

Histopathological findings in horses with and without clinical signs of rhabdomyolysis with special reference to polysaccharide storage myopathy

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ABSTRACT: Objective of the study was to assess histopathological changes in horses with a clinical history of exertional rhabdomyolysis (ER) with special reference to polysaccharide storage myopathy and to compare histopathological findings in horses with and without a clinical history of ER. In total 39 muscle samples were collected, from horses with a history of repeated episodes of exertional rhabdomyolysis (test group, 10 horses) and from horses without clinical signs of muscular disorders in their history (control group, 29 horses). Frozen muscle samples were stained with haematoxylin and eosin and periodic acid-Schiff with and without amylase digestion. Histopathologic changes (amylase resistant polysaccharide, subsarcolemmal glycogen, intracytoplasmic masses, subsarcolemmal vacuoles, fibre size variation and internal nuclei) were evaluated. There was a statistically significant difference between groups in the presence of subsarcolemmal amylase sensitive glycogen deposits ($P \leq 0.0001$), the risk ratio was 5.22. Statistically significant differences between groups were not found regarding the presence of intracytoplasmic masses, subsarcolemmal vacuoles, amylase resistant polysaccharide, fibre size variation and internal nuclei. Presence of amylase resistant polysaccharide within muscle fibres of apparently healthy horses could be a manifestation of different phenotype expression of PSSM but also the insufficient specificity of this diagnostic criterion.

Keywords: muscle; glycogen; horse

Exertional rhabdomyolysis (ER) is a common and economically relevant problem in horses. Clinical signs include muscle stiffness, muscle fasciculations, sweating, exercise intolerance, weakness, reluctance to move, lameness, swollen muscles and muscle wasting (Valberg et al., 1997; Hunt et al., 2008). Fatal rhabdomyolysis with the acute onset was described in Belgian and Percheron draught horses (Valentine et al., 1997).

Establishing the cause of ER is a diagnostic challenge. The most common cause of ER is polysac-

charide storage myopathy (PSSM). PSSM was first described in 1992 in nine Quarter Horse-related horses with a history of repeated ER. The disorder is characterized by abnormal subsarcolemmal and intracytoplasmic accumulation of amylase sensitive glycogen and amylase resistant polysaccharide within muscle fibres (Valberg et al., 1992). The most often affected breeds are Quarter Horse-related breeds and draught horses (McCue et al., 2006). PSSM was also reported in warmbloods, Standardbreds, Morgan, some pony breeds,

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Thoroughbreds and Arabian horses (Valentine et al., 2000; McCue et al., 2006; Hunt et al., 2008).

Diagnostic criteria of PSSM are not consistent among individual authors. Most often the diagnosis is made on the basis of amylase resistant polysaccharide presence within muscle fibres (Valberg et al., 1997; McGowan et al., 2003; Firshman et al., 2005). Some authors also consider subsarcolemmal and intracytoplasmic accumulation of amylase sensitive glycogen to be diagnostic for PSSM (Valentine et al., 2001; Valentine and Cooper, 2005). McCue et al. (2006) concluded that the accumulation of amylase sensitive glycogen as a diagnostic criterion might increase the sensitivity of histopathology but conversely decrease specificity of the diagnosis. For this reason, they recommend using this criterion only for horses with clinical signs of PSSM and in the breeds that are commonly affected with PSSM. Chronic non-specific myopathic changes including excessive fibre size variation and internal nuclei count are also found in some horses (Valentine et al., 2001; Firshman et al., 2005).

However, histopathologic finding of amylase resistant polysaccharide accumulations was described in some horses without clinical signs of myopathy (Firshman et al., 2005; Valentine and Cooper, 2005). Recently, mutation of the *GYS1* gene for glycogen synthase was identified in some horses with histopathological features of PSSM (McCue et al., 2008).

The purposes of this study were:

(1) To assess histopathological changes in muscle tissue of horses with a clinical history of ER with special reference to polysaccharide storage myopathy.

(2) To compare histopathological findings in horses with and without a clinical history of ER.

MATERIAL AND METHODS

Horses

Thirty-nine horses older than one year of age were included in the study. Twenty-eight horses were sampled at the Equine Clinic, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic, whereas eleven horses were sampled at the Equine Clinic of the Department of Equine Sciences, Utrecht University, The Netherlands. History connected with signs of ER and the use of horses was obtained from the own-

ers of all horses. On the basis of clinical history, the horses were divided into two groups: test and control group.

Test group

The test group consisted of 10 horses with a mean \pm SD age of 6.3 ± 2.0 years. Five horses were Quarter Horse-related breeds (three Quarter Horses, one Paint Horse, one Appaloosa), three warmbloods, one Czech Moravian Belgian draught horse (CMB) and one Thoroughbred. Use of horses included western riding, eventing, heavy draught, flat racing and pleasure riding.

Nine of them were sampled in Brno, one in Utrecht. All of them had had repeated episodes of ER in their history and repeatedly confirmed elevation of muscle enzyme activities and/or a positive result following an exercise test (20 min of trotting on the lunge or usual training session resulted in more than doubling of the basal creatine kinase activity four hours after exercise). Clinical signs included stiffness and reluctance to move in all horses. Sweating was observed in four horses, myoglobinuria in four horses, gait abnormality (short stride) in one horse and mild colic in two horses. Stiffness lead to recumbency in one horse and the horse was euthanized.

Glutathione peroxidase (GSH-Px) activity in the whole blood was determined in all horses from the test group. All horses had GSH-Px activity higher than the reference value ($> 12\,000$ IU/l) that had been established previously (Ludvikova et al., 2005).

Control group

The control group consisted of 29 horses with a mean \pm SD age of 9.0 ± 7.6 years. Twenty-four horses were euthanized for diseases unrelated to the neuromuscular system and five were two and half year old healthy CMB horses. None of these horses had a history or clinical signs of myopathy. Ten horses were warmbloods, seven CMB horses, three Thoroughbreds, four Friesian horses, two Arabian horses, one Shetland pony, one Standardbred and one Hucul horse. Nineteen of them were sampled in Brno, ten in Utrecht. Use of horses within the control group included pleasure riding, show-jumping and dressage. Two horses were used for heavy work

in the forest. Five CMB horses were in light draught training. Four young horses were not used for the work and kept on the pasture only. Exercise test was not performed in the control group.

Muscle tissue sampling

Gluteus medius muscle was sampled in nineteen horses (eight horses from the test group, eleven from the control group). Vastus lateralis muscle was sampled in one horse from the test group. Semitendinosus muscle was sampled in nineteen horses (one horse from the test group and eighteen horses from the control group). Horses from the control group that were euthanized were sampled immediately after euthanasia.

Needle biopsy from the gluteus medius muscle was performed using a modified Bergstrom needle (6 mm diameter) at two thirds of the distance on a line from the tail head to the tuber coxae (nearer to tuber coxae) from a depth of 6 cm. Vastus lateralis muscle was sampled using a modified Bergstrom needle (7 mm diameter) at a point located 15 cm ventral from the centre of the tuber coxae and 10 cm caudal from the cranial border of the vastus lateralis muscle from a depth of 5 cm. If necessary, sampling was repeated twice or three times until approximately 500 mg of muscle tissue was obtained.

Sampling from the semitendinosus muscle involved an open surgical method, through a 5 cm vertical skin and fascia incision at a site between the head of tail and tuber ischium. Local anaesthesia with bupivacaine was used in all live horses. Two parallel incisions were made longitudinal to the muscle fibres. Dorsal and ventral transverse incisions were then made to separate the muscle biopsy specimen.

Muscle tissue processing

The sections of muscle obtained by open surgical method were fixed to a cork plate by pins, wrapped in saline-moistened gauze and transported directly to the laboratories of Equine Clinic, University of Veterinary and Pharmaceutical Sciences Brno or Department of Equine Sciences, Utrecht University. The transverse and horizontal specimens were placed on thin cork discs and plunged into propane-butane (Brno) or isopentane (Utrecht) previously chilled in liquid nitrogen. The specimens

were then wrapped in tinfoil, placed in paper boxes and kept at -80°C until processing.

Staining

Six-micrometer thick sections of frozen muscle were stained with haematoxylin and eosin (HE) and periodic acid-Schiff with (A-PAS) and without (PAS) amylase digestion.

Sample evaluation

Histopathologic changes observed in the muscle samples were evaluated according to Table 1. Intracytoplasmic masses were characterized by central accumulation of intracytoplasmic PAS-positive amylase sensitive homogenous material.

Statistical analyses

Fisher's Exact test was used to test for difference between the test and control groups in the presence of subsarcolemmal glycogen, intracytoplasmic masses, subsarcolemmal vacuoles, PAS-positive amylase resistant polysaccharide, fiber size variation (grade 2–3) and internal nuclei (grade 2–3). Fiber size variation and internal nuclei grade 1 were not used for statistical analyses because of low specificity of these mild histopathologic changes. A risk ratio was calculated. A value of $P \leq 0.05$ was considered significant.

RESULTS

Histopathological findings in the test and control groups are summarized in Table 2. A statistically significant difference was found between groups in the presence of subsarcolemmal amylase sensitive glycogen deposits ($P \leq 0.0001$) (Figure 1), the risk ratio was 5.22. Statistically significant differences between groups were not found regarding the presence of intracytoplasmic masses, subsarcolemmal vacuoles (Figure 2), amylase resistant polysaccharide, grade 2 and 3 fibre size variation and internal nuclei.

Muscle fibre necrosis was observed in one sample from the test group (CMB stallion euthanized for severe rhabdomyolysis and recumbency).

Table 1. Evaluation of histopathological findings in muscle biopsy samples

PAS-positive amylase resistant polysaccharide (A-PAS)	Absent		Present	
Subsarcolemmal glycogen (PAS)	grade 0 absent	grade 1 1–3/100×	grade 2 4–6/100×	grade 3 > 6/100×
Intracytoplasmic masses (PAS)	absent	present		
Subsarcolemmal vacuoles (PAS)	absent	present		
Centrally located nuclei (HE)	grade 0 absent	grade 1 < 1/100×	grade 2 1–2/100×	grade 3 > 3/100×
Fibre size variation (HE)	grade 0 absent	grade 1 mild	grade 2 moderate	grade 3 severe
Necrosis (HE)	absent	present		

Legend: 100× magnification. Subsarcolemmal accumulations of glycogen: 1 = present in 1–3 fibres in one random microscopic field (rmf), 2 = present in 4–6 fibres in rmf, 3 = present in 6–10 fibres in rmf. Centrally located nuclei: 1 = present, but average of fewer than one fibre in rmf, 2 = present in 1–2 fibres in rmf, 3 = present in three or more fibres in rmf

HE = haematoxylin and eosin, PAS = periodic acid-Schiff, A-PAS = PAS with amylase digestion

Table 2. Incidence of histopathological findings in the test and control groups

Histopathological signs	Test group (<i>n</i> = 10)	Control group (<i>n</i> = 29)	<i>P</i>
Amylase resistant polysaccharide	3 (30%)	2 (7%)	N.S.
Subsarcolemmal glycogen	9 (90%)	5 (17%)	<i>P</i> ≤ 0.0001
Intracytoplasmic mass	4 (40%)	4 (14%)	N.S.
Subsarcolemmal vacuoles	3 (30%)	2 (7%)	N.S.
Internal nuclei (grade 2–3)	4 (40%)	4 (14%)	N.S.

N.S. = non significant



Figure 1. Amylase-sensitive subsarcolemmal glycogen (PAS, 400×, frozen section)

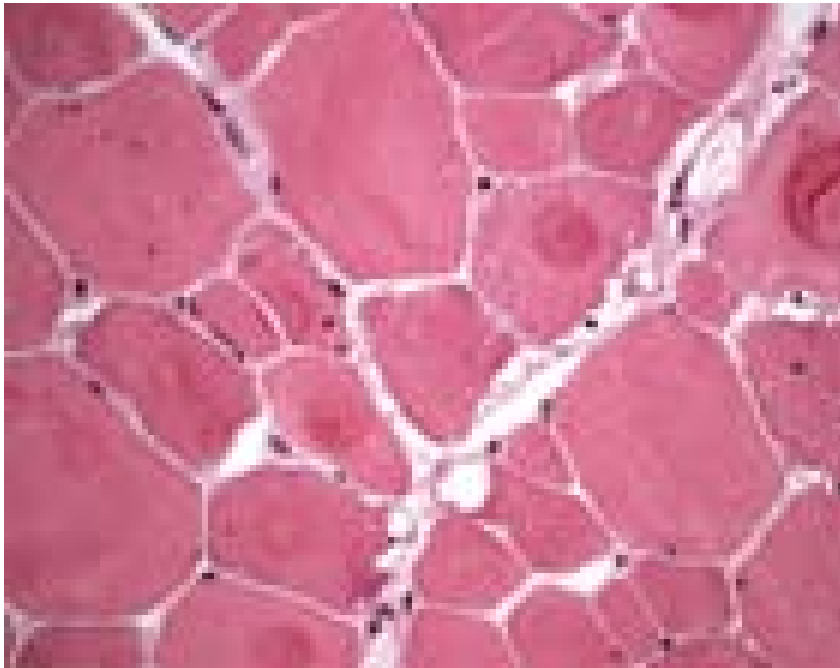


Figure 2. Increased fiber size variation and subsarcolemmal vacuoles (HE, 400×, frozen section)

DISCUSSION

The finding of amylase resistant polysaccharide within muscle fibres is considered as the gold standard diagnostic criterion of PSSM (Valberg et al., 1997; Firshman et al., 2005). In our study, amylase resistant polysaccharide was found in only three horses from the test group.

McCue et al. (2006) used as diagnostic criteria the finding of amylase resistant polysaccharide or subsarcolemmal glycogen in combination with clinical signs. According to these criteria we would establish diagnosis of PSSM in nine horses from the test group. McCue and Valberg (2007) used the finding of amylase resistant polysaccharide for an estimation

of prevalence of PSSM in Quarter Horses (horses without clinical signs of PSSM were examined). According to this rule the diagnosis of PSSM would be established in three horses from the test group and in two horses from the control group.

Valentine et al. (2001) and Valentine and Cooper (2005) established the diagnosis of PSSM on the finding of amylase resistant polysaccharide, subsarcolemmal aggregates of glycogen, central cytoplasmic bodies containing glycogen or any combination of these findings. According to these criteria we would diagnose PSSM in nine horses from the test group and also in nine horses from the control group (five CMB, one Standardbred, one warmblood, one Thoroughbred and one Friesian horse).

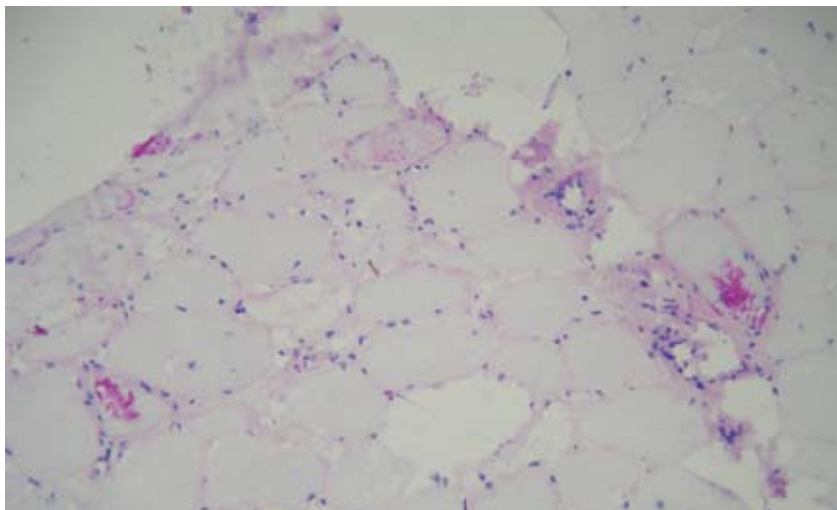


Figure 3. Amylase resistant polysaccharide in CMB mare from the control group (A-PAS, 200×, frozen section)

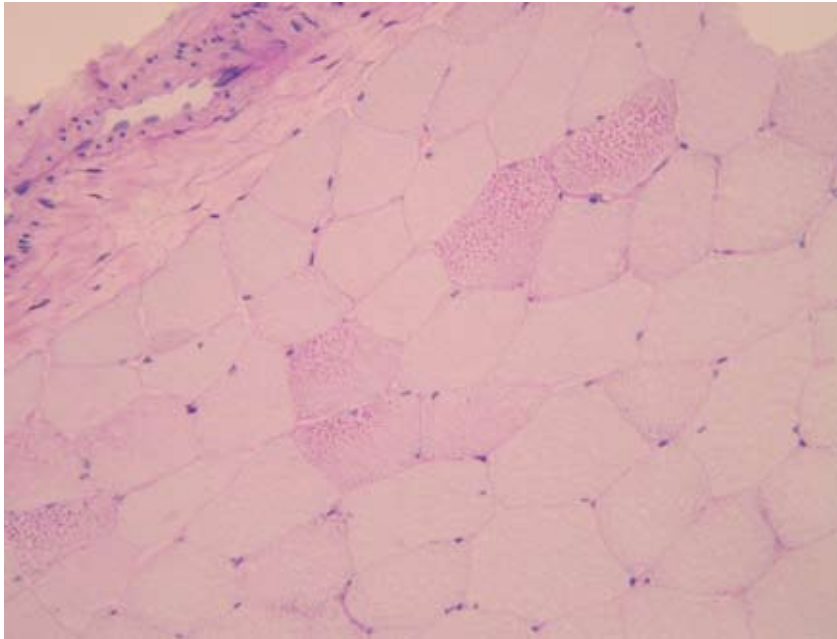


Figure 4. Amylose resistant polysaccharide in Friesian mare from the control group (A-PAS, 200×, frozen section)

Comparing test and control groups in our study, we found a significant difference in the presence of subsarcolemmal glycogen. A risk ratio of 5.22 means that horses with the presence of subsarcolemmal glycogen within muscle fibres have a 5.22 times higher probability of having PSSM. Significant differences in the presence of intracytoplasmic masses and subsarcolemmal vacuoles between groups were not found. There was no significant difference between groups in the presence of amylose resistant polysaccharide in muscle fibres. The lack of significance may be influenced by the small numbers of horses in each group.

Two horses from the control group had histopathologic findings of amylose resistant polysaccharide without any clinical signs of muscle disorder in their history. The first of them was an 11-year-old CMB mare. She was regularly used for heavy work in the field and in the forest and was fed with oats, barley and hay. The mare was euthanized because of acute severe colic. Histopathologic muscle examination revealed mild fibre size variation (grade 1), internal nuclei (grade 3), subsarcolemmal glycogen (grade 3) and amylose resistant polysaccharide within muscle fibres (Figure 3). The second was a 5-year-old Friesian mare. She was in very light training only and was euthanized because of severe colic due to acute grass sickness. The horse did not have increased fibre size variation, internal nuclei, any deposits of subsarcolemmal glycogen or intracytoplasmic mass, but had fibres with amylose resistant polysaccharide (Figure 4). The lack

of clinical signs in these cases was not explained, it could be indication of subclinical myopathy. The finding of amylose resistant polysaccharide without any additional histopathologic features of PSSM was described in Cob Normand draft horses by Larcher et al. (2008).

The presence of amylose resistant polysaccharide in horses without any clinical signs of PSSM was previously described by Valentine and Cooper (2005), Firshman et al. (2005) and Firshman et al. (2006). The lack of clinical signs of PSSM in horses with amylose resistant polysaccharide in the study of Firshman et al. (2005) was explained by the fact that these horses were not actively working. McCue and Valberg (2007) described finding amylose resistant polysaccharide in 20 Quarter Horses without any clinical signs of ER from a group of 164 horses. The absence of clinical signs in these horses was explained by good training and feeding management. Valentine et al. (1997) found amylose resistant polysaccharide within muscle fibres of draught horses suffering by severe fatal attack of exertional rhabdomyolysis as a first sign of myopathy. Possibility of similar acute attack of rhabdomyolysis can not be excluded in apparently healthy horses with presence of amylose resistant polysaccharide.

Occurrence of amylose resistant polysaccharide is reported to be an age-related phenomenon. Abnormal polysaccharide accumulation may take up to three years to be obvious in muscle fibres (De La Corte et al., 2002). Valentine and Cooper (2006) found out that higher occurrence of amylose

resistant polysaccharide inclusions were most often in horses older than 10 years. In the face of the low mean age of horses in the test group of our study (6.3 ± 2 years), age of horses could be a cause of low occurrence of amylase resistant polysaccharide in these animals.

Subsarcolemmal glycogen was found in four apparently healthy two years old control CMB stallions in our study. Muscle fibre size variation or internal nuclei were not observed in them. These stallions were in a light training. It would be of interest to examine these horses in the future. The CMB breed has been bred from a basis of original Belgian Draught Horses since the 19th century. Occurrence of subsarcolemmal glycogen in Belgian Draught Horses was already identified by Valentine et al. (2001), Firshman et al. (2005) and McCue et al. (2006). Because of frequent presence of subsarcolemmal glycogen in draught horses with or without a history of rhabdomyolysis, we suppose that subsarcolemmal glycogen could be a non specific finding in healthy draught horses.

Subsarcolemmal vacuoles were found in three horses with clinical signs of ER (test group) and in two horses without clinical signs (control group) in our study. Intracytoplasmic masses were found in four horses with ER (test group), but also in four horses without clinical signs of ER (control group). These histopathologic changes are often found in PSSM but they are not specific according to some authors (Firshman et al., 2006).

Significant differences in the presence of internal nuclei and fibre size variation between groups were not found. Finding a great number of internal nuclei within fibres would be suggestive of a myopathy but they can also occur in chronic neuropathies. The control group in our study was established on the basis of no history or clinical signs of muscular disorders (especially ER). However, this does not exclude subclinical neurological disease.

Selenium deficiency is a relatively common problem in horses in the Czech Republic (Ludvikova et al., 2005) and nutritional myodegeneration occurs (Ludvikova et al., 2007). However, all horses in the test group of our study had a selenium status within the reference range.

No histopathologic changes supporting the diagnosis of PSSM or other known myopathy were found in the muscle biopsy of one horse from the test group (warmblood mare used for pleasure riding). Further diagnostic screening would be indicated in this case.

Differential diagnosis of exertional myopathies is extensive. The most often published disorder is PSSM. In spite of this fact, its exact cause and heritability are not known so far and diagnostic criteria are still not clear. We can conclude that presence of amylase resistant polysaccharide within muscle fibres of apparently healthy horses could be a manifestation of different phenotype expression of PSSM (from subclinical myopathy with histopathological findings only to severe exertional rhabdomyolysis) but also insufficient specificity of this diagnostic criterion. Histopathologic signs of PSSM are found in some horses without ER signs despite their exercise and grain-based diet.

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