set of measurements with SBP higher than the subsequent, any set of values differing >20% in SBP and obviously faulty registrations were discarded, in line with a recommendation from small animal medicine (Brown *et al.*, 2016). Mean values for MAP, SBP, DBP and HR were calculated from the remaining measurements.

Scoring of body condition

Subjective scoring of body condition (BCS) and cresty neck score (CNS) were made by the same experienced veterinarian. BCS was graded from 1 to 9 with 1 being extremely emaciated and 9 being extremely obese (Henneke *et al.*, 1983). CNS was graded from 0 to 5 with 0 being no presence of a neck crest and 5 being a neck crest large enough to permanently fall to one side (Carter *et al.*, 2009a).

Blood samples and calculation of insulin sensitivity

Plasma insulin from EHC was analyzed using a human commercial insulin ELISA (Mercodia Insulin ELISA, Mercodia AB) since exogenous human insulin was used during the infusion. A commercial insulin analysis kit (Mercodia Diabetes Antigen Control (Low/High), Mercodia AB) was used as an additional control test. Plasma glucose was analyzed with a clinical chemistry analyzer (YSI 2300 Stat Plus Analyzer, YSI Incorporated).

Insulin sensitivity was quantified by calculating the M/I-ratio (metabolized glucose per unit insulin, unit [mg/kg/min x 10^3] / [mU/I]) modified from Defronzo (1979), M being the metabolized glucose which is defined as follows:

$$M = GIR - SC$$

Where GIR = glucose infusion rate ($mg \cdot kg^{-1} \cdot min^{-1}$) and SC is the space corrector, used to compensate for glucose removed from circulation by other means than metabolization, defined as follows:

$$SC = (G1 - G2)x\ 0{,}019$$

Where G1=glucose concentration in the previous test, G2=glucose concentration in the subsequent test. M/I ratio is calculated during the last 60 minutes of the EHC, starting at 120 minutes of insulin infusion. At this time, blood glucose levels have reached steady state.

Statistical analysis

Data was analyzed with a commercial statistical software (JMP Pro 12.2.0, SAS Institute Inc.) with statistical significance set at $P<0.05$. Means and standard errors (SE) were calculated and compared between groups for descriptive data (except M/I ratio). BCS, CNS and age were evaluated using the Student t-test and sex was analyzed using a Pearson test. Baseline HR and BP were compared using a Student t-test. Due to non-normality, M/I-ratio was analyzed by Wilcoxon test and presented by median and interquartile range.

JMPs repeated measures mixed model analysis of variance (ANOVA) with AR(1) used as a repeated covariance structure (with horse set as subject) were used to compare heart rates and diastolic blood pressures over time and between groups of horses (IR horses vs controls). AR(1) is a repeated covariance structure which assumes measurements more closely related in time are more correlated than those further apart. Due to unequal variances between groups of horses for SBP and MAP, these data were analyzed separately for each group of horses using a repeated mixed model of ANOVA.

Because of non-normality and heteroscedasticity of temperature data, HWST measurements for every 15 mins were transformed into temperature responses over 1 hour for 3 different time periods using area under the curve (AUC) (GraphPad Prism, Version 6.0, GraphPad Software Inc.). AUC data were then expressed as change from baseline calculated from measurements taken before infusion start. Missing HWST values (n=3, from group IR) were extrapolated from the last obtained time points. Hoof temperature changes (ΔHWST-AUC) were analyzed using a mixed model, with group, time and group-time interaction as fixed effects, while room temperature changes (ΔRT) were analyzed by group with time as fixed effects.

Tukey-Kramer was used as a post hoc test to compare least square means (LSMs) where statistical significance was found. Residuals were visually checked for normality.

Because of difference in baseline HR, a multiple regression analysis was made to find a model explaining the differences. In the multiple regression analysis model, analyses were performed in a backward stepwise manner, with age, M/I ratio, BCS, CNS and stress levels included as explanatory variables and HR as a dependent variable in the initial model. Due to unequal distribution between groups, sex was not included in the model. Variables with $P<0.05$ were removed until all that remained were significant. R^2 of the resulting model is presented.

RESULTS

Horses

Horse data for IR and control group are presented in table 1.

Table 1. *Characteristics of insulin resistant and control horses*

	Control horses	Insulin resistant horses
N	4	10
Sex: Mares	1*	9*
Sex: Geldings	3*	1*
Age	9.3 ± 2.1	13.9 ± 1.7
BCS	5.3 ± 0.1*	6.8 ± 0.4*
CNS	2.4 ± 0.1***	3.8 ± 0.2***
M/I [i]	6.7 (6.2-7.8)*	1.7 (1.4-2.4)*

Table 1: Number of horses, sex, age (years), body condition score (BCS), cresty neck score (CNS) and M/I ratio (unit [mg/kg/min x 10^3] / [mU/l]) for insulin resistant horses and controls. Values are expressed as means ± standard error, except i = median and interquartile range, */*** significantly different between groups, * = $P<0.05$, *** = $P<0.0001$.

Heart rate

Mean heart rates were significantly higher in the insulin resistant group compared to controls as a main effect ($P<0.05$). Although mean heart rate increased numerically during insulin infusion in both controls and insulin resistant horses the increase was not statistically significant. Values are presented in table 2.

In the multiple regression analysis, the only remaining significant factor was BCS, and a positive correlation between BCS and baseline heart rate was found. R^2 for the final model was 0.54 ($P<0.05$).

Table 2. *Heart rate in insulin resistant horses and controls before and during the insulin infusion*

	Control horses		Insulin resistant horses	
Time	LSM	SE	LSM	SE
0	28.2	2.2	35.8	1.7
45	29.8	2.5	37.8	1.7
105	30.0	2.9	38.3	1.7
165	32.0	2.9	38.6	1.7

Table 2. Least square means for heart rate (beats/min) in insulin resistant horses and controls at the start of insulin infusion (0), and at 45, 105 and 165 min of the insulin infusion. LSM: Least square means, SE: Standard error.

Blood pressure

No significant differences in baseline systolic (SBP), mean arterial (MAP) or diastolic pressure (DBP) could be found between groups. In controls, systolic blood pressure decreased significantly during insulin infusion ($P<0.05$), but no significant decrease was seen in MAP or DBP. No significant change was seen during insulin infusion in the IR group.

Measurement errors commonly occurred in horses in both groups, necessitating several refittings of tail cuffs. Refittings were made in accordance with measurements of cuff overlap and distance to tail base.

Table 3. *Blood pressures in insulin resistant horses and controls before and during the insulin infusion*

		Control horses		Insulin resistant horses	
Time		LSM	SE	LSM	SE
0	SBP	113	6.7	111	4.2
	MAP	83	4.7	81	3.0
	DBP	66	4.3	64	2.8
45	SBP	104*	6.7	113	4.2
	MAP	77	4.7	83	3.0
	DBP	61	4.3	66	2.7
105	SBP	102*	7.7	108	4.2
	MAP	76	5.4	80	3.0
	DBP	62	5.0	64	2.7
165	SBP	99*	7.7	114	4.2
	MAP	74	5.4	85	3.0
	DBP	58	5.0	69	2.7

Table 3. Least square means for systolic (SBP), mean arterial (MAP)- and diastolic blood pressure (DBP) in mmHg in insulin resistant horses and controls before insulin infusion (0) and at 45, 105 and 165 minutes during insulin infusion. * - significantly changed from baseline ($P<0.05$). LSM = Least square means, SE = standard error.

Hoof wall surface temperature

In one of the IR horses, the hoof wall temperature sensor failed to give readings, and the result from this horse was therefore excluded.

Response of HWST from baseline during insulin infusion did not reach significance in the individual groups. However, when comparing the groups, insulin infusion had a significantly different effect ($P<0.05$) on HWST in controls and insulin resistant horses at the last two time periods (2 and 3 h into the infusion), with a decrease seen in controls and a slight increase seen in IR horses. Data is shown in Fig. 1.

Fig.1. *Alterations in mean hoof wall surface temperatures in insulin resistant horses and controls during the insulin infusion*

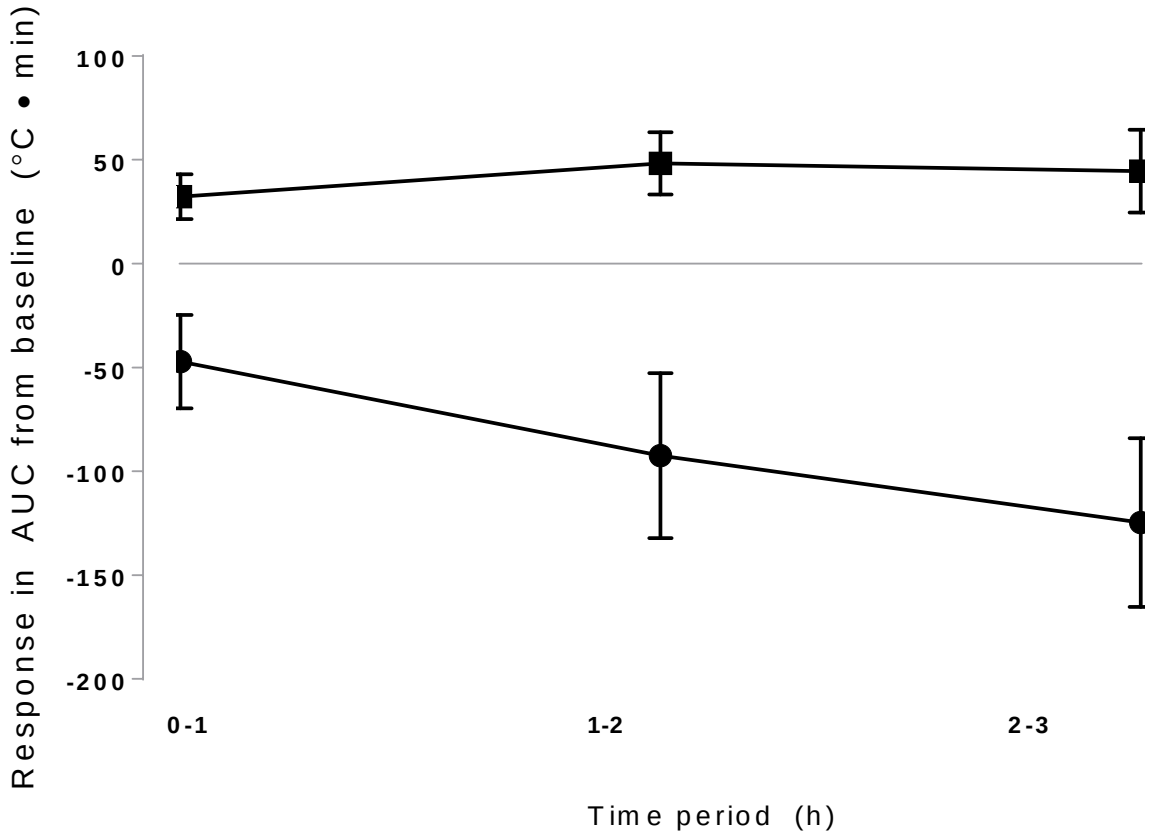

Fig 1. Mean response in area under the curve from baseline for hoof wall surface temperature (measured in °C · min) during time period 0-1 h, 1-2 h and 2-3 h. Square markers represent insulin resistant horses and round markers represent control horses. Means ± standard error. Changes from baseline *P*= NS in the individual groups.

DISCUSSION

Heart rate, systemic blood pressure and HWST in insulin resistant horses and controls

The results of this study show that baseline heart rate was higher in IR horses compared to controls and that the discrepancy between groups could be attributed to the differences in BCS. Many studies performed on people, rats or dogs include both IR and obese subjects, which makes separation of factors for comparison difficult. Obesity and IR are strongly related in people; one study in people found that higher insulin resistance was associated with higher body

mass index (BMI), and that 84% of subjects in the top IR tertile were either obese or overweight (McLaughlin *et al*., 2004). The results obtained in the present study is in agreement with studies on dogs and rats with induced obesity and IR where elevated baseline heart rates were seen compared to lean subjects (Bourgoin *et al.*, 2008; Hall *et al.*, 1995). Reports regarding obesity in people are conflicting; obese subjects have been reported to have equal to or slightly elevated baseline heart rates compared to lean individuals (Alpert *et al*., 2016). The pathophysiology of cardiovascular changes in hemodynamics related to obesity are decreased parasympathetic tone, increased sympathetic tone but also increased blood flow demands to non-adipose tissue as well as adipose tissue (Alpert *et al.*, 2016; Hall, 2000).

Obesity has also been demonstrated to be strongly related to the development of hypertension in people (Alpert *et al*., 2016; Hall, 2000). In addition to the increase in sympathetic activity mentioned above, hypertension in obesity and IR is believed to be caused by increased renin-angiotensin-aldosterone system (RAAS) activation, increased renal natrium resorption as well as endothelial dysfunction and increased inflammatory activity in adipose tissue (Alpert *et al*., 2016; Hall, 2000; Kim *et al*., 2006). Higher baseline blood pressure has been shown in dogs and rats with induced obesity and IR compared to lean controls (Bourgoin *et al*., 2008; Hall *et al*., 1995). The prevalence of hypertension in obese people is 50-60 % (Alpert *et al.*, 2016). In the present study, there were no baseline differences in systemic blood pressure between insulin resistant horses and controls, even though heart rates were increased in the former group. However, the individual variation in blood pressure has a greater impact on the result in a study performed on a small material of horses. Therefore, the fact that the study was performed on a small material of horses makes it difficult to detect differences in blood pressure between groups which also might have influenced the results obtained. Studies on a larger material of insulin resistant horses and controls are necessary. Hypertension has been reported in IR horses, but few studies on equine obesity and hypertension exists to elucidate if the pathophysiology matches that reported in people and other animals (Bailey *et al*., 2008; Rugh *et al*., 1987). Bailey *et al.* (2008) showed that insulin resistance and blood pressure was higher in laminitis-prone ponies in June than in November-December, and that this difference was not evident in healthy controls. Pasture-associated laminitis is often seen in EMS horses. The pathophysiology behind this is still unknown but is thought to be related to increased intake of non-structural carbohydrates leading to hyperinsulinemia in IR subjects (Geor, 2008).

No difference between baseline HWST was seen in IR horses compared to controls in the present study. One study has shown difference in baseline HWST between laminitic or laminitis-prone ponies and healthy controls, but also reported that ambient temperatures potentially confounded the results (Carter *et al*., 2009b). Baseline room temperatures were not different between groups in the present study, and did not change significantly during infusion in either group.

Effects of hyperinsulinemia on heart rate, systemic blood pressure and HWST

In line with our hypothesis and with results from de Laat (2010; 2012), no significant increase in heart rate during insulin infusion could be seen. Studies on insulin sensitive people as well as dogs have shown that insulin infusion causes an increase in heart rate (Anderson *et al.*, 1991;

Baron and Brechtel, 1993; Hall *et al.*, 1995; van de Borne *et al.*, 1999). No corresponding change in HR (or BP) was seen in IR people by Baron & Brechtel (1993). In the present study, a numerically small but non-significant increase of HR from baseline was apparent in both groups. A greater number of study subjects might have elucidated the difference between groups, which might be masked by large individual variations.

Insulin infusion caused a decrease in SBP in controls but not IR horses. This decrease in SBP during insulin infusion was also shown in a study by Nostell *et al.* (2016). The difference in the cardiovascular response to insulin between groups might be attributed to the effect of insulin resistance on NO-mediated vasodilation (Scherrer and Sartori, 1997). Increased peripheral blood flow during insulin infusion has been seen in many studies on people (Anderson *et al.*, 1991; Baron and Brechtel, 1993; Baron *et al.*, 1995). Activation of PI-3 signaling pathways have been shown to be strongly decreased in IR rats and people compared to controls, potentially inhibiting vasodilation and subsequent decrease in blood pressure (Cusi *et al.*, 2000; Jiang *et al.*, 1999). Insulin resistant subjects have been reported to require four times higher serum insulin concentrations to achieve the same peripheral blood flow as IS subjects (Laakso *et al.*, 1990). However, increased vasodilation does not always lead to decreased systemic blood pressure, as showed during EHC in healthy people (van de Borne *et al.*, 1999). The complexity of the effect of insulin on hemodynamics and vasculature makes prediction of response to hyperinsulinemia difficult, and there might be great variation between subjects depending on insulin sensitivity.

In the present study, insulin infusion did not cause a simultaneous decrease in MAP or DBP. This is in contrast with another study in horses where insulin infusion caused a concurrent decrease in MAP and DBP (Nostell *et al.*, 2016). In addition to this, a study in people showed decreases in MAP and DBP, but not SBP, during an EHC (Anderson *et al.*, 1991). The reason for this discrepancy in results is likely to related to the blood pressure measurement device used in the present study, which has been shown to have a higher CV (a higher between-measurements variance) for diastolic than systolic measurements (Söder *et al.*, 2012). Several horses in both IR and control group had low diastolic values (DBP < 60). Low diastolic values might be a common trait in the breed, with earlier studies on Icelandic horses reporting SBP 105±14 mmHg and DBP 66 ±17 mmHg (Söder *et al.*, 2012). The values obtained in the present study are however in the normal interval reported in horses (Parry *et al.*, 1984). Furthermore, Söder *et al.*, (2012) showed that Icelandic horses had lower serum cortisol levels than Standardbreds. Cortisol is involved in blood pressure regulation, with excessive secretion leading to hypertension (as seen in human Cushing's syndrome) and impaired secretion leading to hypotension (as seen in human Addison's disease) (Hammer and Stewart, 2006; Whitworth *et al.*, 2005). This could implicate that the lower serum cortisol level seen in Icelandic horses could have a correlation to their lower blood pressures. Since low DBPs were a feature in both groups, this is not likely to have influenced the results. The low values could also be attributed to difficulties in measurements. The fact that all horses in the study were of a breed known for its thick tail sometimes made cuff placement difficult and several refittings were required for many subjects, increasing the risk of inconsistent placement. However, care was taken to ensure consistency by measuring and noting the cuffs' overlap and distance to tail base. Blood pressure

values might be underestimated if the cuff is too wide, which (due to the thickness of the tails) seems less likely in this case (Latshaw *et al.*, 1979).

A significantly different effect of insulin infusion on hoof wall surface temperature was seen between groups (with controls having a decrease of HWST and IR a slight increase). This decrease of PBF in healthy individuals is contrary to what was seen by de Laat (2010), and in many human studies (Anderson *et al.*, 1991; Baron and Brechtel, 1993; Baron *et al.*, 1996; Baron *et al.*, 1995; Eggleston *et al.*, 2007; van de Borne *et al.*, 1999). However, the result in HWST in the individual groups should be interpreted carefully since the responses in the individual groups from baseline are nonsignificant, and the number of subjects in the control group (n=4) is low. Sources of error in HWST measurements are erroneous placement of probes, detachment of probes over time due to adhesive tape coming loose or variability between individuals due to weight shifting. Differences in insulin infusion rates have been shown to influence tissue insulin sensitivity and serum insulin concentrations in healthy horses (Urschel *et al.*, 2014). The infusion rate in de Laat (2010) was twice the rate in the present study. Different insulin rates have been used during EHC in the human studies quoted, all resulting in variations in serum insulin concentrations and potentially different results (Anderson *et al.*, 1991; Laakso *et al.*, 1990). In addition, time aspects are of importance when comparing studies. In the study by de Laat (2010), no differences in HWST between groups were seen until 11-15 h into EHC. If the lower insulin infusion rate used in the present study resulted in lower serum insulin concentrations (and consequently a slower increase in PBF), no differences in HWST might have been detected over the 3 h the present study lasted. The time course for insulin's cardiovascular effects have not been established in horses, but in people, increases in PBF have been reported at 30-100 minutes into EHC (Baron *et al.*, 1996; Eggleston *et al.*, 2007; Laakso *et al.*, 1990). Indirect measurement methods like HWST generally have decreased sensibility to detect changes in PBF compared to direct measurements (plethysmography) used in human studies (Anderson *et al.*, 1991; Baron and Brechtel, 1993). HWST might have greater risk of measurement errors (e.g. by misplacements of sensors) than other indirect measurement of blood flow (Doppler ultrasonography) used in equine studies (Bailey *et al.*, 2004; Menzies-Gow *et al.*, 2008).

Hyperinsulinemia (obtained by either prolonged EHC or glucose infusion) has been shown to induce laminitis in healthy horses (Asplin *et al.*, 2007; de Laat *et al.*, 2010; de Laat *et al.*, 2012). Both increased and decreased HWST has been shown when laminitis was induced by hyperinsulinemia, carbohydrate overload or lipopolysaccharide-infusions in healthy horses (Hood *et al.*, 2001; Menzies-Gow *et al.*, 2004; Menzies-Gow *et al.*, 2008; Pollitt and Davies, 1998). However, although *in vivo* studies are lacking, several *ex vivo/in vitro* studies has been made on IR horses during hyperinsulinemia, where an increase of vascular resistance have been shown in IR tissues compared to controls (Gauff *et al.*, 2013; Venugopal *et al.*, 2011). In addition, several studies has shown that equine digital veins are more responsive to vasoconstriction by ET-1 than arteries (Katz *et al.*, 2003; Peroni *et al.*, 2006; Stokes *et al.*, 2006; Venugopal *et al.*, 2014). This tendency for increased venoconstriction is hypothesized to be of importance in laminitis pathophysiology as venous vasoconstriction could lead to stasis of digital vasculature, increase hydrostatic pressure and subsequent edema and digital ischemia

(Stokes *et al.*, 2006). Also, increased activity of arterio-venous anastomoses shunting blood away from the lamellar microvasculature is hypothesized to lead to digital ischemia despite evidence of increased blood flow (Asplin *et al.*, 2007). These theories are interesting in regards to HWST because theoretically, vascular disturbances causing lamellar tissue hypoperfusion leading to laminitis could lead to HWST either decreasing (due to vasoconstriction of digital blood vessels) or increasing (due to an increase in arterio-venous anastomoses that shunts blood from microvasculature despite increased DBF, or due to stasis and edema).

Limitations of the study

Small sample sizes are an issue in this study. Cohesive groups were prioritized over number of participants and therefore only Icelandic horses were included to minimize between-breed difference, since characteristics of IR has been shown to vary among horse breeds (Bamford *et al.*, 2014). Starting out with six control horses, two were eliminated due to decreased insulin sensitivity which became apparent when M/I ratios were calculated. Ideally, since HWSTs are known to physiologically vary over time (for example due to weight shifting) additional IR and healthy horses could have been used as a separate control group receiving a placebo infusion. This was done in the study by de Laat (2010), where horses receiving saline infusion were shown to have a larger variation in HWST compared to EHC horses.

Preferably, the groups should have been better matched in several aspects, including sex, although no sex predilection has been found (Johnson, 2002). Inherent to the study recruitment method characteristics of horses differed between groups. Control horses belonged to Wången National Center for Education in Trotting and were used in training therefore required to be healthy and athletic. In contrast, IR horses were recruited with the aid from field active veterinarians and consequently were privately owned with varying health and athleticism. Feed also differed between individuals and between groups.

Control horses were studied in their home environment while IR horses were housed at the Equine clinic. Although horses were allowed minimum 2 days of acclimatization before EHC was performed, baseline stress could still be higher in IR horses due to the new environment, which could affect HR and BP.

CONCLUSION

This study indicates that horses with insulin resistance have higher baseline heart rates compared to controls and that heart rate is associated with BCS. There are also indications that insulin resistant horses might have an altered cardiovascular response to hyperinsulinemia, apparent as a decrease in SBP in control horses compared to IR. Since few other studies exist on EHC in IR horses compared to controls, further studies are needed to validate this result, preferably with greater sample sizes and using different breeds. Hopefully, more research on this subject could lead to a deepened understanding of cardiovascular influence of IR and take us a step closer towards management and prevention of EMS and endochrinopathic laminitis.

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