Aus der Klinik für Pferde, Allgemeine Chirurgie und Radiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Intracellular magnesium concentration in healthy horses and horses with insulin dysregulation

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Judith Christine Winter
Tierärztin aus Frankfurt am Main

Berlin 2020
Journal-Nr.: 4143

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Dekan: Univ.-Prof. Dr. Jürgen Zentek

Erster Gutachter: Univ.-Prof. Dr. Heidrun Gehlen

Zweiter Gutachter: PD Dr. Friederike Stumpff

Dritter Gutachter: Univ.-Prof. Dr. Robert Klopfleisch

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List of abbreviations

ATP	Adenosine triphosphate
BCS	Body condition score
cAMP	Cyclic adenosine monophosphate
CGIT	Combined glucose insulin tolerance test
CNS	Cresty neck score
EHC	Euglycaemic hyperinsulinaemic clamp
EMS	Equine metabolic syndrome
$FSGITT_{MM}$	Frequently sampled glucose insulin tolerance test with minimal model analysis
G_0	Glucose at time point 0
HOMA-IR	Homeostasis model assessment for insulin resistance
I_0	Insulin at time point 0
I_{45}	Insulin at time point 45
I_{45-0}	Difference of insulin at time point 45 and 0
ID	Insulin dysregulation
IFG	Impaired fasting glycaemia
IGT	Impaired glucose tolerance
IL	Interleukin
IR-PI3K	Insulin receptor phosphoinositol 3-phosphate act/protein kinase B
$[Mg^{2+}]_i$	Intracellular magnesium concentration
MIRG	Modified insulin-to-glucose ratio
OGTT	Oral glucose tolerance test
OST	Oral sugar test
PDE-3B	Phosphodiesterase 3B
PKA	Protein kinase A
PKB	Protein kinase B
RISQI	Reciprocal inverse square of insulin
SLC41A1	Solute carrier 41 member 1
TNF α	Tumor necrosis factor alpha

1. Introduction

The equine metabolic syndrome (EMS) is currently one of the most prevalent metabolic diseases in the horse. Affected horses suffer from insulin dysregulation or peripheral insulin resistance.

A study at our clinic showed that about 80% of all patients presented for various reasons were overweight and a large part of these horses also suffered from EMS (Liertz, dissertation, unpublished). Similar to small animals and humans, the disease appears to have profound effects on other organ systems, thereby affecting the performance of the "athlete" horse.

The electrolyte magnesium is important for insulin receptor signal transduction and glucose metabolism. In addition, insulin increases intracellular magnesium shifts and can thereby influence the serum and intracellular magnesium concentration.

The clinical picture of EMS shows similarities to diabetes mellitus type II in humans (Frank et al., 2010). In human medicine, these patients often exhibit a magnesium deficiency in the serum or at the cellular level, and supplementation with magnesium has led to improved insulin sensitivity in numerous studies (Barbagallo et al., 2003). These findings suggest that magnesium metabolism may play a role in EMS horses and a further evaluation of the intracellular magnesium concentration in healthy and insulinresistant horses may provide new insights into the disease and a promising therapeutic approach.

2. Literature

2.1. Equine metabolic syndrome

The term equine metabolic syndrome (EMS) was first used by Johnson in 2002. He described a relationship between obesity, insulin resistance and endocrinopathic laminitis, which displayed similarities to the human metabolic syndrome (Johnson et al., 2002). EMS is a widespread endocrine disorder and is more common in younger horses. The phenotype of a horse with EMS typically exhibits the following characteristics:

• Regional or generalized obesity: regional fat accumulation, especially at the neck ("cresty neck"), above the tail, behind the scapula or in the area of the udder or prepuce.

• Insulin resistance: characterized by hyperinsulinemia or an abnormal glycemic/insulinemic response to oral or intravenous glucose or glucose/insulin tests.

• Predisposition to laminitis: in many, but not all cases, horses are predisposed to develop laminitis without apparent direct triggers (such as endotoxemia or massive concentrate intake) (Frank et al., 2010).

In EMS, dysfunction of the adipose tissue seems to be an important pathophysiological factor. In human medicine, a clear link has been made between obesity, insulin resistance, altered adiponectin production and an inflammatory response (Antuna-Puente et al., 2008). In this case, an overload of the storage capacity of adipocytes triggers an inflammatory reaction and cellular stress (Goossens et al., 2008). Especially the endocrine function of the adipose tissue is affected. In addition to an altered production of various hormones (adipokines like adiponectin and leptin), adipocytes and macrophages release cytokines such as tumor necrosis factor (TNF) α, interleukin (IL)-1 and IL-6. These cytokines induce a self-sustaining, chronic inflammatory response (Rasouli et al., 2008). Compared to human medicine, scientific evidence of the important role of adipocytes in the pathophysiology of EMS are less numerous. Vick et al. (2006) found a positive correlation between body condition score, mRNA expression of TNFα and interleukin 1 as well as TNFα protein expression in the blood of obese horses. Regional fat pads assessed by cresty neck score were also associated with an elevated plasma TNFα concentration. Ponies with a history of laminitis had significantly higher plasma TNFα protein concentrations, higher cresty neck scores, and insulin concentrations than ponies with no history of laminitis (Carter et al, 2009). The oxidative burst of neutrophil

granulocytes was significantly higher in the blood of overweight horses, suggesting an influence of obesity on immune function (Holbrook et al., 2012). The expression of interleukin 1-β and interleukin 6 was significantly higher in the neck fat than in other fat deposits, suggesting that regional obesity leads to an increased risk of laminitis (Burns et al., 2010). Experimental infusion of insulin for six hours resulted in a significant increase in plasma TNFα and IL-6 protein concentrations, demonstrating the association between increased insulin concentration and inflammatory response (Suagee et al., 2011). In humans, omental fat plays a special role in this inflammatory response while in the horse the neck fat is particularly endocrinologically active (Bruynsteen et al., 2013). Thus, overweight horses show a leptin resistance resulting in an increased leptin production in the adipocytes and increased blood leptin levels. The leptin concentration is positively correlated with the body condition score in horses, ponies, and donkeys (Buff et al., 2002, Carter et al., 2009, Frank et al., 2006). Leptin is therefore also proposed as an indirect marker of adipocyte function (Frank and Tadros, 2014). The anti-inflammatory hormone adiponectin is produced in the adipocytes as well and is seen as an antagonist of TNFα. Lower concentrations are found in overweight horses (Kearns et al., 2006). In addition, there is a negative correlation between adiponectin and insulin concentrations in overweight horses (Wooldridge et al., 2012). Adipose tissue and insulin dysregulation thus plays a central role in the proinflammatory events in metabolic syndrome in humans and in horses. Hyperinsulinemia has direct effects on laminar epidermal basal cells and can induce alterations in their extracellular matrix (Pollitt et al., 1999). What is more, insulin modulates vascular compliance and the laminar perfusion. Insulin attenuates the phosphatidylinositol-3-kinase signaling pathway with a decreased production of nitric oxide (vasodilator) and preserves the mitogen-activated protein-kinase signaling pathway, which leads to an increased production of endothelin-1 (vasoconstrictor) (Kim et al., 2006). This mismatch between the vasodilators and vasoconstrictors can lead to a reduced local perfusion with hypoxia and ischemia and is likely to play a role in the development of laminitis.

In horses, these factors combined could favor the development of laminitis. Laminitis is clinically one of the most serious consequences of the EMS and in extreme cases can be fatal. Due to this clinical relevance, it is necessary to gain a better understanding of the underlying mechanisms of insulin resistance and possible therapeutic actions.

In overweight EMS patients the first treatment recommendation would be weight loss through diet and exercise programs. A treatment with metformin (a human preparation for the treatment of type 2 diabetes) has shown improvements of the insulin sensitivity in some studies (Durham et al., 2008, Rendle et al., 2013); however, no change in insulin sensitivity was noted in other

studies (Vick et al., 2006). One possible explanation for the inconsistent results could be the low oral bioavailability of metformin in horses (Hustace et al., 2009). Weight loss can be increased with orally administered levothyroxine sodium at a dosage of 48 mg per 500 kg horse. Total thyroxine concentrations increase during treatment with levothyroxine but concentrations vary considerably. Clinical signs of hyperthyroidisms have not been observed in horses so far. Feed intake is subjectively increased in horses with levothyroxine treatment and horses should additionally be kept on a hypocaloric diet (Frank, 2010).

Therapeutic approaches with magnesium supplementation are scarce and have gained differing results. They are discussed in further detail in section 2.3. Magnesium homeostasis in the horse.

2.2. Magnesium deficits in diabetes mellitus type II patients

Magnesium plays an important role in the development of various diseases, amongst others diabetes mellitus. Low concentrations of magnesium in the serum or at the cellular level have been documented in diabetes mellitus type I and II. In patients with metabolic syndrome, 23,3 % had a serum magnesium deficit and 36,1 % had a reduced intracellular magnesium concentration (Lima Mde et al., 2009). In humans, the supplementation with magnesium reduced the risk to develop diabetes (Yang et al. 1999; He et al. 2006). It has a positive influence on different parameters related to the control of blood glucose concentration and counteracts the development of an insulin resistance (Volpe 2008; Günther 2010). The lower the serum magnesium concentration, the more insulin is secreted to metabolize the same amount of glucose (Kolterman et al., 1981). Causative for that could be a reduced insulin sensitivity and/or an altered intracellular glucose metabolism. In addition, glucose can modify the intracellular magnesium concentration independent of insulin. Hyperglycemia can reduce the intracellular magnesium concentration in erythrocytes in vivo and in vitro (Barbagallo and Dominguez, 2007). Low magnesium concentrations, on the other hand, can lead to hyperglycemia, hyperinsulinemia, hyperlipemia and hypertonia (Chaudhary et al., 2010). A magnesium deficit can induce a reduced signal transduction at the insulin receptor, a diminished intracellular glucose metabolism and ATP production (Kandeel et al., 1996).

2.3. Magnesium homeostasis in the horse

The daily requirement for elemental magnesium in adult horses is approximately 12.5 mg/kg per day. For performance horses (pregnancy, lactation, sports), demands can increase to as much as 30 mg/kg per day (National Research Council Committee on Nutrient Requirements of Horses, 2007). Oral supplementation at the following dosages are recommended for the horse: magnesium oxide, 30-50 mg/kg per day; magnesium sulfate, 80-100 mg/kg per day Toribio, 2007); magnesium aspartate hydrochloride, up to 300 mg/kg per day. Since magnesium aspartate hydrochloride is a compound with 9.9% magnesium (Mg^{2+}) content, 300 mg/kg magnesium aspartate hydrochloride corresponds to 30 mg/kg Mg^{2+}, a level that meets the daily needs of the competitive sports horse. Thus, the dosages are below the amount used for laxation, namely 500-1000 mg/kg magnesium sulfate (Toribio, 2007). Magnesium aspartate hydrochloride is characterized by excellent oral bioavailability. Studies in cats and rats have shown that Mg^{2+} from the aspartate hydrochloride formulation is significantly better absorbed from the gastrointestinal tract than that from magnesium sulfate, chloride, or aspartate (Classen et al., 1973). Magnesium aspartate hydrochloride has a demonstrated better oral bioavailability in humans than has magnesium oxide (Mühlbauer et al., 1991). No comparable investigations in horses have been carried out to date. Magnesium aspartate hydrochloride is currently offered as a dietary supplement for horses. Its administration is not doping-relevant.

Chameroy et al. (2011) investigated the influence of a Mg^{2+}- and Cr^{3+}-containing feed additive on insulin resistance and found no changes. However, they used a dosage of 8.8 g Mg^{2+} as an oxide/proteinate per animal per day meaning that only about 18 mg/kg/day were actually administered, based on a mean body weight of 454 kg of the animals. In addition to the relatively poor bioavailability, only half of the dosage recommended for performance horses was used. An additional study showed improvements in the combined glucose insulin tolerance test (CGIT, shorter positive phase duration of the glucose curve) in 3/5 horses and a trend towards a lower fructosamine concentration after three months of supplementation with 30 mg/kg magnesium as magnesium-aspartate hydrochloride (Winter et al., 2016). Nevertheless, the results of the last-mentioned study should be interpreted with care, because of the small sample size and the limited repeatability of the glucose curve. Horses with insulin dysregulation (ID) might have an increased requirement for magnesium, and a dosage above the usual requirements might thus be necessary to induce changes in insulin sensitivity.

2.4. Summary of the literature and aims of our studies

- The metabolic syndrome of the horse shows many similarities with type II diabetes mellitus.
- Insulin resistance in type 2 diabetes mellitus favors a lack of Mg^{2+} at the intracellular level, which in turn has a negative influence on insulin sensitivity.
- Oral Mg^{2+} supplementation improves insulin sensitivity in diabetic patients.
- In humans, the measurement of the intracellular magnesium concentration is much more compelling than the measurement of the serum magnesium concentration. The measurement of the intracellular magnesium concentration, however, is expensive and is currently not commercially available.
- In horses with insulin resistance, no serum magnesium deficiency has been described so far. Measurements of the intracellular magnesium concentration have not been performed and are also not established.
- The beneficial effect of a magnesium supplementation on insulin sensitivity has rarely been studied in horses

This results in the following questions:

- Is it possible to measure the intracellular magnesium concentration in equine blood lymphocytes?
- Is there an intracellular magnesium deficiency in horses with insulin resistance?
- Is the intracellular magnesium concertation correlated with other parameters of insulin resistance?

3. Paper I

"Intracellular free magnesium concentration in healthy horses"

Journal of *Animal Physiology and Animal Nutrition*

Vol. 102, Issue 5, 2018 Oct., pages 1351-1356

DOI: 10.1111/jpn.12921

4. Paper II

"Relationship between intracellular magnesium concentration and the degree of insulin resistance in horses with equine metabolic syndrome"

Pferdeheilkunde – Equine Medicine

Vol. 36 (2020), 4 (July/August), 325–332

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5. Paper III

"Oral supplementation of magnesium aspartate hydrochloride in horses with Equine Metabolic Syndrome"

Pferdeheilkunde – Equine Medicine

Vol. 32 (2016), 4, 372-377

DOI: 10.21836/PEM20160410

6. Discussion

6.1. Diagnosis of equine metabolic syndrome

In the present study baseline glucose and insulin levels were within the reference ranges in most horses with EMS. In addition, two different proxies, RISQI (reciprocal inverse square of insulin) and MIRG (modifies insulin-to-glucose ratio) were calculated from glucose and insulin baseline concentrations. They have first been described in horses in 2005 and can be used as a screening method. RISQI ($1/\sqrt{\text{insulin}}$) represents the degree of insulin sensitivity, while MIRG ($[800 – 0.3 \times (\text{insulin} – 50)^2] / [\text{glucose}–30]$) describes the degree of ß-cell secretion in the pancreas (Treiber et al., 2005 and 2006). Both proxys have to be interpreted with care as reference values were established for a specific group of animals (Frank, 2010). EMS is therefore most commonly diagnosed using various dynamic tests whose advantages and disadvantages are described in detail in the literature (Frank et al., 2010). In practice, the combined intravenous glucose insulin tolerance test (CGIT) is used (Eiler et al., 2005). Here, glucose and insulin are administered intravenously and the glucose curve (1-150 minutes) and insulin at time point 45 minutes are measured. Benefits of this test include ease of use and the ability to make statements about both insulin and glucose responses. In comparison, the frequently sampled glucose insulin tolerance test with minimal model analysis ($FSGITT_{MM}$) and the EHC (euglycaemic hyperinsulinaemic clamp) techniques are more precise but also more elaborate and are therefore not routinely used in practice (Knowles et al., 2012). In the $FSGITT_{MM}$, blood samples are taken for glucose and insulin determination at very short intervals and then evaluated with a special software using minimal model analysis (Radziuk, 2000). In the EHC technique, patients are given a continuous infusion of insulin and from the amount of glucose needed to keep glucose levels stable, insulin sensitivity is calculated. This method is considered to be the gold standard for determining insulin sensitivity in human medicine (DeFronzo et al., 1973).

In our study, we considered it of importance to find out whether alterations in insulin sensitivity as determined with an easy-to-use test could be correlated with magnesium metabolism. Similar tests include the Oral Glucose Tolerance Test (OGTT) or the Oral Sugar Test (OST). In such tests, horses receive a glucose solution (OGTT) or corn syrup (OST) orally. Subsequently, the blood glucose and insulin concentrations are examined, and the insulin sensitivity is calculated (Pratt-Phillips et al., 2015). However, results of these tests are influenced by gastric emptying, glucose absorption, and hepatic glucose trapping. Furthermore, the OST is primarily a test for the β-cell response rather than a test for insulin sensitivity (Lindåse et al., 2017). This test was found to be highly specific but had a poor

sensitivity for diagnosing insulin resistance. Some authors report a good correlation of OGTT with intravenous glucose tests, but others describe strong variations in the insulin response. The sensitivity of the CGIT (positive phase duration of the glucose curve >45 minutes) was 85.7%, and its specificity was 40%, whereas for insulin at 45 minutes >100 µIU/mL, sensitivity and specificity were 28.5% and 100%, respectively, when compared with the $FSIGTT_{MM}$ (Bröjer et al., 2013). Hereby, the CGIT showed better agreement with the $FSIGTT_{MM}$ than the OST in previous studies. Calculated parameters obtained for the glucose curve of the CGIT should however be interpreted with care, as they had low repeatability, whereas the parameters for the insulin curve had high repeatability. An effect of breed (Standardbred versus Icelandic horse) and of stress was noted with regard to the glucose dynamics, but not to the insulin dynamics of the CGIT (Bröjer et al., 2013). The insulin concentrations therefore seem to be a more reliable parameter for diagnosing insulin dysregulation. In our study, glucose dynamics have been used as criteria for the diagnosis of ID, and the influences mentioned above might have led to imprecise classifications. In the multivariable model, only MIRG had a significant influence on $[Mg^{2+}]_i$. This is interesting, since proxy measures are usually recommended as screening tests, whereas dynamic tests are preferred for the diagnosis of insulin resistance (Frank and Tadros, 2014). Nevertheless, proxies showed a better correlation with results of the $FSIGTT_{MM}$ than did the basal insulin concentration and HOMA-IR. An examination of the influence of a magnesium supplementation on parameters of ID, including MIRG, would be thus of interest.

6.2. Magnesium deficits in diabetes patients and in horses

Magnesium deficiency and insulin resistance in human diabetes mellitus type II have over the last few years been shown to be interrelated. In a large multicenter study, Kao et al. described an inverse correlation between serum magnesium concentration and the incidence of type 2 diabetes mellitus for the first time (Koa et al., 1999). Patients with a high magnesium intake had a 15% lower risk to develop diabetes mellitus type II (Hruby et al., 2017). Total serum magnesium concentration and the ionized serum magnesium fraction were reduced in 25-39% of diabetes patients (de Valk, 1999; Lima et al., 2009). The suggested reasons for this magnesium deficit in human diabetes type 2 patients are diverse. According to studies in Denmark and USA, the dietary habits in these patients lead to an inadequate magnesium intake (Paolisso and Barbagallo, 1997). Furthermore, hyperglycemia might lead to an increased renal tubular flow with a reduced absorption of magnesium and an increased magnesium excretion in urine (Ponder et al., 1990). At the cellular level, insulin physiologically stimulates the phosphodiesterase (PDE)-3B and decreases the intracellular cAMP

concentration. This decreased intracellular cAMP concentration constrains the magnesium efflux from the cells. Insulin resistance can therefore induce an increased magnesium efflux and lead to an intracellular magnesium deficit (Mastrototaro et al., 2015). Stress can potentiate this intracellular magnesium deficit via β-adrenergic effects (Romani and Maguire, 2002). Furthermore, an established magnesium deficit influences the tyrosine kinase activity of the insulin receptor and the signal transduction at the post receptor level and can thereby worsen the insulin resistance (Barbagallo et al. 2003) resulting in a vicious circle.

The diet of horses consists of grass, hay, and various grains that are relatively rich in magnesium, and equines are thus unlikely to suffer from a dietary Mg^{2+} deficit. Long-term studies carried out by Stewart et al. showed that horse diets need to be artificially deprived of magnesium to produce magnesium deficiency (Stewart et al., 2004). Increased Mg^{2+} excretion via the urine in hyperglycemia (hyperglycemic polyuria) is possible, but horses with EMS usually do not show resting hyperglycemia. As a result, horses are unlikely to develop total magnesium deficiency a priori (Stewart, 2011). On the other hand, a disturbance of magnesium homeostasis is conceivable because of a shift of the magnesium from intracellular to extracellular compartments with the consequence of an intracellular Mg^{2+} deficiency. Such intracellular Mg^{2+} deficiencies have been described in human diabetes mellitus patients; these patients respond positively to Mg^{2+} supplementation above demand (Barbagallo and Dominguez, 2007).

Furthermore, magnesium deficiency can lead to a mismatch of calcium and magnesium. This results in increased vascular tone and increased secretion of catecholamines by sympathetic nerve endings. Ultimately, these changes trigger hypertension in humans and might also play a role in equine laminitis.

6.3. Intracellular magnesium concentration

A low serum magnesium concentration is a reliable but insensitive marker for a deficit in whole-body magnesium (Chaudhary et al. 2010). The emptying of intracellular and bone reservoirs can stabilize the serum magnesium concentration and lead to intracellular or tissue Mg^{2+} deficits (Vormann 2003).

Importantly, patients with insulin resistance can also exhibit Mg^{2+} depletion at the cellular level, even in cases of a supposedly adequate Mg^{2+} supply and an apparently physiological serum magnesium concentration. Insulin regulates, amongst other characteristics, the cellular magnesium balance and, in healthy individuals, leads to an increased intracellular Mg^{2+} concentration (compared with the extracellular space). The Na^{+}/Mg^{2+} exchanger SLC41A

(solute carrier 41 member 1) plays a central role in these events (Mastrototaro et al., 2015). SLC41A1 is the major export mechanism for Mg^{2+} from the cell and is regulated by the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA). PKA phosphorylates the exchanger and thereby increases its activity. Insulin stimulates the phosphodiesterase (PDE)-3B via the insulin receptor phosphoinositol 3-phosphate akt/protein kinase B (IR-PI3K-Akt/PKB) signal transduction pathway, which in turn reduces the intracellular cAMP concentration. The resulting inhibition of PKA leads to a decreased release of Mg^{2+} from the cells. Conversely, insulin resistance leads to an increased release of Mg^{2+} from the cells and thus to intracellular Mg^{2+} depletion. Stress conditions can further accelerate this cellular Mg^{2+} depletion via β-adrenergic effects (adrenaline) (Romani and Maguire, 2002).

These findings suggest that the intracellular magnesium concentration is superior to the serum magnesium concentration for the evaluation of the magnesium status in diabetes mellitus type 2 (Saris et al., 2000) and in EMS patients. $[Mg^{2+}]_i$ levels in blood lymphocytes, erythrocytes, or platelets have been measured as proxies of the intracellular magnesium status in human medicine since the 1980s. Of these techniques, platelet and lymphocyte magnesium measurements are time-consuming and have not been established as a routine procedure in human medicine.

Erythrocyte magnesium concentrations can be performed faster and with less effort and have thus become more popular. Direct and indirect measurements have been proposed (Elin and Hosseini 1985; Gueux et al. 1988; Al-Khursany et al. 1992; Millart et al. 1995; Stelmasiak et al. 1995; Widmer et al. 1995a; Widmer et al. 1995b; Guerin et al. 1996; Corica et al. 1997; Moorkens et al. 1997). For direct methods, erythrocytes are isolated from whole blood (Deuster et al. 1987; Gueux et al. 1988; Millart et al. 1995). For indirect methods, the magnesium concentration of lysed whole blood and plasma is measured, and the erythrocyte concentration is calculated in relation to the hematocrit value of the samples (Basso et al. 2000). Unfortunately, the erythrocyte magnesium concentration is influenced by the age of the erythrocytes (Elin and Hosseini 1985; Deuster et al. 1987; Flatman 1988), the HLA phenotype (Millart et al. 1995; Nadler and Rude 1995), and the activity of the erythrocyte sodium-magnesium exchanger (Widmer et al. 1995a). Basso et al. (2000) have pointed out that erythrocyte magnesium changes might not reflect the systemic magnesium status, but rather the activity of metabolic determinants such as the sodium-magnesium exchanger. Another factor that needs to be considered is that the total (but not free) $[Mg^{2+}]_i$ is measured when erythrocytes are lysed. However, as erythrocytes are practically devoid of magnesium-storing organelles (mitochondria, endoplasmic reticulum), the latter is likely to be of minor relevance.

In humans, the reference range of the free $[Mg^{2+}]_i$ in blood lymphocytes ranges in the literature between 0.24 mmol/L (Huijgen et al. 1998) up to approximately 0.65 mmol/L (Maj-Zurawska

1994). Values as low as 0.16 mmol/L [Mg2+]i have been detected in the mononuclear blood cells of healthy volunteers (Huijgen et al. 1998). In human patients with diabetes mellitus type 2, an increase in $[Mg^{2+}]_i$ from 0.43 ± 0.06 mmol/L to 0.61 ± 0.06 mmol/L has been achieved by oral magnesium supplementation, a change determined by fluorometric measurements (Kurth 2013).

In the present study, we have chosen lymphocytes that have magnesium-containing organelles and may therefore more closely resemble magnesium homeostasis in most body tissues. We have measured the biologically active, free ionized magnesium for which two different probes can be used, namely mag-indo 1 and mag-fura 2. Mag-indo 1 is a dual-emission probe, whereas mag-fura 2 is a dual-excitation probe. The advantage of the latter probe is its wider ratio from minimum to maximum magnesium concentration, thereby theoretically allowing better precision of the magnesium measurement (Huijgen et al. 1998).

When processing the lymphocytes for the measurement of $[Mg^{2+}]_i$, care has been taken to maintain a physiological extracellular magnesium concentration (0.8 mmol/L) in order to avoid artificial Mg^{2+} loading or artificial Mg^{2+} bleeding from cells. The mean free intracellular magnesium concentration measured in the present study in healthy horses was slightly lower than the mean values described in healthy humans. Depending on the employed method, various intracellular magnesium concentrations have been detected in healthy humans, ranging from 0.16 mmol/L (Huijgen et al. 1998) to 0.65 mmol/L (Maj-Zurawska 1994). Maj-Zurawska et al. (1994) used ion-selective electrodes, whereas Huijgen et al. (1998) compared the two fluorescent probes mag-indo 1 and mag-fura 2. The use of mag-fura 2 in the present study might explain the comparably low values observed in our study population. Other possible influencing factors on the $[Mg^{2+}]_i$ might be the quantity and vitality of isolated lymphocytes. Between $0.8 \times 10^6 – 4.2 \times 10^6$ cells/mL have been isolated here, with an average percentage of 80% vital cells. Other authors have used 0.3×10^6 to 0.8×10^6 cells/mL (Huijgen et al. 1998) or 5×10^6 cells/ml (Reinhart et al. 1987), representing a similar cell count. The percentage of vital cells was not mentioned in these previous studies.

The coefficients of variation showed acceptable levels of variation within the horses, although the number of samples per horse was rather small. This agrees with the finding of no differences between the time points in the analysis of variance with repeated measurements. In conclusion, the results of our study indicate that the method presented are repeatable and reliable.

6.4. Intracellular magnesium in EMS horses and association with insulin dysregulation

In this study, horses with equine metabolic syndrome exhibited $[Mg^{2+}]_i$ values that were significantly lower than in healthy control horses, but still within the established reference range. Another important finding was the positive correlation between $[Mg^{2+}]_i$ and β-cell function within this group of EMS horses. Regression analysis revealed that, if MIRG increased by 1, then $[Mg^{2+}]_i$ increased by 0.015 mmol/L. This means that EMS horses with a higher insulin secretion from β-cells have a higher $[Mg^{2+}]_i$. One possible explanation for the relatively small difference in $[Mg^{2+}]_i$ might be the comparatively high magnesium intake with the usual equine diet. Another possible explanation for the relatively small drop in $[Mg^{2+}]_i$ in EMS patients might be related to the severity of ID. In human medicine, a distinction is made between impaired glucose tolerance (IGT), impaired fasting glycaemia (IFG), which is also called "non-diabetic fasting hyperglycemia", and diabetes mellitus with hyperglycemia (Alberti et al., 1998). In the above-mentioned studies on the relationship between magnesium and ID in humans (Paolisso et al., 1997; Pham et al., 2007; Chaudhary et al., 2010; Rasheed et al., 2012, Kao et al., 1999; de Valk, 1999), patients were classified as diabetes mellitus type II. Hence, these patients showed hyperglycemia at rest and a more pronounced ID than was found in the horses in our study. Glucose baseline concentrations layed within the reference range in 92.1% (35/38) of our horses, and all exhibited physiological baseline concentrations of insulin. In addition, factors that contribute to magnesium deficiency in humans (renal wasting and reduced intake) are not relevant in horses. A more severe ID or insulin resistance might be associated with a more distinct magnesium deficiency. In addition to altered glucose and insulin levels, laminitis is an important consequence of pronounced ID. Only 7.9% (3/38) of the horses showed acute laminitis when admitted to our clinic; they were examined for our study before discharge. A history of laminitis was present in only 18.4% (7/38) of the horses.

The hypothesis that mild ID is responsible for the small decreases in $[Mg^{2+}]_i$ in the EMS horses of our study is supported by the finding that the reduced $[Mg^{2+}]_i$ in EMS horses is partially reversed by the increased secretion of insulin from pancreatic β-cells. This can be postulated because horses with a higher MIRG suffered less from lower $[Mg^{2+}]_i$. A higher MIRG is, in turn, an indicator of the ability of the pancreas to alleviate the consequences of insulin resistance by the increased secretion of insulin. The increased secretion of insulin might subsequently rescue the insulin-signaling pathway, thus leading to the increased activation of the phosphoinositide 3-kinase (PI3K) by the insulin receptor. PI3K then further activates protein kinase B (PKB), which induces higher activity of the phosphodiesterase. As a result, the cAMP concentration decreases, and protein kinase A (PKA) becomes less active. A less active PKA results in a reduction in the phosphorylation of the Na^+/Mg^{2+} exchanger SLC41A1 and

eventually in reduced efflux of Mg^{2+} out of the cell (Mastrototaro et al., 2015; Zmuda-Trzebiatowska et al, 2006; Ahmed et al., 2010). By means of this pathway, insulin application is able to retain magnesium in the intracellular compartment in non-diabetic humans with a decline in plasma and an increase in intracellular magnesium concentration (Paolisso et al., 1986). Vice versa, the inhibition of SLC41A1 by insulin becomes ineffective in decompensated insulin resistance, with an increased glucose baseline concentration leading to an intracellular magnesium deficit.

Finally, this study has not been able to identify any sex differences in the magnesium status of EMS horses. In human medicine, a higher incidence of hypomagnesemia has been shown in women compared with men at a 2:1 ratio (Pham et al., 2005; Sheehan, 1991). In addition, men with diabetes seem to have higher levels of ionized magnesium (Mikhail and Ehsinapoor, 1999). However, in the horses of the present study, no differences in $[Mg^{2+}]_i$ were observed among sexes. Whether the latter is attributable to all the male horses of the present study (15/38) being geldings and, as such, not under the metabolic influence of testosterone remains to be shown.

7. Summary

Magnesium metabolism is closely connected with insulin, glucose homeostasis and the diseases metabolic syndrome and diabetes mellitus in humans. Especially the free intracellular magnesium concentration $[Mg^{2+}]_i$ is of interest in these patients, as it resembles the status in the tissue and is more reliable than the serum magnesium concentration. The equine metabolic syndrome is an important and widespread disease that shows many similarities to human diabetes mellitus.

Human diabetes mellitus patients often display a magnesium deficit in the serum or at the cellular level and benefit from magnesium supplementation. Reasons for these deficits are versatile and include a low magnesium content in the diet, renal losses due to hyperglycemic glycosuria and magnesium shifts resulting in an intracellular magnesium deficit. Horses' diets typically contain high amounts of magnesium and baseline hyperglycemia with glycosuria and urinary losses of magnesium rarely occur. A whole body magnesium deficit is therefore unlikely to develop. However, shifts from the intracellular to the extracellular compartment are reasonable and studies comparable to those in human medicine have not been performed in horses yet.

To establish reference ranges in healthy horses, the free intracellular magnesium concentration was measured by mag-fura 2 spectrophotometry in blood lymphocytes in 12 non-obese horses at 9 a.m., 12 a.m. and 4 p.m. according to a protocol designed for human blood lymphocytes. Additionally, the serum magnesium concentration was measured. In all horses, the total serum magnesium concentration was within the reference range. The mean free magnesium concentration in blood lymphocytes of all horses was 0.291 ± 0.067 mmol/L with no significant difference among the time points. The reference range for the free intracellular magnesium concentration in equine lymphocytes was set at 0.16–0.42 mmol/L. The established values are slightly lower than those in healthy humans.

The second part of the study included 38 obese horses with insulin dysregulation, diagnosed by a positive combined glucose insulin tolerance test (CGIT) and phenotypic signs of equine metabolic syndrome.

$[Mg^{2+}]_i$ was significantly lower in horses with EMS (P=0.015) than in healthy horses, but still within the established reference range. We further demonstrated a positive correlation between the intracellular magnesium concentration and the proxy MIRG (modified insulin-to glucose ratio). The multivariate model revealed that only MIRG was significantly associated

with $[Mg^{2+}]_i$, whereas all other factors had no additional influence. Thus, MIRG increase by 1 represents a $[Mg^{2+}]_i$ increase by 0.015 mmol/L. This means, the decreased $[Mg^{2+}]_i$ in EMS horses can be partially reversed by increased secretion of insulin from pancreatic β-cells. These findings substantiate the crucial role of insulin in regulating intracellular magnesium homeostasis and its relevance in EMS. Further studies should examine the role of magnesium metabolism in horses with metabolic syndrome, especially in horses with more pronounced insulin resistance.

8. Zusammenfassung

Intrazelluläre Magnesiumkonzentration bei gesunden Pferden und Pferden mit Insulindysregulation

Der Magnesiumstoffwechsel steht in engem Zusammenhang mit Insulin, der Glukosehomöostase und den Erkrankungen metabolisches Syndrom und Diabetes mellitus des Menschen. Bei diesen Patienten ist vor allem die intrazelluläre Magnesiumkonzentration $[Mg^{2+}]_i$ von Interesse, da diese eher die Magnesiumkonzentration des Gewebes wiederspiegelt und zuverlässiger ist als die Serummagnesiumkonzentration.

Das Equine Metabolische Syndrom (EMS) ist eine weit verbreitete Erkrankung mit enormer Relevanz in der Pferdepopulation, die viele Parallelen zum humanen Diabetes mellitus Typ II zeigt. Menschen mit Diabetes mellitus haben häufig einen Magnesiummangel im Serum oder auf zellulärer Ebene und profitieren von einer Magnesiumsupplementierung. Die Gründe für diesen Magnesiummangel sind vielfältig und beinhalten eine niedrige Magnesiumkonzentration in der Nahrung, renale Verluste durch eine hyperglykämische Glukosurie und Magnesiumverschiebungen, die in einem intrazellulären Magnesiummangel resultieren. Die Futtermittel von Pferden sind typischerweise reich an Magnesium und eine basale Hyperglykämie mit Glukosurie kommt ebenfalls selten vor. Ein totaler Magnesiummangel ist daher unwahrscheinlich. Verschiebungen von intra- nach extrazellulär könnten allerdings vorkommen und sind bisher in der Pferdemedizin noch nicht untersucht worden.

Um Referenzbereiche gesunder Pferde zu erstellen, wurde die freie intrazelluläre Magnesiumkonzentration mittels Mag-Fura 2 Spektrometrie in den Blutlymphozyten von 12 normalgewichtigen Pferden zu den Zeitpunkten 9, 12 und 16 Uhr in Anlehnung an ein Protokoll aus der Humanmedizin gemessen. Zusätzlich wurde die Serummagnesiumkonzentration bestimmt. Diese war bei allen Pferden innerhalb des Referenzbereichs. Die mittlere freie intrazelluläre Magnesiumkonzentration der Blutlymphozyten aller Pferde lag bei 0,291 ± 0,067 mmol/L ohne signifikante Unterschiede zwischen den verschiedenen Zeitpunkten. Der Referenzbereich für $[Mg^{2+}]_i$ gesunder Pferde wurde daher bei 0,16-0,42 mmol/L festgelegt.

Der zweite Teil der Untersuchung schloss 38 übergewichtige Pferde mit Insulindysregulation (diagnostiziert durch einen positiven kombinierten Glukose-Insulin-Toleranztest (CGIT)) und phänotypischen Anzeichen von EMS ein. $[Mg^{2+}]_i$ war bei Pferden mit EMS zwar signifikant

niedriger als bei gesunden Pferden (P=0,015), aber noch immer innerhalb des erstellten Referenzbereichs. Wir konnten außerdem eine positive Korrelation zwischen $[Mg^{2+}]_i$ und dem Proxy MIRG (modifizierte Insulin-Glukose Ratio) zeigen. Das multivariate Model zeigte, dass nur MIRG signifikant mit $[Mg^{2+}]_i$ assoziiert war, während alle anderen Faktoren keinen zusätzlichen Einfluss hatten. Eine Erhöhung von MIRG um den Faktor 1 hängt mit einer $[Mg^{2+}]_i$ Erhöhung um 0,015 mmol/L zusammen. Dies könnte bedeuten, dass erniedrigte $[Mg^{2+}]_i$ bei EMS Patienten durch eine verstärkte Insulinsekretion aus den pankreatischen ß-Zellen revidiert werden können.

Diese Ergebnisse bestätigen die wichtige Rolle von Insulin in der Regulation der intrazellulären Magnesiumkonzentration und deren Bedeutung bei EMS. Weitere Studien sollten die Rolle des Magnesiumstoffwechsels bei Pferden mit EMS, insbesondere mit einer deutlicher ausgeprägten Insulinresistenz untersuchen.

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10. List of publications

1. Winter JC, Sponder G, Merle R, Aschenbach JR, Gehlen H. Intracellular free magnesium concentration in healthy horses. *J Anim Physiol Anim Nutr*. 2018;00:1-6.

2. Winter JC, Müller E, Sponder G, Merle R, Aschenbach JR, Gehlen H. Relationship between intracellular magnesium concentration and the degree of insulin resistance in horses with equine metabolic syndrome. *Pferdeheilkunde – Equine Medicine* 2020; 36: 1-8.

3. Winter JC, Liertz S, Merle R, Aschenbach JR, Gehlen H. Orale Supplementierung von Magnesiumaspartat-hydrochlorid bei Pferden mit Equinem Metabolischen Syndrom. *Pferdeheilkunde – Equine Medicine* 2016; 32: 372-377.

4. Winter JC, Sponder G, Aschenbach JR, Gehlen H. Expression of magnesium-sensitive genes and intracellular magnesium content in equine lymphocytes. Tagung der DVG-Fachgruppe "Physiologie und Biochemie" – DVG; 2016; Gießen: Verlag der DVG Service GmbH, S. 118–119.

5. Winter JC, Sponder G, Aschenbach JR, Gehlen H. Supplementierung mit Magnesiumaspartat-Hydrochlorid bei Pferden mit Metabolischem Syndrom – Einfluss auf die Insulinsensitivität? 37. Abstracts; Magnesium-Symposium der Gesellschaft für Magnesium-Forschung e.V. Herne, 2016; S. 19.

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12. Founding

This work was partly founded by the research committee of the academic senate and the presidium of the Free University of Berlin.

13. Declaration of academic honesty

I hereby confirm that I have prepared the present work independently. I assure that I have used only the sources and help indicated.

Judith Christine Winter, 31.03.2020

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