Assessment of (patho)physiological alterations in equine muscle metabolism

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Assessment of (patho)physiological alterations in equine muscle metabolism

Bepaling van (patho)fysiologische veranderingen in het spiermetabolisme van het paard

(met een samenvatting in het Nederlands)

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Voor Jerre

Contents

Chapter 1	General introduction	1
Chapter 2	Equine metabolic myopathies with emphasis on the diagnostic approach; Comparison with human myopathies, a review	9
Chapter 3	Equine biochemical Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) as a cause of rhabdomyolysis	35
Chapter 4	Acquired Multiple Acyl-CoA Dehydrogenase Deficiency in 10 horses with atypical myopathy	53
Chapter 5	Creatine metabolism, carbohydrate metabolism, purine metabolism and oxidative phosphorylation in healthy horses and horses suffering from atypical myopathy	73
Chapter 6	The influence of exercise, training and intensified training on plasma concentrations of amino acids in Standardbreds	91
Chapter 7	Plasma acylcarnitine and fatty acid profile during exercise and training in Standardbreds.	107
Chapter 8	The effect of oral tocopherol acetate administration on equine glucose metabolism, peripheral insulin sensitivity, and antioxidant status	123
Chapter 9	Discussion	139
Summary		151
Samenvatting		155
Samenvatting voor niet ingewijden		159
Dankwoord		163
Curriculum Vitae		167
List of Publications		169
Coloured illustrations		173

Chapter 1

General introduction

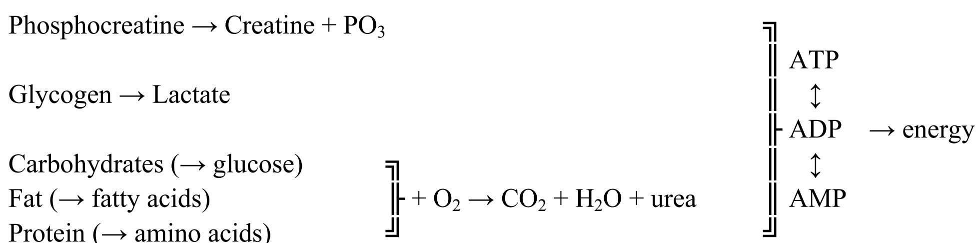
The horse has had a special place in the life of man for many centuries. Its strength, agility and ability to learn and trust have contributed to the life of humans by assisting in warfare, agriculture, business, travel and recreation.
Skeletal muscle is critical for a horse's mobility. Depending on nutritional status, breed and build around 44 to 53% of the horse's body weight consists of muscle [1]. The muscular system is closely associated with cardiovascular, pulmonary and endocrine systems, and disorders of the neuromuscular system have dramatic effects on health and performance of the horse.
It is therefore of the utmost importance to obtain as much information about the anatomy, physiology, pathophysiology and diseases of muscle as possible. This thesis aims to make a contribution to the knowledge base in this area.
In this chapter, muscle metabolism as well as physiological and pathological changes within muscle will be described. In addition, possible therapeutic options for alterations in muscle metabolism will be discussed. Finally, the structure and scope of the thesis will be presented.

Muscle metabolism

Muscle contraction depends on energy provided by adenosine triphosphate (ATP). The concentration of ATP in the muscle fiber is sufficient to maintain full contraction for only one to two seconds at most. After this, ATP is split to form adenosine diphosphate (ADP), which is rephosphorylated to form new ATP within a fraction of a second. There are several sources of energy for this rephosphorylation (Figure 1) [2].
The first source of energy used is phosphocreatine, which carries a high-energy phosphate bond similar to the bonds of ATP. The total amount of phosphocreatine in the muscle fibre is limited, allowing a maximum muscle contraction of five to eight seconds. The reaction is phosphocreatine + ADP ↔ creatine + ATP [2].
ADP may also be rephosphorylated to ATP through the myokinase reaction, which leads to increased adenosine monophosphate (AMP) production (2 ADP↔ ATP + AMP). AMP is deaminated into inosine monophosphate (IMP) and ammonia (NH_3). Further deamination leads to purine synthesis [3,4].
The second important source of energy is glycogen. The enzymatic breakdown to glucose (glycogenolysis) and subsequently to pyruvate (glycolysis) and lactic acid liberates energy that is used to reconstitute both ATP and phosphocreatine. This glycogenolysis and glycolysis can occur both in the presence (aerobic) and absence (anaerobic) of oxygen. Anaerobic glycogenolysis and glycolysis can sustain maximum muscle contraction for about one minute due to accumulation of its many end products [2].

The third and final group of energy sources includes glucose, fatty acids and amino acids in oxidative metabolism. This means the combining of oxygen with these various cellular metabolites to liberate ATP. The relative contribution of different substrates to fuel metabolism during exercise is determined by a number of factors, including intensity and duration of exercise, training and diet [5]. During the early phases of moderate-intensity exercise the use of carbohydrate predominates, but if exercise is continued for longer periods there is increased utilization of fat. The utilization of plasma glucose and muscle glycogen continues throughout both phases, and is reflected by lowered blood glucose concentrations, and depletion of muscle glycogen after endurance exercise [5].
The citric acid cycle is responsible for the last step of the dissimilation of proteins, lipids and glucose into carbon dioxide (CO_2), thereby generating ATP, nicotinamide adenine dinucleotide (NADH) and 1,5-dihydro-flavin adenine dinucleotide ($FADH_2$). The NADH and $FADH_2$ will be used for the production of ATP via the electron transport chain in the oxidative phosphorylation.
In this thesis an addendum is placed with graphic explanation of general metabolism.

Figure 1: Origin of ATP



Changes in muscle metabolism

The function and metabolism of skeletal muscle may be influenced by a variety of factors, of which disease and exercise exert the greatest effect.

The disease-based group of disorders occurs as a result of a disorder in a metabolic reaction and the exercise-based group consists of a physiological or sometimes pathological reaction as a result of exercise and training.

1 Disease based muscle metabolism
A wide variety of conditions can be classified as metabolic myopathies. They result from an underlying abnormality in muscle that interferes with the tissue's ability to produce or maintain adequate energy levels.
Metabolic diseases of muscle can be divided into those that are primary or inherited and those that are secondary or associated with an acquired condition. The primary forms are generally classified according to the altered biochemistry or intercellular component involved rather than by clinical presentation [6]. With this categorisation, metabolic myopathies include disorders of carbohydrate metabolism, lipid metabolism, mitochondrial myopathies (other than those described in lipid metabolism), disorders of the purine metabolism, primary disorders involving ion channels and electrolyte flux and secondary or acquired metabolic myopathies [6].

2 Exercise based muscle metabolism
Physical conditioning is perhaps the most effective means of maximizing muscle function as skeletal muscle can adapt to various kinds of training [7]. High intensity training causes adaptations primarily to the anaerobic metabolic pathways, whilst endurance training causes adaptations primarily to the aerobic metabolic pathways [5,8] .
Factors that adversely affect muscle function generally cause fatigue or weakness. Several mechanisms, such as the accumulation of lactic acid and other metabolic by-products or substrate depletion lead to neuromuscular fatigue [5]. Overreaching is the short-term fatigue associated with the increases in exercise load that are common in any training program [9]. In contrast, overtraining is a state of prolonged fatigue, caused primarily by an imbalance between training and recovery. This means excessive training loads with inadequate periods for regeneration, which reduce performance capacity. Even with complete rest, recovery from overtraining can take weeks or months [1].
Training results in an increase in the ability of a fiber to use oxygen by increasing the number of mitochondria within the sarcoplasma, a decrease in the use of muscle glycogen and blood glucose with a greater reliance on fat oxidation to supply ATP and a decrease in the amount of lactate produced per given intensity of exercise [1].

Therapy for altered muscle metabolism

In situations of normal training and exercise, horses are often provided with all kinds of supplements to replenish the consumed elements, whether this is necessary or not. If a pathologic condition is present, such as a metabolic myopathy, or if a shortage of nutrients is to be expected, as in intensified training, a therapeutic or preventive drug or supplement would be desirable.

Nutrition can be extremely important, depending on the type of problem identified. If, for instance, a disorder in the glycogenolysis is found, a diet focussed on addition of lipid ingredients might be helpful. If a disorder in lipid metabolism is identified, avoidance of fasting and stress, and supplementation of carbohydrates, medium chain triglycerides and carnitine supplements could be prescribed [10-12].
Deficiency in riboflavin (vitamin B2) may contribute to or cause muscle myopathy. Riboflavin is a precursor in the synthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both products are the prosthetic groups of numerous enzymes (called flavoproteins) that catalyze the various electron-transferring reactions in energy-producing, biosynthetic, detoxifying, and electron-scavenging pathways. Most of these flavoproteins are found in mitochondria. ETF and ETF-QO (Electron Transfer Flavoprotein Ubiquinone Oxidoreductase) are among them [13,14]. In cases of riboflavin deficiency, a diet or therapy including riboflavin would be advisable.

Antioxidants have become increasingly popular for horses in recent years. Vitamin E is the most frequently used antioxidant. Antioxidants can neutralize or reduce damage caused by free radicals. There is a correlation between strenuous exercise and decreased antioxidant status [15,16]. This condition may be alleviated by administration of vitamin E, which affects the free radical defence system [17]. Moreover, vitamin E improves the insulin sensitivity of man and rats [17-19]. The extrapolation of such results to the horse in relation to muscle metabolism is interesting, as it would suggest that vitamin E might induce a more economic use of energy in equines.

Structure and scope of this thesis

This thesis focuses on the diagnostic use of metabolic products and enzymes found in plasma, urine and muscle of the horse. Such metabolic products, metabolites and enzymes can reveal changes in muscle metabolism, either physiologically or pathologically, by their accumulation or deficiency.
In the studies described in this thesis, analyses of metabolites of carbohydrate-, lipid- and protein metabolism have been performed as well as analyses of the purine nucleotide cycle, the creatine metabolism and the oxidative phosphorylation. With these analyses we studied metabolic myopathies in the horse as well as metabolic effects of training, intensified training and exercise.

Chapter 2 gives an overview of the known human and equine metabolic myopathies and possibilities for their diagnosis.

In **Chapters 3 and 4** the application of human diagnostic methods in an equine myopathy reveals the pathogenesis of a new equine metabolic disorder of lipid metabolism that appears to have a large and emerging prevalence in Europe and elsewhere. Several hypothetical options for prevention and treatment of this disorder are presented.
Chapter 5 describes the diagnostic analysis of creatine-, carbohydrate- and purine metabolism as well as oxidative phosphorylation, performed in healthy horses and horses with atypical myopathy. The goal of this study was to explore several diagnostic metabolic routes, normally used in human medicine, to see if the exogenic toxic insult associated with atypical myopathy disrupted metabolic cycles or reactions other than the lipid metabolism. Altered parameters were evaluated for usefulness as diagnostic tools in practice.
In **Chapters 6 and 7** the effects of intense exercise and (intensified) training on protein (amino acids) and lipid (organic acids, carnitines) metabolism are studied. Ten Standardbred horses were trained for 32 weeks, divided into four phases, including a phase of intensified training for five horses. At the end of each phase, a standard exercise test, SET, was performed. Plasma amino acid, fatty acid and carnitine concentrations before and after each SET were measured. The objective of this study was to assess the influence of acute exercise, training and intensified training on the plasma amino acid, fatty acid and acylcarnitine profile. If significant differences between trained and intensified trained horses would occur, they could be evaluated as possible markers for overtraining. The study also evaluated the hypothesis that alterations in skeletal muscle carnitine metabolism during exercise and/or training lead to changes in plasma concentrations of acylcarnitines**.**
Chapter 8 describes the influence of the antioxidant vitamin E on carbohydrate metabolism. The objective of this study was to determine the effect of vitamin E on glucose metabolism, peripheral insulin sensitivity and anti-oxidant status as measured using five different variables. Our hypothesis was that oral supplementation with vitamin E, at the dosage used, is effective in improving insulin action and reducing oxidative stress. Both effects contribute to better performance, the first by, amongst other effects, optimizing energy metabolism, and the second by reducing muscle damage.
In **Chapter 9** the results of the thesis are integrated and discussed.

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Chapter 2

Equine metabolic myopathies with emphasis on the diagnostic approach

Comparison with human myopathies

A review

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Abstract

This review gives an overview of the presently known human and equine metabolic myopathies with emphasis on the diagnostic approach. Metabolic myopathies are muscle disorders caused by a biochemical defect of skeletal muscle energy system, which results in inefficient muscle performance. Myopathies can arise in different levels of the metabolic system. In this review the metabolic myopathies are categorized in disorders of the carbohydrate metabolism, lipid metabolism, mitochondrial myopathies (other than those described in lipid metabolism), disorders of purine metabolism, primary disorders involving ion channels and electrolyte flux and secondary or acquired metabolic myopathies.

Keywords

Comparative study, Diagnostics, Equine diseases, Horses, Humans, Metabolic myopathies, Muscular diseases

Introduction

Metabolic myopathies (MMs) are a group of muscle disorders caused by a biochemical defect of skeletal muscle energy system, which results in inefficient muscle performance [1]. The first described myopathy in humans, a case of myophosphorylase deficiency, was found in 1951 and identified in 1959 [2]. Subsequently, additional defects of carbohydrate metabolism and disorders of lipid and purine metabolism have been recognised, and after discovery of the sequence of human mitochondrial DNA in 1981, many mitochondrial myopathies have been diagnosed [2].
In equine medicine much less is known about the aetiology of muscle metabolic myopathies. Although muscle disorders are a common cause of suboptimal performance or even disability to perform, research is limited compared to human medicine. The first equine metabolic myopathy, associated with abnormal storage of glycogen, was recognised in 1979 [3,4].

A biochemical defect of the skeletal muscle energy system can involve several mechanisms [5,6]:
Adenosine triphosphate (ATP) is the universal intracellular vehicle of chemical energy within skeletal muscle. Energy is generated during hydrolysation to adenosine diphosphate (ADP). However, the limited amount of ATP present in skeletal muscle is only sufficient for a few seconds. If continued muscular contraction has to be maintained, rapid resynthesis of ATP must occur. This can be done via two distinct processes: The oxidative, aerobic phosphorylation and the anaerobic phosphorylation. Depending on the type of exercise, a balance occurs between the contributions of oxidative and anaerobic phosphorylation. The major substrates for aerobic phosphorylation are circulating nonesterified fatty acids (NEFA's) and glucose, as well as intramuscular glycogen and triglycerides. These substrates are used in the fatty acid oxidation (1 mol creates 129 ATP) and the glucose oxidation (1 mol creates 36 ATP). In the anaerobic phosphorylation ATP is generated from creatine phosphate (CP), circulating glucose, and local glycogen stores. The phosphocreatine reaction, converting CP + ADP to creatine + ATP creates 1 mol ATP and supports exercise for only a few seconds. The myokinase reaction catalyses the reaction of 2 molecules of ADP to one molecule of ATP and one AMP, therefore producing only little energy. If these reactions are insufficient, anaerobic glycolysis is stimulated, producing per molecule glucose derived from glycogen 3 molecules ATP, and lactate [5,6].

Myopathies can originate from different levels of the metabolic system. A way to categorise the metabolic myopathies is in disorders of carbohydrate metabolism (figure 1), lipid metabolism (figure 2), mitochondrial myopathies (other than those described in lipid metabolism) (figure 3), disorders of the purine metabolism (figure 4), primary disorders

involving ion channels and electrolyte flux and secondary or acquired metabolic myopathies.
The purpose of this review is to give an overview of the known human metabolic myopathies and the way they are diagnosed. Thereafter an overview will be given of the known equine metabolic myopathies and their diagnosis.

Overview of human metabolic myopathies and the route to their diagnosis

A list of known human metabolic myopathies is shown in Table 1. To come to a diagnosis of metabolic myopathy the following steps are routinely performed: [1,7,8]

- History: The age of onset is of importance, as well as the medical history including prescribed drugs and the family history. In addition if the problems arise at rest, shortly after exercise, or after sustained or prolonged exercise. It is important to make a distinction between weakness and fatigue. Fatigue describes the inability to continue performing a task after multiple repetitions. In contrast a patient with primary weakness is unable to perform the first repetition of the task. Asthenia is a sense of weariness or exhaustion in the absence of muscle weakness [9].
- Physical examination with emphasis on muscle tone, symmetrical or asymmetrical appearance, atrophy or hypertrophy, respiratory-, neurological or cardiac disorders.
- Laboratory tests: all patients suspected of a metabolic myopathy should receive the following assessments: serum creatine kinase (CK) at rest and after exercise, serum chemistry (lactate, ammonia, AST, etc.), urinary and serum myoglobin levels [10], urinary excretion tests and sedimentation rate.
- Electrophysiological studies can detect dystrophinopathies or other muscle diseases in patients with little or no symptoms and can also differentiate primary myopathies, myotonia, and neurological diseases. The electromyogram shows a myogenic pattern in patients with acute rhabdomyolysis and can help to localise a biopsy site.
- Imaging studies: In difficult cases with little or no physical findings or focal abnormalities, magnetic resonance imaging (MRI), computed tomography [11], ultrasonography and scintigraphy can provide diagnostic orientation.
- Muscle biopsy: can be used for morphological, histoenzymological and ultrastructural studies. A large fragment can be used for in vitro contraction tests [12].
- Noninvasive metabolic investigation: Nuclear magnetic resonance (NMR) spectroscopy with phosphorus 31 provides direct and strictly non-invasive images of high-energy compounds present within the muscle cell [13].
- Ischemic forearm test (LAER): Blood lactic acid and ammonia levels are measured during a forearm exercise test (exercise during temporarily circulatory occlusion with

a cuff). The increase in blood lactic acid is abnormally small in patients with glycogen storage disease and abnormally large in those with mitochondrial dysfunction [6].

- To exclude a secondary metabolic myopathy systemic investigation is necessary as is ancillary investigation, to evaluate the organs that might be involved with the metabolic myopathy.

Table 1: List of known human metabolic myopathies [1,2,7,16-18,75]

1) DISORDERS OF CARBOHYDRATE METABOLISM
Acid Maltase deficiency (Pompe disease) (Glycogen Storage Disease II = GSD II)
Debrancher Enzyme deficiency (Cori-Forbes) (GSD III)
Branching enzyme deficiency (Andersen) (GSD IV)
Myophosphorylase Deficiency (McArdle's disease) ((GSD V)
Phosphofructokinase deficiency (Tarui) (GSD VII)
Phosphorylase b Kinase deficiency (GSD VIII)
Phosphoglycerate Kinase Deficiency (GSD IX)
Phosphoglycerate mutase (GSD X)
Lactate Dehydrogenase Deficiency (GSD XI)

2) DISORDERS OF LIPID METABOLISM
(Fatty acid transport and β-oxidation myopathies)
(Systemic Primary) Carnitine Deficiency (CDSP)
Carnitine Palmitoyltransferase Deficiency (CPT)
Carnitine-Acylcarnitine Translocase deficiency (CACT)
Very-long-chain Acyl-CoA Dehydrogenase Deficiency (VLCAD)
Long-chain Acyl-CoA Dehydrogenase Deficiency (LCAD)(more than 12 C's)
Medium-chain Acyl-CoA Dehydrogenase Deficiency (MCAD)(4-14 carbons)
Short-chain Acyl-CoA Dehydrogenase Deficiency (SCAD)(4-6 carbons)
Multiple Acyl-CoA Dehydrogenase Deficiency (MADD)

3) MITOCHONDRIAL MYOPATHIES
Mitochondrial DNA mutations
    Mitochondrial DNA Deletions en duplications
        Kearns-Sayre syndrome
        Chronic progressive external opthalmoplegia syndromes (CPEO)
        Pearson's syndrome
        Diabetes mellitus and deafness
        Mitochondrial myopathy

        - Fahr's syndrome variants
    - Missense Mutations
        - Leber's hereditary optic neuropathy
        - Leber's hereditary optic neuropathy plus dystonia
        - Leigh's disease (=subacute necrotizing encephalopathie).
        - Pigmentary retinopathy, ataxia, and neuropathy syndromes
    - Transfer RNA mutations
        - Myoclonic epilepsy and ragged red fiber disease (MERRF)

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS)

        - Diabetes mellitus (usually with deafness)
        - Hypertrophic cardiomyopathy plus mitochondrial myopathy
        - Mitochondrial myopathy
    - Ribosomal RNA Mutations
        - Maternally inherited deafness with aminoglycoside sensitivity
        - Cardiomyopathy

- Nuclear DNA mutations
    - Abnormal mtDNA copy number regulation
        - MtDNA depletion diseases
    - Nuclear DNA mutations causing multiple mtDNA rearrangements
        - Autosomal dominant chronic progressive external opthalmoplegia
        - Autosomal recessive chronic progressive external opthalmoplegia
        - Wolfram syndrome
        - MERRF variant
    - Thymidine phosphorylase
        - Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)
    - Complex I
        - Leigh disease
        - Alexander disease
    - Complex II
        - Leigh disease
    - Complex IV
        - Leigh disease
    - Possible abnormal nuclear regulation of mitochondrial protein assembly
        - Hereditary spastic paraplegia
    - Abnormal mitochondrial iron homeostasis
        - Friedreich ataxia

4) DISORDERED PURINE METABOLISM
Myoadenylate Deaminase Deficiency

5) PRIMARY DISORDERS INVOLVING ION CHANNELS AND ELECTROLYTE FLUX
Disorders of Sodium Channels
- Hyperkalemic Periodic Paralysis (Hyper-PP)
- Paramyotonia Congenita (PC)
- Other Sodium Channel diseases

Disorders of Chloride Channels (dominant and recessive myotonia congenital, DMC/RMC)
Disorders of Calcium Channels and Calcium Distribution
- Hypokalemic Periodic Paralysis (Hypo-PP)
- Malignant Hyperthermia (MH)
- Sarcoplasmic Reticulum Calcium-ATPase Deficiency (SRCAD)

6) SECONDARY (OR AQUIRED) METABOLIC MYOPATHIES
Endocrine Diseases
- Acromegaly
- Hypothyroidism
- Hyperthyroidism
- Hypoparathyroidism
- Addisson's disease
- Cushing's disease and syndrome
- Hyperaldosteronism
- Carcinoid syndrome
- Pheochromocytoma
- Diabetic neuromyopathy

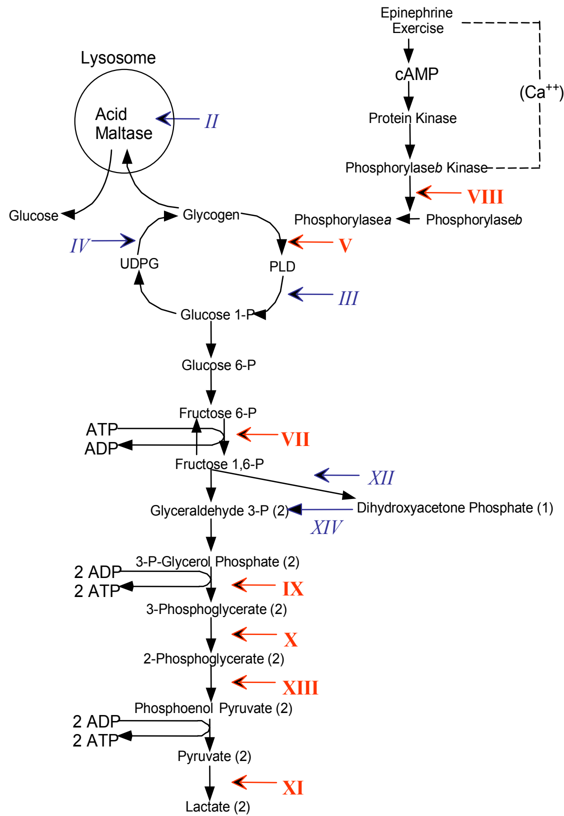
Metabolic-nutritional
- Uremia
- Cirrhosis
- Malabsorption
- Vitamine D deficiency
- Vitamine E deficiency
- Lipid metabolism disorders
  - Acquired carnitine deficiency
  - HMG-CoA reduction inhibitor use

Electrolyte imbalance

Table 2: The diagnosis of the disorders of human carbohydrate metabolism [1,2,7,15,16,76]

Disorder	Age at onset	Clinical presentation	Tissues Manifesting defect	Bloodwork	EMG	Biopsy	Extra
GSD II (Pompe)	Infantile Childhood Adult	Floppy baby Cardiomyopathy Proximal weakness Respiratory problems	Muscle, liver, heart	CK↑, AST↑, LDH ↑	Abnormal (myopathic/ myotonic)	Glycogen storage (all forms) Vacuoles	LAER test normal rise lactate, leucocyte glycogen level, urine tetraglucose ↑
GSD III (Cori-Forbes)	Infantile Adult	Hepatosplenomegaly Failure to thrive Distal weakness Myoglobinuria	Muscle, liver	CK↑, fasting non responsive hypoglycemia	Abnormal (myopathic/ myotonic, decreased nerve conduction	Vacuolar myopathy with diastase-sensitive PAS^+ material	Abnormal ECG, LAER test no or small rise lactate, urine tetraglucose ↑
GSD IV (Anderson)	Childhood Juvenile Ádult	Cirrhosis Progressive weakness Neuropathy	Muscle, heart, liver, brain	CK inconsistently↑		Deposits of abnormal basophilic, PAS^+ filamentous polysaccharide, partially diastase-sensitive	
GSD V (McArdle)	Childhood Juvenile Adult	Exercise intolerance Myoglobinuria Proximal weakness	Muscle	CK inconsistently↑	Abnormal (no electr. act. during cramps)	In 66% subsarcolemmaal glycogen accumulation	LAER test no rise lactate, leucocyte DNA analysis
GSD VII (Tarui)	Childhood Adult	Exercise intolerance Myoglobinuria Proximal weakness Hemolytic anemia	Muscle	CK inconsistently↑ bilirubine↑	Non-diagnostic	subsarcolemmaal glycogen accumulation diastase-insensitive PAS^+ material	LAER test no rise lactate, reticulocytosis
GSD VIII	Childhood Adult	Hepatomegaly Exercise intolerance Myoglobinuria	Muscle	CK inconsistently↑		Glycogen concentration increase 2 to 3 times	LAER test no rise lactate
GSD IX	Childhood Adult	Hemolytic anemia Exercise intolerance Myoglobinuria	Muscle, blood	CK inonsistently↑	normal	Mild rise glycogen	LAER test no rise lactate
GSD X	Adult	Exercise intolerance Myoglobinuria	Muscle	CK↑	Non-diagnostic	Normal or inconsistent rise glycogen	LAER test reduced rise lactate
GSD XI	Adult	Exercise intolerance Myoglobinuria	Muscle				

Figure 1: Scheme of glycogen metabolism and glycolysis. The roman numerals refer to glycogen storage diseases resulting from deficiencies of the various enzymes.
With courtesy of Prof. S. DiMauro



1) Disorders of carbohydrate metabolism

Most disorders of the carbohydrate metabolism identified in man are caused by inherited deficiencies in glycogenolytic, glycolytic or lysosomal enzymes [14].

In Table 2 the diagnostics are given for the disorders of carbohydrate metabolism. To fully understand how the enzymes work Figure 1 is included. This is a scheme of glycogen metabolism and glycolysis. The roman numerals refer to glycogen storage diseases resulting from deficiencies of the various enzymes [15]. As shown in this figure, all enzymes have a function in the glycogen metabolism and a deficiency will result in inefficient energy metabolism.

Specific investigations are: the ischemic forearm test, assessing lactate, ammonia and pyruvate; urine myoglobin; muscle histochemistry; enzyme assays of muscle, blood and fibroblast; leucocyte glycogen level, leucocyte DNA analysis, and phosphorus MR spectroscopy [1,16]. The disorders can be confirmed by biochemical quantitative measurements of the enzymes in fresh and frozen material and genetic investigation of patient and family.

2) Disorders of lipid metabolism

Plasma free fatty acids are the major source of energy while fasting, at rest, and exercising at lower intensity and for longer durations [2]. There are three fundamental steps in lipid metabolism: uptake and activation of FFA in skeletal muscle, transport of FFA through the mitochondrial membrane and β-oxidation (Figure 2). Medium- and short chain fatty acids can enter the mitochondrion with the help of acyl-CoA synthetase, long chain fatty acids need carnitine to enter. For this process multiple enzymes are necessary: acylCoA-synthetase, carnitine palmitoyl transferase I and II, carnitine translocase and acylcarnitine translocase. Once inside the mitochondrium, β-oxidation repeatedly shortens the chain with two carbons that emerge as acetyl-CoA. This process is essential in the lipid metabolism and is helped by long, medium or short chain acyl-CoA dehydrogenase (LCAD, MCAD, SCAD). In Table 1 the enzyme deficiencies in lipid metabolism are shown. In Table 3 the diagnostics are given for the disorders of lipid metabolism. Also, the disorders can be confirmed by biochemical quantitative measurements of the enzymes in fresh and frozen material and genetic investigation.

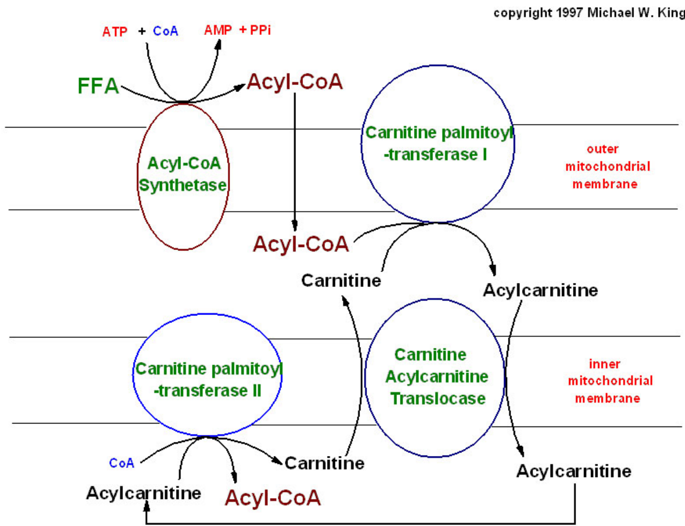
Specific investigations are: prolonged fasting, assessing free fatty acids, uric acid, ammonia, lactate, creatine kinase, glucose; urinalysis for organic acids, myoglobin, acylcarnitine; muscle histochemistry; enzyme assays of muscle, fibroblasts, liver; carnitine assay of muscle and blood [1,17].

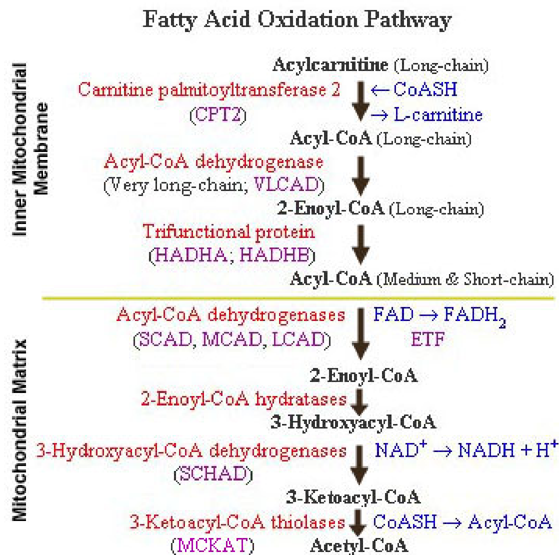
Table 3: The diagnosis of the disorders of human lipid metabolism [1,2,17]

Disorder	Age at onset	Clinical presentation	Tissues Manifesting defect	Bloodwork	EMG	Biopsy	Extra
CDSP	Infantile	Acute hypoketotic hypoglycemia, cardiomyopathy, muscle weakness, hypotonia	Muscle, heart, kidney	carnitine↓ ammonia ↑ glucose ↓	myopathic	Lipid storage	
CPT-I	Infantile	L-CPT-I: lethargy, coma, seizures, hepatomegaly M-CPT-I: myopathy, cardiomyopathy	Liver (L-CPT-I), muscle (M-CPT-I)	carnitine↓ acylcamitines↑			Dicarboxylic aciduria
CPT-II	Neonatal Infantile Adult	Hypoketotic hypoglycemia, encephalopathy, arrhythmias, cardiomyopathy, congenital anomalies Similar, but less severe Exercise-induced pain, stiffness, myoglobinuria	All tissues	Infantile: acylcamitines↑ in plasma, low total Adult: normall acylcarnitine, CK↑(normal between attacks)	Normal between attacks	Seldom lipid accumulation	LAER-test normal
CACT	Neonatal	Hypoketotic hypoglycemia, hepatomegaly, cardiac symptoms, muscle weakness	Muscle, heart, liver	Low plasma free carnitine with increased acylcamitines		Lipid storage in liver, kidney, muscle, heart	Dicarboxylic aciduria
VLCAD	Childhood Adult	Hypoketotic hypoglycemia, hepatocellular disease, hypertrophic cardiomyopathy, hypotonia Exercise-induced pain, myoglobinuria, +/- hypoglycemia	Muscle, heart, liver	Acyl-carnitine accumulation, CK↑		Lipid storage in multiple tissues	Dicarboxylic aciduria
LCAD		Hypotonia, muscle weakness, hypoketotic hypoglycemia				Lipid storage in type I fibres	Decreased total carnitine in muscle

MCAD		Lethargy, vomiting, coma, hypoglycemia, sudden death		carnitine↓. midchain acylcarnitines↑			Decreased total carnitine in muscle, dicarboxylic aciduria, urinary glycine conjugates
SCAD	Infantile Adult	Nonketotic hypoglycemia, failure to thrive, hypotonia, seizures Chronic myopathy	Muscle, liver	C4-carnitine↑		Lipid storage	Decreased total carnitine in muscle, increased excretion of urinary ethylmalonic and methylsuccinic acids

Figuur 2: Lipid metabolism; uptake and activation of FFA in skeletal muscle, transport of FFA through the mitochondrial membrane and β-oxidation.
With courtesy of Prof. M.W. King (first image) and Dr A. Pestronk (second image)





3) Mitochondrial myopathies

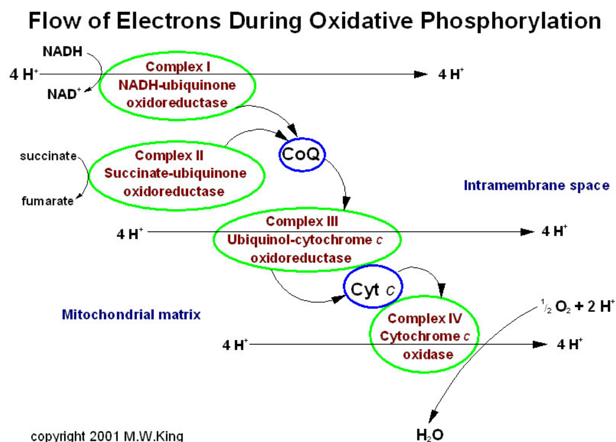
Mitochondria provide the energy required for all biosynthetic and motor activities through the processes of oxidative metabolism and energy transfer [2]. The function of mitochondria is critical to every organ system, but skeletal muscle and brain are often most affected by mitochondrial disorders. In Table 1 an overview of known human mitochondrial myopathies is listed. As shown mitochondrial disorders are mostly due to genetic mutations, and by finding the mutations the diagnosis is made. Other ways to come to a diagnosis are [18]: family history, physical examination, metabolic testing (blood: quantitative organic acids, amino acids, carnitine/acylcarnitine profile; urine: quantitative organic acids, amino acids; CSF: organic and amino acids), skeletal muscle biopsy with histology (with multiple stainings), electron microscopy and enzymology using mitochondria that were immediately isolated from fresh muscle.

Symptoms that are found in human mitochondrial myopathies are among many others exercise intolerance, muscle weakness and extraocular muscle dysfunction.

The most typical morphological change in mitochondrial diseases is the ragged red fiber, a dark appearing cell resulting of aggregates of abnormal mitochondria [2]. They are found primarily in type I and, to a lesser degree, in type IIa fibers.

Important enzymes in mitochondrial function are the “reductases” that take care of electron transport while generating ATP. The main ones are: Complex I (NADH:Q reductase), Complex II (Succinaat:Q reductase), Complex III (QH2: cytochrome c reductase) and Complex IV (cytochrome oxidase). Figure 3 shows the mitochondrial respiratory chain for oxidative phosphorylation.

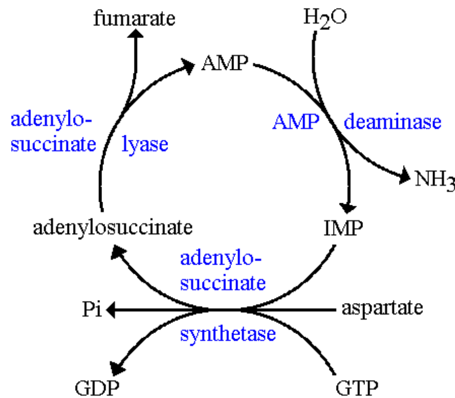
Figure 3: Mitochondrial respiratory chain for oxidative phosphorylation
With courtesy of Prof. M.W. King



4) Disordered purine metabolism

Myoadenylate deaminase (or muscle AMP deaminase) deficiency was reported as a new muscle disease in 1978 [19]. Myoadenylate deaminase catalyzes the irreversible deamination of AMP to IMP and so plays an important role in the purine nucleotide cycle (Figure 4)[2]. Symptoms of the deficiency are muscle weakness, decreased muscle mass, cramping after exercise, hypotonia and exertional myalgia. The deficiency is also reported in patients with other neuromuscular disorders, but in these cases a higher residual enzyme activity can be measured. The primary deficiency is autosomal recessive inherited. Diagnosis is based on the lack of an exertional increase in plasma ammonia in patients while lactate does rise. Diagnosis can be confirmed in muscle biopsy by histochemical stain, direct enzymatic assay or genotyping.

Figure 4: Purine Metabolism
With the courtesy of Dr. J.A. Illingworth



5) Primary disorders involving ion channels and electrolyte flux

An other name for these disorders is channelopathy [2]. The disorders are characterized by a disturbance in the excitation of muscle fiber membranes. The diseases are autosomal dominant inherited or the result of new mutations. Table 1 gives an overview of these myopathies. Symptoms vary between the disorders and Table 4 shows symptoms and diagnostics [2,20,21]. A combination of symptoms, electrolyte concentrations, electromyography, histology, response to treatment and special tests usually leads to the diagnosis. A special test is the caffeine/halothane test where a muscle bundle is exposed to an increasing concentration of caffeine and halothane. Caffeine and halothane act directly at the sarcoplasmatic reticulum to release calcium [22].

Table 4: The diagnosis of the human disorders of ion channels and electrolyte flux [2](Gutmann 2000)(Cannon 02)

Channel	Disorder	Age at onset	Clinical presentation	Bloodwork	EMG	Biopsy	Extra
Sodium	Hyperkal.PP	Infantile Childhood	Myotonia Episodic weakness	K↑, except during attacks CK usually↑	Myotonia in some patients		
	Param.Cong.		Myotonia that worsens with activity, face, neck, hands	K usually low during attacks CK may be↑	Cold- induced alterations		
	Normok.PP		Resembles Hyperkal. PP	K always normal			
Chloride	DMC	Birth till childhood	Generalized painless myotonia, disapp. when warm		Myotonia		
	RMC	Juvenile	Like DMC, but more severe, including weakness		Myotonia		
Calcium	Hypokal.PP	6-30 yr.	Episodes of painless paralysis, eyelid myotonia	K↓ during attacks	Mostly normal	Numerous vacuoles, mostly during attacks	
	Mal.Hyperth.	variable	Intense muscle rigidity, tachycardia, other arrhythmias, hyper- and hypotension, fever	CK↑	66% of patients: ↑polyphasic a.p., short-duration a.p., fibrillation	Usually normal	EM: mitochondrial changes Pos. caffeine halothane test
	Sarc. RCAD	childhood	Exercise-induced stiffness, cramps, difficulty relaxation	CK normal or mildly elevated,	Normal	Normal or type II atrophy	Diagnosis by biochemical/immunohistochemical analysis

6) Secondary (or acquired) metabolic myopathies
Myopathy can result from a variety of diseases involving the endocrine system, nutritional state, or electrolyte imbalance. This is easily understood because hormones play an important role in energy metabolism and electrolytes are crucial for the processes of nerve and muscle function. These myopathies are typically manifested by weakness, exercise intolerance resulting from fatigue and postexertional myalgias, cramps and stiffness. The onset is usually insidious. The myopathic changes will almost always disappear with successful treatment of the underlying disorder. However, some of these disorders can cause rhabdomyolysis [2]. In Table 1 an overview is given of these myopathies.

Overview of equine metabolic myopathies and the route to their diagnosis

Equine metabolic disorders have received increasing attention over the past decades [23]. Exertional rhabdomyolysis, literally the dissolution of muscle with exercise, is a common cause of disability in horses [24,25]. Numerous and conflicting causes for the condition have been proposed, including vitamin E and selenium deficiency, electrolyte imbalances, heat exhaustion, viral infection, transport and anesthesia [23,26,27]. Because in humans more than 50% of cases of recurrent rhabdomyolysis are caused by errors in skeletal muscle energy metabolism, a similar approach to diagnosis in equine medicine was undertaken [24]. Identified specific equine exertional myopathies are several variations of PSSM (polysaccharide storage myopathy), EPSM (Equine Polysaccharide Storage Myopathy) and RER (Recurrent Exertional Rhabdomyolysis), NADH CoQ reductase (Mitochondrial Complex I) deficiency, and a defect in skeletal muscle excitation-contraction couple resembling malignant hyperthermia [24]. Further equine metabolic myopathies that are described are HPP (hyperkalemic periodic paralysis), congenital myotonia, postanaesthetic myopathy, GBED (Glycogen Brancher Enzyme Deficiency) and MADD (Multiple Acyl-CoA Dehydrogenase Deficiency)[28].

The disorders will be described using the same order as in the human part, to make clear what the differences are in diagnostic knowledge.

1) Disorders of carbohydrate metabolism
Most disorders of the carbohydrate metabolism identified in the horse are caused by inherited deficiencies in glycogenolytic, glycolytic or lysosomal enzymes [14]. In 1985, various metabolic enzymes were measured by van den Hoven et al in normal horses and in 1988 in horses with neuromuscular problems [29,30]. In 1998, Valberg *et al.* demonstrated the first enzyme deficiency of the carbohydrate metabolism in horses with myopathic conditions [31].
Polysaccharide storage myopathy (PSSM) is a metabolic myopathy that results in the accumulation of high muscle-glycogen concentrations and an abnormal polysaccharide in

the type II fibers of skeletal muscle. [24]. It is a common cause of exertional rhabdomyolysis in Quarter Horses and Quarter Horse-related breeds such as the Paint Horse and Appaloosa [24,32], but also occurs in warmbloods [24], Morgan, Arabian, Welsh-cross ponies and standardbred related horses [33].

Diagnosis is based on:

- Clinical signs (sweating, pain, stiffness, anxiety, spasm, atrophy, reluctance to move, rarely recumbency or even death)[4,34].
- Serum activities of CK, LDH and AST (increased)[34]. Lactate concentration is normal, suggesting there is no enhanced anaerobic glycolysis as cause of the rhabdomyolysis [35]. The ability, however, to produce lactate and ammonia during near-maximal-exercise indicates that deficiencies in glycogenolytic and adenylate kinase enzymes are not probable causes of rhabdomyolysis in horses [35].
- Muscle biopsy with various stainings (HE, Gomori's trichrome, NADH, periodic acid-Schiff (PAS), acid phosphatase and ATP-ase)[24]. Pathognomic for PSSM are PAS positive, amylase resistant inclusions [33]. Biopsies are preferably taken from the semimembranosus or semitendinosus muscle, and longitudinal as well as transverse sections are to be used [36]. There is discussion whether aggregates of stored glycogen and chronic myopathic changes are also characteristic enough for the diagnosis [4].
- Enzymology in search of the glycogen storage diseases as known in human PSSM resembles GSD VII most, according to Valberg et al [31], but evidence of a complete enzyme deficiency has not been found yet [25,31].
- Exercise tests [24]

  During maximal exercise tests Quarter Horses utilise muscle glycogen and produce lactic acid, suggesting a functional glycolytic pathway [37]. De La Corte et al (1999) found that the glucose uptake is enhanced in Quarter Horses with PSSM [38], maybe as result of enhanced affinity to insulin. Lacombe et al tested that if after multiple strenuous exercises the glycogen stores were at least 55% depleted, anaerobic power generation and high intensity exercise capacity diminished. Intravenous glucose administration increased both again [39].
- Genetic testing

  A beginning has been made in genetic testing. Collinder et al (1997) found gene frequencies for several markers in the equine rhabdomyolysis syndrome (RHA). A rhabdomyolysis risk group could be characterized using four or five genetic marker loci [40].

Equine Polysaccharide Storage Myopathy (EPSM) is the term currently used for a disorder similar to PSSM described in Draft-breed horses. Although methods of diagnosis including histopathologic abnormalities of muscle biopsy samples are similar with PSSM, clinical signs and apparent incidence in the two breeds are different [41,42]. Possible clinical signs of

EPSM include: weakness, abnormal gait, muscle atrophy, exertional rhabdomyolysis, post-anesthetic myopathy, poor performance, spontaneous recumbency or sudden death [41].
Glycogen Brancher Enzyme Deficiency (GBED) was first reported in 1999 by Render et al [43]. He reported a storage disease in fetal and neonatal Quarter Horses with findings similar to those of glycogen storage disease type IV, suggesting a severe inherited loss of glycogen brancher enzyme (GBE) activity. In 2001 and 2003 additional cases were described in which the GBE activity was actually measured and found reduced [44,45].
Equine GBED is a simple autosomal recessively inherited, fatal muscle disease in Quarter Horse foals and American Paint horses. GBE deficiency results in the formation of long, unbranched α-1,4-linked chains of glucose with few α-1,6 branch points, resembling amylopectin. Symptoms are abortion, stillbirth, cardiac or respiratory arrest, recurrent convulsions, weakness at birth or sudden death on pasture. Diagnosis can be made with PAS staining of muscle biopsies showing big clumps of abnormal glycogen, reduced GBE activity can be measured in blood samples and some tissues, and DNA analysis can be performed showing a deficiency encoded by the GBE1 gene [43-47].

2) Disorders of lipid metabolism
Two of the three fundamental steps in lipid metabolism, uptake and activation of FFA in skeletal muscle and transport of FFA through the mitochondrial membrane have not led to known disorders in the horse yet. An explanation can be that, in contrast to human food, the basic equine diet is low in carnitine. In order to have a sufficient amount of carnitine it is synthesised by the animal itself in the liver, by a multistep biosynthetic pathway from lysine and S-adenosyl-methionine via deoxycarnitine to carnitine. No difference was measured in plasma free carnitine and acylcarnitine between healthy horses and horses predisposed to exertional rhabdomyolysis [48]. Scholte et al [49] have determined the activity of the enzymes active in the lipid metabolism. No difference was found between healthy horses and horses predisposed to exertional rhabdomyolysis. So no myopathic carnitine deficiency is found in horses yet. The concentration of carnitine does rise in the older and more trained horse, but this is because the mitochondrial density rises in these situations [50].
With regard to the β-oxidation a multiple acyl-CoA dehydrogenase deficiency (MADD) has recently been reported by Westermann *et al* [28]. Diagnosis was performed with blood biochemistry, gas chromatography-mass spectrometry (GC/MS) profiling, electrospray tandem mass spectrometry and measurement of muscle enzyme activities.

3) Mitochondrial myopathies
There are relatively few studies of potential mitochondrial myopathies in horses [51]. Scholte et al (1991) have determined the activity of complex I, II, III and IV of the mitochondrial respiratory chain (figure 3)[49]. No difference was found between healthy horses and horses predisposed to exertional rhabdomyolysis. The only disorder found was a deficiency in

complex I: NADH Co Q reductase [51,52]. Diagnosis was made by the signs (exercise intolerance, lactic acidosis because of failure of oxidative mechanism and shift to anaerobic system), oxygen consumption test (inadequate use of O_2), muscle biopsy with normal and electron microscopic histology (ragged red fiber appearance). Enzymology, measuring all enzymes involved, was used for confirmation of the diagnosis [52].
In human beings definite diagnosis is possible by mutational analysis of mitochondrial or genomic DNA. Maybe in the future this will also be applicable to horses, since the complete equine mitochondrial DNA sequence is available now [46,53].

4) Disordered purine metabolism
Disorders in purine metabolism have not been identified in equine medicine yet.

5) Primary disorders involving ion channels and electrolyte flux
More is known about the equine myopathies due to primary disorders involving ion channels and electrolyte flux. The first horse with hyperkalemic periodic paralysis was described in 1985 [51], malignant hyperthermia was mentioned in a case report in 1975 [51], post anaesthetic myopathy is known as long as there are operations performed on horses, and amongst others Jamison et al described a case of congenital myotonia [54]. Recurrent exertional rhabdomyolysis in the Thoroughbred population can also be included in this group, because the pathogenesis is most likely a disruption in the calcium regulation [55].
Hyperkalemic periodic paralysis, a muscular disorder, predominantly confined to Quarter Horses, was first reported in 1985. It apparently emerged as a naturally occurring genetic mutation in the stallion Impressive, and has been passed on by selective breeding.
Diagnosis can be made by [51,56]:

- Clinical signs: brief myotonia, muscle fasciculations and spasms and sometimes prolaps of the nictitating membrane, along with a sardonic grin, pharyngeal and laryngeal dysfunction [57]. If more severe: swaying, sweating, staggering, dog-sitting or even collapse into involuntary recumbency. There is fear, but no pain. Horses with this problem are often well-muscled and may have no apparent abnormalities between attacks of weakness. Various stimuli such as transport and other types of stress may precipitate clinical onset of the problem. Episodes last for variable periods, but most resolve in 15-60 minutes. Some horses may die during attacks [58].
- Laboratory evaluation: Sometimes the potassium concentration is elevated and the sodium concentration decreased during an attack. The oral potassium chloride challenge test can induce hyperkalemia and clinical signs in “carrier horses”. However, because of the detrimental effects of hyperkalemia on the heart, provoking clinical signs with this protocol is not without risks and is not longer routinely performed [46,59,60]. Bain et al (1990) mention that the potassium concentration measured extracellularly often differs from intracellular values [61]. Especially in myopathies

correct measurement is very important. Measuring intra-erythrocyte-potassium is a good option [62].

- EMG: findings are myotonic discharges [63]. Affected horses can also show spontaneous discharges, including fibrillation potentials and positive waves [64].
- ECG: various changes indicative of hyperkalemia can be found during an attack. The ECG reveals smaller, wider P waves, increased amplitude of T waves and widening of QRS complexes. Between episodes the ECG is normal [39 56].
- Histological pictures vary from normal to slight degeneration to vacuoles in type IIb fibres. EM shows distension and proliferation of sarcoplasmatic reticulum [65].
- DNA test: the most sensitive and specific test for HPP, only requiring blood or hair. DNA testing can differentiate if a horse is heterozygous or homozygous for HPP by using a polymerase chain reaction-restriction fragment length polymorphism-test the abnormal gene. The entity is caused by one gene mutation (a Phe-Leu mutation in a subunit of the sodium channel) in 30.000 genes and is inherited in a dominant autosomal trait. Most affected horses are heterozygote [46,59,60].

Myotonia was first described by Jamison in 1987. A congenital form was found in a Quarter Horse colt. No abnormalities were found in blood analysis. Post-mortem examination showed a mixed metabolic/dystrophic myopathy [54]. Montagna (2001) also described a horse (5 years old) with a mixture of muscular dystrophy and myotonia [66].
Myotonia, rare in the horse, is characterized by sustained contraction and difficulty in relaxation of muscle following stimulation [58]. The biochemical defects responsible for the condition are not known explicitly, but seem to be inherent within the muscle rather than the nervous system, maybe involving the chloride channels. An inherited basis for this condition in the horse is suspected, based on familial incidence; however this has not been proven. Myotonia in horses is usually detected in the first year of life.
Symptoms are mild hind end stiffness, gait abnormalities, bulging of thigh and rump muscles and contraction (muscle dimpling) of affected muscles after being stimulated by percussion for up to a minute [67]. The symptoms get worse following a period of rest [46]. There is no significant and sustained elevation in CK and AST activities. Elevations are only seen especially after stress due to transportation or intramuscular injections [46]. Definitive diagnosis requires electromyography (EMG), revealing fibrillation potentials, positive sharp waves and myotonic discharges [68]. Histologic examination will likely be normal or show mild myopathic changes including excessive fiber size variation and internally located nuclei. The latter is the main differential with myotonic dystrophy which is characterized by severe muscle histopathological changes [46].
Malignant hyperthermia (MH) is a syndrome seen amongst others in horses [58]. It is clinically characterized by the rapid onset of elevated body temperature and skeletal muscle rigidity following the administration of anesthetics and/or muscle relaxants. Other clinical

signs are tachypnea, tachycardia, dysrhythmias, acidosis, myoglobinuria, elevated muscle enzyme activities and blood lactate concentration and even death with acute rigor mortis. Mutations in the ryanodine receptor 1 (RyR1) gene cause the dysfunction of the RyR1, also known as the calcium release channel of the sarcoplasmic reticulum in the skeletal muscle [69].

There does not seem to be any predisposition to sex, age, weight, or condition for this problem. There may be a familial history of tying up in these patients. Clinical pathology shows an elevation in muscle enzymes and blood lactate [70]. The generalized form of post-anesthetic myositis (muscle inflammation and necrosis) similar to severe tying-up may be caused by a condition similar to MH.

Recurrent exertional rhabdomyolysis [71] affects approximately 5% of the Thoroughbred population, is most common in young fillies and is associated with various forms of stress. Symptoms range from slight stiffness to immobility and may include anxiety, sweating and contracture of major muscle groups. [46,72].

Lentz et al (1999) [22,73] have done tests on muscle contraction in Thoroughbreds with recurrent exertional rhabdomyolisis [71] showing that the regulation of muscle contraction in Thoroughbreds with RER is abnormal, probably due to abnormal intracellular calcium regulation, resembling malignant hyperthermia. Threshold for contraction induced by potassium depolarisation was lower compared with normal muscles and thresholds for contracture induced by caffeine and halothane was also lower [22,73]. However, despite the similarities between RER and malignant hyperthermia, RER does not resemble the SR ryanodine receptor defect responsible for MH, so RER in thoroughbreds may represent a ditinct neuromuscular disorder involving calcium regulation [72]. Diagnosis is based on a more than 250% rise in plasma CK activity 4 hours post exercise compared to the pre-exercise measurement [46], muscle biopsies (H&E staining, non specific, centrally located nuclei in mature muscle fibers rather than nuclei underneath the cell membrane, mostly in type II fibers). Contracture testing of muscle and calcium fluorescence testing of cultured muscle can only be performed in highly specialized laboratoria [32,74]

6) Secondary (or acquired) metabolic myopathies

There is a group of secondary or acquired metabolic myopathies with a wide variation of diseases resulting in myopathies which will not be discussed here.

Conclusion

Currently it is clear that many more metabolic diseases are known in the human than the equine field. However, since the horse has sports as its main use, the value of diagnosing

muscle disorders is enormous. This is the reason that much research is focussing on equine metabolic muscle disorders. Human diagnostic tools are of importance in this research. At this moment electrophysiological and imaging studies are gaining terrain quickly. Muscle biopsy has become a well known additional route of diagnosis, but should be used more often in practice as a standard procedure when muscle problems arise. The use of gas chromatography-mass spectrometry (GC/MS) profiling, electrospray
tandem mass spectrometry and measurement of muscle enzyme activities will bring out more diagnoses of equine metabolic myopathies in the near future. A promising future will also be in the DNA research of mutational analysis of mitochondrial or genomic DNA.
The next challenge will be to try to find a cure for some metabolic myopathies. At this stage the horse might become interesting for man as a model animal.

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Chapter 3

Equine biochemical Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) as a cause of rhabdomyolysis

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Abstract

Two horses (a 7-year-old Groninger warmblood gelding and a six-month-old Trakehner mare) with pathologically confirmed rhabdomyolysis were diagnosed as suffering from multiple acyl-CoA dehydrogenase deficiency (MADD). This disorder has not been recognised in animals before. Clinical signs of both horses were a stiff, insecure gait, myoglobinuria, and finally recumbency. Urine, plasma, and muscle tissues were investigated. Analysis of plasma showed hyperglycemia, lactic acidemia, increased activity of muscle enzymes (ASAT, LDH, CK), and impaired kidney function (increased urea and creatinine). The most remarkable findings of organic acids in urine of both horses were increased lactic acid, ethylmalonic acid (EMA), 2-methylsuccinic acid, butyrylglycine (iso)valerylglycine, and hexanoylglycine. EMA was also increased in plasma of both animals. Furthermore, the profile of acylcarnitines in plasma from both animals showed a substantial elevation of C4-, C5-, C6-, C8-, and C5-DC-carnitine. Concentrations of acylcarnitines in urine of both animals revealed increased excretions of C2-, C3-, C4-, C5-, C6-, C5-OH-, C8-, C10:1-, C10-, and C5-DC-carnitine. In addition, concentrations of free carnitine were also increased. Quantitative biochemical measurement of enzyme activities in muscle tissue showed deficiencies of short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and isovaleryl-CoA dehydrogenase (IVD) also indicating MADD. Histology revealed extensive rhabdomyolysis with microvesicular lipidosis predominantly in type 1 muscle fibers and mitochondrial damage. However, the ETF and ETF-QO activities were within normal limits indicating the metabolic disorder to be acquired rather than inherited. To our knowledge, these are the first cases of biochemical MADD reported in equine medicine.

Abbreviations used: SCHAD, short-chain hydroxy acyl-CoA dehydrogenase MADD, multiple acyl-CoA dehydrogenase deficiency; SCAD, short-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; ETF-α, electron transfer flavoprotein-α; ETF-β, electron transfer flavoprotein-β; ETF-QO, electron transfer flavoprotein ubiquinone oxidoreductase; ETFDH, electron transfer flavoprotein dehydrogenase; GA-II, glutaric acidemia type II; NADH CoQ, nicotinamide adenine dinucleotide coenzyme Q; FAD, flavin adenine dinucleotide; ASAT, aspartate aminotransferase; CK, creatinine phosphokinase; LDH, lactate dehydrogenase.

Keywords

Horse; Rhabdomyolysis; Myopathy; MADD; ETF; ETF-QO

Introduction

Muscle disorders are a common cause of suboptimal performance or even disability to perform. In comparison to human medicine, the etiology of muscle disorders in equine medicine is less explored. In addition to some glycogen storage diseases [1-7] an equine mitochondrial myopathy, NADH CoQ reductase deficiency [8], several metabolic myopathies due to primary disorders involving ion channels and electrolyte flux and some secondary or acquired metabolic myopathies [9,10] have been observed.

Multiple acyl-CoA dehydrogenase deficiency (MADD) (also known as glutaric acidemia type II (GA-II)) (McKusick 231680) is a severe inborn error of metabolism, which can lead to early death in human patients. This autosomal recessive disease, first reported in 1976 by Przyrembel et al. is associated with a deficiency of several mitochondrial dehydrogenases that utilize flavin adenine dinucleotide (FAD) as cofactor [11,12]. These include the acyl-CoA dehydrogenases of fatty acid β-oxidation and enzymes that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid, and sarcosine (a precursor of glycine). During these dehydrogenation reactions, reduced FAD donates its electrons to the oxidized form of electron transfer flavoprotein (ETF), then to ETF-ubiquinone oxidoreductase [ETF-QO, also known as ETF dehydrogenase (ETFDH)] and finally to the respiratory chain in order to produce ATP. The reduced form of ETF is recycled to oxidized ETF by the action of ETF-QO. Since electrons from FAD are transferred to ETF, deficiency of ETF or ETF-QO results in decreased activity of many FAD-dependent dehydrogenases and the combined metabolic derangements similar to those observed in MADD [13]. Heterogeneous clinical syndromes of human ETF- and ETF-QO deficiency have been described. These clinical features fall into three classes: a neonatal-onset form with congenital anomalies (type I), a neonatal-onset form without congenital anomalies (type II), and a late-onset form (type III). The latter form is also called ‘ethylmalonic-adipic aciduria’ or ‘late-onset glutaric aciduria type II’ [14,15]. The neonatal-onset forms are usually fatal and are characterized by severe nonketotic hypoglycemia, metabolic acidosis, multisystem involvement, and excretion of large amounts of fatty acid- and amino acid-derived metabolites. Symptoms and age at presentation of late-onset MADD are highly variable and characterized by recurrent episodes of lethargy, vomiting, hypoketotic hypoglycemia, strong ‘sweaty feet’ odour, hyperammonemia, metabolic acidosis, and hepatomegaly often preceded by metabolic stress [14,16,17]. Muscle involvement in the form of pain, weakness, and lipid storage myopathy also occurs. The organic aciduria is less clear in the milder or episodic forms of the disease. Some only manifest increased excretions of EMA and adipic acid [18]. In others, abnormal organic acid profiles are only found during periods of illness or catabolic stress.

It has been shown that riboflavin treatment and therefore elevation of FAD may alleviate the enzymatic and biochemical phenotype as well as the clinical symptoms in late-onset riboflavin-responsive MADD [19-22].
Diagnosis of human MADD is based on medical and family history, clinical examination, a characteristic organic aciduria [11,23], histopathologic abnormalities (increased lipid deposition in myofibers) as well as enzymatic and molecular characterization [16].
Several experiments have been carried out in order to obtain mice or rats with MADD like diseases. Although riboflavin-deficient rats mimicking the human disorder of MADD have been described, there are no reports of acquired MADD yet [24]. White et al. have mapped the genes for the mouse ETF-α, ETF-β, and ETFDH, determining localization of these mouse genes to chromosomes 3, 7, and 13. However, there are no mutations that might be considered as a model of human MADD [25]. To the authors' knowledge, MADD is diagnosed in no other species than man so far.
The present study describes two horses with rhabdomyolysis due to MADD. As a consequence, this animal model might be an option for further comparative research with special reference to riboflavin-responsive MADD.

Materials and methods

Case reports

Case 1 (gelding)
A seven-year-old Groninger warmblood gelding was presented at the Utrecht University Equine Clinic with a history of moderate pain following exercise. An episode of myopathy had been reported previously. Upon arrival at the clinic the horse was recumbent while shaking and sweating. There was a willingness to eat, but this caused trembling and sweating too. The horse walked straddle-legged and insecure. Further symptoms were depression and preference for lateral recumbency. Shaking and sweating developed after every minor physical activity. Myoglobinuria was also observed. Clinical examination including neurological examination and rectal exploration revealed no further abnormalities. The next day the horse was able to stand up reluctantly. However, one day later recumbency became permanent. Because of the poor prognosis the horse was euthanized at the owner's request.
Case 2 (foal)
A six-month-old Trakehner mare was sent to the Utrecht University Equine Clinic with a suspicion of colic. On arrival, the foal had a stiff gait and extremely firm gluteal, quadriceps, longissimus, and triceps muscles. She became recumbent shortly after arrival. Rectal temperature was 36.0 °C and the heart rate elevated to 56 beats per minute. The foal

was dehydrated. No abnormalities were found in the digestive and neurological systems. Myoglobinuria was also observed. A tentative diagnosis of rhabdomyolysis was made. After a small improvement in the evening the condition of the foal deteriorated during the following hours. Due to the poor prognosis the horse was euthanized at the owner's request too.

Blood biochemistry

Biochemical analysis of blood from the patients was performed using a CBC Analyzer from Sysmex Inc. (white blood cell count), a Synchron CX5 from Beckman Coulter Inc (concentrations of urea, creatinine and glucose as well as activities of ASAT, CK, LDH), and an ABL-605 Radiometer from Radiometer Copenhagen (pH, pCO_2, BE, , and lactate). Results were compared with the laboratory's validated reference values for horses.

Analysis of organic acids and acylglycines

Identification-analyses of organic acids and glycine conjugates were carried out by gas chromatography–mass spectrometry (GC–MS) on a Hewlett Packard 5890 series II gas chromatograph linked to a HP 5989B MS-engine mass spectrometer. Prior to this GC–MS analysis, the organic acids and glycine conjugates were trimethylsilylated with *N*,*N*-bis(trimethylsilyl)trifluoracetamide/pyridine/trimethylchlorosilane (5:1:0.05 v/v/v) at 60 °C for 30 min. The gas chromatographic separation was performed on a 25 m × 0.25 mm capillary CP Sil 19CB column (film thickness 0.19 mm) from Arian/Chrompack, Middelburg, The Netherlands. The results were compared with values from 12 healthy control horses.

Analysis of free and acylcarnitines

Free and acylcarnitines in plasma and urine were analyzed as their butyl ester derivatives by electrospray tandem mass spectrometry (ESI-MS–MS) on a Micromass Quattro Ultima system equipped with an Alliance HPLC system. Results were compared with values from 12 healthy control horses. Acylcarnitine concentrations in muscle tissue were measured as described previously [26].

Muscle enzyme activities

Measurement of muscle enzyme activities was performed in lateral vastus muscle tissue of the patients, collected immediately after euthanasia in liquid nitrogen and stored at −80 °C. Lateral vastus muscle tissue of two healthy control horses was used for control measurements. The activities of medium-chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD) and isovaleryl-CoA dehydrogenase (IVD) were measured according to methods, which are based on the use of the substrate phenylpropionyl-CoA (for MCAD), butyryl-CoA (for SCAD) and isovaleryl-CoA (for

IVD). Shortly, incubations were performed at 25 °C in a buffered medium, containing an aliquot of the muscle homogenate plus ferricenium hexafluorophosphate as the electron acceptor. After termination of the reactions by acidification, the acidified samples were centrifuged and the protein-free supernatants neutralized, followed by HPLC-analysis to separate the different acyl-CoA esters. The activity of short-chain 3-hydroxy-acyl-CoA dehydrogenase (SCHAD) was measured as described previously [27].

Assessment of muscle ETF(-QO) activities

ETF activity was measured with reduction of the artificial electron acceptor (dichlorophenol indophenol) using octanoyl-CoA as substrate and purified MCAD on the 100,000 g supernatant of sonicated muscle tissue according to a previously published protocol [28].

ETF-QO activity was revealed from homogenized muscle membranes. ETF-QO activity was measured in the reverse reaction, using succinate as substrate under anaerobic conditions at pH 8.6. Succinate dehydrogenase generates electrons which are transferred to purified oxidized ETF through Coenzyme Q and ETF-QO. Reduction of ETF was followed spectro-fluorimetrically. SCHAD was used as a control enzyme and results were compared with data from 3 healthy control horses.

Pathology

A post-mortem examination of both animals was performed macroscopically, microscopically and by electron micrography. Routine microscopical analysis of various muscles was performed using haematoxylin and eosin (H&E) stained paraffin embedded sections. In order to identify lipid droplets formaldehyde fixed specimens of *M. vastus lateralis* of the gelding were snap frozen in liquid nitrogen and cut on a Leica CM3050 microtome. Thin cryosections (10 µm) were attached to Superfrost Plus slides and incubated in 0.02 µg/µl Bodipy 493/503 (Molecular Probes, Invitrogen, Breda, The Netherlands) for 15 min in a humidified environment in order to stain neutral lipids. Lipid droplets were visualised by a Leica DMR fluorescence microscope equipped with a Photometrics Coolsnap CCD digital photo camera. Images were processed using IP-laboratory image analyses software. For muscle fiber typing a monoclonal antibody was used specific for type 1 MyHC isoform kindly provided by prof. A.F.M. Moorman, Academic Medical Centre, Amsterdam, The Netherlands.

Table 1. Biochemical parameters in plasma of gelding and foal

	Reference range	**Unit**	**Gelding**					**Foal**		
			Day 1	**Day 2**	**Day 2**	**Day 3**	**Day 3**	**Day 1**	**Day 1**	**Day 2**
				08.00	**20.00**	**08.00**	**14.00**	**20.00**	**22.00**	**08.00**
pH	7.35–7.45		7.35	7.42	7.26	7.44	7.35	7.12	7.33	7.22
pCO_2	4.7–6.0	kPa	6.1	6.3	6.7	6.3	6.1	6.8	7.0	5.3
BE	−3 to +3	mmol/l	−0.4	5.8	−5.0	6.2	6.1	−13.9	0.3	−11.0
Lactate	<1.0	mmol/l	3.5	3.8	12.4		3.3	15.9	7.8	10.6
WBC	7–10	G/l		6.2		7.3		7.2	5.2	4.1
ASAT	125–275	U/l	6,800			17,500		19,100		
CK	<200	U/l	180,000			237,000	240,000	100,000		
LDH	150–420	U/l	18,000					30,800		
Urea	<8	mmol/l		9.2		15.1			10.9	15.0
Creatinine	106–168	µmol/l		227					189	219
Glucose	3.9–5.6	mmol/l	13.1	6.6	9.9	8.1			12.5	

Results

Blood biochemistry

Table 1 shows the biochemical parameters in plasma of the gelding and foal. The first day of hospitalisation revealed hyperglycemia (13.1 and 12.5 mmol/l for gelding and foal, respectively, reference range 3.9–5.6), high lactic acidemia (3.5 and 15.9 mmol/l, respectively, reference <1.0) and very high activities of CK (180,000 and 100,000 U/l, respectively, reference range 200), ASAT (6800 and 19,100 U/l, respectively, reference ranges 125–275), and LDH (18,000 and 30,800 U/l, respectively, reference ranges 150–420 U/l). On the first day of hospitalisation for the foal and on the second day of hospitalisation for the gelding, a period of metabolic acidosis occurred (gelding, pH 7.26, BE −5.0 mmol/l, lactate 12.4 mmol/l and foal, pH 7.12, BE −13.9 mmol/l, lactate 15.9 mmol/l).

Table 2. Organic acids and glycine conjugates in urine

Acid or conjugate	**mmol/mol creatinine**		
	Gelding	**Foal**	**Upper limit of reference range (*n* = 12)**
Lactic acid	180	20,606	141
Pyruvic acid	40	483	10
3-OH-Butyric acid	17	392	133
3-OH-Isobutyric acid	13	294	138
Ethylmalonic acid	106	278	5
2-Methylsuccinic acid	47	114	5
Succinic acid	n.d.	5	12
Adipic acid	n.d.	13	4
Glutaric acid	n.d.	10	136
Butyrylglycine	++++++	++	n.d.
(iso)Valerylglycine	+	+	n.d.
Hexanoylglycine	++	+	n.d.

n.d.: not detectable.

Analysis of organic acids, acylglycines and free and acylcarnitines

Metabolic screening of plasma and urine obtained several hours before euthanasia was performed. The most remarkable findings of organic acids in urine of gelding and foal were increased ethylmalonic acid (EMA), 2-methylsuccinic acid, lactic acid, butyrylglycine (iso)valerylglycine and hexanoylglycine (Table 2). Surprisingly, the concentration of glutaric acid in urine was normal. EMA was also increased in plasma of both animals (Table 3). Furthermore, the profile of acylcarnitines in plasma from both animals showed a substantial elevation of C4-, C5-, C6-, C8-, and C5-DC-carnitine (Table 4). Concentrations of acylcarnitines in urine of both animals revealed increased excretions of C2-, C3-, C4-, C5-, C6-, C5-OH-, C8-, C10:1-, C10-, and C5-DC-carnitine. In addition, concentrations of free carnitine were also increased.

Table 3.
Organic acids in plasma

Acid	**Gelding (µmol/l)**	**Foal (µmol/l)**	**Upper limit of reference range (n = 12) (µmol/l)**
3-OH-Butyric acid	534	176	391
3-OH-Isobutyric acid	47	72	111
Glutaric acid	13	30	3
cis-4-Decenoic acid	6	20	5
3-Oxobutyric acid	10	81	13
Decanoic acid	5	82	10
Ethylmalonic acid (EMA)	38	54	1

Muscle enzyme activities
In muscle tissue of the gelding, deficiencies of three acyl-CoA dehydrogenases, namely SCAD MCAD and IVD were found. Measurement of SCHAD as a control enzyme was within normal limits. In muscle tissue of the foal, deficiencies of SCAD and MCAD were found. The activity of IVD in the foal was within normal limits (Table 5).

Acylcarnitine profiling in muscle
The profile of acylcarnitines in muscle tissue of the gelding showed a substantial elevation in C4-, C5-, C6-, C8-, C10:1-, C4-DC-, and C5-DC-carnitine. Results for the foal were comparable, with even higher values for C4-, C5-, C8-, and C10:1-carnitine. In addition the

free carnitine concentration in the foal muscle was remarkably reduced. Values are shown in Table 4.

Table 4.
Relevant acylcarnitines in urine, plasma, and muscle tissue

	Urine (mmol/mol creatinine)			**Plasma (µmol/l)**			**Muscle tissue (pmol/mg protein)**			
	Gelding	**Foal**	**Upper limit of reference range (*n* = 12)**	**Gelding**	**Foal**	**Upper limit of reference range (*n* = 12)**	**Gelding**	**Foal**	**Results of healthy controls (*n* = 2)**	
Free carnitine	753	298	8	322	53	41	563	30	483	686
C2-Carnitine	458	316	1	116	12	5	1769	247	871	333
C3-Carnitine	24	30	0	4	4	1	18	101	32	9
C4-Carnitine	325	15	1	63	57	1	954	5469	112	9
C5-Carnitine	290	46	0	55	61	0	1332	9068	65	14
C6-Carnitine	40	19	0	11	9	0	126	171	11	1
C5-OH-Carnitine	2	4	0	1	1	0	20	6	2	4
C8-Carnitine	12	10	0	3	3	0	19	51	4	1
C10:1-Carnitine	2	5	0	1	2	0	10	28	1	0
C10-Carnitine	1	3	0	1	1	0	4	17	5	2
C4-DC-Carnitine	0	1	0	0	0	0	26	8	2	1
C5-DC-Carnitine	9	11	1	2	1	0	1	1	0	0

Assessment of muscle ETF(-QO) activities

Measurements of ETF and ETF-QO activities as well as the activity of the control enzyme (SCHAD) in muscle biopsy of the gelding were slightly decreased compared to controls. ETF and ETF-QO activities in muscle biopsy of the foal were in the control range. In both foal and gelding the ratios (ETF/SCHAD and ETF-QO/SCHAD) were normal (Table 6).

Table 5.
Enzyme activities of SCAD, MCAD, IVD, and SCHAD (control enzyme) in muscle

(nmol/min/mg protein)	Gelding	Foal	Healthy control 1	Healthy control 2
Acyl-CoA dehydrogenase				
SCAD	0.24	0.32	2.27	1.72
MCAD	0.21	0.40	5.54	3.78
IVD	0.41	1.21	1.61	1.36
3-Hydroxy acyl-CoA dehydrogenase				
SCHAD	249	270	294	295

Table 6.
ETF, ETF-QO, and SCHAD activities (nmol/min/mg protein) in muscle

	Gelding	Foal	Healthy control 1	Healthy control 2	Healthy control 3
ETF	0.42	0.62	0.62	0.69	0.98
SCHAD	509	899	706	751	961
Activity ratio ETF/SCHAD (1000×)	0.83	0.69	0.88	0.92	1.02
ETF-QO	0.076	0.188	0.185	0.202	0.188
SCHAD	30	183	143	203	164
Activity ratio ETF-QO/SCHAD (1000×)	2.55	1.03	1.22	0.99	1.15

Pathology

In the Groninger gelding as well as in the foal pathologic examination confirmed an acute rhabdomyolysis with pale, degenerated looking musculature in various muscles. Microscopically, in haematoxylin and eosin (H&E) stained paraffin sections, muscle fibers with loss of striations, floccular degeneration and myolysis were found. Some fibrotic areas were visible as were slight infiltrations with macrophages and neutrophils (Fig. 1 and Fig. 2).

Electron micrography showed subsarcolemmal accumulation of mitochondria and severe loss of mitochondrial cristae and numerous extensively damaged mitochondria (Fig. 3). Fluorescence microscopy showed microvesicular lipidosis predominantly in type 1 fibers (Fig. 4).

Fig. 1. Vastus lateralis muscle fibers from a seven-year-old Groninger warmblood gelding showing loss of striations, floccular degeneration and myolysis as well as slight infiltration with macrophages. At the lower section normal striated fiber (H&E stained paraffin section, objective 10times).

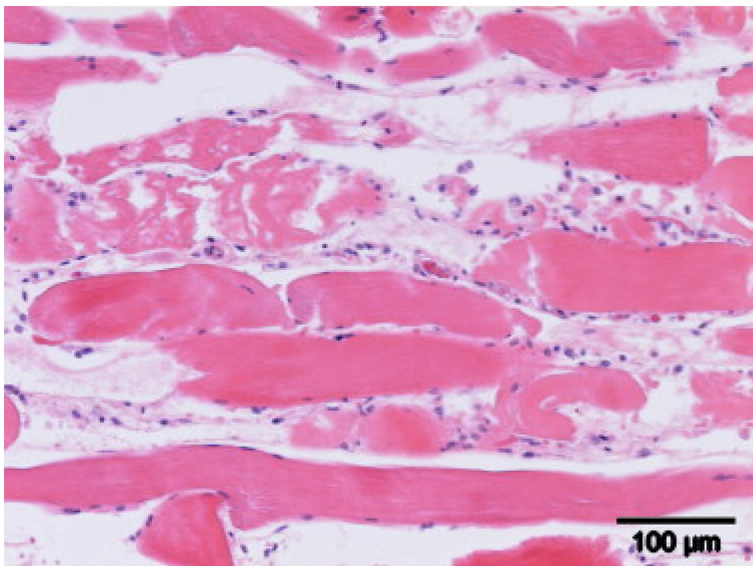


Fig. 2. Detail of Fig. 1 (H&E stained paraffin section, objective 20 times).

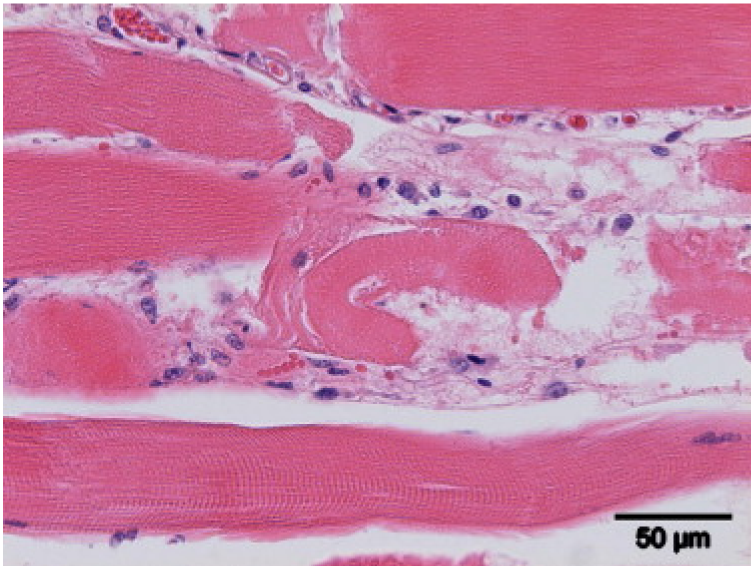


Fig. 3. Electron micrograph of lateral vastus muscle from a seven-year-old Groninger warmblood gelding illustrating severe mitochondrial damage.

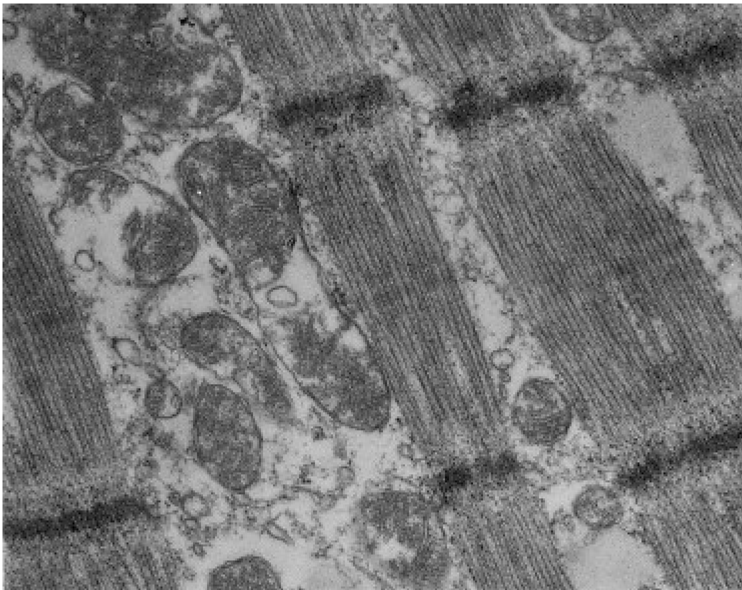
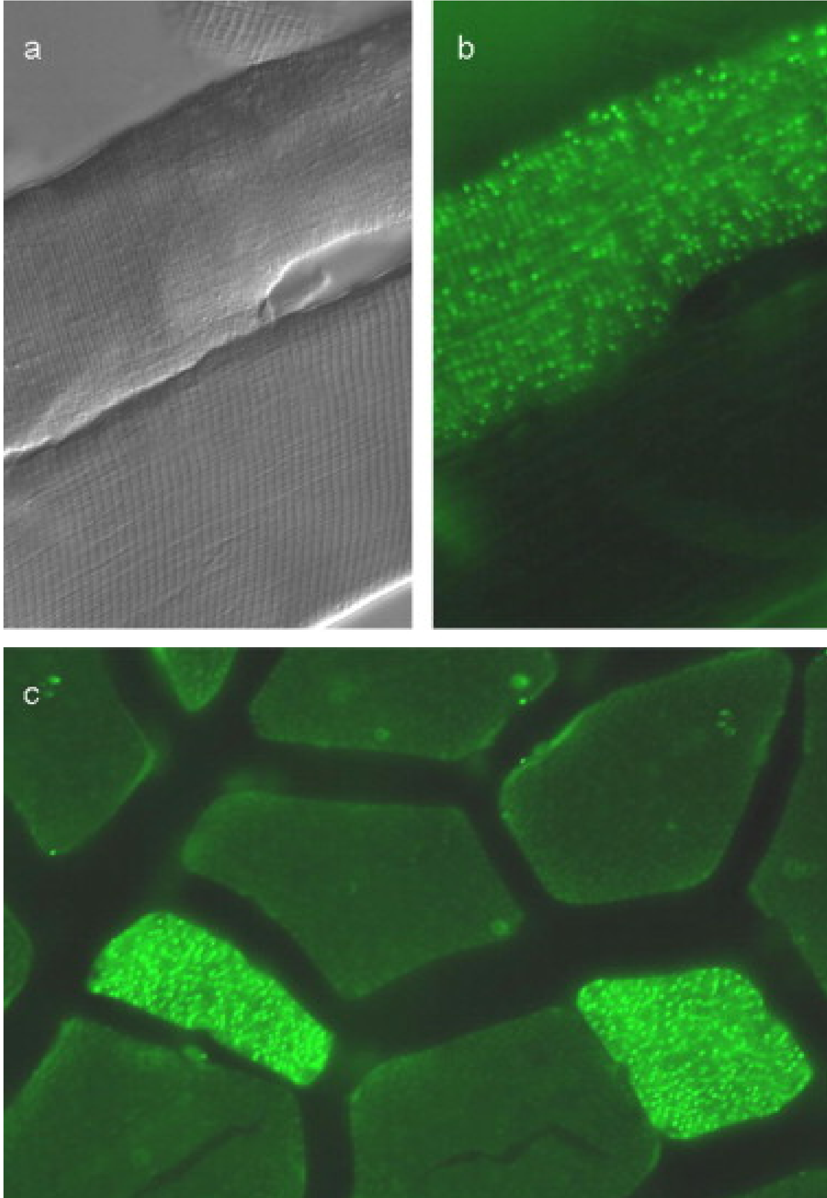


Fig. 4. Neutral lipid staining of lateral vastus muscle from a seven-year-old Groninger warmblood gelding showing microvesicular lipidosis in type 1 fibers longitudinally (b), the corresponding differential interference contrast microscopy image (a), and microvesicular lipidosis in type 1 fibers cross-sectionally (c).



Discussion

This report describes for the first time biochemical MADD in equine medicine as a cause of pathologically confirmed rhabdomyolysis. The diagnosis is based on characteristic profiles of organic acids and acylcarnitines in urine and plasma. In urine, EMA and methylsuccinic acid, as well as the glycine conjugates of (iso)valerate, butyrate and hexanoate were increased as observed in human MADD patients. In contrast, 2-methylbutyric acid was not found in the urine of the horses while glutaric acid excretions were not elevated. In horses glutaric acid appears to be a normal constituent in urine in contrast to man (Table 2). We have no clear explanation for the observation that the equine patients had elevated concentrations of glutarate in plasma, but not in urine.
Acylcarnitine analyses in muscle showed increased concentrations of the short- and mid-chain carnitine esters. The strongly reduced enzyme activities of SCAD, MCAD, and IVD found in muscle tissue of the gelding supported the diagnosis MADD. The muscle tissue of the foal showed reduced enzyme activities of SCAD and MCAD while IVD appeared to be normal. This may be due to biochemical heterogeneity of the disease. This phenomenon of reduced acyl-CoA dehydrogenase activities has been described before in rats on a riboflavine-deficient diet [29].
The decreased enzyme activities suggest a defect in the electron transfer flavoprotein (ETF) system or the riboflavin synthesis system. Surprisingly, ETF and ETF-QO activities in muscle of the gelding were slightly decreased while these activities were normal in muscle of the foal. The ratios (ETF/SCHAD and ETF-QO/SCHAD) were normal for both gelding and foal, thereby excluding an (inherited) deficiency of either ETF or ETFDH. We therefore speculate that the biochemical MADD in the two horses may be caused by an exogenous factor e.g. a riboflavin deficiency or blocking, predominantly affecting SCAD, MCAD, and IVD.

When comparing clinical symptoms of both horses with those described for human MADD, the horses must have suffered from the ‘late-onset’ form. Similar symptoms are weakness and myopathy, though in the horses the disorder seems to be more acute and severe. Pathological investigations revealed lipid accumulation in muscle fibers and damaged mitochondria /ragged red fibers in the affected horses similar to observations in humans. Although only muscle tissue was collected immediately post mortem and available for fluorescence microscopy in order to study the presence of lipid droplets, it cannot be excluded that other organs had similar fatty changes similar to human patients. In contrast to findings in man where rhabdomyolysis is very unusual in late-onset MADD, skeletal muscle seems to be the main target organ of MADD in the equine species. However, it cannot be excluded that the enzyme deficiencies and the acute myopathy in the horses have a common cause.

Interestingly, the horses were hyperglycemic. This is in contrast with the observed hypoglycemia seen in human cases with MADD [16]. Hypoglycemia may occur as a result of increased utilization of glucose because of the block in fatty acid oxidation and the subsequent inability to synthesize ketone bodies. In addition, hepatic gluconeogenesis may be impaired. However, hypoketotic hypoglycaemia in man can also be absent. This may reflect stimulation of gluconeogenesis and ketogenesis by unimpaired oxidation of long-chain and medium-chain fatty acylCoA's [30]. We hypothesize that β-adrenergic mechanisms in stressed horses results in hyperglycemia [31]. Furthermore, ketogenesis is a very unlikely metabolic pathway in the equine species [32]. Also at odds with human MADD patients is the elevated free carnitine in the horse, which remains unexplained.
In humans, MADD is an autosomal recessive inherited disorder. Since not all patients suffering from MADD have mutations in the genes encoding the α- or β-subunit of ETF or ETFDH, other as yet unidentified exogenic factors may play an important role in the initiation of this disease. Since a proper mitochondrial flavin balance is maintained by a mitochondrial FAD transporter, a defect of this transporter or its precursor riboflavin could also cause a MADD-like phenotype [33].
In conclusion, a new type of equine acute myopathy is described. This may stimulate the performance of more metabolic investigations on equines suffering from rhabdomyolysis.
There are several reports concerning treatment of MADD in humans [17,34-36]. Biochemical MADD in horses might be of importance with reference to treatment options in the elusive types of human riboflavin-responsive MADD as well as to study the function of the mitochondrial FAD transporter in (equine) patients.

Acknowledgments

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Chapter 4

Acquired Multiple Acyl-CoA Dehydrogenase Deficiency in 10 horses with atypical myopathy

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Abstract

The aim of the current study was to assess lipid metabolism in horses with atypical myopathy.
Urine samples from 10 cases were subjected to analysis of organic acids, glycine conjugates and acylcarnitines, revealing increased mean excretion of lactic acid, ethylmalonic acid, 2-methylsuccinic acid, butyrylglycine, (iso)valerylglycine, hexanoylglycine, free carnitine, C2- , C3- , C4- , C5-, C6-, C8:1-, C10:1- and C10:2-carnitine as compared with 15 control horses (12 healthy and three with acute myopathy due to other causes). Analysis of plasma revealed similar results for these predominantly short-chain acylcarnitines. Furthermore, measurement of dehydrogenase activities in lateral vastus muscle from one horse with atypical myopathy indeed showed deficiencies of Short-Chain Acyl-CoA Dehydrogenase (0.66 as compared with 2.27 and 2.48 nmol min^{-1} mg^{-1} in two controls), Medium-Chain Acyl-CoA Dehydrogenase (0.36 as compared with 4.31 and 4.82 nmol min^{-1} mg^{-1} in two controls) and Isovaleryl-CoA Dehydrogenase (0.74 as compared with 1.43 and 1.61 nmol min^{-1} mg^{-1}).A deficiency of several mitochondrial dehydrogenases that utilize Flavin Adenine Dinucleotide as cofactor including the acyl-CoA dehydrogenases of fatty acid β-oxidation, and enzymes that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid and sarcosine was suspected in 10 out of 10 cases as the possible etiology for a highly fatal and prevalent toxic equine muscle disease similar to the combined metabolic derangements seen in human Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) also known as Glutaric Acidemia Type II.

Introduction

So-called atypical myopathy is an acute myopathy that appears in grazing horses [1-3]. To the authors' knowledge, the first case reports of myopathy in grazing horses concerned outbreaks that occurred in the autumn of 1939 in the North of Wales, UK [4]. Since the recognition of the syndrome, outbreaks of atypical myopathy have been reported in several European countries and case reports prior to the syndrome's identification suggest that the condition has also been encountered in Australia, Canada and the United States of America [2,5]. For example, in the autumn of 1995, over one hundred horses died from this condition in Northern Germany [1,2]. In the autumn of 2000, Belgium recognised its first cases of atypical myopathy and large outbreaks were recorded during cold periods in the autumn and spring of 2002, 2004, and 2006 in Belgium and France. Since 2004, the syndrome has been recognised in more than ten European countries, suggesting its emerging nature. The syndrome is associated with a mortality rate of about 90% and death usually occurs within 72 hours [2,3].

To date, a number of potential aetiological and contributing factors have been considered, but neither the exact aetiology nor the pathophysiology has been resolved. Whatever the cause, particular weather conditions seem to trigger the appearance of the syndrome [2,5].

The main feature of this syndrome is the sudden onset of clinical signs characterized by acute generalised progressive myopathy. Serum biochemical abnormalities usually include markedly elevated muscle enzyme activities, indicating severe muscle damage. At *post mortem* examination, widespread myodegeneration is found in both skeletal muscle and myocardium [1-3]. It has been shown previously that in equine atypical myopathy, predominantly type 1 muscle fibres were degenerated, associated with the accumulation of neutral lipids [1].

The aim of the current study was to perform metabolic screening of lipid metabolism in horses with a tentative diagnosis of atypical myopathy.

Materials and Methods

Horses

Five horses sampled during the outbreak of the autumn of 2006 in the Netherlands and 5 Belgian cases from outbreaks in 2003, 2004, and 2006 with a tentative diagnosis of atypical myopathy were used. In nine of 10 horses (except case 5, which survived) the disease turned out te be fatal. The description and clinical course of disease in these 10 horses (indicated as 1-10) is given in Table 1. Peak blood values are shown in Table 2. Inclusion criteria included access to pasture, no previous anaesthesia, nonexertional and nonrecurrent acute progressive rhabdomyolysis, absence of nonmuscular pathology and plasma creatine kinase activity > 2000 IU L^{-1}.

Table 1: Details from 10 horses (1-10) with a tentative diagnosis of atypical myopathy and 3 diseased controls (11-13).

Horse	Breed	Age	Gender	Outcome
1	Fjord	2 years	mare	Death within 3 days
2	Belgian draft	6 months	mare	Death within 1 day
3	Standardbred	1.5 years	mare	Death within 3 hours
4	Ardennais	0.5 years	mare	Death within 10 hours
5	Draft crossbreed	10 years	mare	Survived
6	Draft crossbreed	11 years	mare	Death within 1 day
7	Pony	1 year	mare	Death within 3 days
8	Friesian	10 months	mare	Death within 1 day
9	Arabian	2 years	stallion	Death within 1 day
10	Warmblood	3.5 years	mare	Death within 2 days
11	Tinker	14 years	gelding	Death within 2 days
12	Thoroughbred	4 years	mare	Survived
13	Warmblood	10 years	mare	Survived

Table 2: Peak blood values in 10 horses with a tentative diagnosis of acute myopathy (1-10) and 3 controls (11-13).

Horse	CK	AST	LDH	Lactate	Glucose	Total triglycerides
1	38,600	22,300	41,400	1.3	9.5	15.2
2	939,000					3.2
3	146,600		23,250	8.8	11.4	1.0
4	171,600	5,060	1,935	15.9	6.5	2.4
5	69,000	6,017	26,160	2.5		
6	400,000	12,376	35,541	5.4	8.2	
7	409,400	2,790		11.7	8.5	
8	>2,000	>1,000		14.7	12.4	
9	162,800	3,701	8,010			
10	410,000	11,522	36,350			
11	12,039	441	2,681	5.3	7.1	
12	132,730	5,091	14,318			
13	63,673	2,721	8,781			

Values include creatine kinase (CK upper limit of reference range 200 IU L^{-1}), aspartate aminotransferase (AST upper limit of reference range 275 IU L^{-1}), lactate dehydrogenase (LDH upper limit of reference range 600 IU L^{-1}), glucose (upper limit of reference range 5.6 mmol L^{-1}), lactate (upper limit of reference range 1.0 mmol L^{-1}), and total triglycerides (upper limit of reference range 1.0 mmol L^{-1}).

Twelve clinically healthy warmblood mares belonging to Utrecht University housed in boxes and accustomed to frequent handling were used as healthy controls. These horses were 3.7 to 20.5 years of age (mean age ± SD, 9.6 ± 5.0 years) and weighed 470 to 758 kg (mean weight, 596 ± 84.7 kg). Their diet consisted of grass silage supplemented with concentrate and met nutrient requirements for maintenance and performance. The total diet contained 10% ash, 14.5% crude protein, 1.3% crude fat, 20% crude fiber, and 56.2% other carbohydrates. Water was provided ad libitum.

In addition, three horses suffering from acute myopathy due to other causes (including one case of post-anaesthetic myopathy (horse 11) and two cases of recurrent exertional rhabdomyolysis (horses 12 and 13)) without a tentative diagnosis of atypical myopathy were used as diseased controls. The description and clinical course of disease for these 3 horses (indicated as 11-13) is given in Table 1. Peak blood values in these horses are shown in Table 2.

Muscle pathology

Nine of 10 horses (except case 5, which survived) with a tentative diagnosis of atypical myopathy were submitted for pathology. For histologic examination, muscle specimens were fixed in 4% (w/v) phosphate-buffered paraformaldehyde, sectioned at 5 μm, and stained with H&E. In addition, NADH, cytochrome c oxidase, succinate dehydrogenase,

acid phosphatase, periodic acid Schiff, ATP-ase (pH 4.3 and 9.4), Sudan black B, and Oil red O stains were performed on various muscle tissues frozen in isopentane that was precooled in liquid nitrogen, and stored at –80 ºC.

Analysis of organic acids and glycine conjugates

Urine samples from all 10 cases with a tentative diagnosis af atypical myopathy were subjected to analysis of organic acids and glycine conjugates, and results were compared with urine samples from 15 control horses (12 clinically healthy warmblood mares and three horses suffering from acute myopathy due to other causes without suspicion of atypical myopathy) by using the 95th percentile as the upper limit of the reference range based on the values obtained from the 12 healthy control horses. In addition, heparinised plasma samples from 8 cases with a tentative diagnosis af atypical myopathy were subjected to analysis of organic acids and results were compared with samples from the 15 control horses. Identification-analyses of organic acids and glycine conjugates in urine and plasma were carried out by gas chromatography-mass spectrometry (GC-MS) on a Hewlett Packard 5890 series II gas chromatograph linked to a HP 5989B MS-Engine mass spectrometer. Prior to this GC-MS analysis, the organic acids and glycine conjugates were trimethylsilylated with N,N-bis(trimethylsilyl)trifluoracetamide/ pyridine/trimethylchlorosilane (5:1:0.05 v/v/v) at 60 °C for 30 min. The gas chromatographic separation was performed on a 25 m x 0.25 mm capillary CP Sil 19CB column (film thickness 0.19 mm) from Arian/Chrompack, Middelburg, The Netherlands. The glycine conjugates were measured qualitatively only. Urinary concentrations are expressed as either organic acid: creatinine or glycine conjugate: creatinine ratios.

Analysis of free and acylcarnitines

Urine samples from all 10 cases with a tentative diagnosis of atypical myopathy were subjected to analysis of acylcarnitines and results compared with urine samples from the 12 clinically healthy control horses. In addition, heparinised plasma samples from 7 cases with a tentative diagnosis of atypical myopathy were available for analysis of acylcarnitines and results compared with samples from the 15 control horses (12 clinically healthy warmblood mares and three horses suffering from acute myopathy due to other causes without suspicion of atypical myopathy). Free and acylcarnitines in urine and plasma were analysed as their butyl ester derivatives by electrospray tandem mass spectrometry (ESI-MS-MS) on a Micromass Quattro Ultima system equipped with an Alliance HPLC system. Urinary concentrations are expressed as acylcarnitine: creatinine ratios.

Measurement of muscle dehydrogenase activities

Measurement of muscle dehydrogenase activities was performed in lateral vastus muscle tissue of one horse (number 6) with a tentative diagnosis of atypical myopathy, collected immediately after euthanasia in liquid nitrogen and stored at -80 °C. Lateral vastus muscle

tissue of two clinically healthy control horses was used for control measurements. The activities of medium-chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD) and isovaleryl-CoA dehydrogenase (IVD) were measured according to methods, which are based on the use of the substrate phenylpropionyl-CoA (for MCAD), butyryl-CoA (for SCAD) and isovaleryl-CoA (for IVD). Short-Chain Hydroxy Acyl-CoA Dehydrogenase (SCHAD) was used as a control enzyme in order to assess tissue viability. In brief, incubations were performed at 25° C in a buffered medium, containing an aliquot of the muscle homogenate plus ferricenium hexafluorophosphate as the electron acceptor. After termination of the reactions by acidification, the acidified samples were centrifuged and the protein-free supernatants neutralized followed by HPLC-analysis to separate the different acyl-CoA esters.

Fluorescence Microscopy

In order to clearly identify the presence of muscular lipid droplets, *M. vastus lateralis* specimens of one horse (number 6) with a tentative diagnosis of atypical myopathy were snap frozen in liquid nitrogen and cut on a Leica CM3050 microtome. Lateral vastus muscle tissue of two clinically healthy control horses was used for control fluorescence microscopy. Thin cryosections (10 µm) were attached to Superfrost Plus slides and incubated in 0.02 µg/µl Bodipy 493/503 (Molecular Probes, Invitrogen, Breda, the Netherlands) for 15 minutes in a humidified environment in order to stain neutral lipids. Lipid droplets were visualised by a Leica DMR fluorescence microscope equipped with a Photometrics Coolsnap CCD digital photo camera. Images were processed using IP-lab image analyses software. For muscle fiber typing a monoclonal antibody was used specific for type 1 MyHC isoform kindly provided by prof. A.F.M. Moorman, Academic Medical Centre, Amsterdam, the Netherlands.

Riboflavin assay

Heparinised plasma samples from 7 cases with a tentative diagnosis of atypical myopathy were subjected to analysis of riboflavin and results compared with samples from 6 control horses. Riboflavin was assessed using HPLC. The detection limit of the HPLC was 13 nmol L^{-1}, and the intra-assay and interassay coefficients of variation were 1.86 and 8.97%, respectively.

Statistical analysis

Normality of the organic acids, acylcarnitines, and the glycine conjugates data was analysed using normal P-P Plot by means of Blom method and the Kolmogorov-Smirnov test. Given the fact that these data not always showed a normal distribution, the 95th percentile was calculated using the corresponding data from the 12 healthy control horses. As a consequence, the 95th percentile was always used as the upper limit of the reference range rather than the 95% confidence interval. The cut-off method used for acylcarnitines

in urine was defined as a value above 5 mmol creatinine^{-1} following the subtraction of the 95th percentile from the average value of each acylcarnitine in urine. The significance of differences between groups with reference to plasma riboflavin concentrations was assessed by the Mann-Whitney test (two-tailed). Values of $P < 0.05$ were considered significant. Results from riboflavin assay are reported as mean ± SD.

Results

Blood analysis supported the tentative diagnosis of acute myopathy in all 13 myopathy cases. The clinicopathological diagnosis of acute myopathy was confirmed *post mortem* in all 9 cases with a tentative diagnosis of atypical myopathy illustrated by pale, degenerated looking musculature in various muscles. Microscopic findings were floccular degeneration, necrosis and myolysis predominantly affecting type 1 muscle fibres, increase of internally located nuclei, subsarcolemmal vacuolation, subsarcolemmal accumulation of mitochondria, slight infiltrations with macrophages and neutrophils and histochemically in some type 1 fibres there was a slight increase of fat. More specific with reference to an 11-year-old Draft horse mare (number 6) suffering from fatal atypical myopathy, histopathological examination of a muscle biopsy from lateral vastus muscle showed myopathic changes with signs of regeneration and fibrotic areas. The extent and severity of the degenerative process varied between cases and muscles.
Fluorescence microscopy from lateral vastus muscle from the same horse (number 6) showed microvesicular lipidosis predominantly in type 1 fibers (Fig. 1). In addition, electron micrography of lateral vastus muscle from the same horse (number 6) showed subsarcolemmal accumulation of mitochondria (Fig. 2a) and absence of mitochondrial cristae associated with increased matrix density (Fig. 2b).

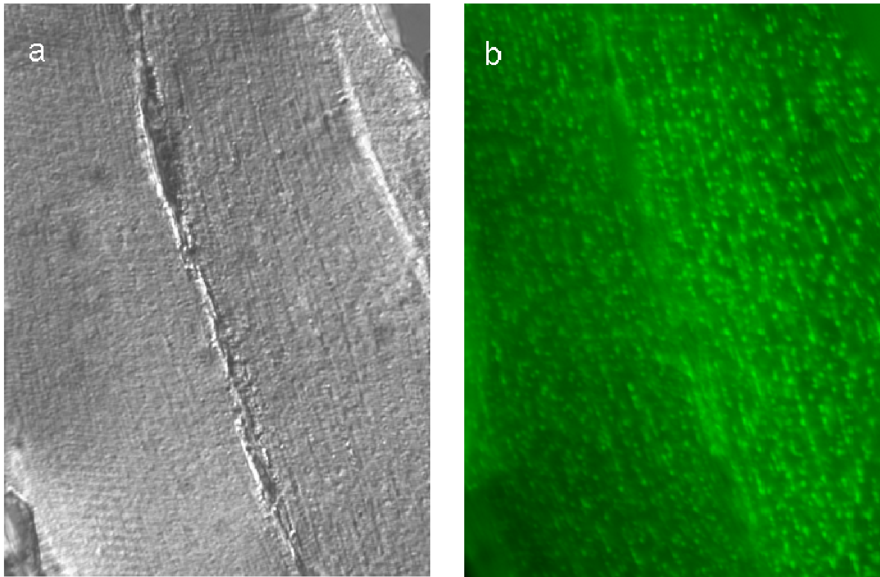

Figure 1: Neutral lipid staining from lateral vastus muscle from an 11-year-old Draft horse mare suffering from fatal atypical myopathy showing microvesicular lipidosis in type 1 fibers (right) and the corresponding differential interference contrast microscopy image (left).

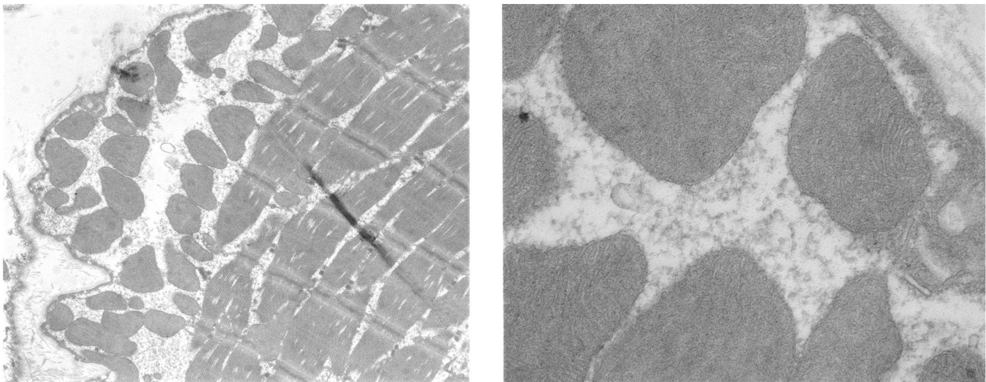

Figure 2 a&b: Electron micrograph from lateral vastus muscle from an 11-year-old Draft horse mare suffering from fatal atypical myopathy showing subsarcolemmal accumulation of mitochondria (top) and absence of mitochondrial cristae associated with increased matrix density (bottom).

Table 3a: Organic acids (mmol mol creatinine^{-1}) and glycine conjugates (mmol mol creatinine^{-1}) in urine from 10 horses with a tentative diagnosis of atypical myopathy (1-10) and 3 diseased controls (11-13). The glycine conjugates and 2-methylsuccinic acid were measured qualitatively only and their presence (+) or absence (-) indicated. Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Horse number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**P95**	**11**	**12**	**13**
Lactic acid	66	4824	2086	2093	182	173	3157	2720	3772	143	133	3375	138	9
Glycolic acid	16	38	23	17	24	23	n.d.	55	64	12	22	25	17	21
Pyruvic acid	38	143	72	49	49	29	85	n.d.	n.d.	42	9	76	3	3
2-OH-butyric acid	1	18	2	2	1	n.d.	22	38	12	3	5	25	n.d.	n.d.
3-OH-butyric acid	44	74	30	16	22	12	383	144	295	43	131	216	3	4
3-OH-isobutyric acid	20	49	21	23	18	17	210	74	141	25	127	82	11	12
3-OH-isovaleric acid	15	23	4	5	1	34	16	76	42	2	34	5	4	3
Methylmalonic acid	2	9	2	1	3	2	15	9	13	4	7	n.d.	4	2
Ethylmalonic acid	74	194	76	122	324	231	381	270	491	604	5	4	3	2
2-methylsuccinic acid	39	69	32	66	90	98	78	132	93	105	-	-	-	-
Succinic acid	3	2	1	2	6	5	5	116	4	n.d.	10	n.d.	2	3
Glutaric acid	6	n.d.	n.d.	n.d.	57	39	n.d.	170	n.d.	n.d.	122	7	4	n.d.
Fumaric acid	6	1	4	5	8	n.d.	19	n.d.	11	8	12	6	n.d.	n.d.
2-ketoglutaric acid	10	11	3	12	4	3	16	74	29	5	56	n.d.	n.d.	n.d.
Unsaturated suberic acid	39	18	9	12	33	31	35	63	23	36	10	2	1	1
Suberic acid	39	20	8	13	23	21	30	78	18	33	6	n.d.	1	1
Homovanillic acid	2	2	1	6	1	1	5	5	3	25	37	1	8	7
Adipic acid	n.d.	n.d.	3	n.d.	n.d.	39	289	16	n.d.	401	4	4	1	1
Butyrylglycine	+	+	+	+	+	+	+	+	+	+	-	-	-	-
(Iso)valeric glycine	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Hexanoylglycine	+	+	+	+	+	+	+	+	+	+	-	-	-	-

Table 3b: Organic acids (µmol/l) in heparinised plasma from 8 horses with a tentative diagnosis of atypical myopathy (3-10) and 3 diseased controls (11-13). Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Horse number	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**P95**	**11**	**12**	**13**
Lactic acid	606	24831	1112	5778	6861	6242	8484	7941	2162	9356	1260	1488
2-OH-butyric acid	14	14	2	38	66	105	32	69	24	63	6	6
3-OH-butyric acid	15	103	182	313	375	275	327	317	379	202	227	154
3-OH-isobutyric acid	20	44	18	25	54	48	68	79	107	37	39	21
3-ketobutyric acid	n.d.	52	5	40	82	106	90	70	11	n.d.	n.d.	n.d.
Cis-4-decenoic acid	n.d.	1	n.d.	5	4	9	2	4	4	n.d.	n.d.	n.d.
Glutaric acid	4	22	n.d.	37	26	56	19	16	5	n.d.	n.d.	n.d.
Decanoic acid	3	12	n.d.	n.d.	17	21	7	n.d.	n.d.	n.d.	n.d.	n.d.
Lauric acid	2	9	1	n.d.	n.d.	17	n.d.	3	1	n.d.	n.d.	n.d.
Myristicacid	6	16	2	15	11	50	9	19	1	10	n.d.	n.d.
Palmitoleic acid	21	30	3	44	17	89	13	49	1	40	3	n.d.
Palmitinezuur	133	105	29	154	114	414	95	258	5	93	22	13
Oliezuur	76	80	34	142	97	418	77	252	3	97	16	11
Linolzuur	121	80	17	105	109	309	95	201	4	33	15	20
Stearinezuur	68	33	26	34	192	184	146	120	4	16	12	5

Table 4a: Acylcarnitines in urine (mmol mol creatinine^{-1}) from 10 horses with a tentative diagnosis of atypical myopathy (1-10). Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Horse number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**P95**
Free carnitine	369.41	250.29	328.65	131.08	339.43	318.12	196.64	327.88	289.81	762.91	12.5
C2-carnitine	379.53	151.60	104.79	68.78	226.74	128.33	177.14	442.19	210.28	385.91	1
C3-carnitine	7.89	13.58	10.30	4.75	17.51	12.79	26.41	22.12	29.77	22.08	0.1
C4-carnitine	28.29	167.66	25.91	78.74	587.63	240.03	98.31	199.72	288.98	164.96	1
C5:1-carnitine	0.12	0.16	0.11	0.10	0.08	0.17	0.27	0.55	0.22	0.20	0.01
C5-carnitine	17.64	341.07	23.03	73.57	388.86	226.67	233.19	395.46	676.69	250.61	0.1
C4:3-carnitine	7.87	1.85	0.70	0.95	1.11	1.81	2.56	6.37	3.45	2.50	0
C6-carnitine	2.28	44.12	7.47	11.40	72.19	53.07	14.95	25.68	23.70	17.81	0.02
C5-OH-carnitine	0.31	2.81	0.94	0.84	4.42	2.82	2.71	9.21	5.15	1.69	0.1
C8:1-carnitine	0.49	19.43	3.96	11.48	23.53	21.46	11.37	32.53	28.24	16.49	0.01
C8-carnitine	0.66	21.04	5.38	10.27	29.30	20.19	9.40	30.83	16.11	14.05	0.03
C10:2-carnitine	0.34	29.71	5.00	14.40	25.78	16.11	13.87	23.53	26.42	14.86	0.02
C10:1-carnitine	0.26	7.19	2.84	4.72	11.16	10.21	6.78	11.18	9.44	7.43	0.01
C10-carnitine	0.40	5.76	2.60	3.21	1.81	1.61	4.12	8.64	3.80	3.16	0.02
C4DC-carnitine	0.11	0.24	0.36	0.15	0.44	0.37	0.24	0.50	0.40	0.28	0.4
C5DC-carnitine	0.95	5.29	4.24	6.81	11.35	6.97	2.15	5.19	6.13	2.49	0.5
C12:1- carnitine	0.14	0.47	0.39	0.28	0.14	0.13	0.51	0.96	0.39	0.55	0.01
C12-carnitine	0.05	0.14	0.08	0.10	0.04	0.05	0.11	0.23	0.12	0.11	0.04
C6-DC- carnitine	0.43	0.43	0.42	0.16	0.96	0.74	0.51	1.61	0.67	0.62	0.1
C14:2- carnitine	0.01	0.03	0.02	0.02	0.06	0.05	0.04	0.09	0.06	0.08	0
C14:1- carnitine	0.02	0.03	0.04	0.04	0.06	0.10	0.04	0.04	0.10	0.11	0.01
C14-carnitine	0.04	0.05	0.06	0.03	0.16	0.19	0.09	0.18	0.12	0.10	0.03
C8-DC- carnitine	0.13	0.20	0.18	0.08	0.29	0.25	0.24	0.58	0.28	0.25	0.05
C14-OH- carnitine	0.04	0.02	0.03	0.02	0.04	0.03	0.03	0.07	0.05	0.02	0.01
C16:1- carnitine	0.02	0.02	0.02	0.01	0.03	0.04	0.03	0.04	0.04	0.02	0
C16-carnitine	0.03	0.03	0.05	0.03	0.14	0.16	0.09	0.14	0.11	0.14	0.02
C10-DC- carnitine	0.05	0.07	0.06	0.07	0.09	0.09	0.10	0.22	0.10	0.10	0.02
C16:1-OH- carnitine	0.01	0.02	0.01	0.01	0.03	0.03	0.04	0.04	0.05	0.01	0
C16-OH- carnitine	0.03	0.02	0.03	0.03	0.08	0.08	0.04	0.10	0.06	0.05	0.01
C18:2- carnitine	0.02	0.04	0.02	0.03	0.01	0.01	0.01	0.02	0.01	0.02	0
C18:1- carnitine	0.01	0.02	0.01	0.01	0.01	0.02	0.03	0.04	0.03	0.04	0

Horse number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**P95**
C18-carnitine	0.06	0.05	0.04	0.04	0.08	0.08	0.08	0.16	0.07	0.09	0
C18:2-OH- carnitine	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01	0.00	0
C18:1-OH- carnitine	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.04	0.03	0.01	0
C16-DC-carnitine	0.00	0.01	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.01	0
C18:1-DC- carnitine	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0

Table 4b: Acylcarnitines in plasma (µmol/l) from 7 horses with a tentative diagnosis of atypical myopathy and 3 diseased controls (11-13). Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Horse number	**3**	**4**	**6**	**7**	**8**	**9**	**10**	**P95**	**11**	**12**	**13**
Free carnitine	27.46	44.84	54.40	25.66	75.11	32.75	131.06	44	111.91	70.23	37.43
C2-carnitine	7.09	11.92	21.30	9.63	26.97	10.88	46.98	5.6	50.54	21.89	3.47
C3-carnitine	0.51	1.62	1.66	1.66	3.44	1.94	3.13	0.95	1.53	1.19	0.42
C4-carnitine	2.09	27.74	41.55	13.69	60.84	17.60	31.05	0.7	1.82	0.78	0.30
C5:1-carnitine	0.01	0.03	0.06	0.05	0.15	0.06	0.08	0.02	0.04	0.04	0.03
C5-carnitine	2.64	34.57	29.47	23.98	92.77	39.06	30.44	0.5	0.92	0.59	0.26
C4:3-OH-carnitine	0.08	0.50	0.58	0.38	1.53	0.59	0.81	0.03	0.66	0.10	0.03
C6-carnitine	0.43	5.72	5.87	1.56	16.90	1.94	6.66	0.02	0.30	0.01	0.01
C5-OH-carnitine	0.07	0.35	0.44	0.36	1.54	0.37	0.44	0.04	0.03	0.01	0.01
C8:1-carnitine	0.06	1.20	1.00	0.60	4.89	1.12	0.30	0.02	0.12	0.02	0.02
C8-carnitine	0.01	1.64	1.67	0.57	5.38	0.77	1.72	0.02	0.11	0.03	0.02
C10:2-carnitine	0.07	1.43	1.21	0.65	3.54	0.81	0.16	0.02	0.10	0.04	0.03
C10:1-carnitine	0.05	0.55	0.75	0.41	2.03	0.39	0.43	0.02	0.06	0.03	0.04
C10-carnitine	0.01	0.76	0.93	0.30	2.06	0.30	0.67	0.03	0.13	0.04	0.04
C4DC-carnitine	0.01	0.08	0.13	0.04	0.09	0.05	0.14	0.05	0.15	0.07	0.06
C5DC-carnitine	0.13	1.87	0.88	0.19	0.96	0.45	0.71	0.06	0.10	0.06	0.07
C12:1- carnitine	0.01	0.26	0.22	0.12	0.36	0.08	0.07	0.02	0.13	0.05	0.05
C12-carnitine	0.01	0.63	0.22	0.13	0.39	0.14	0.16	0.02	0.12	0.06	0.05
C6-DC- carnitine	0.04	0.06	0.10	0.06	0.39	0.07	0.15	0.02	0.08	0.05	0.06
C14:2- carnitine	0.01	0.06	0.07	0.05	0.14	0.05	0.14	0.02	0.04	0.01	0.02
C14:1- carnitine	0.04	0.40	0.25	0.14	0.34	0.11	0.09	0.03	0.17	0.03	0.02
C14-carnitine	0.01	0.78	0.24	0.13	0.30	0.13	0.12	0.02	0.05	0.02	0.01
C8-DC- carnitine	0.01	0.03	0.04	0.03	0.13	0.04	0.04	0.02	0.02	0.01	0.01
C14-OH- carnitine	0.01	0.14	0.04	0.04	0.06	0.06	0.02	0.01	0.01	0.01	0.01
C16:1- carnitine	0.01	0.53	0.22	0.09	0.21	0.11	0.07	0.02	0.08	0.01	0.01
C16-carnitine	0.06	1.54	0.56	0.35	0.61	0.34	0.29	0.02	0.08	0.02	0.02
C10-DC- carnitine	0.01	0.06	0.03	0.02	0.07	0.03	0.07	0.01	0.02	0.01	0.01
C16:1-OH- carnitine	0.01	0.25	0.09	0.10	0.16	0.17	0.04	0.01	0.01	0.01	0.01
C16-OH- carnitine	0.01	0.23	0.05	0.04	0.07	0.09	0.03	0.01	0.02	0.01	0.01

Horse number	**3**	**4**	**6**	**7**	**8**	**9**	**10**	**P95**	**11**	**12**	**13**
C18:2-carnitine		0.33	0.16	0.13	0.26	0.19	0.03	0.01	0.02	0.01	0.02
C180.25:1- carnitine	0.05	1.19	0.68	0.36	0.71	0.38	0.08	0.02	0.15	0.02	0.03
C18-carnitine	0.04	0.30	0.28	0.25	0.33	0.29	0.18	0.01	0.03	0.01	0.01
C18:2-OH- carnitine	0.01	0.11	0.05	0.07	0.06	0.13	0.01	0.01	0.01	0.01	0.01
C18:1-OH- carnitine	0.01	0.13	0.04	0.08	0.12	0.14	0.03	0.01	0.01	0.01	0.01
C16-DC-carnitine	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.01
C18:1-DC- carnitine	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01

Metabolic screening of the horses with a tentative diagnosis of atypical myopathy (numbers 1-10) using urine obtained *ante mortem* revealed an increased excretion (above the 95th percentile) of ethylmalonic acid, 2-methylsuccinic acid, butyrylglycine, (iso)valerylglycine and hexanoylglycine in all 10 cases and lactic acid in 9 of 10 as shown in Table 3a. Organic acid concentrations in plasma were not elevated except for lactic acid (Table 3b). Furthermore, the profile of acylcarnitines in urine showed a substantial elevation above the cut-off value for free carnitine, C2-, C3-, C4-, C5-, C6-, C8:1, C10:1 and C10:2-carnitine (Table 4a). Metabolic screening of plasma revealed similar results for C2-, C4-, C5-, and C6-, C8:1, C10:1 and C10:2-carnitine in all 7 cases studied and free carnitine in 4 of 7 and C3- and C8-carnitine in 6 of 7 as shown in Table 4b.
Repeated metabolic screening of the only surviving horse (number 5) fifteen days later, revealed normal urinary excretion of lactic acid (6 mmol mol creatinine^{-1}), ethylmalonic acid (4 mmol mol creatinine^{-1}) and acylcarnitines without detectable glycine conjugates and 2-methylsuccinic acid associated with a normal acylcarnitine profile in plasma.
In comparison, metabolic screening of 3 diseased control horses suffering from acute myopathy without a tentative diagnosis of atypical myopathy (indicated as 11-13) revealed normal urinary excretion of ethylmalonic acid and 2-methylsuccinic acid as well as other organic acids (except for lactic acid) without detectable glycine conjugates (Table 3a). Organic acid concentrations in plasma were not elevated except for lactic acid (Table 3b). Metabolic screening of heparinised plasma revealed substantial elevation of free carnitine, C2-, C3-, and C4-carnitine in 2 out of 3 cases.
Furthermore, quantitative biochemical measurement of dehydrogenase activities in lateral vastus muscle from one horse (number 6) with a tentative diagnosis of fatal atypical myopathy indeed showed a deficiency of Short-Chain Acyl-CoA Dehydrogenase (SCAD; 0.66 as compared with 2.27 and 2.48 nmol min^{-1} mg^{-1} in two controls), Medium-Chain Acyl-CoA Dehydrogenase (MCAD; 0.36 as compared with 4.31 and 4.82 nmol min^{-1} mg^{-1} in two controls) and Isovaleryl-CoA Dehydrogenase (IVD; 0.74 as compared with 1.43 and 1.61 nmol min^{-1} mg^{-1} in two controls). Short-Chain Hydroxy Acyl-CoA Dehydrogenase showed similar results in all three animals (343 as compared with 387 and 307 nmol min^{-1} mg^{-1} in two controls) indicating similar tissue viability.
Mean heparinised plasma riboflavin concentrations in 7 horses with a tentative diagnosis of atypical myopathy did not differ significantly from those in 6 clinically healthy control horses (78.9±17.6 versus 68.3±11.8 nmol L^{-1}).

Discussion

The 10 horses with a tentative diagnosis of atypical myopathy in this study suffered from a rapidly progressing muscular disease with a mortality rate of 90% associated with degeneration and necrosis primarily affecting type 1 muscle fibres in agreement with the

findings on this disease as reported earlier [2]. The signs of regeneration and fibrotic areas as seen on histopathology in only one case were similar to a previously described case (case 7 in [2]) and might suggest some subclinical chronicity of the syndrome.

In the body, fat serves as an efficient source of tissue fuel as compared with carbohydrates. The central role of the mitochondrion is immediately apparent, since it acts as the focus and cross-roads of carbohydrate, lipid, and amino acid metabolism. In particular, it houses the enzymes of the citric acid cycle, of the respiratory chain and ATP synthase, of β-oxidation of fatty acids, and of ketone body production [6]. The main function of muscle mitochondria is oxidative phosphorylation using fatty acids (besides carbohydrates) as the chief substrate to concentrate potential energy. The type 1 fibers have cytochemical features that indicate a mainly aerobic-oxidative metabolic profile associated with numerous mitochondria [7].

Based on the characteristic urinary profiles of organic acids (ethylmalonic acid and 2-methylsuccinic acid), glycine conjugates ((iso)valerate, butyrate and hexanoate) and predominantly short-chain acylcarnitines (acylgroups less than 10 carbon atoms) in all 10 horses with a tentative diagnosis of atypical myopathy, a deficiency of several mitochondrial dehydrogenases that utilize Flavin Adenine Dinucleotide (FAD) as cofactor was suspected. Several mitochondrial dehydrogenases utilize Flavin Adenine Dinucleotide as cofactor including the acyl-CoA dehydrogenases of fatty acid β-oxidation, and enzymes that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid and sarcosine (a precursor of glycine)[6]. Decreased activity of many FAD-dependent dehydrogenases results in the combined metabolic derangements seen in human Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) also known as Glutaric Acidemia Type II (GA-II) [8,9].

Riboflavin is a precursor in the synthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both products are the prosthetic groups of numerous enzymes (called flavoproteins) that catalyze the various electron-transferring reactions in energy-producing, biosynthetic, detoxifying, and electron-scavenging pathways. Most of these flavoproteins are found in mitochondria [10,11]. Mean plasma riboflavin concentrations in horses with a tentative diagnosis of atypical myopathy did not differ significantly from those in control horses indicating no riboflavin deficiency.

Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) is a severe inborn error of metabolism, which can lead to early death in human patients. This autosomal recessive disease was first reported in 1976 by Przyrembel and co-workers [12]. The clinical presentation of MADD is very heterogeneous and ranges from neonatal death to late-onset myopathy. In the majority of human cases, MADD is caused by mutations in the genes encoding the α- or β-subunit of ETF or ETF-DH [13-15]. Based on the epizootic occurrence of the disease, we hypothesize that the MADD seen in these horses may be caused by an exogenous factor predominantly affecting SCAD, MCAD and IVD directly or indirectly via Flavin Adenine Dinucleotide (FAD) as cofactor.

Since not all patients suffering from human MADD have mutations in the genes encoding the α- or β-subunit of ETF or ETF-DH, other as yet unidentified genes are predicted to be involved as well. Because all affected mitochondrial flavoproteins in MADD have FAD as a prosthetic group, the underlying defect in these patients may be due to a thus far undisclosed disturbance in the metabolism of FAD. Since a proper mitochondrial flavin balance is maintained by a mitochondrial FAD transporter [9], a defect of this transporter could also cause a MADD-like phenotype.

Here we identify the biochemical defect in 10 out of 10 horses leading to fatal atypical myopathy based on the characteristic urinary profiles of organic acids, glycine conjugates, and predominantly short-chain acylcarnitines as well as additional quantitative biochemical measurement of dehydrogenase activities in lateral vastus muscle in a single case as a deficiency of several mitochondrial dehydrogenases that utilize Flavin Adenine Dinucleotide as cofactor. As a consequence, atypical myopathy in grazing horses reflects a MADD-like phenotype. With reference to future cases, the characteristic urinary profiles of organic acids, glycine conjugates, and acylcarnitines might be helpful in the diagnosis of atypical myopathy. However, glutaric acid excretion was not elevated possibly due to the fact that glutaric acid appears to be a normal constituent in equine urine in contrast to man [16]. We have no clear explanation for the observation that the equine patients had elevated concentrations of glutarate in plasma, but not in urine.

The disease is characterized by a very high mortality rate of about 90% and death usually within 72 hours due to acute generalised progressive myopathy. As the exact pathophysiology of the disease was unknown till so far effective treatments were not available. Although the mean plasma concentration of riboflavin found in diseased horses was not different from control horses this does not rule out riboflavin as a possible treatment option given the possibility that there might be competition between riboflavin and a toxic compound. Our findings could aid in developing effective treatments by improving efficacy of carbohydrate metabolism rather than fat metabolism in diseased horses. As mentioned before, carbohydrates and fatty acids are the main metabolic fuels for skeletal muscle. Glucose transport in equine muscle is mediated by the glucose transporter 4 protein, which is stimulated by insulin and muscle contraction [17]. Hence, we suggest intravenous administration of fluids enriched with especially insulin (besides some glucose given the presence of hyperglycaemia upon admission) to be most likely candidate for potential treatment of the condition via increased cellular glucose uptake due to enhanced glucose transporter 4 protein translocation. With reference to improvement of efficacy of lipid metabolism, intravenous carnitine might be a likely candidate.

In humans, MADD is an autosomal recessive inherited disorder. However, this seems to be unlikely in equine atypical myopathy given the epizootic occurrence and the recovery of a 10-year-old Draft horse mare following hospitalisation. It should be realized also that humans with fatty acid oxidation genetic defects may have normal biochemistry between episodes of decompensation. As a consequence, the equine disease might be considered as

an acquired (rather than inherited) model of the human counterpart. Despite several experiments in order to obtain mice or rats with MADD like diseases, there are no mutations that might be considered as a model of human MADD yet. To the authors' knowledge, MADD is diagnosed in no other species than man so far except for two anecdotal case reports in horses not associated with atypical myopathy prospectively [16]. It might be of importance to apply new therapeutic approaches of possible benefit to man on horses with atypical myopathy.

As has been mentioned before, particular weather conditions seem to trigger the appearance of the syndrome [2,5] suggesting that an exogenic factor may play an important role in the pathogenesis of this disease. It has been hypothesized that (myco)toxins might be regarded as potential aetiological factors [2]. Interestingly, on a pasture with a severe outbreak of atypical myopathy in the Netherlands, fungi of the species Cortinarius type Dermocybe uliginosus were found. The subsarcolemmal vacuolisation seen at histopathology also suggests toxic muscular changes given the absence of glycogen storage disorders. Furthermore, a new myopathy syndrome affecting humans has recently been attributed to fungi of the group of Tricholoma equestre. Repeated consumption of this wild mushroom may cause fatal myopathy [18,19]. In Japan, another mushroom, Russula subnigricans, has also been reported as being myotoxic [20]. In addition, toxic alkaloidal substances in the seeds of *Galeopsis ladanum* via ingestion of quail also caused rhabdomyolysis in man [21]. To the authors' knowledge, neither histopathology for lipid storage nor metabolic screening of lipid metabolism has been performed yet in these human toxic myopathies.

With reference to these potential aetiological factors and possible public health consequences further research is necessary. The possibility that the catalytic efficiency of several mitochondrial dehydrogenases that utilize Flavin Adenine Dinucleotide (FAD) as cofactor is impaired due to the attachment of (myco)toxins thereby causing atypical myopathy might be of help in the potential isolation of these (myco)toxins. Future studies might reveal the exact aetiology of the condition in horses as preceeded by its elucidated pathophysiology.

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Chapter 5

Creatine metabolism, carbohydrate metabolism, purine metabolism and oxidative phosphorylation in healthy horses and horses suffering from atypical myopathy

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Abstract

Objective
The objective of the current study was to assess creatine, purine and carbohydrate metabolism as well as oxidative phosphorylation in horses with atypical myopathy and to compare the results with healthy control horses.
Animals
Urine of five and muscle of two horses with a tentative diagnosis of atypical myopathy was used as well as urine of five and muscle of two to seven healthy control horses.
Procedures
A large panel of analyses of creatine, purine and carbohydrate metabolism as well as oxidative phosphorylation was performed.
Results
In patients, the mean concentrations of creatine and uric acid in urine were 6-36 and 4 times as high as in controls, respectively. The patient with the most clinically severe myopathy was most glycogen depleted and had more active glycolysis than other patients or controls.
In one patient, activity of phosphoglycerate mutase was reduced to less than 10% of reference values. Most complex activities in patients were 70-80% of the activity in control horses, with the exception of complex II which was lowered to 55% of normal activity.
In one patient ATP-synthase activity was reduced to less than 40% of activity in control horses.
Conclusions
Examples of general metabolic indicators for myopathic damage are changes in creatine kinase, lactic acid, creatine and uric acid levels. Analysis of carbohydrate, lipid and protein metabolism as well as oxidative phosphorylation is recommended as tool to obtain more detailed information about the cause of the myopathy metabolic,
Clinical Relevance
This study expands options for diagnosis of equine myopathies.

Keywords

Horse, atypical myopathy, creatine, purine, carbohydrate, oxidative phosphorylation, respiratory chain, PGAM-deficiency, complex V deficiency

Introduction

Atypical myopathy is a frequently fatal, acute myopathy that appears in grazing horses [1-3]. Horses with atypical myopathy suffer from severe rhabdomyolysis. Clinical symptoms are muscular weakness, recumbency, sweating and myoglobinuria. Routine biochemical analysis of plasma shows markedly elevated activities of muscle enzyme such as creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate amino transferase (ASAT), indicating severe muscle damage [4]. Typical histopathological lesions are degeneration of type I fibers and accumulation of lipid droplets [2].
Particular weather conditions seem to trigger the appearance of the atypical myopathy syndrome suggesting that an exogenic factor, for instance a (myco-)toxin may play an important role in the aetiology of this disease [4].
Research on ten horses suffering from atypical myopathy, described in chapter three and four of this thesis, has shown that a disorder of beta-oxidation, MADD (Multiple Acyl-CoA Dehydrogenase Deficiency), is the biochemical background of atypical myopathy [5]. To obtain this diagnosis, additional analyses were performed, including analysis of organic acids, acylglycines and acylcarnitines in urine and plasma, acylcarnitine profiling in muscle and analysis of muscle dehydrogenase activities.
In horses, an exogenic factor is thought to cause MADD, in contrast to the situation in humans, where the disease has an autosomal recessive inheritance as aetiology. It would be of interest to analyse other metabolic pathways to find out if the presumed toxin has induced additional deficiencies.
This study describes analyses of the (phospho) creatine metabolism, carbohydrate metabolism, purine metabolism and oxidative phosphorylation of healthy horses and horses suffering from acute rhabdomyolysis as a result of atypical myopathy due to MADD. For these analyses, diagnostic methods that are normally used in human medicine were used.
As described in chapter three and four, lipid metabolism is damaged by atypical myopathy, an example of a condition resulting in acute, progressive rhabdomyolysis. We hypothesize that other metabolic pathways may also be affected by the toxic insult, resulting in non-specific degenerative effects.

First, the metabolic pathways will be explained. Muscle contraction depends on energy supplied by ATP (adenosine triphosphate). The concentration of ATP in muscle fiber is sufficient to maintain full contraction for 1 to 2 seconds at most. After this, ATP is split to form ADP, which is rephosphorylated to form new ATP within a fraction of a second. There are several sources of energy for this rephosphorylation [6]. The first source of energy used is phosphocreatine, which carries a high-energy phosphate bond similar to the bonds of ATP. The total amount of phosphocreatine in the muscle fiber is very small, sufficient for maximal muscle contraction for 5-8 seconds. The reaction is phosphocreatine + ADP ↔ creatine + ATP [6].

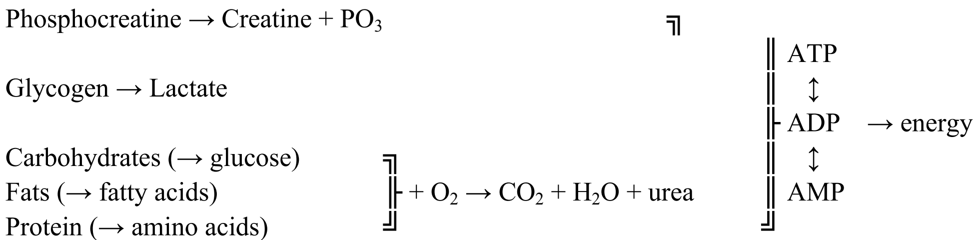
ADP may also be rephosphorylated to ATP through the myokinase reaction, which leads to increased AMP production (2 ADP↔ ATP + AMP). AMP is deaminated into inosine monophosphate (IMP) and NH_3. Further deamination leads to purine catabolism [7,8].

The second important source of energy is glycogen. The enzymatic breakdown of glycogen to pyruvate and lactic acid liberates energy that is used to reconstitute both ATP and phosphocreatine. This glycolysis can occur both in the presence (aerobic) and absence (anaerobic) of oxygen. Anaerobic glycolysis can sustain maximum muscle contraction for about 1 minute [6]. For periods of maximal muscle activity lasting 2 to 4 hours, as much as one half of the energy can come from stored glycogen before the glycogen is depleted [6].

The third and final source of energy is oxidative metabolism. This means the combining of oxygen with various cellular nutrients to liberate ATP. The foodstuffs that are consumed are carbohydrates, fats and protein. For extremely long-term maximal muscle activity (over a period of many hours) by far the greatest proportion of energy comes from fats [6]. All substrates used in oxidative metabolism are converted to Acetyl-CoA, carbohydrates (glycogen and glucose) by glycogenolysis and glycolysis, fats (fatty acids) by lipolysis and β-oxidation and protein by proteolysis and deamination of amino acids [9].

In the citric acid cycle and the respiratory chain, the last step of the dissimilation of proteins, lipids and glucose into CO_2 takes place. The NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (1, 5-dihydro-flavin adenine dinucleotide) generated in the citric acid cycle is used for ATP synthesis, using the electron transport chain in the oxidative phosphorylation.

Figure 1: Origin of ATP



Creatine metabolism

Creatine is mainly synthesized in the liver and pancreas from arginine and glycine that form ornithine and guanidinoacetate. Guanidinoacetate reacts with methionine to form homocysteine and creatine. Creatine is then actively and preferentially transported into brain and muscle tissue to be utilized in the creatine/creatinephosphate system [10,11].

The enzyme necessary for the dephosphorylation of phosphocreatine to creatine and the associated production of ATP, is the muscle iso-enzyme creatine kinase (CK-MM,

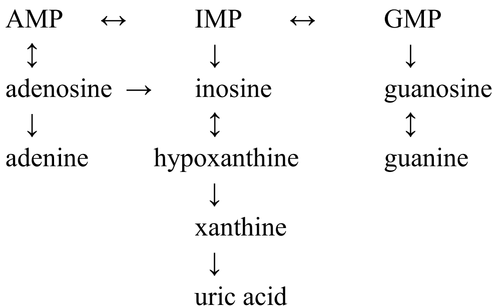
henceforth referred to as CK).When muscles are at rest, creatin is rephosphorylated by mitochondrial CK. Most serum CK is of muscle origin. It is a sensitive indicator of striated muscle damage and generally regarded as the enzyme of choice to determine this damage [12]. An increase in serum CK is due to leakage out of the muscle cell into the surrounding blood and lymph, after disruption of muscle cell membranes as result of degeneration, necrosis or inflammation [12]. In chapter four, it can be seen that myopathy patients have very high activity of plasma creatine kinase CK, which is due to the damaged muscle cells.
After dephosphorylation, creatine is transformed into creatinine. A rather constant amount of creatine is converted to creatinine and excreted in the urine at a daily rate that is essentially proportional to muscle mass [9,13]. Our hypothesis is that myopathic patients have higher concentrations of creatine than control animals due to the fact that creatin is not rephosphorylated and leaves the muscle cells. Under this hypothesis, the concentration of creatine in urine could be used to determine the severity of disease.
The concentration of guanidinoacetate is not expected to increase or decrease, because it is a precursor of creatine, not involved in the CK reaction and not even present in muscle.

Purine metabolism

During intense exercise, the rate of muscle ATP utilisation is higher than the rate of ATP regeneration, which leads to accumulation of ADP and AMP. In addition to creatine kinase mediated ATP regeneration, ADP may be rephosphorylated to ATP through the myokinase reaction. This leads to increased AMP production. To avoid large accumulations of AMP within the cell, AMP is deaminated to IMP by the enzyme AMP deaminase [7,8]. Further deamination leads to purine catabolism (Figure 2) [14] .
Purines and pyrimidines make up the two groups of nitrogenous bases. These bases are crucial components of deoxyribonucleotides and ribonucleotides, and form the basis for the universal genetic code. The general term purine also refers to substituted purines and their tautomers such as adenine, guanine, hypoxanthine, xanthine and uric acid. In humans, uric acid is the end product of purine catabolism. Uric acid is eliminated by two routes: about a quarter to a third of the uric acid produced daily enters enteric secretions and is destroyed by intestinal flora (intestinal uricolysis); the remaining two-thirds to three-quarters are excreted in urine [15].
Uric acid (UA) accumulation from adenine nucleotides is of importance when the energy balance is critical and sustained. Due to the relative speed of the uricase reaction, uric acid is the metabolite that suffers the biggest increase when the metabolic pathway is overloaded in nonmaximal exercises [16-18].
It is suggested that the UA increments, which reflect exhaustion of energy resources, are related to a loss of plasmatic membrane selectivity followed by cellular damage. In horses that are maximally exercised, there is a high correlation between UA, CK and lactate production[16].

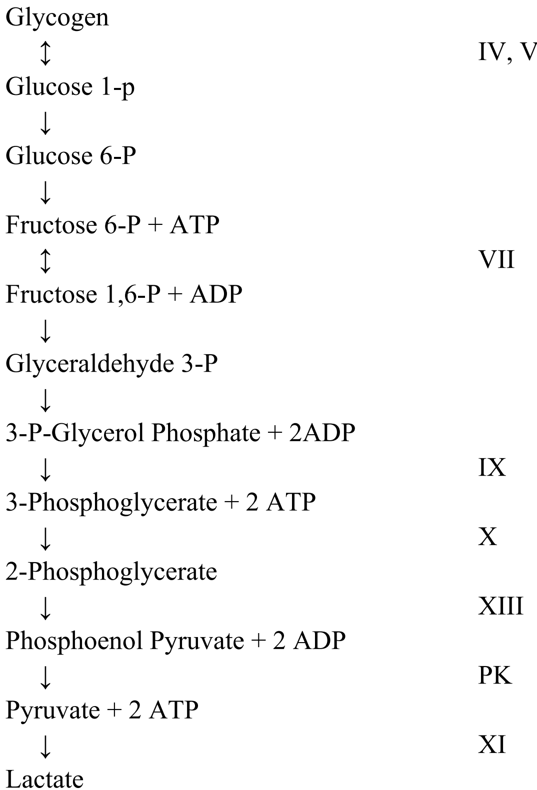
Muscle exercise to a degree that does not exceed the anaerobic threshold is also the threshold for the acceleration of purine nucleotide degradation [19]. Higher concentrations of purines and pyrimidines are expected to be found if this threshold is exceeded.
Our hypothesis is the biggest increase in UA concentration will be seen in equine patients with the highest CK activity and lactate concentration.

Figure 2: Purine catabolism



Carbohydrate metabolism
Under rested and fed conditions, the enzymes for glycogenolysis are in an inactive form so that glycogen will remain stored. During exercise, prolonged fasting or disease, glycogenolysis enzymes are necessarily active. Figure 3 shows the glycogen metabolism and glycolysis. In Table 4 the names of the analysed enzymes can be found.
Rhabdomyolysis, as present in the muscles of patients, can be an energy demanding illness. Our hypothesis is that glycogen concentrations will be decreased in muscle because glycogenolysis and glycolysis are activated. Concentrations of enzymes are expected to show only small changes.

Figure 3: Glycogen metabolism and glycolysis. The roman numerals refer to glycogen storage disease resulting from deficiencies of the various enzymes that were analysed in this study.



Oxidative phosphorylation

One of the functions of the mitochondrion is oxidative phosphorylation in the respiratory chain. NADH originates mostly from the TCA cycle and is oxidized by complex I (NADH-ubiquinone oxidoreductase). $FADH_2$ also originates from the TCA cycle but most $FADH_2$ is generated through beta-oxidation of fatty acids and oxidized by complex II (succinate-ubiquinone oxidoreductase). The redox reactions in complexes I, III (ubiquinol-cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase) generate a proton gradient across the inner mitochondrial membrane that drives complex V (=ATP synthase), creating energy necessary for functioning [14]. The localization of this system of enzymes is in the inner mitochondrial membrane.

At rest and in steady-state exercise, oxidative phosphorylation meets most energy demands. With exercise, the rate of oxidative phosphorylation increases so that the ATP supply meets ATP demand, but there is a transition time in which ATP supply is limited. Inhibition of

mitochondrial respiration by nitric oxide could be partially responsible for the delayed activation of oxidative phosphorylation [20,21].
Phosphocreatine is one of the contributors to ATP in these early seconds of exercise to reduce the effect of this transition time.
It is difficult to predict the effect of rhabdomyolysis on oxidative phosphorylation. Defects in the oxidative phosphorylation can cause rhabdomyolysis themselves.

Materials and methods

Animals

For analysis of creatine and purine metabolism, urine from five horses with a tentative diagnosis of atypical myopathy was used (Tables 1 and 2). The control horses were five healthy warmblood mares, aged 7, 11, 11, 12 and 14 years, respectively.

For analysis of carbohydrate metabolism and oxidative phosphorylation, muscle tissue from the *M. vastus lateralis* of two horses with a tentative diagnosis of atypical myopathy was used (P 2 and P 3 in Table 1 and 2).

Table 1: Details from five horses with a tentative diagnosis of atypical myopathy

Horse number	**Breed**	**Age**	**Gender**	**Clinical course**
P 1	Draft crossbreed	10 years	mare	Survived
P 2	Draft crossbreed	11 years	mare	Death within 1 day
P 3	Pony	1 year	mare	Death within 3 days
P 4	Belgian draft	0.5 year	mare	Death within 1 day
P 5	Standardbred	1.5 years	mare	Death within 3 hours

Table 2: Peak blood values in five horses with a tentative diagnosis of acute myopathy

Horse number	**CK**	**AST**	**LDH**	**Lactate**
P 1	69,000	6,017	26,160	2.5
P 2	400,000	12,376	35,541	5.4
P 3	409,400	2,790	n.a.	11.7
P 4	939,000	n.a.	n.a.	n.a.
P 5	146,600	n.a.	23,250	8,8

Values include creatine kinase (CK, upper limit of reference range 200 IU L^{-1}), aspartate aminotransferase (AST, upper limit of reference range 275 IU L^{-1}), lactate dehydrogenase (LDH, upper limit of reference range 600 IU L^{-1}) and lactate (upper limit of reference range 1.0 mmol L^{-1}). n.a. = not analysed

For carbohydrate metabolism the two control horses were healthy warmblood mares, aged 7 and 10 years, respectively.

For analysis of glycogen, phoshoglycerate kinase (PGK, IX) and phosphoglycerate mutase (PGAM, X), muscle tissue of the *M. vastus lateralis* from an additional five healthy warmblood control horses, aged 9, 9,12, 12 and14 years, was used.
For analysis of oxidative phosphorylation, six healthy warmblood mares were used as control, aged 7, 9, 9, 10, 12 and 14 years.

Creatine metabolism

Chemicals

Guanidino acetate, creatine and creatinine were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). D_3-Creatin and D_3-Creatinine were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). $^{13}C_2$-Guanidinoacetate was obtained from H.J. ten Brink, Ph.D., Free University of Amsterdam. All chemicals used were HPLC-grade or of higher quality.

Sample preparation

Frozen urine samples were thawed at 37°C prior to analysis and mixed with an equal volume of internal standard solution. Samples were deproteinised by the addition of acetonitrile, mixed thoroughly and centrifuged for 10 min at 25000 × *g*. The supernatant was transferred to a clean vial and evaporated to dryness under vacuum. Derivatisation was performed by butylation with N-butanol and acetylchloride for 20 min at 65°C. After evaporation of excess butylation agent, samples were dissolved in acetonitrile and ready for HPLC-MS/MS analysis.

HPLC-MS/MS analyses

All analyses were performed using a Waters Micromass Quattro Micro Tandem Mass Spectrometer equipped with a Waters Alliance 2695 Separation Module HPLC-system (Milford, MA, USA). Samples were separated by reversed-HPLC using a Waters Symmetry C18, 3.5 µm, 2.1 × 100 mm column with a gradient system of ammonium formiate buffer pH 6.7 (0.1 % v/v) and acetonitrile. Relevant compounds were detected in ESI-positive mode using multiple reaction monitoring (MRM).

Purine metabolism

Chemicals

All standards were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). $^{15}N_2$-Uracil, $^{15}N_2$-Thymine, $^{15}N_2$-Orotic acid, ^{13}C-Uridine, ^{13}C-Thymidine, ^{13}C-Adenine, $^{15}N_4$-Inosine, $^{15}N_5$-2'-Deoxyadenosine and $^{15}N_2$-Uric acid were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). $^{13}C_5$-adenosine was purchased from Omicron Biochemicals (South Bend, IN, USA) All chemicals used were of HPLC-grade or higher quality

Sample preparation

Frozen urine samples were thawed at 37°C prior to analysis. Samples were diluted to a concentration of creatinine between 0-2.0 mM and mixed vigorously. Urine (50 µl) was combined with 200 µl of 0.25 M ammonium formiate pH 5.0 and 75 µl of internal standard

solution. The mixture was filtered using a 0.22 µm Costar Spin-X centrifuge tube filter (Costar, Corning, NY, USA) for 5 min at 4°C at 25000 × *g*. The resulting filtrate was ready for HPLC-MS/MS analysis.

HPLC-MS/MS analyses

All analyses were performed using a Waters Micromass Quattro Micro Tandem Mass Spectrometer equipped with a Waters Alliance 2695 Separation Module HPLC-system (Milford, MA, USA). The samples were separated by reversed-HPLC using a Waters Xterra RP18, 3.5 µm, 2.1 × 100 mm column with a gradient system of a 0.05 M ammonium formiate buffer pH 5.0 and acetonitrile. Purines were detected in both ESI-positive mode and ESI-negative mode using multiple reaction monitoring (MRM).

Carbohydrate metabolism

Chemicals used were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Enzymes were obtained from Hoffman-La Roche (Nutley, NJ, USA). Tissue glycogen content was determined in homogenates using a previously described enzymatic method with minor modifications [22]. Phosphorylase activity was measured in the reversed direction in the presence of absence of AMP by coloric measurement of the inorganic phosphate released from glucose-1-phosphate essentially according to Maire et al.[23]. Branching enzyme activity was measured as described by Howell et al, adapted for leucocytes and with the use of purified fosforylase b in the presence of AMP instead of fosforylase a [24]. The activities of the glycolytic enzymes were measured by kinetic spectroscopic methods mostly coupled to the formation or disappearance of NAD(P)H according to standard methods as described by Beutler [25].

Oxidative phosphorylation

Tissue homogenates were prepared from frozen muscle in 0.25 M sucrose, 10 mM N-[2-hydroxyethyl] piperazine-N'-[2 ethylsulfonic acid](HEPES) and 1 mM Ethylene diamine tetra-acetic acid (EDTA), pH 7.4. Enzyme activities were measured in the frozen homogenates. Citrate synthase, a measure of the amount of mitochondria, was measured according to Srere (1969) [26]. Activities of the complexes of the mitochondrial respiratory chain were measured in muscle homogenates by spectrophotometric methods as described by Scholte et al. [27,28]. Complex I or NADH-Coenzyme Q reductase was measured kinetically by following the rotenon sensitive decrease in the amount of NADH. Complex II or Succinate Coenzyme Q reductase was measured by following the TTFA dependent reduction of DCPIP after addition of succinate. Complex III or Ubiquinol-cytochrome c reductase was measured by following the Antimycin A sensitive reduction of cytochrome c by reduced decylubiquinol as described by Trounce et al. [29]. Complex IV or cytochrome c oxidase was measured by following the formation of oxidized cytochrome c. Complex V or ATP synthase was measured as the oligomycin sensitive Mg-ATPase [27,28].

Results

Creatine metabolism

A clear difference in the creatine excretion was found between patients and controls. The creatine concentration in urine from patients was increased from 6 times the control level in patient P2 and P5, to 8 times in P1 and P3, and up to 36 times in P4. In P5, the excretion of creatinine increased as well. In all patients, the creatine/creatinine ratio was increased (Table 3). No hypoxanthine or guanine was detected in patient or control samples.

Table 3: Results of creatine metabolism and purine analysis in μmol/mmol creatinine

	P1	P2	P3	P4	P5	P mean	P SD	C mean*	C SD**	C P 95
Guanidino-acetaat	66	60	66	83	58	67	10	59	37	104
Creatine	1739	674	1890	3609	632	1709	1212	91	76	182
Creatinine	9530	8945	8003	8689	17757	10585	4047	9591	4149	13182
Creatine/creati-nine ratio	1.74	0.67	1.89	3.61	0.63	1.71	1.21	0.09	0.07	0.20
Uric acid	236	137	724	630	86	363	294	84	42	121
Xanthine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.4	0.8
Inosine	1.0	0.0	0.0	0.0	0.0	0.2	0.4	0.0	0.0	0.0
Adenine	0.0	1.0	2.0	1.0	0.0	0.8	0.8	0.8	1.1	2.0
Guanosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.9	1.6
Adenosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.5	1.0

P is patient
C is control
* Mean from 5 healthy control horses
** SD from 5 healthy control horses

Purine metabolism

To investigate the purine catabolism in patients, the excretion of Adenosine, Guanosine, Inosine, Adenine, Guanine, Xanthine, Hypoxanthine and Uric acid was measured. Table 3 shows that the only difference between patients and controls is found in the excretion of uric acid, from nearly normal in P5 to an increase of about 7 times the control level in P3 and P4. The mean concentration of uric acid increased 4 times for the patients.

Carbohydrate metabolism

To evaluate carbohydrate metabolism, we measured both glycogen and the activities of the enzymes involved in the glycogenolysis and glycolysis. From Table 4, it can be seen that patient P3 is most glycogen depleted. Activities of most enzymes involved in glycolysis were increased in P3 compared to controls and the other patient. The most striking result was the very low activity of the enzyme PGAM in patient 2 (less than 10% of reference values).

Table 4: Enzyme activities in muscle from carbohydrate metabolism and oxidative phosphorylation

	Units	**P2**	**P3**	**C mean***	**C SD****	**C P 95**
Glycogen *	µg/mg protein	102	**1**	94	26	134
Phosphoglycerate mutase (PGAM, X) *	nmol/mg protein	**503**	8650	5847	3054	8815
Phosphoglycerate kinase (PGK, IX) *	nmol/mg protein	3790	5930	3733	989	4520
Phosphofructokinase (PFK, VII) *	nmol/mg protein	2210	2010	2355	802	
Phosphorylase (V) *	nmol/mg protein	674	946	621	105	
Phosphorylase +/- AMP #*	nmol/mg protein	2	2	1.7	0.4	
	Units	**P2**	**P3**	**C1**	**C2**	**C P 95**
Enolase (XIII)	nmol/mg protein	735	1420	803	738	800
Pyruvate kinase (PK)	nmol/mg protein	8500	16700	9680	9470	9670
Lactate dehydrogenase (XI)	nmol/mg protein	8420	14100	9030	8170	8987
Branching enzyme (IV)	nmol/mg protein	n.a.	566	651	622	650
	Units	**P2**	**P3**	**C mean^**	**C SD^^**	**C P 95**
Protein	mg/g muscle	78	66	136	46	181
Citrate synthase (CS)	nmol/mg protein	367	295	344	135	535
Citrate synthase (CS)	% of control	107	86	100	39	155
Complex I	nmol/mg protein	71	65	93	28	130
Complex I	% of control	77	70	100	30	140
CI/CS		0,19	0,22	0.28	0.03	0.30
Complex II	nmol/mg protein	42	42	71	39	129
Complex II	% of control	58	59	100	56	182
CII/CS		0,11	0,14	0.20	0.04	0.24
Complex III	nmol/mg protein	177	187	223	36	260
Complex III	% of control	79	84	100	16	116
CIII/CS		0,48	0,63	0.71	0.19	0.91
Complex IV	nmol/mg protein	272	221	307	110	458
Complex IV	% of control	89	72	100	36	150
CIV/CS		0,74	0,72	0.90	0.06	0.97
Complex V	nmol/mg protein	178	89	230	164	472
Complex V	% of control	77	**39**	97	66	195
CV/CS		0,49	0,30	0.62	0.18	0.86

* Mean from 7 healthy control horses
** SD from 7 healthy control horses
^ Mean from 6 healthy control horses
^^ SD from 6 healthy control horses
# This is an indirect measurement for phosphorylase kinase; normally ratio < 3.6
n.a. = not analysed

Oxidative phosphorylation

To assess the functioning of the oxidative phosphorylation, we measured the amount of protein, the amount of citrate synthase, and the activity of the different complexes. Table 5 shows that both patients had a reduced amount of muscle protein. Citrate synthase, CS, an indicator for the amount of mitochondria per muscle is similar for patients and controls, indicating that the oxidative capacity based on protein is not different between the control animals and the patients. Activities of the complexes are compared per amount of protein and based on the amount of CS to make objective comparison possible.

Results show that all complex activities are lower in the patients than in the control horses. The activity of complex I, III and IV of the respiratory chain are about 75% of the activity in controls. The activity of complex II however, a flavin dependent complex, is only 59% of the activity in control horses. The activity of complex V or ATP synthase is also about 75 % in patient 2. In patient 3, complex V activity (ATP synthase) is lowered to 39% of controls. In humans with an ATP synthase deficiency, residual complex V activities can be reduced to as little as 40% of normal values.

Discussion

Results are in agreement with our hypothesis that myopathic patients have higher concentrations of creatine than than healthy control horses. However, if Table 2 and 3 are compared, there is some, but no complete linearity between the plasma levels of CK and the urine creatine excretion or the creatine/creatinine ratio. This may be due to the fact that creatine is not rephosphorylated by the mitochondrial CK in a timely manner because of reduced oxidative phosphorylation capacity in the patients (MADD), as described in chapter four for atypical myopathy patients. Creatine can leave the intact cell whereas CK is only released after rhabdomyolysis.

Creatinine concentration seems to be a response to the changes in renal blood flow rather than a reflection of the emergency metabolism [30], because not much variability in concentration is present in the rhabdomyolysis patients.

As expected, an increase in UA excretion was found in all patients, due to energy resource exhaustion and damage to the cell membrane [31]. However, the hypothesized correlation between UA, CK and lactate was not found. For instance, patient 2 has a lower concentration of uric acid while Table 2 shows that its CK, LDH and AST are much higher. This may be due to the fact that patient 2 has a lower capacity for anaerobic glycolysis and ATP production, leading to an earlier damage to the cell membrane and leakage of CK, LDH and AST. Uric acid is able to leave the intact cell in the other patients. We conclude that creatine and UA excretion, like CK and lactate excretion, can be used as a parameter to indicate myopathy. Which parameter is the most reliable marker for the degree of damage or disease, is not yet clear [32] [31]. Large intersubject variability in the rise in circulating CK

activity exists, indicating that CK alone is not an accurate predictor of skeletal muscle damage [31]. However, if CK activity is increased the cells are damaged, and if uric acid or creatine is increased the cells can still be intact. If myopathy is suspected, combined analysis of CK, UA and creatine recommended.

A remarkable finding was the glycogen depletion in one of the myopathic patients. Almost complete glycogen depletion (1 µg/mg protein compared to 94 µg/mg in control horses) as seen in a myopathic patient in chapter V is rare in horses. Studies of Lacombe et al. (2003) showed that exercise of various durations and intensities resulted in a reduction of up to 60% of the muscle glycogen concentrations in horses [33]. In a study on humans, glycogen was measured after bicycle exercise of varying intensity. Depletion increased with increasing exercise intensity. The percentage of depletion was higher than in the horse [34]. The increased activities of both glycogenolysis and glycolysis in the equine myopathy patient indicate a greater demand for anaerobic energy. This demand, combined with cell damage due to the extreme rhabdomyolysis, probably explains the glycogen depletion in the patient.

Because of the very low activity of the enzyme PGAM in patient 2 (less than 10% of reference values), this patient seems to be PGAM deficient. In human medicine, this condition is known as muscle-specific phosphoglycerate mutase (PGAM-M) deficiency, glycogenosis type X. It is an autosomal recessive disorder resulting in a metabolic myopathy characterised by exercise intolerance and cramps [35,36]. It is a rare disorder in man and it has not been described in equine literature so far. There are three possible explanations for the occurrence of PGAM deficiency in patient 2. The first is that this horse had an inherited form of glycogenosis type X. To find out if inheritance plays a role, both parents of this patient would have to be examined. Both would be expected to have around 50% activity of the enzyme if this were an autosomal recessive disorders. Unfortunately these horses not unavailable for further analyses. The second explanation for PGAM deficiency is that the exogenic toxin that caused the myopathy also damaged PGAM in this horse. It is unclear why the toxin would only affect PGAM activity and not the activity of other glycolytic or glycogenolytic enzymes, unless PGAM-M enzyme is more labile than the other enzymes. Finally, leakage of the PGAM enzyme from the muscle because of the extreme rhabdomyolysis may have occurred. This has been described in Duchenne muscular dystrophy patients in man [37] but so far not in equine patients.

The reduced amount of muscle protein in both patients is possibly a result of damage due to the rhabdomyolysis.

A decrease of ATP synthase activity, as seen in patient 3, would be called a respiratory chain defect in human medicine. Complete loss of ATP synthase activity is probably not

compatible with life, but partial loss reflected by a lower amount of functional ATP synthase has been associated with human disease[38]. This disease seems to have an inherited etiology in humans.
In our patient with reduced activity of complex V, we observed glycogen depletion and increased activities of glycolysis and glycogenolysis enzymes that compensate to meet the energy demands. This horse had to obtain most energy through anaerobic glycolysis, which is reflected by the high lactate concentration. In addition to increased lactate levels, we found an induction of enzyme activities in glycolysis and glycogenolysis. Anaerobic pathways are very often induced when enzyme activities in aerobic oxidative phosphorylation pathways are reduced for prolonged periods. Therefore, patient 3 may have a respiratory chain complex V deficiency in addition to MADD as a result of the toxin.

Conclusion

Using the analyses described in this chapter, combined with analyses described in chapters 2, 3 and 4, a wealth of information on a variety of metabolic pathways in myopathic patients can be obtained. A single patient can suffer from multiple metabolic disturbances. The challenge is to find out which problem is primary and which secondary. Examples of general metabolic indicators for myopathic damage are CK, lactic acid, creatine and UA. For more detailed information on the cause of the myopathy, metabolic analysis of carbohydrate, lipid and protein metabolism as well as oxidative phosphorylation is recommended.

In the authors' opinion it is still not possible to state the exact main cause of all metabolic defects of the patients with atypical myopathy. It is possible that the presumed exotoxin that seems to have caused MADD also caused other enzyme deficiencies, such as PGAM deficiency or Complex V deficiency, that were found in this study. The low activities of complex II may be due to MADD itself, because flavin is needed in complex II. The observed MADD may be due to a lowering in FAD because the ETF enzyme activities were normal. Alternatively, the toxin caused MADD leading to extensive rhabdomyolysis, which in turn caused other metabolic pathways to be disturbed, predominantly in fat oxidation. The lowered PGAM activity observed in patient 2 may well be due to the rhabdomyolysis.

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Chapter 6

The influence of exercise, training and intensified training on plasma concentrations of amino acids in Standardbreds

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Abstract

The objective of this study is to assess the influence of acute exercise, training and intensified training on the plasma amino acid profile of horses. In a 32-week longitudinal study using ten Standardbred horses, training was divided into four phases, including a phase of intensified training for five horses. At the end of each phase, a standardised exercise test, SET, was performed. Plasma amino acid concentrations before and after each SET were measured. Training significantly reduced mean plasma aspartic acid concentration, whereas exercise significantly increased the plasma concentrations of alanine, taurine, methionine, leucine, tyrosine and phenylalanine and reduced the plasma concentrations of glycine, ornithine, glutamine, citrulline and serine. There was no statistically significant difference in plasma concentration of various amino acids between normally and intensified trained horses. To our knowledge, this is the first report on quadruplicate longitudinal monitoring of the almost complete equine plasma amino acid panel.

Keywords

Horse, Standardbred, exercise, training, amino acids

Introduction

Amino acids serve as the monomer units from which the polypeptide chains of proteins are constructed. Much attention has been paid to amino acid supplementation in humans and horses in association with training and/or exercise. Protein metabolism is suggested to comprise 5-15% of the energy expenditure during exercise [1]. Since storage of large quantities of free amino acids does not occur in the muscle cells, amino acids are mainly stored as structural/functional proteins. If necessary, as in exercise, they can rapidly be decomposed into amino acids and transported via the blood to other tissues [2].

Plasma levels of free amino acids either from diet, protein breakdown or carbohydrates and fatty acid metabolites that are synthesized from amino acids via transamination reactions, are in a dynamic equilibrium that is altered by daily protein intake, protein accretion in the body mass and physical activity [3].

The effect of exercise on the concentration of amino acids in blood has been investigated intensively in horses using a wide variety of exercise intensities and durations, from long-lasting low intensity exercise like an endurance race to short-lasting high intensity exercise like our standardised exercise test [3-14]. In contrast to the present study, in which a large panel of amino acids was analysed, most of the studies mentioned above only investigated the effects of exercise on a few parameters of apparent importance. For instance, medium-lasting low-intensity exercise has been shown to increase overall free amino acid serum concentrations as a result of mobilisation, whereas long-lasting low-intensity exercise has been shown to decrease these concentrations as a result of amino acid catabolism [3].

In man and horse, much research has been performed to find an ideal training method that would improve performance capacity while avoiding overtraining. Intense training methods are now widely accepted in both human and equine practice. Overtraining is a state of prolonged fatigue that is caused primarily by an imbalance between training and recovery. Overtraining, also known as intensified training or overload training, is distinct from overreaching, which is a relatively short-term state of fatigue that lasts 2-6 weeks and that is associated with the increases in exercise load that are common in most training programs [15]. Several amino acids are related to fatigue, e.g. glutamine, tryptophan, tyrosine and the branched-chain amino acids (BCAA) valine, leucine and isoleucine [3,4,7,14,16-19]. The cause of fatigue is complex, influenced by events occurring in both the periphery and the central nervous system (CNS) [20]. The central fatigue hypothesis is a theory based on the observation that exercise promotes an increase in plasma free fatty acids (FFAs) and a decrease in plasma large neutral amino acids (LNAA), such as leucine, methionine, valine, phenylalanine and tyrosine, due to uptake by skeletal muscle. As both conditions favour tryptophan entering the CNS, increased production of brain serotonin might be expected. This could account for the decreased motor drive and increased

sensation of fatigue, as experienced after a period of strenuous exercise. However, there is much controversy about the role of tryptophan in central fatigue[17].

To date, it is impossible to diagnose human and equine overtraining and overreaching, at least in part due to lack of suitable diagnostic tools, variable results in previous studies, a lack of well controlled studies, and variations in individual responses to training [21]. A possible basis for the development of such a diagnostic tool seems to be the multiple assessment of the plasma concentration of various amino acids in relation to long-term training and intensified training. The objective of this study is to assess the influence of acute exercise, training and intensified training on the plasma amino acid profile with special reference to large neutral amino acids.

Materials and methods

Animals

Ten Standardbred geldings aged 20 ± 2 months (mean ± SD) and weighing 384 ± 42 kg (range: 331-485 kg) were used. Horses were housed individually. Their diet consisted of grass silage supplemented with concentrate according to the daily estimated energy requirements of 58 MJ NE (range 54-66). Salt blocks and water were available ad libitum. During the intensified training period, a supplement with vitamin E, selenium, electrolytes and other vitamins was added to the diet of all horses (Pavo-Eplus[a] and Equitop® Forte[b]). All horses had a body condition score between 4 and 5 during the study [22].

Prior to commencing training, horses were acclimated to exercising on a treadmill[c] for 4 weeks. They were trained for 24 weeks and detrained for 4 weeks. All training and exercise tests took place on a high-speed treadmill. At the end of each training phase, a SET was performed. Prior to the SETs, food was withheld for approximately one hour. The horses were divided into pairs based on age. Of each pair, one horse was randomly assigned to the intensified training program (IT) and the other horse served as an age matched control (C).

Training protocol

Figure 1 shows the training and testing schedule.

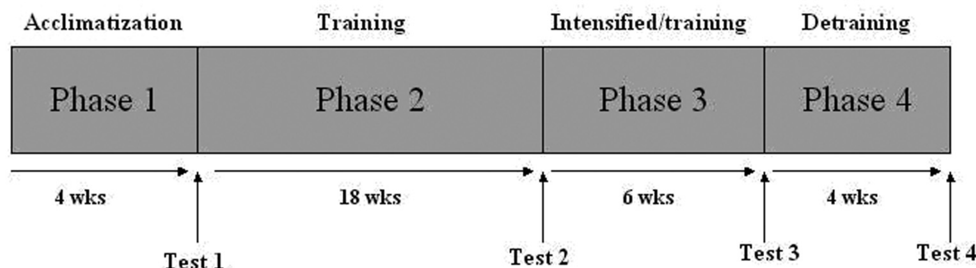

Figure 1. Schedule of the study

Monitoring training

To standardise training to the individual exercise capacity of the horses, training and exercise intensity were adapted to the maximal heart frequency as based on horses that successfully performed an incremental exercise test on a high-speed treadmill reaching a plateau in maximal heart frequency. The incremental exercise test started with a warm-up. Horses then trotted for 2 mins at 5 m/s, followed by 2 mins at 6 m/s. Intensity was increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed at which the horse could not keep up with the treadmill despite human encouragement. Heart frequency was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0[d] and heart rate meter Polar S610i[e]).

Training was divided into four phases (Figure 1): During Phase 1, the horses were introduced to the high-speed treadmill for four weeks. Each exercise session was preceded by a 30 min warm-up in a walking machine followed by an 8 min warm-up on the high-speed treadmill. The latter consisted of 4 min at 1.6 m/s and 4 min at 3.0-4.0 m/s, with no incline. The program during Phase 1 consisted of endurance exercise: week 1, 30% HF_{max} for 20-30 min 3 days/week; week 2, 30% HF_{max} for 25-45 min 4 days/week; week 3, 40% HF_{max} for 30-45 min 4 days/week; and week 4, 50% HF_{max} for 35-45 min 4 days/week. Each exercise session ended with a cooling down period, consisting of a 5 min walk on the treadmill followed by a 30 min walk in the walking machine.

During Phase 2, horses received an 18 week training program of mixed endurance training (ET) and high intensity training (HIT) [23]. Days of ET were alternated with days of HIT. Each training session was preceded by a 30 min warm-up in the walking machine followed by an 8 min warm-up (4 min at 1.6 m/s and 4 min at 4.5 m/s) on the treadmill. The endurance running included 20-24 min of continuous speed running at 60% HF_{max} or 16-18 min at 75% HF_{max}. The interval training included three 3-min bouts at 80-85% HF_{max} or

four 2-min bouts at 80-85% HF_{max} interspersed with 3-min or 2-min periods at 60% HF_{max}. Each training session ended with a cooling down session consisting of a 5 min walk on the treadmill followed by 30 mins walk in the walking machine. The horses exercised 4 days/wk throughout Phase 2.

During Phase 3, the control group continued training for six weeks at the volume and intensity they received in Phase 2. For the IT regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks followed by 7 days/week for the last 3 weeks according to a validated protocol in Standardbreds for overtraining that is characterised by abolishment of resting days [24]. Each training session was preceded by a 30 min warm-up period in the walking machine followed by an 8 min warm-up (4 min at 1.6 m/s and 4 min at 4.5 m/s) on the treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 min at 60-75% HF_{max}. High intensity exercise gradually increased to five 3-min bouts at 80-85%HF_{max} interspersed with 2-min periods at 60% HF_{max} or six 2-min bouts at 80-85% HF_{max} interspersed with 1-min or 2-min periods at 60% HF_{max}.

In Phase 4, the horses received a 4 week detraining program of light endurance exercise. The horses performed endurance training for 20 min at 60% HF_{max} for 3 days and 70% HF_{max} for 1 day a week.

On the resting days in all phases, the horses walked for 60 minutes in the walking machine.

At the end of each phase, a SET (SET 1,2,3 and 4 respectively), was performed at ~ 80% HF_{max} (equivalent to 7.5-8.5 m/s and 1-4% incline) for 20 minutes. Heart rate was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0[d]) and heart rate meter (Polar S610i[e]) during the SET.

Horses were trained based on individual fitness levels, the goal being to keep them at a work rate of about 80% of their HF_{max}. Heart rate was monitored daily to ensure that they were working at the proper level and every week the speed or duration of exercise was adjusted based on those results.

Sample collection

Blood for analysis was taken immediately before and 60 minutes after ending each SET, and collected in lithium-heparinised tubes. Blood samples were centrifuged for 10 minutes at 6,000 X *g*. Plasma was harvested, stored at –20°C and analysed at the end of the study. Samples from one pair of horses taken prior to the second SET were lost to follow-up.

Biochemical analysis

Amino acids were analysed by means of automated ion-exchange chromatography with post column ninhydrin derivatization. Plasma amino acid analyses were performed on a JEOL AminoTac [8]. All amino acids except tryptophan could be analysed with this equipment. Heparinised blood samples were kept on ice until heparinised whole blood lactate had been analysed [9].

Statistical analysis

The statistical program SPSS was used for statistical analysis [10]. Results during training and detraining were compared using a two-way repeated-measures analysis of variance (ANOVA) with 'training group' as between subject variable and 'before/after' and 'time' as a repeated-measures factor. Post hoc tests with Bonferroni correction were performed where F-values were significant (P<0.05). The significance of differences between groups in trotting time during SET 3 was assessed by the independent t-test. Results are presented as means ± SD. P-values < 0.05 were considered significant.

Results

SETs were performed throughout the study to monitor performance. A right shift of the lactate curve during SET 3 compared to SET 2 was observed in both groups (Figure 2). The right shift of the lactate curve was still observed during SET 4 for the intensified trained group but not for the control group.

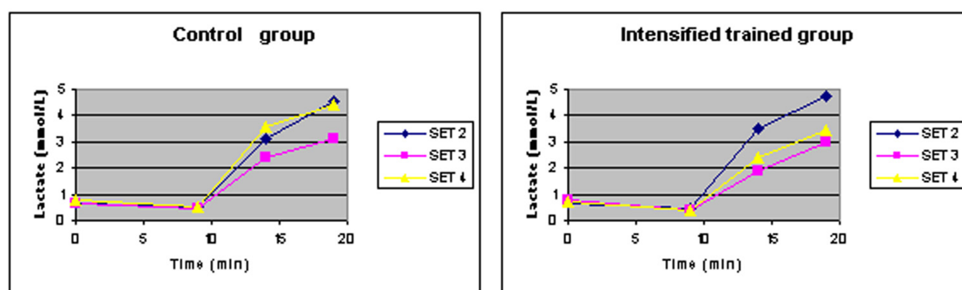

Figure 2. Lactate curves of standardised exercise test (SET) 2, 3 and 4 presented as mean concentrations at the first 10 minutes of trot (t = 9, 14 and 19 mins) in the control or intensified trained group. The first 9 min of the SET consist of a warming up.

A right shift of the lactate curve during SET 3 compared to SET 2 was observed for the IT group, with a significant difference in blood lactate concentration at t=14 min (P = 0.034), t = 19 min (P = 0.043) and t = 24 min (P = 0.011) compared with control horses (data not

shown). Heparinised blood lactate concentrations during SETs did not differ significantly between Phases 3 and 4. The IT horses maintained trotting at high speeds during SET 3 for 16.1 ± 2.3 min as compared with 19.8 ± 0.4 min in control horses. The mean duration of trotting during SET 3 was decreased significantly ($P = 0.012$) by 19%in the IT group as compared with control horses, indicating early overtraining.

To assess the effect of training, the results for all amino acids before and after all SETs were compiled (Table 1). Results for amino acids that fell below detection limits, i.e. hydroxyproline, alpha-amino butyric acid and cysteine, were excluded.

The results of all SETs indicate that there was no statistically significant difference between the control group and the intensified training group. Therefore, results of both groups were combined.

A statistically significant effect of training ($P = 0.014$) was only found for aspartic acid, the concentration of which decreased significantly throughout the whole experimental period.

Exercise significantly increased the plasma concentrations of alanine ($P = 0.001$), taurine ($P = 0.013$), methionine ($P = 0.022$), leucine ($P = 0.038$), phenylalanine ($P = 0.039$) and tyrosine ($P = 0.041$), whereas exercise significantly reduced the plasma concentration of glycine ($P = 0.001$), ornithine ($P = 0.003$), glutamine ($P = 0.004$), citrulline ($P = 0.005$) and serine ($P = 0.018$) over all SETS.

Table 1: Mean ± SD of free amino acid concentrations in blood plasma (µmol/l) before and after the standardized exercise test for treatment and control groups combined.

PLASMA	SET 1 before	SET 1 after	SET 2 before	SET 2 after	SET 3 before	SET 3 after	SET 4 before	SET 4 after
taurine	40±10	43±13	41±10	47±9	39±8	49±14	39±7	44±10
aspartic acid	16±5	14±3	12±2	12±3	11±4	11±2	11±3	10±2
threonine	115±37	95±24	79±11	88±15	85±29	93±29	85±22	84±20
serine	259±48	229±45	281±68	230±57	235±58	216±54	236±47	205±49
asparagine	22±6	26±10	34±6	28±4	30±12	30±13	35±11	29±9
glutamic acid	58±20	48±20	44±6	42±8	45±11	39±11	48±6	37±6
glutamine	392±62	362±69	431±35	394±30	416±74	392±68	422±86	384±59
proline	200±88	157±57	150±23	153±29	139±34	154±34	132±30	128±28
glycine	560±95	500±68	523±42	467±69	491±107	462±8	516±56	475±52
alanine	212±57	261±74	208±45	321±56	194±45	297±64	177±37	237±67
citrulline	139±26	126±19	132±27	123±25	126±19	119±13	130±21	123±15
valine	230±38	211±42	185±22	191±21	183±41	200±38	198±56	207±38
methionine	26±4	25±6	26±3	30±6	25±4	31±6	27±5	28±5
isoleucine	73±19	67±15	57±33	58±12	58±19	64±17	62±21	61±19
leucine	114±32	123±24	98±55	113±21	96±29	121±32	112±31	120±31
tyrosine	61±12	70±13	70±40	77±16	65±15	78±19	73±18	76±22
phenylalanine	66±11	72±10	70±39	75±14	64±11	76±14	70±14	73±15
ornithine	76±15	69±13	77±44	66±13	72±13	64±12	74±12	66±14
histidine	78±14	83±18	78±48	81±9	86±17	83±16	90±14	81±11
lysine	91±23	88±13	76±44	82±18	81±25	86±20	78±24	74±20
arginine	110±23	115±21	124±68	115±22	121±20	116±21	123±22	109±17

Results for amino acids that fell below the detection limits have been excluded (hydroxyproline, alpha-amino butyric acid and cysteine).
All amino acids except tryptophan could be analysed with the available equipment.

Discussion

The right shift of the lactate curve during SET 3 compared to SET 2 that was observed in both groups possibly indicates an adaptation to long-term training (Figure 2). The right shift of the lactate curve was still observed during SET 4 for the intensified trained group but not for the control group. This might indicate that the IT group adapted better to training than the control group as a result of the intensified training program during Phase 3. Alternatively, the right shift may be a manifestation of the lactate paradox, which is seen in the parasympathetic form of overtraining [25, 26]. The lactate paradox may have been seen in the IT group as a result of overtraining if the recovery phase was not long enough to compensate for the overtraining effects.

This is, to the best of our knowledge, the first study that investigated the longitudinal effect of long-term training and intensified training on a large panel of plasma amino acid concentrations. Our results showed that only plasma concentrations of aspartic acid decreased significantly during a training period.

Aspartic acid is a glycogenic amino acid, converted by transamination to oxaloacetate, which is one of the intermediates of the citric acid cycle. It has an important function in energy metabolism during sustained exercise and intense training [27,28]. The effect of aspartic acid is said to be an increase in FFA concentration and a decrease of lactate concentration [29,30].The effects of exogenous supplementation of aspartic acid on endurance performance in man has been investigated by Abel et al. [29]. In their study, aspartate, the salt of aspartic acid, did not lead to improved endurance performance [29]. In contrast, studies with rats supplemented with amongst others aspartate, did improve performance through a reduction in muscular fatigue and an increase in maximal oxygen uptake [31]. The findings in rats suggest that further research on the possible beneficial effect of supplementing aspartic acid to horses in training might be of interest.

The amino acids predominantly involved in energy metabolic processes are alanine and the BCAAs leucine, valine and isoleucine. Alanine increased in all of our SETS, as in studies by others [6-9,13,32]. During exercise, alanine is synthesised in muscle and other tissues from other amino acids or by transamination of pyruvate derived from glucose. The *de novo* synthesis of alanine explains why the output of alanine during exercise is larger than its concentration in muscular protein [9]. Meanwhile, BCAAs are required for the production of alanine via transamination reactions [33]. Earlier studies have shown that exercise mobilizes BCAA from muscle tissue resulting in a rise in plasma concentrations, which indicates that protein degradation is higher than its rate of utilisation. By contrast, prolonged exercise gives rise to greater utilization, which reduces the plasma concentration [4,5]. Excessive oxidation of BCAA may lead to fatigue by depleting the glutamate and ketoglutarate pool, which decreases the influx into the citric acid cycle [13].

Amino acids involved in the urea cycle, which allows for the disposal of excess nitrogen, are ornithine, citrulline and arginine. In the present study, we found decreased

concentrations of these three amino acids. Although the analysis of amino acid concentrations in plasma does not say much about the concentrations in the liver or the function of the urea cycle, the decrease in plasma levels might be a reflection of shortage as a result of the exercise-induced protein breakdown.

The decrease of glutamine in intense exercise that was observed in this study, possibly reflects an increased use for tissue repair. In addition, it could be a result of increased activity in glutamine dependent tissues, including cells of the immune system. Alternatively it may reflect decreased synthesis with utilisation remaining constant [11]. Glutamine is utilized at a very high rate by cells of the immune system and it is considered to be an important source of fuel for these cells. The major source of glutamine is skeletal muscle. BCAA are thought to be an important source of nitrogen for glutamine synthesis in muscle and they have been shown to stimulate the rate of glutamine release from muscle. The immunosuppressive effect of a single bout of exercise or the overtraining syndrome could be caused, at least in part, by a decrease in plasma glutamine level. The mechanism for the decrease in plasma glutamine concentration is not known. In the study of Parry-Billings et al (1992) glutamine concentration decreased only after long lasting exercise [34]. Miller-Graber et al. (1991) and Routledge (1999) however, found that intense exercise caused a very short period (minutes) of increase of glutamine concentration with decrease after this period [7,11]. This is in accordance with our findings.

The increase in plasma taurine concentration might be associated with damaged muscle cells. Plasma taurine concentrations could be used as a biochemical indicator of early muscle damage [35]. The increase in plasma methionine might be due to enhanced adrenaline production in acute exercise [27]. Glycine, the smallest amino acid, is of importance in the production of creatine and as a consequence might be expected to decrease following acute exercise [2,36-38]. Finally, serine, which plays a role in purine synthesis, can be expected to be decreased given the need for DNA repair in muscle tissue following a bout of acute exercise [37,38].

Factors of potential influence on the amino acid concentration in this study include the feeding regimen or age of the horses, the decision to analyse amino acid concentration from blood and not from muscle tissue and the time of blood sampling. In our study, feeding was standardised and the results are unlikely to be affected by nutritional aspects. [39]. The influence of growth is not apparent, because a longitudinal change in amino acid concentration is not present.

In our study, the concentration of amino acids was analysed in plasma and not in muscle. Because there is no storage depot for amino acids to act as a buffer between the structural molecules in muscle and circulating plasma pool, differences between the production, use, or excretion of amino acids are reflected by changes in the concentrations of amino acids in plasma [40]. This fact and the ease of plasma collection and analysis determined our choice.

The time of blood sampling relative to exercise may have affected our results. In previous studies, various sampling times have been used, and it seems that concentrations vary

greatly in the first minutes and hours after exercise. Bergero et al. (2005), Assenza et al. (2004) and Vervuert et al. (2005) collected blood samples immediately after exercise. Essen et al. (2002) sampled 30 to 60 minutes after exercise and Trottier (2002) during, immediately after and 24, 48 and 72 hours after exercise [3-5,13,14]. These differences make comparison between the different studies difficult.

Overtraining has been defined as an accumulation of training and/or non-training stress resulting in long-term decrease in performance capacity with or without related physiological and psychological signs and symptoms of overtraining. Restoration of performance capacity may take several weeks or months [21]. The intensively trained group in the current study showed a 19% reduction in performance compared with the control horses [11]. The drop in performance seen in the current study is similar to the average reduction in treadmill run-time-to fatigue of 14% as reported by others [41]. Intensified training did not result in alterations in amino acid concentrations in general or large neutral amino acids in particular. As a consequence, no particular marker for intensified training could be identified and intensively trained horses were unlikely to be associated with defective protein metabolism. The drop in performance was possibly due to overtraining at another level than the amino acid metabolism, such as the mental or endocrine level. Unfortunately, the role of tryptophan could not be studied because all amino acids except tryptophan could be analysed with the available equipment.

Conclusion

The training schedule in this study significantly reduced mean plasma aspartic acid concentration, whereas acute exercise significantly increased the plasma concentrations of alanine, taurine, methionine, leucine, tyrosine and phenylalanine and reduced the plasma concentrations of glycine, ornithine, glutamine, citrulline and serine. Based on the current study, further research into the beneficial effects of supplementation of these amino acids on training and bouts of acute exercise seems warranted.

No amino acid, however, presented itself as an obvious candidate for an overtraining marker. To our knowledge, this is the first report in the literature of quadruplicate longitudinal monitoring of the almost complete plasma amino acid panel in the equine species.

Footnotes

[1]Pavo, Boxmeer, the Netherlands
[2]Boehringer Ingelheim, Alkmaar, the Netherlands
[3]Treadmill, Kagra, Graber A, Fahrwangen, Switzerland.
[4]Cardio Perfect Inc, Atlanta, GA, USA
[5]Polar 610i, Polar Electro Oy, Kempele, Finland.
[6]Polar 810i, Polar Electro Oy, Kempele, Finland.
[7]Hettich zentrifugen, Tuttlingen, Germany.
[8]JEOL AminoTac JLC-500/V, Tokyo, Japan.
[9]ABL-605 Radiometer Copenhagen, Westlake, Ohio, USA
[10]SPSS Version 12 for Windows Inc., Chicago, USA
[11]Thesis: De Graaf-Roelfsema E. Endocrinological and behavioural adaptations to experimentally induced physical stress in horses. *Faculty of Veterinary Medicine*. Utrecht: Utrecht University, 2007

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Chapter 7

Plasma acylcarnitine and fatty acid profile during exercise and training in Standardbreds

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Abstract

Objective
The current study evaluates the hypothesis that alterations in skeletal muscle carnitine metabolism during exercise and/or training lead to changes in plasma concentrations of acylcarnitines.
Animals
Ten Standardbred geldings aged 20 ± 2 (SD) months with a mean bodyweight of 384 kg were used.
Procedures
In a longitudinal study of 32 weeks, training on a treadmill was divided into four phases: acclimatisation for 4 weeks, 18 weeks with alternating endurance and intensity training, increased training volume and intensity for another 6 weeks and detraining for 4 weeks. In Phase 3, the horses were randomly divided into two groups: control and intensified training. At the end of each phase, a standardized exercise test was performed. Acylcarnitine profile before and one hour after each SET was assessed.
Results
Concentrations of lactic acid, total nonesterified fatty acids, 3-OH-isobutyric acid, and C2-carnitine significantly increased in all SETs following exercise, whereas β-hydroxybutyric acid, C3- and C4-carnitine were significantly decreased. No significant effect of (intensified) training was found.
Conclusions
Exercise was associated with alterations in plasma concentrations of short-chain acylcarnitines. In the equine species, carnitine forms esters predominantly with short- and medium-chain fatty acids. Despite decreased ketogenesis as reflected by a lower level of β-hydroxybutyric acid, the ketogenic pathway activity using valine was increased following exercise.
Clinical Relevance
Our findings suggest that plasma acylcarnitine profiles in the equine species reflect skeletal muscle carnitine metabolism following exercise, which may provide a practical method to investigate potential disorders in carnitine metabolism in horses suffering from myopathy.

Keywords

lipid metabolism, sports medicine equine

Introduction

In humans, skeletal muscle carnitine metabolism changes with exercise in normal subjects. High-intensity exercise was characterized by an increase in acylcarnitines and a decrease in free and total carnitine in vastus lateralis muscle [1]. In addition, exercise in humans has been associated with increases in the plasma concentration of acylcarnitines and the urine excretion of free carnitine [2,3].

L-carnitine, a betaine derivative of β-hydroxybutyrate, is found in virtually all cells of higher animals and also in some microorganisms and plants. In animals, it is synthesized almost exclusively in the liver. Carnitine is released into the circulation by the liver primarily as acetylcarnitine (C2) and the actual ester pattern is a result of the uptake/release action of peripheral tissues. Two essential amino acids, i.e. lysine and methionine, serve as primary substrates for its biosynthesis. The primary biochemical function of carnitine is related to its ester-forming capability which plays a role in β-oxidation of the long-chain fatty acids. For the transfer of activated long-chain fatty acids across the inner mitochondrial membrane, acyl-CoA esters are transesterified to form acylcarnitine. Short-chain acylcarnitines (acyl groups less than 10 carbon atoms) are formed from, and are in equilibrium with, the corresponding intracellular short-chain acyl-CoA esters. Thus, changes in the distribution of total carnitine between acylcarnitines and free carnitine reflect similar changes in the acyl-CoA pool [4-7]. Carnitine can also form esters with several short- and medium-chain endogenous or exogenous fatty acids [7-9]. Under some metabolic conditions, carnitine serves as a "buffer" of the metabolically critical mitochondrial acyl-CoA pool [10]. Furthermore, carnitine is used as a therapeutic agent to improve exercise performance [11].

Exercise in two Thoroughbreds resulted in a marked fall in free carnitine and an almost equivalent rise in acetylcarnitine (C2) in middle gluteal muscle [12]. However, to the authors' knowledge, plasma concentrations of various acylcarnitines following exercise in horses have not been assessed. In contrast to other studies, in which total content of short-chain acylcarinitines was measured, the current study addresses the whole acylcarnitine profile monitored in a longitudinal study of 32 weeks duration.

The aim of the current study was to evaluate the hypothesis that alterations in skeletal muscle carnitine metabolism during exercise and/or training lead to changes in plasma concentrations of acylcarnitines.

Materials and methods

Animals

Ten Standardbred geldings aged 20 ± 2 months (mean ± SD) and weighing 384 ± 42 (range: 331-485) kg were used. Horses were individually housed.
Their diet consisted of grass silage supplemented with concentrate according to the daily estimated energy requirements of 58 MJ NE (range 54-66). Salt blocks and water were available *ad libitum*. During the intensified training period, a supplement with vitamin E, selenium, electrolytes and other vitamins was added to the diet of all horses (Pavo-Eplus[a] and Equitop® Forte[b]). All horses had a body condition score between 4 and 5 during the study [13].

Prior to commencing training, horses were acclimated to exercising on a treadmill[c] for 4 weeks. They were trained for 24 weeks and detrained for 4 weeks. All training and exercise tests took place on a high-speed treadmill. At the end of each training phase, a SET was performed; prior to the SETs food was withheld for around one hour. The horses were divided into pairs based on age. Of each pair, one horse was randomly assigned to the intensified training program (IT) and the other horse served as an age matched control (C).

Training protocol

In Figure 1 the schedule of the study is shown.

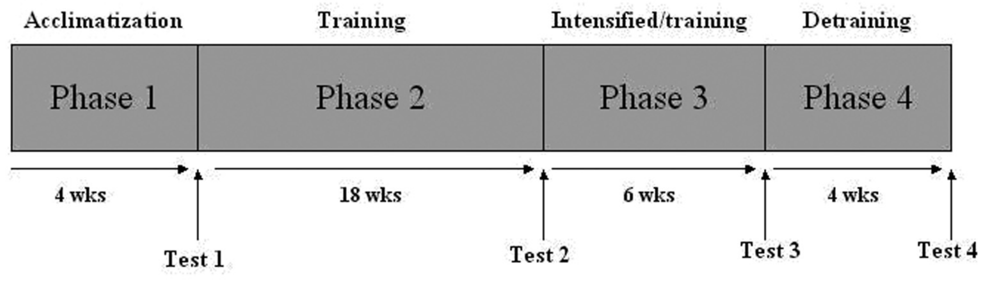

Figure 1. Schedule of the study

Monitoring training

To standardise training to the individual exercise capacity of the horses, training and exercise intensity were adapted to the maximal heart frequency as based on horses that successfully performed an incremental exercise test on a high-speed treadmill until they reached a plateau in maximal heart frequency. The incremental exercise test started with a

warm-up and horses then trotted for 2 mins at 5 m/s, followed by 2 mins at 6 m/s. Intensity was increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed at which the horse could not keep up with the treadmill despite humane encouragement. Heart frequency was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0[d] and heart rate meter Polar S610i[e]).

Training was divided into four phases (Figure 1):
During Phase 1, the horses were introduced to the high-speed treadmill for four weeks. Each exercise session was preceded by a 30 min warm-up in a walking machine followed by 8 mins warm-up on the high-speed treadmill, which consisted of 4 min at 1.6 m/s and 4 min at 3.0-4.0 m/s, with no incline. The program during Phase 1 consisted of endurance exercise: week 1, 30% HF_{max} for 20-30 min 3 days/week; week 2, 30% HF_{max} for 25-45 min 4 days/week; week 3, 40% HF_{max} for 30-45 min 4 days/week; and week 4, 50% HF_{max} for 35-45 min 4 days/week. Each exercise session ended with a cooling down period, consisting of a 5 min walk on the treadmill followed by a 30 min walk in the walking machine.

During Phase 2, the horses received an 18 week training program of mixed endurance training (ET) and high intensity training (HIT) [14]. Days of ET were alternated with HIT. Each training session was preceded by a 30 min warm-up in the walking machine followed by an 8 min warm-up (4 min at 1.6 m/s and 4 min at 4.5 m/s) on the treadmill. The endurance running included 20-24 min of continuous level running at 60% HF_{max} or 16-18 min at 75% HF_{max}. The interval training included three 3-min bouts at 80-85% HF_{max} or four 2-min bouts at 80-85% HF_{max} interspersed with 3-min or 2-min periods at 60% HF_{max}. Each training session ended with a cooling down session consisting of a 5 min walk on the treadmill followed by 30 mins walk in the walking machine. The horses exercised 4 days/wk throughout phase 2.

During Phase 3, the control group continued training at the volume and intensity they received in the second phase for six weeks. For the IT, the intensified training regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks followed by 7 days/week for the last 3 weeks according to a previously validated protocol in Standardbreds for overtraining characterised by abolishment of resting days [15]. Each training session was preceded by a 30 min warm-up period in the walking machine followed by an 8 min warm-up (4 min at 1.6 m/s and 4 min at 4.5 m/s) on the treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 min 60-75% HF_{max}. High intensity exercise gradually increased to five 3-min bouts at 80-85%HF_{max} interspersed with 2-min periods at 60% HF_{max} or six 2-min bouts at 80-85% HF_{max} interspersed with 1-min or 2-min periods at 60% HF_{max}.

In Phase 4, the horses received a 4 week detraining program of light endurance exercise. The horses performed endurance training for 20 min at 60% HF_{max} for 3 days and 70% HF_{max} for 1 day a week.

On the resting days in all phases, the horses walked for 60 minutes in the walking machine.

At the end of each phase, a SET (SET 1,2,3 and 4 respectively), was performed at ~ 80% HF_{max} (equivalent to 7.5-8.5 m/s and 1-4% incline) for 20 minutes. Heart rate was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0[d]) and heart rate meter (Polar S610i[e]) during the SET.
Horses were trained based on individual fitness levels, the goal being to keep them at a work rate of about 80% of their HF_{max}. Heart rate was monitored daily to ensure that they were working at the proper level and every week their program was adjusted (speed or duration) based on those results.

Collection of samples
Blood was sampled from the jugular vein and put in lithium-heparinized tubes immediately before and 60 minutes after each SET. For the analysis of β-hydroxybutyric acid (BHBA), non esterified fatty acids/ total of free fatty acids (NEFA/FFA) and lactic acid blood was collected before, immediately after and 60 minutes after ending the SET in lithium-heparinized tubes (FFA/lactate) and serum tubes (BHBA). Blood for analysis of additional lactic acid was sampled during the SET at t = 9, 14 and 19 mins. Blood samples were centrifuged[f] for 10 minutes at 6,000 x *g*; plasma was harvested and stored at –20°C and analyzed after termination of the study. Blood for analysis of lactic acid was stored on ice and analysed immediately. Samples of two horses of each group were lost to follow-up for the first SET after exercise.

Biochemical analysis
Analysis of FFAs: Identification-analyses were carried out by gas chromatography-mass spectrometry (GC-MS) on a Hewlett Packard 5890 series II gas chromatograph linked to a HP 5989B MS-Engine mass spectrometer. Prior to this GC-MS analysis, the fatty acids were trimethylsilylated with N,N-bis(trimethylsilyl)trifluoracetamide/pyridine/ trimethylchlorosilane (5:1:0.05 v/v/v) at 60 °C for 30 min. The gas chromatographic separation was performed on a 25m x 0.25mm capillary CP Sil 19CB column (film thickness 0.19mm)[g].
Analysis of free and acylcarnitines: Free carnitines and acylcarnitines in plasma were analysed as their butyl ester derivatives by electrospray tandem mass spectrometry (ESI-MS-MS) on a Micromass Quattro Ultima system equipped with an Alliance HPLC system. The coefficient of variation for the determined acylcarnitines was 10-15%.

Plasma total FFA concentrations were measured by use of a commercial kit[h] that had been validated for use in samples obtained from horses. The intra-assay and interassay coefficient of variation (CV) was 7.3% and 16.0%, respectively. Curves obtained with serial dilutions were parallel to the standard curve.
Serum β-hydroxybutyrate concentrations were measured by use of a commercial kit[i] that had been validated for use in samples obtained from horses. The intra-assay and interassay CV was 7.8% and 10.9%, respectively. Curves obtained with serial dilutions were parallel to the standard curve.
Blood lactate concentration was determined with a blood-gas analyser[j].

Statistical analysis

The statistical program SPSS was used for statistical analysis[k]. Results during training and detraining were compared using a two-way repeated-measures analysis of variance (ANOVA) with 'training group' as between subject variable and 'before/after' and 'time' as a repeated-measures factor. Post hoc tests with Bonferroni correction were performed where F-values were significant. The significance of differences in trotting time during SET 3 between both groups was assessed by the independent t-test. Results are presented as means ± SD. P-values < 0.05 were considered significant.

Ethics

The studies were approved by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

Results

A right shift of the lactate curve during SET 3 compared to SET 2 was observed in both groups (Figure 2). The right shift of the lactate curve was still observed during SET 4 for the intensified trained group not for the control group.

Concentrations of FFAs and acylcarnitine esters in plasma collected before and one hour after the SET are shown in Table 1 and 2. Concentrations of FFA, BHBA and lactic acid, all collected before, immediately after and one hour after the SET are shown in Table 3. Because no statistical differences between the control group and the intensively trained group were found, results are presented as mean ± SD of all ten horses. Exercise had a significant effect on the volatile fatty acid 3-OH-iso-butyric acid (Table 1; $P = 0.003$) with higher concentrations 60 minutes after every SET. There was no significant effect of training and exercise on the concentrations of other organic acids.

As shown in Table 2, exercise significantly decreased C3- and C4-carnitine (P = 0.046 and 0.010, respectively), whereas the concentration of C2-carnitine increased

significantly ($P = 0.002$) following exercise. Training did not affect FFAs and acylcarnitine esters.

The concentrations of FFA and lactic acid increased significantly ($P = 0.004$ and $P = 0.000$, respectively) immediately after exercise followed by a significant decrease ($P = 0.000$ for both compounds) one hour after exercise without reaching pre-exercise values ($P = 0.020$ and $P = 0.000$, respectively) (Table 3). In comparison, BHBA concentration decreased significantly ($P = 0.000$) immediately after exercise without reaching pre-exercise values one hour post-exercise ($P = 0.009$). There was no significant effect of training on the concentrations of FFA, BHBA and lactic acid.

The intensively trained horses maintained trotting at high speeds during SET3 for 16.1 ± 2.3 minutes only as compared with 19.8 ± 0.4 minutes in control horses (equivalent with a 19% reduction; $P = 0.012$)[l].

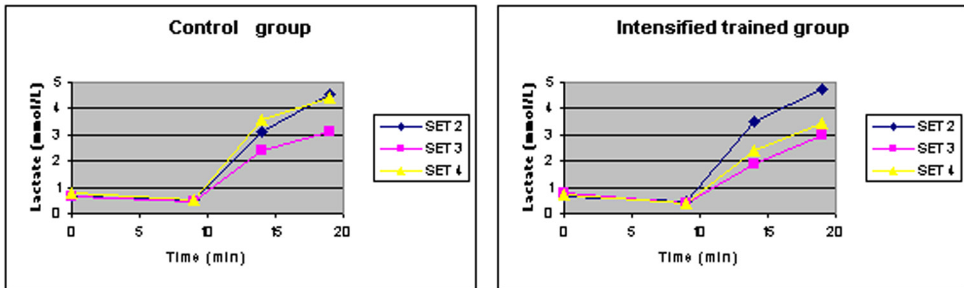

Figure 2. Lactate curves of SET 2, 3 and 4 presented as mean concentrations at the first 10 minutes of trot (t = 9, 14 and 19 mins) in the control or intensified trained group. The first 9 min of the SET consist of a warming up.

Table 1. Fatty acid concentrations in plasma (µmol/L) of horses (n = 10) before and one hour after a standardized exercise test (SET) [32]. Values are expressed as Mean ± SD.

PLASMA	SET 1 before	SET 1 after	SET 2 before	SET 2 after	SET 3 before	SET 3 after	SET 4 before	SET 4 after
2-OH-Butyric acid	4±3	4±2	2±1	6±2	3±1	25±51	2±1	5±3
3-OH-Butyric acid	227±47	243±215	179±35	197±55	231±87	200±62	239±68	218±54
3-OH-Iso-butyric acid *	36±14	42±10	22±4	47±14	27±5	51±13	30±6	44±10
Myristic acid	3±3	7±5	2±1	3±2	2±1	6±10	1±1	2±1
Palmitoleic acid	3±3	4±3	2±1	4±4	2±2	4±2	1±1	3±2
Palmitic acid	25±17	21±21	12±10	22±16	17±12	21±13	14±8	19±15
Oleic acid	26±19	19±20	11±11	21±16	15±13	20±13	12±8	18±15
Linoleic acid	31±20	24±21	19±18	29±19	27±16	31±19	23±14	26±18
Stearic acid	14±7	10±9	9±7	13±8	11±6	13±7	10±6	10±7

*****There was a significant effect of exercise on the volatile fatty acid 3-OH-iso-butyric acid ($P = 0.003$) with higher concentrations 60 minutes after every SET. There was no significant effect of training and exercise on the concentrations of other fatty acids.

Table 2: Free and acylcarnitine concentrations in plasma (µmol/L) of horses (n = 10) before and one hour after a standardized exercise test (SET) [32]. Values are expressed as Mean ± SD.

PLASMA	SET 1 before	SET 1 after	SET 2 before	SET 2 after	SET 3 before	SET 3after	SET 4 before	SET 4 after
Free carnitine	15.12±6.53	11.63±3.11	15.68±5.67	13.38±4.32	16.39±3.70	14.37±2.86	16.63±5.50	15.03±3.80
C-2-carnitine *	2.51±0.70	3.61±1.25	2.59±0.63	4.03±1.28	2.45±0.39	3.99±0.52	2.60±0.42	3.83±0.88
C-3-carnitine *	0.54±0.30	0.45±0.24	0.59±0.32	0.51±0.19	0.56±0.17	0.50±0.13	0.68±0.19	0.56±0.15
C-4-carnitine *	0.41±0.11	0.34±0.11	0.40±0.07	0.39±0.10	0.40±0.06	0.38±0.07	0.47±0.12	0.45±0.10
C5:1-carnitine	0.06± 0.00	0.05± 0.00	0.06± 0.01	0.05± 0.00	0.05± 0.01	0.05± 0.00	0.06± 0.01	0.05± 0.00
C-5-carnitine	0.20±0.05	0.17±0.05	0.20±0.04	0.19±0.05	0.20±0.03	0.20± 0.04	0.22±0.04	0.22±0.03
C4:3-OH-carnitine	0.06± 0.01	0.05± 0.01	0.06± 0.00	0.06± 0.01	0.06± 0.01	0.06± 0.01	0.06± 0.00	0.05± 0.01
C6-carnitine	0.01± 0.00	nd	0.01± 0.00	nd	0.01± 0.00	nd	0.01± 0.00	nd
C5-OH-carnitine	0.02± 0.01	0.01± 0.00	0.02± 0.01	0.01± 0.00	0.03± 0.01	0.01± 0.00	0.03± 0.01	0.01± 0.00
C8-carnitine	0.03± 0.00	0.02± 0.00	0.03± 0.00	0.02± 0.00	0.03± 0.00	0.02± 0.00	0.03± 0.00	0.02± 0.00
C10:2-carnitine	0.04± 0.00	0.04±0.01	0.04± 0.00	0.03± 0.00	0.04± 0.00	0.03± 0.01	0.04± 0.00	0.03± 0.01
C10:1-carnitine	0.04± 0.00	0.03± 0.01	0.04± 0.00	0.03± 0.01	0.04± 0.00	0.03± 0.00	0.04± 0.00	0.03± 0.00
C10-carnitine	0.04± 0.00	0.04± 0.00	0.04± 0.00	0.04±0.01	0.04± 0.00	0.04±0.01	0.04± 0.00	0.04±0.01
C4-DC-carnitine	0.05±0.01	0.04±0.02	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.01
C5-DC-carnitine	0.04±0.01	0.03±0.02	0.05±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
C12:1-carnitine	0.05± 0.00	0.05± 0.00	0.05± 0.00	0.05± 0.01	0.05± 0.00	0.05± 0.01	0.05± 0.00	0.05± 0.01
C12-carnitine	0.06± 0.01	0.05± 0.00	0.05± 0.01	0.05± 0.00	0.05± 0.00	0.05± 0.00	0.05± 0.00	0.05± 0.00
C14:2-carnitine	0.02± 0.00	0.01± 0.00	0.02± 0.01	0.01± 0.01	0.02± 0.01	0.01± 0.01	0.01± 0.01	nd
C14:1-carnitine	0.02± 0.01	0.02± 0.01	0.02± 0.01	0.01± 0.01	0.01± 0.00	0.01± 0.01	0.01± 0.00	0.00± 0.01
C14-carnitine	0.02± 0.01	0.01± 0.01	0.02± 0.01	0.01± 0.01	0.02± 0.01	0.01± 0.01	0.01± 0.01	nd
C8-DC-carnitine	0.01± 0.00	nd	0.01± 0.00	nd	0.01± 0.00	nd	0.01± 0.00	nd
C14-OH-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C16:1-carnitine	nd	0.00± 0.01	nd	0.00± 0.01	nd	nd	nd	nd

PLASMA	SET 1 before	SET 1 after	SET 2 before	SET 2 after	SET 3 before	SET 3after	SET 4 before	SET 4 after
C16-carnitine	0.00± 0.01	0.01± 0.01	0.01± 0.01	0.01± 0.01	0.00± 0.01	0.00± 0.01	0.01± 0.01	0.00± 0.01
C10-DC-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C16:1-OH-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C16-OH-carnitine	nd	0.00± 0.01	nd	nd	nd	0.00± 0.01	nd	0.01± 0.01
C18:2-carnitine	nd	nd	nd	0.00± 0.01	nd	nd	nd	nd
C18:1-carnitine	0.01± 0.01	0.02± 0.01	0.00± 0.01	0.01± 0.01	0.01± 0.01	0.01± 0.01	0.00± 0.01	0.01± 0.01
C18-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C18:2-OH-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C18:1-OH-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C16-DC-carnitine	nd	nd	nd	nd	nd	nd	nd	nd
C18:1-DC-carnitine	nd	nd	nd	nd	nd	nd	nd	nd

*: Exercise decreased C3- and C4-carnitine significantly (p=0.046 and 0.010, respectively), whereas the concentration of C2-carnitine increased significantly (p=0.002) following exercise

nd: not detectable

Table 3: Mean ± SD of total free fatty acids (FFA), β-hydroxybutyric acid (BHBA) and lactic acid concentrations in blood plasma (mmol/l) before, immediately after (T0) and one hour after (T60) the standardized exercise test using 10 Standardbreds.

	FFA	**BHBA**	**Lactic acid**
SET 1 before	0.20±0.10	0.21±0.13	0.78±0.20
SET 1 after (0)	1.01±0.50	0.16±0.04	3.96±1.86
SET 1 after (60)	0.48±0.31	0.18±0.06	1.22±0.34
SET 2 before	0.16±0.12	0.21±0.06	0.64±0.08
SET 2 after (0)	0.67±0.17	0.19±0.03	7.98±2.43
SET 2 after (60)	0.28±0.07	0.21±0.06	1.64±0.54
SET 3 before	0.29±0.46	0.23±0.06	0.64±0.28
SET 3 after (0)	0.41±0.20	0.21±0.08	5.46±2.77
SET 3 after (60)	0.23±0.06	0.22±0.03	1.56±0.61
SET 4 before	0.11±0.04	0.25±0.07	0.73±0.25
SET 4 after (0)	0.60±0.21	0.19±0.04	7.10±4.12
SET 4 after (60)	0.23±0.10	0.22±0.05	1.55±0.77

The concentrations of FFA and lactic acid increased significantly (P = 0.004 and P = 0.000, respectively) immediately after exercise followed by a significant decrease (P = 0.000 for both compounds) one hour after exercise without reaching pre-exercise values (P = 0.020 and P = 0.000, respectively). In comparison, BHBA concentration decreased significantly (P = 0.000) immediately after exercise without reaching pre-exercise values one hour post-exercise (P = 0.009). There was no significant effect of training on the concentrations of FFA, BHBA and lactic acid.

Discussion

The right shift of the lactate curve during SET 3 compared to SET 2, observed in both groups possibly indicates an adaptation to long-term training (Figure 2). However, the right shift of the lactate curve was still observed during SET 4 for the intensified trained group and not for the control group which might indicate that the IT group adapted better to training than the control group due to the intensified training program during Phase 3. Another explanation could be that the right shift shows the lactate paradox seen in the parasympathetic form of overtraining in the IT group and that the recovery phase was not long enough to compensate for the overtraining effects [16,17].

Based on the values assessed prior to SET 1 (Table 1), the predominant FFAs (C ≥ 14) measured in plasma were linoleic acid, oleic acid, palmitic acid and stearic acid, presented in order of decreasing concentration. Together these fatty acids constituted over 93% of the total concentration of C ≥ 14. Other fatty acids, i.e. myristic acid and palmitoleic acid, constituted < 7% of the total concentration of C ≥ 14. These data agree with those presented in the study of Orme et al. (1974), where the predominant FFAs (C ≥

14) in plasma were palmitic (C16:0), linoleic (C18:2), oleic (C18:1), stearic (C18:0) and linolenic acid (C18:3) in normally fed horses. Together these fatty acids constituted over 90% of the total concentration of C ≥ 14. Other FFAs present were myristic acid (C14:0) and palmitoleic acid (C16:1), each of which constituted < 5% of the total concentration of C ≥ 14 [18]. In comparison, the major fatty acids in the horse carcass have been shown to be (in decreasing order): oleate, palmitate and linoleate [19].

Immediately following various SETs, lipolysis occurred as reflected by the increased concentration of FFAs (Table 3) and taking into account that FFA utilisation is considered proportional to blood concentration [20]. Hambleton et al. (1980) showed that four long-chain fatty acids (palmitic, stearic, oleic, and linoleic) measured in plasma initially decreased with exercise (mechanical horse walker for 3.2 m/sec at trot during 2 hours per day and a SET based on trotting at 3.2 m/sec for 6 hours), and increased after training [21]. Palmitic-, stearic-, oleic-, and linoleic acid concentrations in plasma in our study were not significantly affected by exercise or by long-term training despite a trend towards Hambletons' results, which can be seen in Table 1. After detraining, however, an unexpected increase in concentrations of these fatty acids was measured, albeit not statistically significant (Table 1, SET 4). Analysis of total of FFA showed a significant increase after exercise (Table 3). The difference between total FFA and the individual fatty acids may be due to the time of sampling. Total FFAs were sampled before, immediately and one hour after exercise. In contrast, the individual FFAs were sampled only before and one hour after exercise. To obtain better correlation, one should sample blood for analysis of fatty acids before, immediately after and some time after exercise.

With 30 minutes of high-intensity exercise in humans, plasma short-chain and long-chain acylcarnitine concentrations increased by 46 and 23%, respectively [1]. In our study with similar plasma lactic acid concentrations following exercise (Figure 2) in Standardbreds,the short-chain acylcarnitine esters were mainly affected. However, horses were only sampled before and one hour after exercise. Except for the propionylcarnitine (C3), which can also be derived from catabolism of the amino acids methionine, valine and isoleucine, these acylgroups are mainly degradation products of the longer chain fatty acid oxidation [22]. Our study revealed that plasma acetylcarnitine (C2) shows a significant increase in concentration whereas all other short-chain acylcarnitine concentrations decrease, which is in agreement with the rise of acetylcarnitine seen in middle gluteal muscle in exercising Thoroughbreds [12].

Training itself did not alter the concentrations of carnitine esters. Foster and co-workers (1992) described altered skeletal muscle carnitine concentrations in trained men and horses. Well-trained human athletes had slightly higher muscle carnitine concentrations than untrained athletes. This resulted from an adaptive response to an increased demand for carnitines. In an equine study, there was a trend toward higher total carnitines with age and training as a consequence of underlying changes in mitochondria density as indicated by differences in levels of citrate synthase activity [23]. Foster and Harris (1989) also reported

higher concentrations of free carnitines in plasma of trained 3-6 year old horses as compared with plasma of untrained age-matched animals [24]. This is in contrast to results found in the present study, as the mean concentration of free carnitine in plasma was unaffected by training.

Plasma β hydroxybutyrate concentrations provide a crude assessment of the rate of hepatic ketogenesis [1]. Despite reaching the anaerobic threshold following various SETs, plasma BHBA concentrations did not increase, which is in agreement with the fact that ketogenesis is a very unlikely metabolic pathway in the equine species (Table 3) [25,26]. On the other hand, we demonstrated that exercise was associated with an increased mean plasma concentration of the volatile fatty acid 3-OH-iso-butyric acid which can be derived from the catabolism of valine, one of the branched chain amino acids, as substrate of gluconeogenesis [27].

In animals, changes in the plasma and urine carnitine pools may reflect production from tissues other than muscle. Most commonly, enhanced ketogenesis is associated with an elevated plasma short-chain acylcarnitine concentration in humans and animals [4]. Because no increase in plasma BHBA concentration was seen in association with exercise-induced elevation in plasma acetylcarnitine concentration, a hepatic source for the increased plasma acetylcarnitine level is unlikely. Although short-chain (or volatile) fatty acids, mainly acetate (C2), propionate (C3) and butyrate (C4), are produced via bacterial fermentation in the caecum and colon [28], increased production within the digestive-tract following exercise does not seem probable either. We conclude that plasma acylcarnitine profile in the equine species may reflect skeletal muscle carnitine metabolism following exercise.

Acetyl-CoA is the immediate substrate of lipogenesis and palmitic acid is its end product [29]. When comparing levels of palmitic acid (C16:0) and other endogenous or exogenous fatty acids in Tables 1 and 2, taking into account the associated plasma concentration of free carnitine, it is obvious that carnitine predominantly forms esters with short- and medium-chain fatty acids in the equine species.

It is presently impossible to discern acute fatigue and decreased performance experienced in or due to isolated training sessions from overreaching and overtraining. This is partially the result of a lack of diagnostic tools, variability of results of research studies, a lack of well controlled studies, and individual responses to training [30]. Overreaching is the short-term fatigue associated with the increases in exercise load that are usual in any training program [31]. In contrast, overtraining is a state of prolonged fatigue, caused primarily by an imbalance between training and recovery. Even with complete rest, recovery from overtraining can take weeks or months [31]. In our experiment, half of the horses were normally trained and half of the horses received intensified training with the goal of becoming overtrained.

Overtraining has been defined as an accumulation of training and/or non-training stress resulting in long-term decrement in performance capacity with or without related

physiological and psychological signs and symptoms. Restoration of performance capacity may take several weeks or months [30]. The reduction in performance seen in the current study is similar to that presented in other studies, which reported reduction in the average treadmill run-time-to-fatigue by 14% [19]. Intensified training revealed no alterations in FFAs and acylcarnitine esters. As a consequence, no particular marker for intensified training could be identified and intensively trained horses were unlikely to be associated with defective mitochondrial fat metabolism.

The relationship between skeletal muscle carnitine metabolism and recurrent exertional rhabdomyolysis needs further study.

Footnotes

[a]Pavo, Boxmeer, the Netherlands
[b]Boehringer Ingelheim, Alkmaar, the Netherlands
[c]Treadmill, Kagra, Graber A, Fahrwangen, Switzerland.
[d]Cardio Perfect Inc, Atlanta, GA, USA
[e]Polar 610i, Polar Electro Oy, Kempele, Finland.
[f]HeSETich zentrifugen, TuSETlingen, Germany.
[g]Varian/Chrompack, Middelburg, The Netherlands
[h]Randox kit NEFA, FA 115, Randox Laboratories Ltd, Antrim, UK
[i]Randox kit Ranbut, RB 1007, Randox Laboratories Ltd, Antrim, UK
[j]ABL, Radiometer, Rijswijk®, The Netherlands
[k]SPSS Version 12 for Windows Inc., Chigaco, USA.
[l]Thesis: De Graaf-Roelfsema E. Endocrinological and behavioural adaptations to experimentally induced physical stress in horses. *Faculty of Veterinary Medicine*. Utrecht: Utrecht University, 2007

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Chapter 8

The effect of oral tocopherol acetate administration on equine glucose metabolism, peripheral insulin sensitivity, and antioxidant status

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Abstract

The objective of this experiment was to determine the effect of dl-α-tocopherol acetate (vitamin E analog) on glucose metabolism, peripheral insulin sensitivity and anti-oxidant status as measured using five different methods. Six healthy warmblood horses, four mares and two geldings, with a mean age of 10.3 years were used. A 2 x 2 Latin square design for the euglycaemic hyperinsulinaemic clamp experiment was used before and after two weeks of oral supplementation with 4 mg/kg BW of α-tocopherol acetate or a placebo, with a wash-out period of 4 weeks. Five different methods of measuring the antioxidant status were used: glutathionperoxidase (GSH-Px)-activity, vitamin E and selenium-concentration, 3-(4-morpholino)-sydnone imine (SIN)-induced, cypridina luciferin analog enhanced chemiluminescence expressed as Trolox equivalents and the Total Antioxidant Power assay expressed as copper reducing equivalents.
Alpha-tocopherol acetate administration at a dose of 4 mg/kg BW/day did not alter glucose metabolism or peripheral insulin sensitivity. In addition, α-tocopherol acetate administration did not improve the measured parameters of antioxidant status. Positive significant correlations were seen between amount of glucose metabolised (M) and the ratio of amount of glucose metabolised to plasma insulin concentration (M/I)(r = 0.896, P = 0.000), between chemiluminescence and GSH-Px (r = 0.423, P = 0.040) and between GSH-Px and α-tocopherol acetate (r = 0.517, P = 0.012). Significant negative correlations were seen between plasma insulin concentration and M/I (r = -0.640, P = 0.001) and between Total Antioxidant Power and M as well as M/I (both with r = -0.471 and P = 0.020).
The results suggest that further research utilizing a higher dose of vitamin E or combinations of vitamin E with other antioxidants, may be useful. Based on the current knowledge, we would give preference to the GSH-Px activity assay to assess antioxidant status in horses. The (negative) correlation between Total Antioxidant Power and glucose metabolism suggests that the reduced antioxidant status, which is represented by copper reducing equivalents, reflects an increased need for antioxidants with higher glucose metabolism.

Keywords

Antioxidant status, clamp, glucose, horse, insulin sensitivity, vitamin E

Introduction

There is an ongoing search for methods to improve the performance of sports horses without compromising their welfare. One of the methods is feed supplementation with, among other supplements, vitamin E, the most commonly known and most popular antioxidant. In humans, antioxidants affect glucose metabolism. Hyperglycaemia causes generation of reactive oxygen species [2]. This is important in the pathogenesis of diabetes mellitus and syndrome X [3]. In the horse, there are diseases similar to those found in man, of which quine metabolic syndrome with insulin resistance is the most serious problem [4].

Our experiment focuses on the role of α-tocopherol acetate (α-TA), a stabilized derivate of naturally occurring vitamin E, as a potentially useful supplement to improve insulin sensitivity and antioxidant status of the horse. In experiments in rats and man, vitamin E improved insulin sensitivity via an insulin-mediated increase of glucose disposal [5]. Our hypothesis is that oral supplementation with α-TA is effective in improving insulin action and reducing oxidative stress. Both effects contribute to better performance, by optimizing energy metabolism, among other things, and by reducing muscle damage, respectively [6]. The number of different antioxidants and their possible interaction *in vivo* makes it difficult to analyse each antioxidant separately. It also makes measurement of individual antioxidants less representative of the overall antioxidant status [7]. We compared five different methods of assessment of the antioxidant status to assess their potential use in the equine species.

Materials and methods

The Committee on Animal Welfare of Utrecht University, the Netherlands, approved this study.

Animals and diets

Six healthy warmblood horses, four mares and two geldings, with an average weight of 581 ± 12 kg, were used. Their age varied between nine and twelve years, with a mean age of 10.3 ± 1.0 years. They were housed on shavings and fed normal quality hay *ad libitum* with a mean intake of 10 ± 0.5 kg daily. This intake remained constant during the entire experiment. During the whole study, the same batch of hay was used. The concentration of vitamin E analyzed in a sample taken from various parts of the batch of hay was 15 mg/kg as fed and the concentration of selenium in this hay was 0.10 mg/kg as fed. The horses were exercised one hour daily on a walking machine.

Experiment

A 2 x 2 Latin square design was set up for the experiment (Figure 1). All six horses were adapted to the hay, box, and exercise for two weeks. After this period, the horses were divided randomly into two groups. Group one (horse 1, 2, 3) received an oral supplement of 4 mg/kg BW α-TA[a] for two weeks. Group two (horse 4, 5, 6) received a placebo, consisting of a comparable amount of saline. Both treatments were administered orally via a syringe. After this, a four week wash-out period was applied to all horses and, subsequently the treatment scheme was reversed, i.e. group two received the oral supplement of α-TA for two weeks and group one received the placebo.

Euglycaemic hyperinsulinaemic clamp technique

The day before and after each period of α-TA or placebo supplementation (Time points 1, 2, 3 and 4 in Figure 1), insulin sensitivity was examined by use of the euglycaemic hyperinsulinaemic clamp technique as previously described [8-10]. Food was withheld for 12 h prior to the clamp. Before starting the clamp, all horses were weighed. To perform the clamp technique, a catheter was inserted into each jugular vein. One of these catheters was subsequently used for infusion of glucose (as a 50% solution) and insulin[b], whereas the other catheter was used for collection of blood samples to monitor circulating glucose and insulin concentrations. Before each clamp, a blood sample was collected to establish the basal glucose concentration. Within 10 min after collection of the first blood sample, a priming dose of insulin (45 mU/kg BW dissolved in 50 ml of saline solution) was administered IV as a bolus injection. This bolus injection was followed immediately by constant rate infusions of insulin (6 mU/[kg BW x min]) and glucose (mean infusion rate 8.6 µmol/[kg BW x min]). Constant rate infusions were accomplished by use of a pump[c]. Throughout the period of insulin and glucose infusion, blood samples were collected at 10-min intervals into heparinized syringes. Within 2 min after collection, glucose concentration in each sample was measured by use of an automated analyzer[d]. Blood glucose was maintained at euglycemic values in each horse (3.9 to 5.6 mmol/L). When the blood glucose concentration differed from the euglycemic values, the glucose infusion rate was adjusted to compensate. A steady-state condition was presumed to exist when the plasma glucose concentration and glucose infusion rate were concurrently held constant (maximum difference of 0.1 mmol/L) for at least 30 min and the plasma glucose concentration was within the range for euglycemic values at least 90 min following the start of the clamp. Plasma insulin concentration was determined in three blood samples that were collected in tubes that contained lithium heparin at 10-min intervals during the induced euglycemic hyperinsulinaemic condition. These blood samples were centrifuged for 10 min at 6,000 x g; the resulting plasma was harvested and stored at -20°C until insulin concentrations were analysed by use of a radioimmunoassay[e] validated for use in samples

obtained from horses [11]. The clamps took place in the horses' own stables to ensure a minimum of stress.

Calculations

For plasma glucose concentrations to be in a steady-state condition, the amount of glucose infused must equal the amount of glucose being removed from the glucose space (that is the amount of metabolized glucose M), provided that endogenous glucose production is suppressed completely [8,9] . In reality, the plasma glucose concentration is never absolutely constant, and a space correction factor must be included to account for glucose that is added or removed from the glucose space by means other than metabolism [12]. Thus, glucose metabolism (M) in steady state conditions during use of the clamp technique can be expressed by the following equation: M = INF – SC, where INF is the amount of glucose infused and SC is the space correction factor [12]. The space correction was calculated by use of the following equation: SC = ([G2-G1] x GS)/([t2 – t1] x BW) where G1 and G2 are the glucose concentrations at time points t1 and t2, respectively, and GS is the glucose space, which is calculated as 0.19 L/kg BW of the horse. In this study, values of M were calculated for 10-min intervals [12].

Values for insulin (I) were calculated for the three samples recovered during steady-state condition. Those concentrations were used to calculate the mean ratio of glucose metabolism rate to plasma insulin concentration (M/I), which reflects the quantity of glucose metabolized per unit of plasma insulin. This is a good index of tissue sensitivity to exogenous insulin [12].

Assays and analysis of blood samples

At the start of each clamp, additional blood was collected for determination of GSH-Px activity, and for vitamin E and selenium concentrations (lithium-heparinized blood for all assays). Blood samples were also analyzed for Total Antioxidant Power using a chemiluminescence assay as well as a Total Antioxidant Power assay (lithium-heparinized and Na-Citrate blood samples, respectively). The GSH-Px-activity was analyzed as described for cattle by Counotte and Hartmans in 1989, using a Randox test kit validated for the horse [f] [13,14]. For determination of vitamin E concentration, blood collected in lithium-heparinized tubes was centrifuged for 5 min at 6,000 x g and stored at -20°C until vitamin E concentrations were analysed by use of a reversed phase HPLC[g].

The blood selenium concentration was analysed with hybrid generation atomic absorption spectroscopy (HG-AAS) as described by Miksa et al. [15].

For the SIN-induced, CLA-enhanced chemiluminescence, serial dilutions of the plasma samples (1:10 – 1:10,000) in phosphate-buffered saline (pH 7.4) were preincubated at 37°C in white 96-well, flat-bottomed microtiter plates in the presence of cypridina luciferin analog [1h]. After addition of the reactive oxygen- and nitrogen-species donor 3-(4-

morpholino)-sydnone imine (SIN)[i], chemiluminescence was monitored every 30 s for 0.5 s during a 20 min period using a luminometer[j]. The total chemiluminescence signal was quantified by calculation of areas under the curves. A calibration curve of the water-soluble vitamin E analog Trolox[k] was used to calculate the antioxidant capacity of the plasma samples (expressed as Trolox equivalents).
To determine Total Antioxidant Power, blood collected in Na Citrate tubes was centrifuged at 4°C for 12 minutes. Plasma was removed and aliquots were stored at -70°C until further analysis. A colorimetric microtiter plate version of the Total Antioxidant Power assay[l] was performed according to the instructions of the manufacturer. Total Antioxidant Power of the plasma-samples was calculated as copper reducing equivalents.

Statistical analysis
Results at all time points were compared using a two-way repeated-measures analysis of variance (ANOVA) using the multivariate test. Because of the Latin square construction, the chronological time points from Figure 1 were changed into four time points:
1: the time point before administration of placebo
2: the time point after two weeks of placebo
3: time point before treatment with two weeks of vitamin E
4: time point after two weeks of treatment with vitamin E.
The strength of the linear association was assessed by obtaining the correlation coefficient (r), and testing whether it was different from zero by use of the Pearson product moment correlation test (2-tailed). Results are presented as means ± SD. The statistical program SPSS was used [m]. Values of $P < 0.05$ were considered significant.

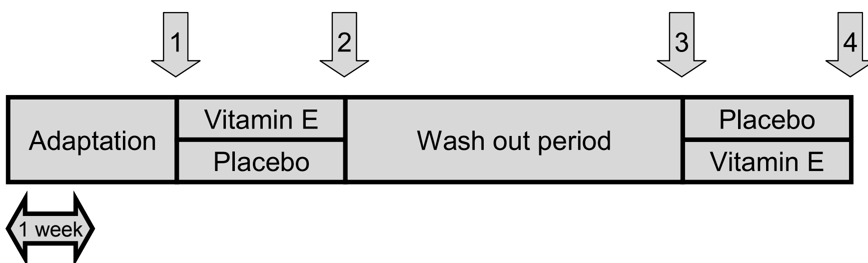

Figure 1: Outline of the experiment Numbers represent chronological timepoints

Results

All results are reported in Figure 2. A highly significant increase in weight was found during the placebo period (*P* = 0.005) together with a significant decrease in the amount of metabolised glucose (*P* = 0.028). There was a significant increase in plasma vitamin E concentration for the placebo period (*P* = 0.030) as well as for the vitamin E-treatment group (*P* = 0.043). GSH-Px showed a significant decrease over the vitamin E-treatment period (*P* = 0.016). The amount of copper reducing equivalents measured in the Total Antioxidant Power assay increased significantly during the placebo period (*P* = 0.015).

Significant positive correlations were seen between the amount of glucose metabolised (M) and the glucose metabolism rate-to-plasma insulin concentration ratio (M/I)(*r* = 0.896, *P* = 0.000), between the Trolox equivalents measured in the chemiluminescence assay and GSH-Px (*r* = 0.423, *P* = 0.040) and between GSH-Px and vitamin E concentration (*r* = 0.517, *P* = 0.012). Significant negative correlations were seen between plasma insulin concentration and M/I (*r* = -0.640, *P* = 0.001) and between Total Antioxidant Power and M as well as M/I (*r* = -0.471, *P* = 0.020).

There were no significant period by day interactions, i.e. there was no difference between horses that received treatment or placebo in the first period and those that received treatment or placebo in the second period.

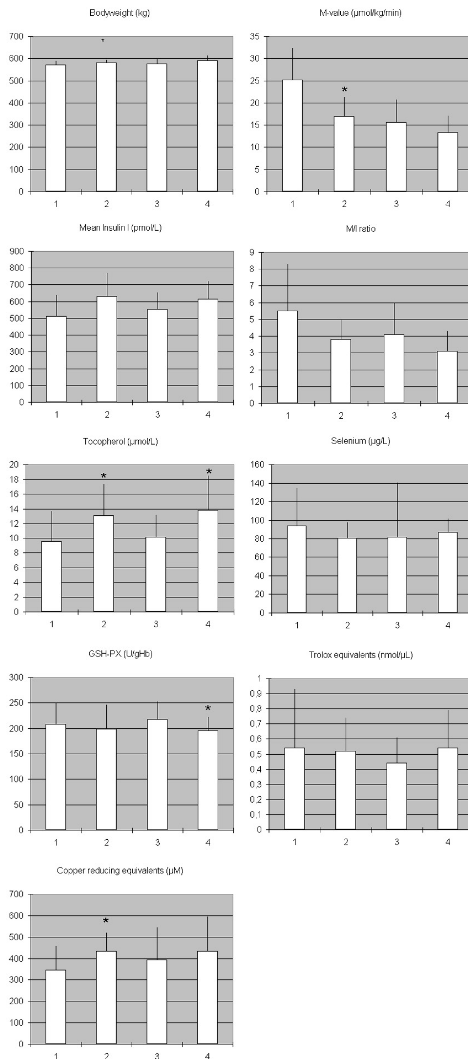

Figure 2. Effects of α-tocopherol acetate supplementation on mean ± SD bodyweight, M-value (the amount of metabolized glucose), mean insulin concentration during steady state I, M/I ratio, vitamin E and selenium concentrations in the blood, GSH-Px (glutathion-peroxidase) activity, Total Antioxidant Power and SIN-induced, CLA-enhanced chemiluminescence (expressed as copper reducing equivalents and Trolox equivalents, respectively)

1 = timepoint 1 = before placebo
2 = timepoint 2 = after placebo
3 = timepoint 3 = before treatment with vitamin E
4 = timepoint 4 = after treatment with vitamin E
* Include significance threshold

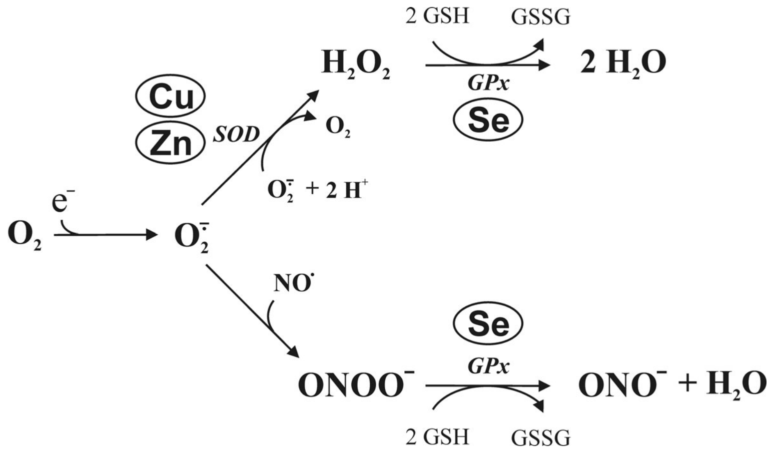

Figure 3. Copper, zinc and selenium in the cytosolic defense against reactive oxygen and nitrogen species. Superoxide anion is generated by reduction of molecular oxygen. The electrons required may leak out of the mitochondrial respiratory chain or may be derived from reactions such as the oxidation of (hypo-)xanthine as catalyzed by xanthine oxidase. Copper, zinc–superoxide dismutase (CuZn–SOD) catalyzes the dismutation of superoxide anion; this reaction is competed for by nitrogen monoxide (NO), if generated in sufficient amounts, to form peroxynitrite. Both the hydrogen peroxide from superoxide anion disproportionation and the peroxynitrite may be reduced at the expense of glutathione (GSH) in reactions that are catalyzed by glutathione peroxidase (GPx).
With permission of Dr L. Klotz, J *Nutr; 2003:* **133**: 1448S-1451S

Discussion

Interesting research in Vitamin E has been performed in man, horse and other species. In rats fed high fructose diets, vitamin E improved the free radical defense system potential [16]. In rats and humans, both healthy and diabetic, vitamin E has improves glucose transport and insulin sensitivity [5]. In horses, research has focused on several topics, such as the pharmacokinetic properties, effects of exercise, oxidative disorders, lung diseases and the relationship between several types of feed and the vitamin E concentration [17-22]. No research has been performed yet on glucose transport and insulin sensitivity in the horse in relation to vitamin E.

Our first hypothesis was that the supplementation with a pharmacologic dose of α-tocopherol acetate will improve insulin action. The results of the current study showed that the administration of α-TA had no significant influence on the amount of glucose metabolized and the ratio of glucose metabolism rate to plasma insulin concentration, i.e. the tissue sensitivity to exogenous insulin. Paolisso (1993) described an experiment where healthy humans received an α-tocopherol acetate supplement that resulted in an insulin-mediated increase of glucose disposal [5]. There are significant differences in experimental design that may explain the differences with our results. Human individuals received 15 mg/kg BW α-tocopherol acetate instead of 4 mg/kg BW as used in our study. In addition, this large amount of α-tocopherol acetate was given for a period of 4 months in the study on humans, instead of two weeks as in the study on horses. Thus, the difference in outcome may be related to dosage, treatment period or species. Administration of the high dose from the human model may not be feasible in horses.

We wanted to test our hypothesis using supplementation of a generally accepted dose of α-tocopherol acetate , that is, 4 mg/kg BW, resulting in a total amount of around 2400 mg dl-α-tocopherol acetate per day. This came on top of the 150 mg/d coming from daily intake of 10 kg of hay containing 15 mg α-tocopherol acetate per kg. The NRC (1989) recommends 50 mg of vitamin E per day per kg hay DM for healthy, normally managed, non-working and non-pregnant horses. Our dose was approximately 4 times as high as the recommended level. This dosage should therefore be sufficient to accomplish a rise in serum concentration of the vitamin. Studies of Roneus et al. (1986) and Kienzle et al. (2003) confirmed this [20,23].

Another reason not to provide more vitamin E is that Williams and Carlucci (2006) found that supplementation above control levels is not more beneficial to antioxidant status in intensely exercising horses. When they administered 10 g of dl-α-tocopherol acetate per day, an amount comparable to that used in the experiment of Paolisso in humans, negative

effects were observed, such as a decrease in beta-carotene, another antioxidant and source of vitamin A [5,22].

Considering the very low concentration in our hay, which was the only source of vitamine E in the placebo group, and the high dosage in the vitamin E-treatment group, we expected to find a significant rise in α-tocopherol acetate concentration in the blood after 2 weeks of supplementation. During the wash out period or the placebo period we expected a steady concentration or decreasing concentration. Figure 2 shows that there was indeed a significant rise in concentration during the treatment period and a decreasing concentration during washout periods, but we also measured a significant rise in concentration during the placebo period that cannot be due to supplementation.

A possible explanation could be that the analyses did not reflect the true vitamin E status. Although the described method is valid, literature mentions that single serum assays are an unsatisfactory indicator of vitamin E status in horses, and it can vary up to 17% in a single animal within a short period. Variables reported to influence normal values of serum vitamin E include age, exercise, breed, type of feed, time of year and physical conditioning [24-26]. In our experiment, these factors were all the same for the whole group which makes large errors unlikely. But as blood contains only 1% of total body vitamin E, this amount can fluctuate [24]. This can indeed be a reason for the varying results regarding plasma vitamin E concentration. Other studies mention a wide range in concentrations of plasma vitamin E too [16,27]. If this is true, all experiments regarding vitamin E, as well as all vitamin E analyses done on patients, should be repeated multiple times to render more reliable results. The economic consequence is clear: this is not affordable. An alternative might be to measure the concentration in other tissues in which vitamin E concentration is more constant, such as adipose tissue [23,26].

Another aim of our study was to compare five different methods of assessment of the antioxidant status to establish their potential usefulness in the equine species. An imbalance between endogenous oxidants and antioxidants in favor of oxidants has been defined by Sies (1991) as 'oxidative stress' [28]. The body's physiological response to oxidative stress is through several antioxidant systems that include enzymes and varying sized molecules [28,29]. There are various ways to measure the antioxidant status in a horse. Because of the multiplicity of antioxidant pathways, there is reason to measure levels of individual (endogenous) antioxidant as well as overall antioxidant capacity or antioxidant power of blood quantitatively [7]. Therefore, we used both the SIN-induced, CLA-enhanced chemiluminescence and Total Antioxidant Power assay. Unfortunately, the correlation between these methods was not significant (r = -0.303, P = 0.150). In addition, a significant decrease was found for GSH-Px activity. This was unexpected because we

found a significant positive correlation between vitamin E concentrations in the blood and GSH-Px activity, as is quite common [15,30-34], and vitamin E has proven to enhance glutathione levels as well as GSH-Px activity [15,30]. A possible explanation in this case could be that the period of two weeks of supplementation was too short to increase GSH-Px because erythropoiesis has to increase and the maturation time for a erythrocyte itself is about one week and the life span six weeks [35]. A possible reason that supplementation with α-tocopherol acetate has not led to a clear effect on antioxidant parameters might be that all antioxidative mechanisms in the body are in a way connected. A change in concentration or activity in one place can give rise to a reaction in another place. An increase of vitamin E might for instance spare another antioxidant [36,37]. Another explanation could be that hay, in addition to the naturally occuring vitamin E, contains other constituents with antioxidant properties. Thus, total antioxidant content of the hay may by far exceed the antioxidant activity attributable to its vitamin E content and, probably, even the antioxidant potential of the supplemented α-tocopherol acetate, with the result that the treatment-effect of α-tocopherol acetate is negligible.

A significant positive correlation between SIN-induced, CLA-enhanced chemiluminescence and GSH-Px and between GSH-Px and vitamin E was found in this study. Correlations between GSH-Px, selenium, and vitamin E have been found multiple times and provide strong support for the synergistic actions between these antioxidants [31,33,34,38,39]. However, in our experiments, these correlations were not always present. The lack of correlation between GSH-Px and selenium cannot be explained, because selenium is used as a cofactor in the reaction catalyzed by GSH-Px (Figure 3). The reported Se content of the hay is acceptable by NRC (1989) standards, i.e. 0,1 mg/kg DM of total ration [40]. Mean plasma concentration of selenium in Fig 2 is around 80-90 μg/L which is normal for the laboratory used (reference value > 70 μg/L). Reference values for selenium concentration in plasma vary from 64-250 μg/L [39,41,42]. Mean plasma concentration of GSH-Px in the study is 200 U/gHb which falls within the reference values of the laboratory used, i.e. 120-300 U/gHb.

We concluded that none of the assays were discriminative enough. This means that it is necessary to do further research to come to a generally accepted method for assessment of antioxidant status. Based on our current observations, we cannot propose evidence-based advice on vitamine E supplementation for horses to meet their needs in various situations. Based on the current knowledge, we would give preference to the GSH-Px activity to assess antioxidant status in horses, because GSH-Px correlates with the SIN-induced, CLA-enhanced chemiluminescence as well as with vitamin E concentration, while the rest of the methods correlate with only one other antioxidant at best. In addition, GSH-Px is involved

in both reactions directed against reactive oxygen and nitrogen species as shown in Figure 3.

In conclusion, given the fact that α-tocopherol acetate administration at an oral dosage of 4 mg/kg BW daily did not alter glucose metabolism or peripheral insulin sensitivity, further research with a higher dose and/or longer administration period is necessary.
Based on our results, we would give preference to the GSH-Px activity to assess antioxidant status in horses.
In the current study, the level of glucose metabolism was assessed four times per horse by means of the euglycaemic hyperinsulinaemic clamp. As a consequence, it was possible to get an impression of the relationship between glucose metabolism and antioxidant status. Preliminary results indicated that the (negative) correlation between Total Antioxidant Power and glucose metabolism suggests that the reduced antioxidant status, which is represented by copper reducing equivalents, reflects an increased need for antioxidants with higher glucose metabolism. Copper reducing equivalants could possibly be a marker of glucose metabolism in individual horses.
The significant decrease in the amount of glucose metabolised during the placebo period associated with an increase in body weight remains unexplained.

Footnotes

[a] dl-α-Tocopherol acetate: 25 ml 1 g/ml dl-alfa tocopherylis acetas Ph Eur; Bufa, IJsselstein, the Netherlands
[b] Actrapid recombinant human insulin (100 U/ml), Novo Nordisk A/S, Bagsvaerd, Denmark
[c] Volumetric pump, Model Argus 414, Argus Medical AG, Heimberg, Switserland
[d] ABL-605, Radiometer Copenhagen, Westlake, Ohio
[e] Coat-A-Count TKIN2 836, Diagnostic Products Corp, Los Angeles, California.
[f] Ransel, Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co.Antrim, United Kingdom, BT29 4QY
[g] A.M.L. bvba, Desguinlei 88, 2018 Antwerpen
[h] Cypridina luciferin analog (5 µM final concentration), TCI-Europe NV, Zwijndrecht, Belgium
[i] 3-(4-morpholino)-sydnone imine (500 µM final concentration), Sigma, St. Louis, MO, USA
[j] Titertek Luminoskan luminometer, TechGen International, Zellik, Belgium
[k] Trolox, Sigma, St. Louis, MO, USA
[l] Oxford Biomedical research Rochester Hills, MI 48309, USA
[m] SPSS Version 12 for Windows Inc., Chicago, USA.

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Chapter 9

Discussion

This discussion aims at deepening several subjects within the thesis without attempting to cover the thesis in its entirety. Selected subjects include “Diagnostics”, which discusses the integrating advanced diagnostic methods in equine medicine; “Myopathies”, which describes different metabolic aspects of atypical myopathy and MADD; “Exercise/Training” which illustrates the influence of exercise on protein and lipid metabolism; and finally “Practical consequences for veterinarians and equine clinics”.

Diagnostics: integrating advanced diagnostic methods in equine medicine

Traditionally only a few tools were used to diagnose myopathy in a horse. Examples of these include the history and clinical signs of a myopathy, increased muscle specific enzymes such as creatine kinase (CK), aspartate amino transferase (ASAT) and lactate dehydrogenase (LDH) and the colour of the urine. A therapy was often initiated without knowing the exact cause and pathophysiology of the myopathy. Additional diagnostic methods that have become more available in recent years are, amongst others, muscle biopsy with various stainings, electromyography (EMG) and exercise tests [1-3]. However, these methods have not gained large popularity as of yet, except in hospital settings, because they are difficult to perform in practice.
Chapter 2 describes how many different causes of metabolic myopathies are discovered in human medicine and, in contrast, how little has been investigated in equine medicine. It is essential that we expand our diagnostic possibilities, not only to discover more diseases in the horse, but mainly to adapt our therapy to the specific type of myopathy. Every route in metabolism has its own special characteristics and if therapy can be more specifically adjusted, great benefit can be obtained. For instance, metabolic intoxication with long chain fatty acids can be diminished by supplementing carnitine that detoxifies these fatty acids by connecting to them and get excreted via the urine as a carnitine-ester [4]; the decreased energy production of one pathway can be compensated for by enhancing another pathway; alternatively if glycolysis is damaged, stimulation of lipid metabolism can be performed, for example by feeding more fat and exercising less intensely [5].

This thesis is carried out in cooperation with human hospitals to explore the possibilities of integrating advanced diagnostic methods in equine medicine.
Most metabolic myopathies are based on a genetic lack of an enzyme or a change in its function leading to a ‘block’ in the metabolic pathway with two possible consequences: accumulation of substrates upstream or a lack of product formation downstream [6].
The accumulated substrates can be toxic metabolites such as organic acids, which cause metabolic intoxication, resulting in a wide variety of signs [6].
A relative shortage of products that should have been produced can also have harmful consequences, such as decreased energy production or a decrease in effectiveness of further

catabolic pathways [6]. The studies in this thesis were based on measuring metabolites in carbohydrate, lipid, protein, creatine and purine metabolism as well as in oxidative phosphorylation. These metabolites were analysed in plasma and urine. By comparing such metabolites from diseased patients with myopathies with a panel of healthy control horses, differences between the two groups could be detected and analysed further.

If a deficiency of an enzyme is suspected due to an accumulation of metabolites, the enzymes involved in the reactions that were disturbed, can be measured in muscle tissue.
The ultimate diagnosis can be made if the genetic mutation can also be detected. Until recently this was not possible, but currently the complete equine genome is unravelled.

Since many myopathies have a genetic origin, and obtaining a diagnosis is not only of value for the diseased horse, but also for its relatives, obtaining material for further diagnostic analysis is important. Urine, plasma and muscle tissue can all be collected for analysis. Urine and plasma may be used to measure the concentration of metabolites. It is best to obtain samples from the horse when the disease is in the acute state, when the concentration of metabolites is at peak. If correctly stored at -20 °C urine and plasma can be used at any subsequent time for analysis. However, in order to measure enzymatic activities muscle tissue is needed. The tissue needs to be frozen immediately in liquid nitrogen and stored at -70 °C for enzyme histochemistry, biochemical evaluation, enzyme studies and mutation analysis. Because of this procedure, only few practitioners have the facilities to collect muscle tissue.
A possible alternative to this is to collect tissue from which fibroblasts may be isolated and cultured. Fibroblasts are stellate or spindle-shaped cells with cytoplasmic processes present in connective tissue, capable of forming collagen fibers. They are the most common cells of connective tissue in animals. A skin biopsy can be made on several areas, after disinfecting with alcohol. The biopsy can be placed immediately in culture medium or, if not available, in saline, and stored at 4 °C until processed. From this skin biopsy fibroblasts can be cultured and stored, and used whenever necessary for metabolic and genetic purposes. The advantage of this is the ease of collecting and the amount of material that will be available from the patient. Offering this facility will increase the amount of material from interesting patients that can be used at any moment. If a new metabolic disease would be found, skin biopsies from family members can easily be performed and fibroblasts cultured for metabolic and genetic analysis.
This is a way to expand the knowledge about metabolic myopathies in horses and to unravel this group of interesting diseases of the horse.

Myopathies: different metabolic aspects of atypical myopathy

With the advanced diagnostic methods available, in the near future new myopathies will be discovered worldwide. Since many of the known human metabolic myopathic diseases have a genetic aetiology and the majority of patients with such disorders are infants, it may be worth extrapolating such a knowledge base to investigate unexpected deaths of foals. and the majority of patients is in its early infancy, special attention must be focussed on foals that die unexplained. If analysis of these foals can produce a diagnosis, this could be valuable for genetic relatives. Like with other, already well known, diseases such as hyperkalemic periodic paralysis (HPP) or glycogen branching enzyme deficiency (GBED), genetic testing can prevent the prevalence of diseased horses to increase [7,8].

The upcoming variety of metabolic myopathies implies that the therapeutic measures taken will also start varying accordingly to the type of myopathy found. Therapy for myopathic patients, such as rapid large volume intravenous or oral fluid administration for rehydration and detoxification, non steroidal anti-inflammatory drug administration, diet measurements and supplementation with, for instance, the antioxidant vitamin E, will have to be refined for each patient, depending on the cause of the myopathy.

As is made clear in chapter 3, 4 and 5, care must be taken in the route towards a diagnosis. If metabolites are found in plasma or urine a putative diagnosis can be made. However, supplementary analyses have to be made to support this diagnosis.
For example, the biochemical defect in horses leading to fatal atypical myopathy was identified as Multiple Acyl-CoA Dehydrogenase Deficiency (MADD), based on the characteristic urinary profiles of organic acids, glycine conjugates and acylcarnitines; the profile of acylcarnitines in plasma; as well as additional quantitative biochemical measurement of dehydrogenase activities in lateral vastus muscle.
Following the biochemical analyses, we tried to identify the genetic mutation causing the disorder; however there was no indication of a genetic aetiology in the horse. In contrast, in man, a genetic variant of Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) has been found. In our study, one patient recovered from MADD, exhibiting normal concentrations of metabolites and normal enzyme activities after recovery. However, like in humans, MADD in horses might show abnormal enzyme activities intermittently. All the data suggest that a non-genetic exogenic factor was more likely to be the cause of the MADD in the horses. However, the exact cause has not yet been identified. , A number of common circumstances all MADD-horses were exposed to should be considered as a possible contribution, such as the weather and field conditions i.e. contamination with mycotoxins.

Even if the metabolic disorder is clear it is wise to subject the patient to a complete analysis of metabolic pathways. Chapter 5 shows that other problems can exist besides the already

clear diagnosis. These problems can be primary or secondary. If they are primary the horse can have a concurrent disorder that may be genetic or caused by the same exogenic factors as the disorder already discovered. If they are secondary, the myopathy caused by the primary disease can damage other pathways, for instance through cell lysis. If a toxin is the cause of a myopathy, several disorders can occur simultaneously. It depends on the stability of the enzymes involved, and of the type of toxin, which enzymes will remain functioning and which will be inactivated [9-11]. Examples of enzymes that are less stable than others include phosphoglycerate mutase (PGAM), that had an activity of less than 10% in one myopathic patient, compared to the control horses and ATP synthase (complex V) that had an activity of 39% of the controls in another patient.

Almost complete glycogen depletion as seen in a myopathic patient in chapter 5 is rare in horses, and not reproduced in human or equine exercise protocols [12,13]. The depletion in the patient must have been the result of a combination of a greater demand for energy and cell damage due to the extreme rhabdomyolysis.

Depletion of glycogen normally increases with increasing exercise intensity. It has been implicated in muscle fatigue since glycogen is the fundamental fuel used to sustain both glycolysis and oxidative phosphorylation [14,15]. The percentage of depletion due to exercise is higher in humans than in the horse [16]. Low glycogen availability has been shown to increase lipid oxidation and amino acid oxidation during exercise [15,17].

Equine medicine can profit from the knowledge obtained by our human colleagues. The pitfall one should not fall in, however, is to assume that diseases in the horse have exactly the same mechanism as in man. The research on MADD proved this, the horse having some different symptoms compared to humans, and having more disturbances in other metabolic pathways than seen in human MADD, as well as it having a non genetic aetiology.

Exercise/training: influence of these on protein and lipid metabolism

The study described in 6 and 7 was part of a larger study in which Standardbred trotters were subjected to an 18 week training program with combined endurance and interval training followed by 6 weeks in which half of the horses continued the training in the same way (C)and half was more intensively trained (IT).

One goal of the study was to assess the influence of exercise on behaviour, hormonal levels, adaptations in skeletal muscle, signal transduction and metabolism. One of the findings in this thesis, containing research on protein and lipid metabolism, was that exercise was associated with alterations in plasma concentrations of short-chain acylcarnitines. In addition, also in the equine species carnitine forms esters predominantly with short- and medium-chain fatty acids. An indication for increased ketogenesis was found in the

significant increase after exercise of 3-OH-iso-butyric acid. This is an intermediary product in the metabolism of valine, one of the branched –chain amino acids, that is transformed, via propionyl-CoA, in Succinyl-CoA, used in the tricarboxylic acid cycle for energy production (Krebs cycle) [18]. Ketone bodies are generated in the liver predominantly from fatty acids via acetyl-CoA, but also to a lesser extent from certain amino acids, like valine [19]. Ketogenesis is relatively unimportant in the horse [20], but as shown in this thesis, in acute, intense exercise a specific pathway of ketogenesis may be more important.
Another finding was that acute exercise alters the plasma concentrations of certain amino acids involved in energy processes, nitrogen transportation and the urea cycle, and we were able to identify a number of these for further research, for instance a project of exercise with pre-exercise dietary supplementation.

A second goal was to assess the influence of training on behaviour, hormonal levels, adaptations in skeletal muscle, signal transduction and metabolism. In this thesis lipid and protein metabolism were analysed.
A prolonged period of training, with an increasing metabolic demand, leads to adaptations in skeletal muscle. Possible adaptations are increases in substrate storage in skeletal muscle, increases in capillary concentration of the muscle and increases in metabolic enzyme activity[21]. The type of training affects whether enzymes of the oxidative pathways or glycolytic pathways are up-regulated, as moderate intensity training up-regulates enzymes of the oxidative pathways, whereas high intensity training affects glycolytic pathways. In our study both types of training were performed. In analogy with research onto the effects of training on humans, it was hypothesised that training leads to a higher usage of free fatty acids, fuelled by increased lipolysis of muscle triglyceride, at the same time having a glycogen-sparing effect [22,23]. In contrast to these findings, in the present study training led to no changes in free fatty acid concentration in plasma, nor to differences in glycogen storage [24].
With regard to amino acids a significant reduction in the concentration of aspartic acid, an important energy substrate, during sustained exercise and intense training was found [25,26]. The results described are indeed significant, but one could question the positive use of supplementation in training programs, while the concentration after a long term training session is significantly lower, but still rather high compared to reference values of (another breed of) control horses as can be seen in the addendum with reference values.
There are conflicting results of the effect of supplementing aspartic acid as described in chapter 6. To the author's knowledge, studies on the effect of this supplement on the horse have not yet been performed.
The horses that were used during the study were relatively young Standardbred horses, chosen because they were not yet trained. The influence of growth could have been a factor in any metabolic changes. However, research in horses has shown that growth does not affect metabolic changes in skeletal muscle related to training [27-29]. In our study, we found

no longitudinal effect on protein and lipid metabolism, so the influence of growth seems to be absent.

The third goal was to reach an overtrained state in the group of horses that was intensively trained and compare this group with the control group of horses with regard to behaviour, hormonal levels, adaptations in skeletal muscle, signal transduction and metabolism.
The horses in the IT group were indeed overtrained as was shown by a 19% reduction in treadmill trotting-time-to-fatigue [30]. found between trained and intensively trained horses [30]. In contrast, based on the activity of key enzymes and fibre typing in muscle biopsies as well as electromyography (EMG) in skeletal muscle no evidence of neuromuscular overtraining was apparent [24].
Results of the present thesis are that (early) overtraining is also not reflected by lipid or protein metabolism as no significant changes were found between the normally trained and intensively trained groups.
If these results are all combined one can conclude that the overtraining syndrome starts at a mental and endocrine level. To reach the metabolic and neuromuscular level of changes, horses should be longer and more intensively trained than was carried out in this study. On the other hand, it is unsure if this level of overtraining would ever be reached or that the psyche of the horse will prevent metabolic overtraining to occur. It is paramount to consider animal welfare and establish markers for overtraining from the mental and endocrinological corner in the future as has been done by de Graaf-Roelfsema and co-workers [30].

In order to expand the studies of chapter 6 and 7, urine was collected to analyse carnitine-, organic acid- and amino acid concentrations. Results of urine analysis offered no significant changes in concentrations of metabolites, so no effect of training could be measured.
Metabolites in urine reflect the metabolic state of the period between the last urination and the moment of collection, therefore urine analysis can be a very valuable diagnostic tool. The influence of many variables in metabolism can be analysed via such analysis. A practical problem in this study was the fact that geldings were used, and that the collection method was not allowed to influence any other measurement performed in the study. This meant that catheterisation was impossible due to the medication necessary. A urine collecting device was used and obtained the first amount of urine after exercise. However, this urine was produced at very different time points after the exercise which could have accounted for the varying results obtained. Variable dilution of urine is standardised as all concentrations of metabolites are correlated with creatinine, which is excreted at a constant rate. The variability lies more in the fact that urine, collected several hours later, might reflect not only the metabolic period of exercise, but also that of post-exercise recovery. If performed again, catheterisation of mares immediately before and after exercise could be a way to obtain better information on this subject.

Practical consequences for veterinarians and equine clinics

Practical consequences for practising veterinarians

If a horse suffers from an acute, life threatening myopathy, there is no time to wait for all results of the additional diagnostic possibilities mentioned above. At such times the practitioner has to perform acute therapeutic help based on his putative diagnosis made after a thorough clinical examination. He might perform some diagnostic tests that can help him at short notice. These tests can consist of determining serum activities of muscle enzymes and concentration of lactate, leucocytes, and electrolytes. The colour of urine can also help obtaining the putative diagnosis. As mentioned before, this will not result in the exact diagnosis of the myopathy and therefore perhaps not the most appropriate therapy.
The accepted opinion is that it is harmful to transport a myopathic horse. The reasons are clear: a horse uses up a lot of energy and has to make a lot of muscle-effort to maintain standing position in a trailer. This can result in a worsening of the situation. However, in regard of diagnostic and therapeutic possibilities it is desirable to offer the horse the best options possible. If the practising veterinarian cannot offer this, a sensible decision is to have a more specialized veterinarian, with good knowledge and equipment, visit the horse. In a way the equine clinic will be moved to the horse instead of the horse to the clinic.
If satisfactory diagnostic options and treatment is unavailable at the location of the horse, a last option might be to transport the horse to the nearest facility where all diagnostic options are available, like extensive blood analysis facilities, muscle biopsy, clamping technique and extensive metabolic analyses, and where the chance of receiving the correct therapy, including glucose, insulin, carnitine and riboflavin, is significantly enhanced. The decision needs careful weighing the disadvantages against the advantages. If transport is considered, the practitioner should take important measures such as to ensure the horse has ample space in the van to be able to lie down on a thick bed of straw, to take the first sample of urine possible and send that to the clinic along with the horse, as well as one or more samples of heparinised plasma taken from different time points at home. Infusions and analgesics prior to transport are recommended.

If a horse suffers from recurrent myopathies the veterinarian can focus on treating the horse and collecting material in the acute phase and perform further diagnostic research after this phase. In this case he has the choice of sending in material for diagnostic purposes, like urine, plasma and muscle, or he can send the horse to a clinic for further analysis along with material collected in the acute stadium. Further analysis could include an assessment of plasma acylcarnitine profile that might reflect skeletal muscle carnitine metabolism following exercise thereby providing a possible practical method to investigate potential disorders in carnitine metabolism in horses suffering from myopathy (Chapter 7).

If a therapy with the antioxidant vitamin E is in order, the practitioner has to keep in mind that the dosage should be high enough, the length of therapy should be long enough and the vitamin E concentration in blood should be analysed multiple times to render reliable results (Chapter 8).

Practical consequences for equine clinics

Equine clinics in the country strive to perform state-of-the-art veterinary health care. The myopathic patients as described in this thesis are a challenging group, but demand a large effort of the veterinarian regarding diagnostic and therapeutic measures. To achieve an optimal situation, some conditions must be met. Firstly, a good understanding of the situation must be obtained, allowing clinicians to visit the myopathic horses or the horses to be transported. Secondly, the diagnosis must be made relatively quickly, in order to start the right therapy as soon as possible. Therefore, facilities as described in "diagnostics" must be available. Third, material and manpower for optimal therapy must be available.
If, for instance, a disease is diagnosed like MADD, a disorder in the fatty acid oxidation, therapy would be differentiated in nutritional, symptomatic and pharmacological therapy [31]. Nutritional treatment would consist of avoiding fasting, restrict fat intake while encouraging carbohydrate intake. Symptomatic treatment would be intravenous glucose infusion under constant evaluation of blood glucose concentration, insulin intravenous and subcutaneous (to enhance the muscular glucose uptake), continuous infusion therapy if the patient is dehydrated, and intravenous sodium bicarbonate if a metabolic acidosis is present. Pharmacological treatment could be intravenous carnitine, that detoxifies these fatty acids by connecting to them and get excreted via the urine as a carnitine-ester [4,31], and riboflavin iv, also known as vitamin B_2. This is a coenzyme of the deficient dehydrogenase enzymes in MADD at the level of electron transfer and can stimulate the enzyme activity sometimes and can therefore have therapeutic value [31,32]. If necessary the horse could be hoisted up and could be helped to remain standing with a supporting device.
While treating the horses, material can be collected, without any harm done to the horse, to perform further research on metabolic myopathies.

Conclusions

In this thesis analyses of metabolites of carbohydrate-, lipid- and protein metabolism have been performed as well as analyses of the purine nucleotide cycle, the creatine metabolism and the oxidative phosphorylation. With these analyses we studied metabolic myopathies in the horse as well as metabolic effects of training, intensified training and exercise.
-Atypical myopathy, a metabolic myopathy with high mortality rates and patients worldwide, is associated with MADD, multiple acyl-CoA dehydrogenase deficiency, a disorder in the β-oxidation (Chapter 3 and 4).

-Examples of general parameters for (atypical) myopathy are increased creatine kinase (CK), lactic acid, creatine and uric acid (Chapter 5).
-Exercise was associated predominantly with alterations in plasma concentrations of shortchain acylcarnitines. In addition, also in the equine species carnitine forms esters predominantly with short- and medium-chain fatty acids. These findings suggest that plasma acylcarnitine profile in the equine species might reflect skeletal muscle carnitine metabolism following exercise thereby providing a possible practical method to investigate potential disorders in carnitine metabolism in horses suffering from myopathy (Chapter 7).
-Early overtraining is not reflected by changes in amino acid, organic acid or carnitine concentration (Chapter 6 and 7).
-Given the fact that α-tocopherol acetate administration at an oral dosage of 4 mg/kg BW daily neither altered glucose metabolism nor peripheral insulin sensitivity, further research with a higher dose is necessary (Chapter 8).

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Summary

This thesis focuses on the diagnostic use of metabolic products and enzymes found in plasma, urine and muscle of the horse. Such metabolic products, metabolites and enzymes can reveal changes in muscle metabolism, either physiologically or pathologically, by their accumulation or deficiency.

In the studies described in this thesis, analyses of metabolites of carbohydrate-, lipid- and protein metabolism have been performed as well as analyses of the purine nucleotide cycle, creatine metabolism and oxidative phosphorylation. With these analyses we studied metabolic myopathies in the horse as well as metabolic effects of training, intensified training and exercise.

In **Chapter 2,** an overview is given of currently known human and equine metabolic myopathies with emphasis on their diagnosis. Metabolic myopathies are muscle disorders caused by a biochemical defect of the skeletal muscle energy system, which results in inefficient muscle performance. Myopathies can arise in multiple components of the metabolic system. In this review, metabolic myopathies are categorized in disorders of carbohydrate metabolism or lipid metabolism, mitochondrial myopathies (other than those described in lipid metabolism), disorders of purine metabolism, primary disorders involving ion channels and electrolyte flux, and secondary or acquired metabolic myopathies.

Chapter 3 describes the extensive diagnostic pathway of two equine myopathic patients to a diagnosis of multiple acyl-CoA dehydrogenase deficiency (MADD). This disorder has not been recognised in animals before. Clinical signs of both horses were a stiff, insecure gait, myoglobinuria, and finally recumbency. Urine, plasma, and muscle tissues were investigated. Analysis of plasma showed hyperglycemia, lactic acidemia, increased activity of muscle enzymes aspartate aminotransferase (ASAT), lactate dehydrogenase (LDH), and creatine kinase (CK), and impaired kidney function (increased urea and creatinine). The most remarkable findings of organic acids in urine of both horses were increased lactic acid, ethylmalonic acid (EMA), 2-methylsuccinic acid, butyrylglycine (iso)valerylglycine, and hexanoylglycine. EMA was also increased in plasma of both animals. Furthermore, the profile of acylcarnitines in plasma from both animals showed a substantial elevation of C4-, C5-, C6-, C8-, and C5-DC-carnitine. Concentrations of acylcarnitines in urine of both animals revealed increased excretions of C2-, C3-, C4-, C5-, C6-, C5-OH-, C8-, C10:1-, C10-, and C5-DC-carnitine. In addition, concentrations of free carnitine were also increased. Quantitative biochemical measurement of enzyme activities in muscle tissue showed deficiencies of short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and isovaleryl-CoA dehydrogenase (IVD), also indicating MADD. Histology revealed extensive rhabdomyolysis with microvesicular lipidosis predominantly in type 1 muscle fibers and mitochondrial damage. However, the electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETF-QO) activities were within normal limits indicating that the metabolic disorder were acquired rather than inherited.

The objective of **Chapter 4** was to assess lipid metabolism in horses with atypical myopathy, an acute myopathy that appears in grazing horses. Urine samples from 10 cases were subjected to analysis of organic acids, glycine conjugates and acylcarnitines, revealing increased mean excretion of lactic acid, ethylmalonic acid, 2-methylsuccinic acid, butyrylglycine, (iso)valerylglycine, hexanoylglycine, free carnitine, C2- , C3- , C4- , C5-, C6-carnitine as compared with 15 control horses (12 healthy and three with acute myopathy due to other causes). Analysis of plasma revealed similar results for acylcarnitines. Furthermore, measurement of dehydrogenase activities in lateral vastus muscle from one horse with atypical myopathy showed deficiencies of Short-Chain Acyl-CoA Dehydrogenase (SCAD, 0.66 nmol min^{-1} mg^{-1}), MCAD (Medium-Chain Acyl-CoA Dehydrogenase, 0.36 nmol min^{-1} mg^{-1}**)** and Isovaleryl-CoA Dehydrogenase (ICD, 0.74 nmol min^{-1} mg^{-1}).

These findings suggest a possible aetiology for a highly fatal and prevalent toxic equine muscle disease. Our data could aid in development of effective treatments by enhancement of carbohydrate metabolism rather than fat metabolism.

The diagnostic analysis of creatine-, carbohydrate- and purine metabolism as well as oxidative phosphorylation is described in **Chapter 5**, in healthy horses and horses with MADD. The goal of this study was to see if, apart from the disorder in lipid metabolism, the exogenic toxic insult associated with MADD disrupted other metabolic cycles or reactions. Examples of general metabolic parameters for myopathic damage found were plasma CK and lactic acid, and urine creatine and uric acid. Very low activity of the enzyme phosphoglycerate mutase (PGAM) and severe lowered activity of ATP-synthase in some patients were found.

Chapter 6 and 7 describe a study on the effects of intense exercise and (intensified) training on protein (amino acids) and lipid (organic acids, carnitines) metabolism. Ten Standardbred horses were trained for 32 weeks, divided into four phases, including a phase of intensified training for five horses. At the end of each phase, a standard exercise test, SET, was performed. Plasma amino acid, fatty acid and carnitine concentrations before and after each SET were measured. Training significantly reduced mean plasma concentration of aspartic acid. Exercise significantly increased the plasma concentrations of alanine, taurine, methionine, leucine, tyrosine and phenylalanine. Exercise significantly reduced the plasma concentrations of glycine, ornithine, glutamine, citrulline and serine. Concentrations of lactic acid, total nonesterified fatty acids, 3-OH-isobutyric acid, and C2-carnitine significantly increased in all SETS following exercise, whereas β-hydroxybutyric acid, C3- and C4-carnitine were significantly decreased. No significant effect of training and intensified training was found. Exercise was associated with alterations in plasma concentrations of short-chain acylcarnitines. In horses, carnitine forms esters predominantly with short- and medium-chain fatty acids. Despite decreased

ketogenesis as reflected by a lower level of β-hydroxybutyric acid, the ketogenic pathway using valine was increased following exercise.
Our findings suggest that plasma acylcarnitine profiles in the equine species reflect skeletal muscle carnitine metabolism following exercise. These profiles may provide practical method for investigation of potential disorders in carnitine metabolism in horses suffering from myopathy.

Chapter 8 describes a Latin square study with six horses using the euglycaemic hyperinsulinaemic clamp method to determine the effect of α-tocopherol acetate (vitamin E) supplementation at a dose of 4 mg/kg BW/day on glucose metabolism, peripheral insulin sensitivity and anti-oxidant status. Antioxidant status was measured using five different methods, i.e. glutathionperoxidase (GSH-Px)-activity, vitamin E and selenium-concentration, 3-(4-morpholino)-sydnone imine (SIN)-induced, cypridina luciferin analog-enhanced chemiluminescence expressed as Trolox equivalents and the Total Antioxidant Power assay expressed as copper reducing equivalents. Results showed that at this dosage neither glucose metabolism nor peripheral insulin sensitivity were altered by vitamin E supplementation. In addition, α-tocopherol acetate administration did not improve the measured parameters of antioxidant status.

In **Chapter 9** the main results and conclusions are discussed, as well as recommendations for practicing veterinarians and equine clinics.

Samenvatting

Dit proefschrift richt zich op het diagnostische gebruik van metabolieten en enzymen, die worden gevonden in plasma, urine en spierweefsel van het paard. Deze metabolieten en enzymen kunnen zowel fysiologische als pathologische veranderingen onthullen in de spierstofwisseling, door te accumuleren ofwel door deficiënt te zijn.

Voor dit proefschrift zijn analyses verricht van metabolieten van het koolhydraat-, vet- en eiwitmetabolisme evenals analyses van het purine- en creatinemetabolisme en de ademhalingsketen. Met deze analyses bestudeerden wij metabole spierziekten bij het paard evenals de metabole effecten van training, intensieve training en arbeid.

In hoofdstuk 2 wordt een overzicht gegeven van de op dit moment bekende spieraandoeningen van mens en paard met nadruk op de diagnostische benadering. Metabole spieraandoeningen zijn spierziekten die door een biochemisch defect van de energievoorziening van de skeletspier worden veroorzaakt. Dit kan resulteren in een inefficiënte spierprestatie. Spieraandoeningen kunnen op verschillende niveaus in de stofwisseling ontstaan. In dit overzicht worden de spieraandoeningen ingedeeld in aandoeningen van de koolhydraatstofwisseling, de vetstofwisseling, mitochondriale aandoeningen (anders dan die al bij vetstofwisseling zijn beschreven), de purine stofwisseling, ionkanaal- en electrolytenafwijkingen en secundaire of verkregen spieraandoeningen.

Hoofdstuk 3 beschrijft de uitgebreide diagnostiek, gedaan bij twee patiënten met een spieraandoening, die leidde tot de diagnose multiple acyl-CoA dehydrogenase deficiëntie (MADD). Deze aandoening is nog niet eerder bij dieren beschreven. Klinische symptomen waren een stijve, onzekere gang, myoglobinurie, en na enige tijd de onmogelijkheid om te kunnen staan. Urine, plasma en spierweefsel zijn onderzocht. Analyse van plasma resulteerde in hyperglycemie, lactaat acidose, een verhoogde activiteit van de spierenzymen ASAT, CK en LDH en een verstoorde nierfunctie (verhoogde ureum- en creatinineconcentraties). De meest opvallende bevindingen van de analyse van organische zuren in urine waren een sterk verhoogde concentratie van lactaat, ethylmalonzuur, barnsteenzuur, butyrylglycine (iso)valerylglycine, and hexanoylglycine. De concentratie van ethylmalonzuur was ook sterk verhoogd in plasma. Verder vertoonde het acylcarnitine profiel in plasma van de patiënten een substantiële stijging van C4-, C5-, C6-, C8-, en C5-DC-carnitine. Concentraties van acylcarnitines in urine waren sterk verhoogd bij C2-, C3-, C4-, C5-, C6-, C5-OH-, C8-, C10:1-, C10-, and C5-DC-carnitine evenals de concentratie van vrije carnitines. Kwantitatieve biochemische bepaling van enzymactiviteiten in spierweefsel toonde deficiënties van kortketen acyl-CoA dehydrogenase (SCAD), mediumketen acyl-CoA dehydrogenase (MCAD), en isovaleryl-CoA dehydrogenase (IVD), wat kenmerkend is voor de aandoening MADD. Het histologische beeld was er een van rhabdomyolyse met mitochondriale schade en kleine vetdruppeltjes, vooral in type 1 vezels. Het feit dat de ETF en ETF-QO activiteiten binnen

de normaalwaarden waren gaf aan dat de stofwisselingsziekte verkregen in plaats van genetisch was.

Het doel van hoofdstuk 4 was om veel facetten van de vetstofwisseling te bepalen in paarden met atypische myopathie, een acute, veelal fatale, spieraandoening van grazende paarden. Urinemonsters van 10 patiënten werden onderworpen aan analyse van de organische zuren, glycine conjugaten en acylcarnitines. Dit leverde verhoogde excretiewaarden op van melkzuur, ethylmalonzuur, barnsteenzuur, butyrylglycine, (iso)valerylglycine, hexanoylglycine, vrij carnitine, en C2- , C3- , C4- , C5-, C6-carnitine, vergeleken met 12 controlepaarden en 3 paarden met een acute spierziekte, anders dan atypische myopathie. Analyse van plasma gaf vergelijkbare resultaten voor acylcarnitines. Er werden ook deficiënties van SCAD, MCAD en IVD gevonden in spierweefsel, wat de diagnose MADD bevestigde. Deze bevindingen suggereren een mogelijke toxische etiologie voor de spierziekte. Zij zouden kunnen helpen bij het ontwikkelen van een effectieve behandeling, bijvoorbeeld door koolhydraatstofwisseling te bevorderen in plaats van vetstofwisseling.

Diagnostische analyse van creatine, koolhydraat, en purine stofwisseling evenals de ademhalingsketen wordt beschreven in hoofdstuk 5, bij gezonde dieren en bij dieren met MADD. Doel van de studie was om te zien of het vermeende toxine naast de eerder beschreven aandoening van het vetmetabolisme, nog andere paden van de stofwisseling zou beschadigen. Voorbeelden van algemene parameters voor spierschade, die werden gevonden, zijn plasma creatine kinase (CK), en melkzuur (lactaat) en creatine en urinezuur in urine. Bij enkele patiënten werd een zeer lage concentratie gevonden van de enzymen phosphoglyceraat mutase (PGAM) en ATP-synthase.

Hoofdstuk 6 en 7 beschrijven een studie die gericht is op de effecten van acute, intense arbeid en (intense) training op eiwit- (aminozuren) en vet- (organische zuren, carnitines) stofwisseling. Tien dravers werden 32 weken getraind, verdeeld over 4 fases, inclusief een fase van erg intense training voor 5 paarden. Aan het eind van elke fase werd een SET, standaard arbeids (exercise) test gedaan. Concentraties van aminozuren, vetzuren en carnitines werden bepaald voor en na elke SET. Training zorgde voor een significante verlaging van de concentratie van asparaginezuur. Arbeid verhoogde significant de concentraties in plasma van alanine, taurine, methionine, leucine, tyrosine and phenylalanine and verlaagde significant de plasma concentraties van glycine, ornithine, glutamine, citrulline and serine. Concentraties van lactaat, totaal niet veresterde vetzuren (vrije vetzuren) en C2-carnitine stegen significant na arbeid, terwijl β-hydroxyboterzuur, C3- en C4-carnitine significant daalde in concentratie. Er werd geen significant effect van training en intensieve training gevonden. Arbeid werd geassocieerd met veranderingen in plasma concentratie van kortketen acylcarnitines. Ook bij het paard vormen carnitine esters zich vooral uit kort- en middenketen vetzuren. Onze bevindingen suggereren dat het acylcarnitine profiel in plasma na arbeid een weergave kan zijn van het carnitine

metabolisme in de spier. Hierdoor ontstaat er mogelijk een praktische methode om mogelijke aandoeningen van het carnitine metabolisme te onderzoeken in paarden met een spierziekte.

Hoofdstuk 8 beschrijft een studie met 6 paarden, die 4 mg vitamine E per kg LG per dag kregen in een opzet met latijns vierkant. Het effect op glucose stofwisseling, perifere insuline gevoeligheid en antioxidant status wordt gemeten met behulp van een euglycemische, hyperinsulinemische clamp. Antioxidant status werd gemeten met vijf methoden, namelijk glutathionperoxidase (GSH-Px)-activiteit, vitamine E and selenium-concentration, 3-(4-morpholino)-sydnone imine (SIN)-geïnduceerd, cypridina luciferin analoog-versterkte chemiluminescentie uitgedrukt in Trolox equivalenten en de Total Antioxidant Power test, uitgedrukt in koper reducerende equivalenten. Resultaten toonden aan dat vitamine E in deze dosering noch invloed had op glucose stofwisseling, noch op perifere insuline gevoeligheid. Tevens verbeterde het niet de parameters van de antioxidant status.

In hoofdstuk 9 worden de belangrijkste resultaten en aanbevelingen voor practici en klinieken bediscussieerd.

Samenvatting voor niet ingewijden

Stofwisseling zorgt ervoor dat het paard kan functioneren. Het paard neemt via de voeding stoffen op. Alles wat gegeten wordt, suiker, vet en eiwit, wordt verkleind en van de darm via het bloed naar de lever vervoerd. Daar vindt de stofwisseling plaats. Overbodig geworden materiaal wordt weer uitgescheiden via (vooral) urine en feces. Van suiker wordt glucose gemaakt, van vet worden vetzuren gemaakt en van eiwit aminozuren. Uit die aminozuren worden de eigen eiwitten gemaakt, enzymen. Die kan het paard dus niet via het voer opnemen. Bij overtollig suiker wordt er glycogeen van gemaakt.

Om glucose, vet of eiwit tot energie te maken zijn er heel veel stappen nodig . Stappen hebben meestal een enzym nodig. Het lichaam kent wel tienduizend verschillende enzymen.
Als het paard door een ziekte een enzym mist, kan de stap niet genomen worden. Dan is het resultaat een ophoping van het product vóór die enzymstap (bv. A) en een relatief tekort aan producten die gevormd zouden hebben moeten worden (bv. B). Een dergelijke ophoping kan schade veroorzaken en een relatief tekort aan een eindproduct kan ook schadelijk zijn.
Als er een foutje zit in het DNA produceert het lichaam levenslang bv. een enzym dat net niet helemaal klopt. Hierdoor hapert dan de stofwisseling.

De stofwisseling zorgt er dus voor dat van de glucose, vetzuren en aminozuren energie wordt gemaakt. Via verschillende mechanismen worden alle onderdelen afgebroken tot producten, vooral acetyl CoA, die in de citroenzuurcyclus omgezet worden in energierijke metabolieten ATP, NADH en FADH2. NADH en FADH2 worden in de oxidatieve fosforylering, de ademhalingsketen, ook nog omgezet in ATP. ATP levert de benodigde energie voor het lichaam.

Dit onderzoek heeft de stofwisseling bij het paard onderzocht met behulp van diverse humane ziekenhuizen (Wilhelmina Kinder ziekenhuis UMC Utrecht, Erasmus Ziekenhuis Rotterdam, AMC Amsterdam en Universitair Ziekenhuis Maastricht). Van zieke paarden, langdurig getrainde paarden, overtrainde paarden en paarden die acute, intensieve arbeid verrichtten, is bloed en urine afgenomen en zijn alle tussenproducten in de stofwisseling gemeten. Tevens zijn in stukjes spierweefsel diverse enzymen gemeten.

Opvallende dingen die gevonden zijn:
-Een nieuwe spierziekte bij het paard (MADD, multiple acyl-CoA dehydrogenase deficiency). Deze aandoening blijkt de oorzaak te zijn van de wereldwijd veel voorkomende spieraandoening, atypische myopathie, met tot nu toe 90% sterfte. We gaan door met onderzoek hiernaar, om tot preventie en optimale therapie te komen en de mortaliteit daarmee te verlagen.

-Afwijkingen in de glucosestofwisseling en de ademhalingsketen. Hierdoor is er een niet-optimale energievoorziening in de bewuste paarden.
-Diverse nieuwe bevindingen over de stofwisselingsveranderingen tijdens arbeid.

Met dit onderzoek kunnen we wellicht in de toekomst een advies op maat geven bij ziekte, training en arbeid over voeding en supplementen als aminozuren, carnitines en vitaminen.

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Curriculum Vitae

Cornélie Martine Westermann was born on July 7, 1968, in Voorschoten, The Netherlands. She graduated from high school (St. Oelbert Gymnasium) in 1986. After one year of studying Biology at Utrecht University, she started her studies in Veterinary Medicine at the Faculty of Veterinary Sciences, University of Utrecht, The Netherlands in 1987. In 1996 she graduated as veterinarian. During her student years, she was on many committees, including the executive committee of the Veterinary Students' Society. From 1996 to 1999 she worked in a mixed veterinary practice in Middelharnis, the Netherlands. In 2000 she started working as junior lecturer in Equine Internal Medicine, Department of Equine Sciences, Faculty of Veterinary Sciences, University of Utrecht, where she subsequently did residency from 2001 until 2004. This lead to the title of Specialist KNMvD (Royal Dutch Veterinary Association) Equine Internal Medicine, which was awarded to her in 2005. From 2005 to 2007 she worked as junior lecturer and researcher at the Equine Internal Medicine, Department of Equine Sciences, Faculty of Veterinary Sciences, University of Utrecht. In 2007 she passed the exam to become a Diplomate of the European College of Equine Internal Medicine (ECEIM). Since 2008, she holds a permanent position in Equine Internal Medicine at Utrecht University. Her responsibilities include clinical work, teaching and research.

Cornélie Martine Westermann is geboren op 7 juli 1968 in Voorschoten, Nederland. In 1986 slaagde zij voor de middelbare school (St. Oelbert gymnasium). Na een jaar de studie biologie te hebben gevolgd aan de Universiteit Utrecht, startte zij in 1987 met de studie diergeneeskunde aan de Faculteit Diergeneeskunde in Utrecht. In 1994 behaalde zij het doctoraal examen, en in 1996 verkreeg ze het dierenartsdiploma. Tijdens haar studententijd zat ze in vele commissies en in het bestuur van de Diergeneeskundige Studenten Kring. Van 1996 tot 1999 werkte ze in een gemengde praktijk in Middelharnis op Goeree Overflakkee. In 2000 werd begonnen met een baan als dierenarts bij de discipline Inwendige Ziekten van de Hoofdafdeling Gezondheidszorg Paard aan de Faculteit Diergeneeskunde te Utrecht, gevolgd door een aanstelling als specialist in opleiding (SIO) van 2001 tot 2004. Dit resulteerde in de titel van Specialist Inwendige Ziekte van het Paard in 2005. Van 2005 tot 2007 werkte ze als junior docent en onderzoeker bij dezelfde discipline, een combinatie van kliniekwerk, onderwijs en promotie onderzoek. Vanaf januari 2008 werkt ze daar in vast dienstverband.
In 2007 werd het Europees specialisten examen gehaald en is ze Diplomate of the European College of Equine Internal Medicine (ECEIM).

List of Publications

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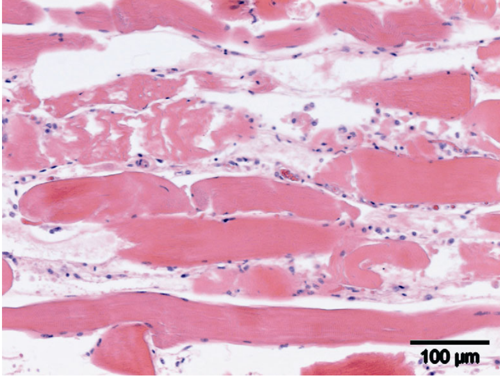
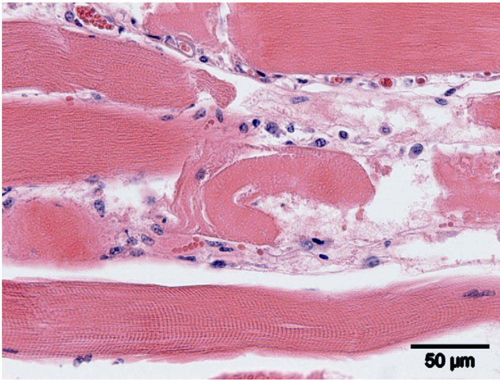
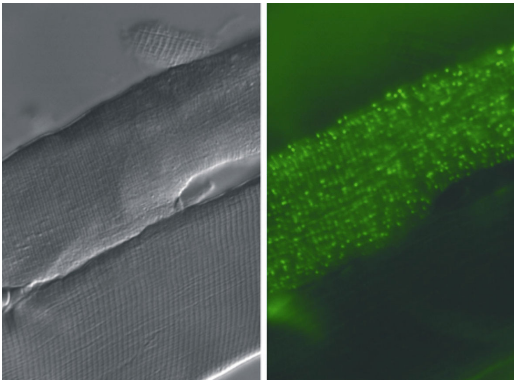


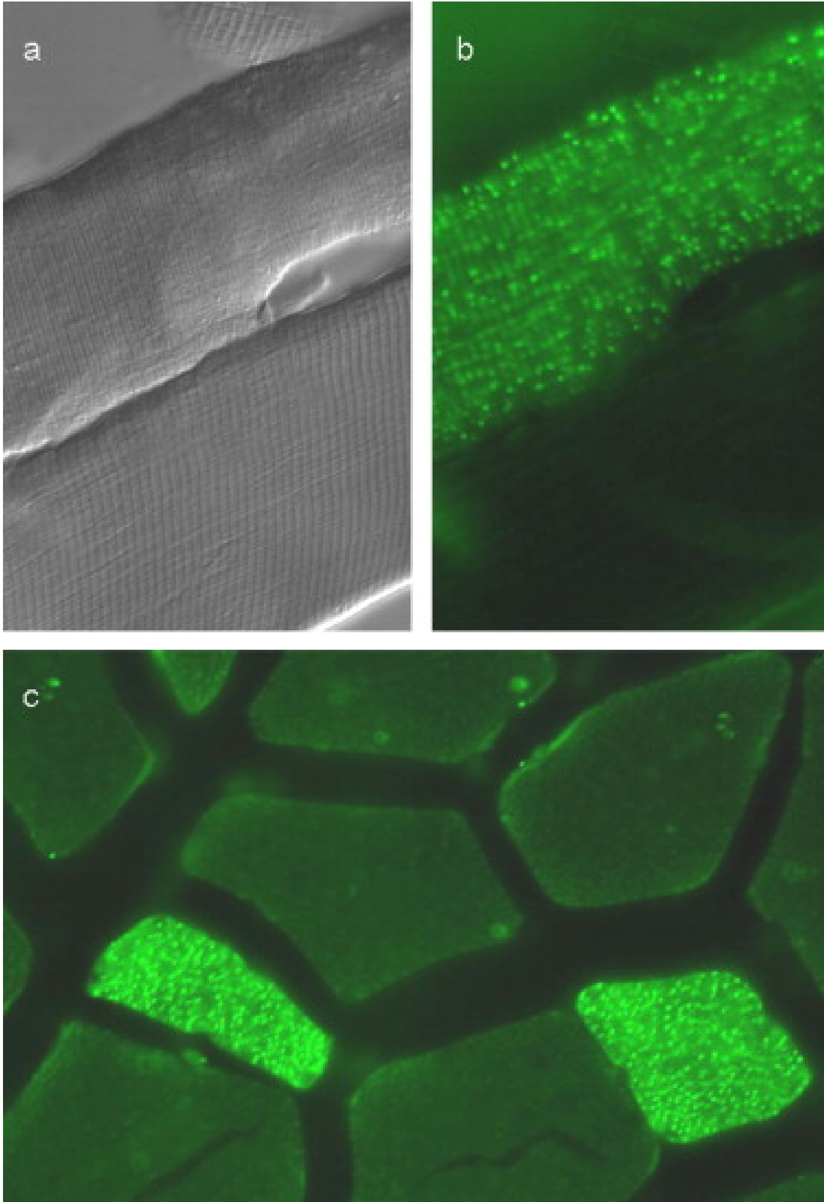
Chapter 3 figure 1



Chapter 3 figure 2



Chapter 3 figure 4



Chapter 4 figure 1

Referentiewaardenkaartje

	Ondergrens (gemiddelde min 2 x SD)	Bovengrens (gemiddelde plus 2 x SD)	Percentiel 95
Organische zuren in plasma (µmol/l) Gebaseerd op 12 gezonde controlepaarden			
melkzuur	16	2158	2162
2-OH-boterzuur	0	24	24
3-OH-boterzuur	62	391	379
3-OH-isoboterzuur	14	111	107
laurinezuur	1	1	1
myristinezuur	1	1	1
citroenzuur	8	139	126
palmitoliezuur	1	1	1
palmitinezuur	2	6	5
oliezuur	1	4	3
linolzuur	2	5	4
stearinezuur	2	4	4
glutaarzuur			5
cis-4-deceenzuur	2	5	4
3-ketoboterzuur	7	13	11

Organische zuren in urine (µmol per mmol kreatinine) Gebaseerd op 12 gezonde controlepaarden			
melkzuur	0	141	133
glycolzuur	3	28	22
pyrodruivenzuur	2	10	9
3-OH-boterzuur	0	133	131
3-OH-isoboterzuur	0	138	127
3-OH-isovaleriaanzuur	0	38	34
methylmalonzuur	0	7	7
ethylmalonzuur	0	5	5
barnsteenzuur	0	12	10
adipinezuur	0	4	4
2-ketoglutaarzuur	10	74	56
3-OH-adipinezuur	0	55	47
onverz. Suberinezuur	0	12	10
suberinezuur	0	6	6
homovanillinezuur	0	39	37
citroenzuur	28	151	130
fumaarzuur	0	17	12
glutaarzuur	0	136	122
appelzuur	0	263	184
2-OH-boterzuur	0	8	5

	Ondergrens (gemiddelde min 2 x SD)	Bovengrens (gemiddelde plus 2 x SD)	Percentiel 95
Aminozuren in plasma (µmol/l) Gebaseerd op 12 gezonde controlepaarden			
taurine	17	49	44
asparaginezuur	3	10	10
hydroxyproline			143
threonine	79	275	233
serine	44	270	236
asparagine	3	83	80
glutaminezuur	0	149	113
glutamine	109	448	353
proline	0	290	261

glycine	126	595	506
alanine	47	314	302
citrulline	22	130	103
alfa-aminoboterzuur	0	228	162
valine	67	657	512
cystine	25	51	48
methionine	3	83	80
isoleucine	42	163	141
leucine	71	292	255
tyrosine	0	198	190
fenylalanine	0	214	189
ornithine	37	84	76
histidine	45	96	90
lysine	72	198	172
arginine	48	154	141
alfa-aminoadipinezuur	64	237	205

Aminozuren in urine (µmol per mmol kreatinine) Gebaseerd op 12 gezonde controlepaarden			
1-CH3-histidine	3	20	17
3-CH3-histidine	7	35	33
alanine	0	103	100
aminoadipinezuur	5	90	80
aminoboterzuur	0	5	4
arginine	0	32	26
asparagine	0	124	110
aspartaanzuur	5	16	14
carnosine	4	9	8
citrulline	0	264	223
cysteine	0	129	117
glutamine	0	696	664
glutaminezuur	0	40	38
glycine	0	584	559
histidine	0	114	110
homocitrulline	0	14	13
leucine	0	10	9
lysine	0	102	87
ornithine	0	24	21
phenylalanine	0	7	7
serine	0	653	610
taurine	0	189	164
threonine	0	1146	928
tyrosine	0	60	50
valine	0	22	22

	Ondergrens (gemiddelde min 2 x SD)	**Bovengrens (gemiddelde plus 2 x SD)**	**Percentiel 95**
Carnitines in plasma (µmol/l) Gebaseerd op 12 gezonde controlepaarden			
vrij carnitine	20,61	47,89	44
C2-carnitine	2,21	5,93	5,55
C3-carnitine	0,33	1,05	0,95
C4-carnitine	0,2	0,72	0,67
C5:1-carnitine	0,02	0,02	0,02
C5-carnitine	0,13	0,57	0,52
C4:3-OH-carnitine	0	0,04	0,03
C6-carnitine	0,01	0,01	0,02
C5-OH-carnitine	0	0,03	0,04
C8:1-carnitine	0	0,03	0,02
C8-carnitine	0,02	0,02	0,02

C10:2-carnitine	0,02	0,02	0,02
C10:1-carnitine	0	0,04	0,02
C10-carnitine	0	0,04	0,03
C4-DC-carnitine	0,02	0,06	0,05
C5-DC-carnitine	0,02	0,06	0,06
C12:1-carnitine	0	0,03	0,02
C12-carnitine	0	0,04	0,02
C6-DC-carrnitine	0,02	0,02	0,02
C14:2-carnitine	0,02	0,02	0,02
C14:1-carnitine	0	0,04	0,03
C14-carnitine	0,02	0,02	0,02
C8-DC-carnitine	0,02	0,02	0,02
C14-OH-carnitine	0,01	0,01	0,01
C16:1-carnitine	0,01	0,01	0,02
C16-carnitine	0	0,04	0,02
C10-DC-carnitine	0,01	0,01	0,01
C16:1-OH-carnitine	0,01	0,01	0,01
C16-OH-carnitine	0,01	0,01	0,01
C18:2-carnitine	0,01	0,01	0,01
C18:1-carnitine	0	0,04	0,02
C18-carnitine	0,01	0,01	0,01
C18:2-OH-carnitine	0,01	0,01	0,01
C18:1-OH-carnitine	0,01	0,01	0,01
C16-DC-carnitine	0,01	0,01	0,01
C18:1-DC-carnitine	0,01	0,01	0,01

Carnitines in urine (mmol/mol kreatinine) **Gebaseerd op 12 gezonde controlepaarden**			
vrij carnitine	0,00	13,13	12,52
C2-carnitine	0,00	1,04	1,01
C3-carnitine	0,00	0,11	0,10
C4-carnitine	0,00	1,14	1,01
C5:1-carnitine	0,00	0,00	0,01
C5-carnitine	0,00	0,14	0,14
C4:3-OH-carnitine	0,00	0,00	0,00
C6-carnitine	0,00	0,03	0,02
C5-OH-carnitine	0,01	0,13	0,13
C8:1-carnitine	0,00	0,00	0,01
C8-carnitine	0,00	0,04	0,03
C10:2-carnitine	0,00	0,03	0,02
C10:1-carnitine	0,01	0,01	0,01
C10-carnitine	0,01	0,01	0,02
C4-DC-carnitine	0,00	0,48	0,44
C5-DC-carnitine	0,04	0,86	0,47
C12:1-carnitine	0,01	0,01	0,01
C12-carnitine	0,00	0,04	0,04
C6-DC-carrnitine	0,01	0,17	0,14
C14:2-carnitine	0,00	0,00	0,00
C14:1-carnitine	0,00	0,00	0,01
C14-carnitine	0,00	0,04	0,03
C8-DC-carnitine	0,00	0,07	0,05
C14-OH-carnitine	0,01	0,01	0,01
C16:1-carnitine	0,00	0,00	0,00
C16-carnitine	0,00	0,03	0,02
C10-DC-carnitine	0,00	0,03	0,02
C16:1-OH-carnitine	0,00	0,00	0,00
C16-OH-carnitine	0,00	0,01	0,01
C18:2-carnitine	0,00	0,00	0,00
C18:1-carnitine	0,00	0,00	0,00
C18-carnitine	0,00	0,00	0,00

C18:2-OH-carnitine	0,00	0,00	0,00
C18:1-OH-carnitine	0,00	0,00	0,00
C16-DC-carnitine	0,00	0,00	0,00
C18:1-DC-carnitine	0,00	0,00	0,00

	Gemiddelde	**Standaard Deviatie**	**Percentiel 95**
Creatine metabolisme and purine analyse in µmol/mmol creatinine Gebaseerd op 5 gezonde controlepaarden			
guanidinoacetaat	59	37	104
creatine	91	76	182
creatinine	9591	4149	13182
creatine/creatinine ratio	0.09	0.07	0.20
urine zuur	84	42	121
xanthine	0.2	0.4	0.8
inosine	0	0	0.0
adenine	0.8	1.1	2.0
guanosine	0.4	0.9	1.6
adenosine	0.4	0.5	1.0

	Gemiddelde	**Standaard Deviatie**	**Percentiel 95**
Enzym activiteiten in spier van koolhydraat metabolisme in nmol/mg eiwit (glycogeen in µg/mg eiwit) Gebaseerd op 7(*) respectievelijk 2 gezonde controlepaarden (*)			
glycogeen *	94	26	134
fosfoglyceraat mutase (PGAM, X) *	5847	3054	8815
fosfoglyceraat kinase (PGK, IX) *	3733	989	4520
fosfofructokinase (PFK, VII)*	2355	802	3335
fosforylase (V) *	621	105	752
fosforylase +/- AMP #*	1.7	0.4	2.1
enolase (XIII)	803	738	800
pyruvaat kinase (PK)	9680	9470	9670
lactaat dehydrogenase (XI)	9030	8170	8987
branching enzyme (IV)	651	622	650

	Gemiddelde	**Standaard Deviatie**	**Percentiel 95**
Enzym activiteiten in spier van oxidatieve fosforylering in nmol/mg eiwit (eiwit in mg/g spier) Gebaseerd op 6 gezonde controlepaarden			
eiwit	136	46	181
citrate synthetase (CS)	344	135	535
complex I	93	28	130
complex II	71	39	129
complex III	223	36	260
complex IV	307	110	458
complex V	230	164	472