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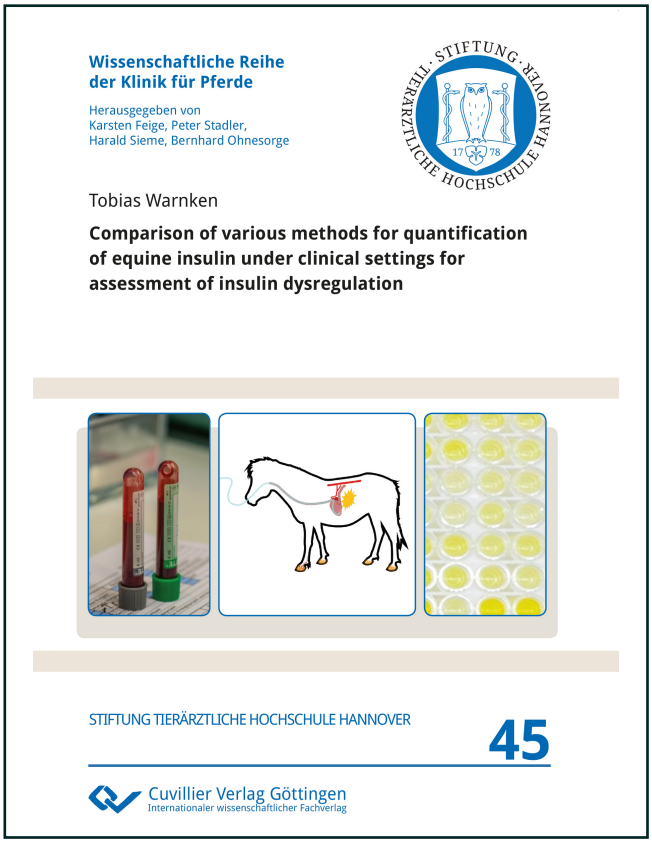
Comparison of various methods for quantification of equine insulin under clinical settings for assessment of insulin dysregulation



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1 INTRODUCTION

Metabolic pathologies, obesity and endocrinopathies play an increasingly significant role in equine veterinary medicine. The prevalence of obesity is high in the equine population with rates ranging between 30 to 48 % (Thatcher et al. 2008; Wyse et al. 2008; Giles et al. 2014). In addition, horses presented to a first opinion hospital for evaluation of laminitis were hyperinsulinemic in 66 % of the cases (Karikoski et al. 2011), indicating the outstanding clinical importance. Impaired insulin regulation reflected by hyperinsulinemia and caused by insulin dysregulation (ID) and/or insulin resistance (IR) is a common feature in equine endocrinopathies with partially severe and life-threating consequences for the individual affected. The assessment of ID or IR by dynamic challenge tests can be difficult under clinical conditions based on complex and time-consuming testing procedures but provides advantages compared to solely analyses of resting insulin and glucose concentrations. Nevertheless, in addition to complex diagnostic testing protocols, most test procedures require analyses of equine insulin in blood samples collected either during or after these dynamic stimulation tests. Quantification of equine insulin is provided by several specialized laboratories using varying immunoassays. However, analyses of basal samples or samples obtained during dynamic diagnostic procedures might require variable laboratory and immunoassay demands for exact quantification of equine insulin. Moreover, reliable references ranges for the combination of commonly used diagnostic procedures, for example, the oral glucose test (OGT) performed via nasogastric tubing (NGT), and analyses with specific immunoassays are lacking.

2 LITERATURE REVIEW

2.1 INSULIN DYSREGULATION

Glucose homeostasis is tightly controlled in physiological conditions to maintain essential metabolic homeostasis of the organism. Imbalances in insulin regulation can occur under either physiological conditions, such as pregnancy and lactation with redistribution of energy sources (Fowden et al. 1984; Maresh 2001), or pathophysiological conditions. The term ID describes impaired regulation in this complex system. The ID can be reflected by basal hyperinsulinemia, excessive or prolonged postprandial hyperinsulinemia and/or by IR (Frank and Tadros 2014; Bertin and De Laat 2017; Durham et al. 2019). The IR is defined as the inability of tissues to respond adequately to insulin (Muniyappa et al. 2008). It can be reflected by a decreased insulin sensitivity, which is characterized by a normal maximal biological effect combined with a need for an increased insulin concentration to achieve this maximal biological effect. By contrast, a decreased insulin responsiveness is characterized by a decreased maximal biological effect combined with normal insulin concentrations (Kahn 1978; De Koster and Opsomer 2013). Underlying pathomechanisms of IR have not been identified completely in humans nor in various animal species. However, several hypotheses have been postulated, including a reduced number of insulin receptors in target tissues due to downregulation, receptor dysfunction or disturbed post receptor signaling (Kahn 1980; Shanik et al. 2008). Horses severely affected by IR have typically increased basal insulin concentrations. However, mild cases may not stand out with basal hyperinsulinemia. This hyperinsulinemia can be symptomatic of tissue IR when it occurs as a compensatory response to peripheral tissue IR. In contrast to the situations in humans, horses normally compensate for reduced insulin sensitivity and develop hyperinsulinemia concurrent with normoglycemia, whereas humans suffer from hyperinsulinemia and hyperglycemia (Divers 2008). However, recent research highlighted the fact that ID can occur independently of tissue IR, reflected by increased insulinemic responses of ponies to PO-applied glucose compared to IV-

applied glucose (De Laat et al. 2016a). It was suggested that the hyperinsulinemia is not just a sequela of IR and that the enteroinsular axis might contribute to ID with intensified insulin secretion mediated by incretine stimulation. Impaired secretion and the action of glucagon-like peptide 1 as one incretine has been identified in humans suffering from diabetes mellitus type II (Toft-Nielsen et al. 2001a) and intravenous glucagon-like peptide 1 infusion lowered plasma glucose in diabetes type II patients (Toft-Nielsen et al. 2001b). Glucagon-like peptide 1 has been analyzed in horses and positively correlated with postprandial insulin concentrations in healthy and in insulin-dysregulated horses after glycemic challenges (Bamford et al. 2015; De Laat et al. 2016a). The hypothesis of impaired action of the enteroinsulinar axis is further supported by several studies reporting weak to missing correlation between direct and indirect measures of tissue IR using enteral glycemic stimulations and, therefore, testing more diverse aspects of ID (Pratt et al. 2005: Banse and Mcfarlane 2014; Pratt-Phillips et al. 2015; Dunbar et al. 2016). However, recent studies were not able to identify differences in incretine concentrations between healthy insulin-sensitive and insulin-dysregulated horses after an oral glycemic challenge test (Chameroy et al. 2016) and healthy and insulin-dysregulated ponies after grazing pasture (Fitzgerald et al. 2019b).

2.1.1 ASSOCIATED EQUINE DISEASES

2.1.1.1 EQUINE METABOLIC SYNDROME

The Equine Metabolic Syndrome (EMS) is more of a collection of risk factors and a symptom complex than an actual disease and is a rising concern in the equine population (Durham et al. 2019). The term EMS was first introduced into veterinary medicine by Johnson (2002) and referred to a cluster of clinical signs predisposing horses and ponies for the development of laminitis. The term and the characteristics were adopted from human medicine where the Human Metabolic Syndrome (MetS) describes a disease pattern in which obesity, diabetes type II and cardiovascular diseases are the major symptoms. Similar symptoms in equids compared to the situation in human medicine had been observed. Generalized obesity or regional

accumulation of fat are frequently observed in affected horses (Treiber et al. 2006; Carter et al. 2009; Giles et al. 2015; Fitzgerald et al. 2019a). The lean EMS phenotype has been described more recently and is increasingly perceived (Durham et al. 2019). A predisposition to laminitis has the major significance, together with obesity. A laboratory key finding and essential for the confirmation of the diagnosis of EMS is the accompanying ID (Frank et al. 2010; Frank and Tadros 2014; Bertin and De Laat 2017; Equine Endocrinology Group 2018; Durham et al. 2019). Additional clinical conditions associated with EMS include hypertriglyceridemia or dyslipidemia (Frank et al. 2006; Treiber et al. 2006; Carter et al. 2009), hyperleptinemia (Cartmill et al. 2003), hypoadiponectemia (Menzies-Gow et al. 2017) and cardiovascular changes, including arterial hypertension (Bailey et al. 2008), and myocardial changes (Heliczer et al. 2017). Altered reproductive cycling in mares (Vick et al. 2006) and generally increased proinflammatory markers (Vick et al. 2007) are further clinical findings being discussed in the context of EMS. In general, EMS seems to occur more commonly in physically inactive and overfed horses and anecdotally in certain native breeds which exhibit the obese, EMS-like phenotype more commonly (Durham et al. 2019). Recent research highlights a possible inherited predisposition for EMS in some breeds, partially based on the assumption that these breeds had genetically adapted to survival under suboptimal nutritional conditions (McCue et al. 2015; Lewis et al. 2017). Interestingly, further studies investigated genetics in EMS horses providing evidence for potential genetic predisposition (Norton et al. 2019a, 2019b).

2.1.1.2 PITUITARY *PARS INTERMEDIA* DYSFUNCTION

Pituitary *pars intermedia* dysfunction (PPID), previously known as Equine Cushing Syndrome, is a severe neuroendocrine equine disease associated with metabolic perturbations and even impaired insulin regulation in some cases (Equine Endocrinology Group 2017). Affected horses are normally over 15 years of age and recent studies have reported prevalence rates of up to 30 % for horses over 15 years (McFarlane 2011). Affected horses generally show clinical signs such as regional adiposity, with atypical adipose tissue accumulation in the neck and tailhead region.

Hirsutism or hypertrichosis is the most unique and frequent clinical sign in horses suffering from PPID occurring in 55 to 80 % of cases. Further clinical signs include polyuria together with polydipsia and concurrent muscle atrophy resulting in poor body condition in some cases (Schott 2002; McFarlane 2011). The underlying etiology of PPID is a specific expansion of the melanotrophic cells of the *pars intermedia* of the pituitary gland (McFarlane and Cribb 2005). The hyperplasia of the *pars intermedia* of the pituitary gland is based on a loss of dopaminergic inhibition by the hypothalamus and uncontrolled release of POMC, mainly adrenocorticotropic hormone (ACTH) (McFarlane 2011). The underlying mechanism for the lack of dopaminergic control is not known. However, there are suggestions that oxidative stress may play a significant role and may led to neurodegeneration (McFarlane and Cribb 2005). Interestingly, some horses with PPID develop mild to severe ID. Studies reporting ranges of 30 to 60 % of PPID horses also being diagnosed with ID (Schott 2002; McFarlane 2011; McGowan et al. 2013; Mastro et al. 2015). The exact potential cross-link between disturbed cortisol regulation and the occurrence of ID is not fully understood. However, the concurrent ID is discussed to dramatically increase the risk of the development of endocrinopathic laminitis in PPID cases which are already being treated with pergolide mesylate.

2.1.2 ENDOCRINOPATHIC LAMINITIS

Laminitis is a life-threatening disease of horses and ponies causing acute or chronic painful conditions of the hooves (Pollitt 2004). It often results in acute or chronic lameness in affected equids, frequently necessitating euthanasia due to welfare aspects. Laminitis is defined as a failure of the laminar tissue of the hooves' lamellar-distal phalangeal attachment apparatus (Pollitt 1996, 2004). Unfortunately, the exact pathomechanisms are still unknown in their entirety despite an excessive research effort. Several etiologies of laminitis have been described and postulated, for example, alimentary and inflammatory induction (Garner et al. 1975; Galey et al. 1991; Van Eps and Pollitt 2006; Pollitt and Visser 2010). However, the etiology of laminitis may be multifactorial in different conditions and is often a result of several

systemic disease entities. Despite well-known causes of laminitis development, such as, endotoxemia or overweight bearing, there is growing evidence of an association with endocrine dysfunction (McGowan 2008, 2010; Patterson-Kane et al. 2018; De Laat 2019). Donaldson et al. (2004) reported the prevalence of PPID defined by a single high plasma ACTH concentration in around 70 % of laminitis cases. Moreover, Karikoski et al. (2011) reported evidence of an endocrinopathy in 89 % of admitted cases presented to a first opinion hospital for evaluation of laminitis. A diagnosis of PPID was made in 33 % of cases, whereas hyperinsulinemia was present in 66 % of cases (Karikoski et al. 2011). Consistent with these findings, De Laat et al. (2019) reported that horses and ponies suffering from laminitis with concurrent endocrinopathies have more marked hyperinsulinemia and that higher basal insulin concentrations in these cases were associated with more severe lameness. In addition, several studies identified elevated serum insulin concentrations as a risk factor for the development of laminitis (Carter et al. 2009; Menzies-Gow et al. 2017). Multiple experimental studies have been performed to prove the relationship between insulin and laminitis in which laminitis was induced under hyperinsulinemic conditions. Prolonged IV infusion of insulin by hyperinsulinemic euglycemic clamps induced clinical laminitis and histopathological changes in the hooves in previously healthy ponies (Asplin et al. 2007, Asplin et al. 2010). Additionally, De Laat et al. (2010) induced laminitis in healthy Standardbred horses within 48 h by prolonged hyperinsulinemia, proving insulin-mediated induction of laminitis even in more insulin-sensitive breeds. Despite the fact that artificial exogenous hyperinsulinemia induced laminitis, De Laat et al. (2012), furthermore, showed that prolonged IV glucose infusions provoking constant endogenous hyperinsulinemia were also able to induce histopathological lamellar changes consistent with laminitis. Despite studies focusing on artificial hyperinsulinemia provoked by IV infusion of exogenous insulin or glucose, Meier et al. (2017) showed the direct link between a pathologically high postprandial insulin response and the occurrence of experimentally induced laminitis by a dietary challenge high in nonstructural carbohydrates (NSC).

2.1.3 ASSESSMENT OF INSULIN DYSREGULATION IN HORSES

Several diagnostic procedures are currently routinely used for the assessment of disturbed insulin regulation in equids. However, different test procedures provide diverse information regarding the aspects of ID. Basal measures of either insulin or combinations of glucose and insulin allow the detection of severe basal hyperinsulinemia but may lack identifying cases with inconspicuous basal insulin and exacerbated postprandial insulin concentrations in response to carbohydrate ingestion. Olley et al. (2019), for example, identified a poor sensitivity of fasted insulin concentrations at conventional cutoff values compared to the IV combined glucose-insulin test. However, studies comparing basal insulin concentrations and results from PO testing are not currently available.

Therefore, dynamic diagnostic tests assessing the horse's response to a glycemic stimulation are currently recommended for the assessment of ID (Bertin and De Laat 2017; Equine Endocrinology Group 2018; Durham et al. 2019). Variable standardized OGT protocols have been established. Based on the physiological mode of action, OGTs are recommended to assess pathological postprandial hyperinsulinemia (Equine Endocrinology Group 2018; Durham et al. 2019). In-feed OGTs can be performed and may offer the most physiological test principle by measuring insulin and glucose response following the ingestion of a meal artificially enriched with NSC, such as glucose or dextrose powder (De Laat et al. 2016a; Smith et al. 2016; Bertin and De Laat 2017; De Laat and Sillence 2017; Meier et al. 2017). The dextrose or glucose dosage for implementation ranges from 0.5 to 1 g/kg bodyweight (BW) (Frank et al. 2010; Frank and Geor 2014; Durham et al. 2019). A clinical decision can be made based on several cutoff values reported in literature. However, the variable cutoff values or reference ranges have to be used based on the glucose dose implemented and the selection of the immunoassay used for the analysis of the equine insulin (Frank and Tadros 2014; Durham et al. 2019). Despite variable dosages and cutoff values, the repeatability of this test procedure is crucial and horses and ponies often refuse to ingest the complete ratio or need variable times for complete ingestion and, therefore, preclude the reliable diagnosis of ID (De Laat and Sillence 2017).

Schuver et al. (2014) described the Oral Sugar Test as a simplified test to assess the insulin response after a defined NSC challenge. Commercially available corn syrup (Karo Corn Syrup®, ACH Food Companies Inc., Memphis, Tennessee, USA) is administered PO for test implementation. The dosages described range from 0.15 ml/kg BW (Schuver et al. 2014) to a recently suggested and recommended 0.45 ml/kg BW (Jocelyn et al. 2018). As a result of the dose comparison studies and further investigations, it was highlighted that increased amounts of corn syrup increase the diagnostic accuracy to assess ID (Manfredi 2016; Jacob et al. 2018a; Jocelyn et al. 2018). However, administration of increased amounts of corn syrup complicates the simple PO application procedure.

Variable reference ranges and cutoff values have been reported based on small numbers of the animals included and use of variable immunoassays, thus, often complicating diagnosing ID based on this test protocol in clinical practice. Diagnostic uncertainty might further occur due to variable composition of the syrup. Multiple analyses indicated controversial results regarding the ingredients and reported marked differences between different lots (Schuver et al. 2014; Jocelyn et al. 2018).

However, OGT can also be performed by glucose application via an NGT and provides the substantial benefit that an exact amount of glucose is administered within a specific period directly into the stomach of the animal tested (Ralston 2002). Although NGT may require trained veterinary personal and is an invasive procedure for diagnostic purposes, it might be the most standardized procedure and is often used in clinical routine when horses refuse to ingest the meal during in-feed OGTs. Nevertheless, there are no reliable cutoff values for the OGT via NGT and, therefore, cutoff values or reference ranges were adopted from different test protocols regardless of the underlying differences in their physiological mode of action and the immunoassay method used for quantification of equine insulin.

2.1.4 ASSESSMENT OF INSULIN RESISTANCE IN HORSES

Despite the assessment of basal hyperinsulinemia or pathologically high postprandial hyperinsulinemia as aspects of ID, assessment of tissue IR as another part of ID can

be performed and achieved by several further test procedures. Measuring responses to IV administered insulin and/or glucose focus on the assessment of peripheral tissue insulin sensitivity and β-cell responsiveness. Tests such as the frequently sampled IV glucose tolerance test (Hoffman et al. 2003; Bailey et al. 2007; Durham et al. 2009) and the hyperinsulinemic euglycemic clamp (Pratt et al. 2005; Pratt-Phillips et al. 2015) are often used to investigate insulin and glucose regulation in experimental and research settings but are too complex and costly for routine clinical use. A simplified procedure such as the combined glucose-insulin test is a more practicable test in routine clinical use and is considered a direct method to assess tissue IR (Eiler et al. 2005). The capacity of the exogenous insulin to shift the injected glucose into the insulin-sensitive tissues is assessed by injection of 150 mg/kg BW glucose followed by an immediately injection of 0.1 IU/kg BW insulin. Whereas glucose concentration is monitored for 45 min, insulin is measured prior to injection and after 45 min. Insulin-sensitive horses show a typical two-phase blood glucose curve with an initial hyperglycemia followed by a second phase with hypoglycemia in which glucose concentrations drop below the initially determined baseline concentration. The first positive phase in insulin-resistant horses is prolonged due to a slower return to baseline. The 45-min value is used as a clinical cutoff value to distinguish between insulin-sensitive and -resistant individuals. Horses should achieve normal glucose concentrations and return to baseline levels within 45 minutes and insulin concentration should remain under 100 µIU/mL if insulin analysis is performed with a human-specific radioimmunoassay (RIA; Coat-A-Count, Diagnostic Products Corp, Los Angeles, California, USA; Eiler et al. 2005). Horses with insulin concentrations above 100 µIU/mL are secreting more insulin than normal or clearing the hormone from the circulation at a slower rate. Therefore, values above this range are interpreted as an indication of reduced insulin sensitivity (Eiler et al. 2005).

2.2 INSULIN

Insulin is the principal important hormone in the regulation of blood glucose homeostasis and essential for the organism's metabolic function (Wilcox 2005, Berg