Evaluation of glucose tolerance and intestinal luminal membrane glucose transporter function in horses with equine motor neuron disease

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Objective—To confirm whether the plasma glucose concentration curve obtained during oral glucose tolerance tests (OGTTs) in horses with equine motor neuron disease (EMND) is decreased, compared with that obtained in clinically normal horses, and determine whether that decrease is a result of defective glucose metabolism or intestinal glucose transport dysfunction.

Animals—8 horses with EMND and 44 matched control horses.

Procedure—Electromyography and OGTTs were performed in all 8 affected horses and 10 control horses. Intravenous GTTs (IVGTTs) were performed in 6 affected horses and another 11 control horses. The activity and levels of jejunal luminal membrane glucose transporter (Na^+/glucose cotransporter isoform 1 [SGLT1]) were measured in 2 affected horses and 23 control horses.

Results—In horses with EMND, generalized neuropathy was detected via quantitative electromyography; the mean increase in plasma glucose concentration during the OGTT was significantly decreased, compared with the value in control horses. During the IVGTT, the mean increase in plasma glucose concentration was significantly lower than that of control horses. The activity and levels of SGLT1 in 2 affected horses were similar to those of control horses. Diagnosis of EMND was confirmed postmortem in all affected horses.

Conclusions and Clinical Relevance—Data suggest that the decreased plasma glucose curve obtained in horses with EMND during OGTTs (compared with control horses) is a result of overall enhanced glucose metabolism or abnormalities in the facilitated glucose transporters; definitive identification of the underlying mechanisms could aid in the development of appropriate treatments of EMND in horses. (*Am J Vet Res* 2005;66:93–99)

Equine motor neuron disease (EMND) is a neurodegenerative disease that affects the lower motor neurons in the brainstem and spinal cord and was first described by Cummings et al[1] in 1990. This disease is characterized by weight loss (a result of generalized neurogenic muscle atrophy), trembling, and weakness combined with a good or ravenous appetite.[1] Although the definitive diagnosis of EMND can be made via histologic examination of specimens of the spinal cord and brainstem as well as biopsy specimens of skeletal muscle or peripheral nerve, a tentative diagnosis can be made on the basis of clinical signs, laboratory findings, and results of needle **electromyographic (EMG)** examination.[1-7,a]

Typically, in glucose tolerance tests, the dose of glucose is fixed and the measure of tolerance is the plasma glucose concentration. Results of previous studies[8,9,b] have indicated that the plasma glucose concentration curve obtained during oral glucose tolerance testing is decreased in horses with EMND, compared with that obtained in clinically normal horses. Generally, it is assumed that this response is a result of intestinal dysfunction.[9,10] However, to the authors' knowledge, convincing evidence to support this theory is scarce.

Thus, the objectives of the study of this report were to confirm whether the plasma glucose concentration curve obtained during **oral glucose tolerance tests (OGTTs)** in horses with EMND is decreased, compared with that obtained in clinically normal horses, and determine whether that decrease is a result of defective glucose metabolism or intestinal glucose transport dysfunction.

Materials and Methods

Animals—Eight adult horses with EMND that were evaluated at the Faculty of Veterinary Medicine, Utrecht University between September 1999 and March 2002 were included in the study. The 8 animals comprised 7 Dutch Warmbloods (4 mares and 3 geldings) and 1 Friesian stallion. The mean age of the horses with EMND was 8.3 ± 1.9 years (range, 6 to 10 years), and their mean weight was 510 ± 42 kg (range, 465 to 569 kg). Rectal temperature and heart and respiratory rates were 38.3 ± 0.3°C, 39 ± 7 beats/min, and 23 ± 8 breaths/min, respectively. For each horse, a CBC, serum biochemical profile, and measurement of serum vitamin E concentration were performed; in some horses, serum glutathione peroxidase activity and serum selenium concentration were measured. The clinical signs of EMND ranged from moderate to severe in the affected animals.

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The inclusion criteria for this study were progressive muscle atrophy, weight loss, and muscle weakness; abnormal postural appearance for unknown reasons; and low serum vitamin E concentration (less than 2 µmol/L) in combination with signs of lower motor neuron disease during EMG examination.

The exclusion criteria for this study were dental problems, protein-losing enteropathy, diarrhea, verminosis, chronic interstitial nephritis, malnutrition, and neoplasia. Horses with clinical evidence of EMND that could not be necropsied were also excluded from the study.

On the basis of findings of examinations, routine blood work, and EMG analyses, 2 groups of matched control horses were identified and included in the study; these groups were used either as the control group for the OGTT evaluation or the **intravenous glucose tolerance test (IVGTT)** evaluation. For the OGTT evaluation, 10 clinically healthy Dutch Warmbloods (4 mares and 6 geldings) were used as the matched control horses; these horses were 6 to 17 years of age (mean age ± SD, 12 ± 3.7 years) and weighed 504 to 712 kg (mean weight, 577 ± 56 kg). For the IVGTT evaluation, an additional 11 Dutch Warmbloods (10 mares and 1 gelding) were used as the matched control horses; these horses were 3 to 11 years of age (mean age, 7.5 ± 2.2 years) and weighed 492 to 701 kg (mean weight, 584 ± 55 kg).

Another group of 23 clinically normal horses maintained on a diet containing hydrolysable digestible carbohydrates (grain) was used to obtain control values for the expression and activity of the Na^+/glucose cotransporter isoform 1 (SGLT1) for comparison with values obtained in 2 of the 8 horses with EMND.

EMG analysis—In 4 horses with EMND, a needle EMG examination was performed and findings were assessed semiquantitatively by determining the presence of pathologic spontaneous activity and the configuration of the **motor unit action potentials (MUPs)**. In addition, in the other 4 horses with EMND, quantitative EMG analysis was performed that included quantitative MUP analysis. As a control for the quantitative MUP analysis in the EMND-affected horses, published data[11,12] that had been obtained from clinically normal horses of similar breed and age were used. The EMG variables were defined on the basis of data included in earlier publications, and examinations were carried out as reported previously.[11-13] In brief, EMG signals were recorded by use of a portable apparatus[c] and concentric needles (length, 50 to 100 mm; diameter, 0.45 to 0.8 mm).[c] The band pass ranged from 5 to 10 kHz, and sweep speed was 10 to 20 ms/division. Amplifier gain was 50 to 100 µV/division for spontaneous activity and 100 to 500 µV/division for MUP recordings. The EMG examination was performed while the horses were held in stocks without restraint; the left subclavian, triceps, and vastus lateralis muscles of each horse were examined. Spontaneous activity was assessed in the same regions from which MUPs were recorded and was only analyzed if present outside the end-plate region. The first 20 to 30 MUPs were used for statistical analysis.

Glucose administration and tolerance testing—The OGTT was performed in all 8 horses with EMND and the 10 matched control horses. Food was withheld from the horses for 12 hours prior to and during the test. To avoid stress, the horses remained undisturbed in their stalls prior to and during the test. Horses were administered 1 g of glucose/kg (dissolved in 2 L of water) via a nasogastric tube; blood samples were collected into tubes containing sodium fluoride[d] just prior to (**time [t]** = 0 minutes) and at 30, 60, 90, and 120 minutes after dosing.

The IVGTT was performed on 6 horses with EMND (food had not been withheld from any of these horses preceding the test). Each horse received 50% glucose solution (0.5 g/kg) administered within 3 minutes via a jugular vein by use of a catheter. During this test, food was withheld from the horses. Blood samples were obtained from the contralateral jugular vein and collected into tubes containing sodium fluoride[d] before (t = 0 minutes) and at 15, 30, 60, 90, 120, 180, 240, 300, and 360 minutes after administration of the glucose solution. At these time points, blood was also collected into heparinized tubes[d] to measure plasma insulin concentrations. Each of 11 matched control horses underwent an IVGTT according to a previously published protocol[14]; blood samples for determination of plasma glucose and insulin concentrations were also collected before (t = 0 minutes) and at 2, 5, 10, 15, 30, 60, 90, 120, 180, 240, 300, and 360 minutes after administration of the glucose solution.

In both GTT evaluations, plasma was separated and stored at –20°C until analysis and plasma glucose concentration was measured within 5 hours after sample collection by use of the hexokinase method. In the IVGTTs, plasma insulin concentration was measured by use of a radioimmunoassay kit[e] validated for use in horses.[15]

Postmortem examination—All 8 horses with EMND were euthanatized via IV administration of an overdose of pentobarbital sodium. Postmortem examination included examination of the ventral horns of the spinal cord and the autonomic ganglia. In addition, from 5 horses with EMND, duodenal, jejunal, ileal, and colonic samples were examined histologically. For histologic examination, the fresh samples of intestine from control horses and horses with EMND were fixed in 4% (wt/vol) phosphate-buffered paraformaldehyde, sectioned, and stained with H&E. In addition, NADH, cytochrome c oxidase, succinate dehydrogenase, acid phosphatase, periodic acid-Schiff, ATPase (pH, 4.3 and 9.4), Sudan black B, and oil red O staining were used to stain sections of vastus lateralis muscle tissues that were frozen in isopentane (precooled in liquid nitrogen) and stored at –80°C.

Isolation of brush-border membrane vesicles—Purified **brush-border membrane vesicles (BBMVs)** were isolated from jejunal mucosal scrapings of 2 of the 8 horses with EMND and 23 healthy control horses. The expression and the activity of SGLT1 were determined in the BBMVs by western blot analysis and glucose transport studies. The BBMVs were prepared from small intestinal mucosal scrapings of control horses and horses with EMND by use of a method based on those of Shirazi-Beechey et al[16] and Dyer et al.[17] Briefly, mucosal scrapings (0.2 g) were thawed in 10 mL of a solution containing 100mM mannitol, 2mM HEPES/Tris (pH, 7.1), and a cocktail of protease inhibitors.[f] The mucosal scrapings were homogenized for 1 minute by use of a polytron tissue homogenizer[g] at setting 5. The volume was made up to 20mL with the same buffer; $MgCl_2$ was added to a final concentration of 10mM, and the homogenate was stirred for 20 minutes on ice. The homogenate was centrifuged at 5,000 × g for 10 minutes in a centrifuge. The pellet was discarded, and the supernatant was centrifuged for 30 minutes. By use of a hand-held homogenizer,[i] the resultant pellet was resuspended in 35 mL of a solution containing 100mM mannitol, 2mM HEPES/Tris (pH, 7.4), and 0.1 mM $MgSO_4$ and centrifuged for 45 minutes. The final pellet containing purified BBMVs was resuspended in a small volume of isotonic buffer (300 mM mannitol, 20 mM HEPES/Tris [pH, 7.4], 0.1mM $MgSO_4$, and 0.02% [wt/vol] NaN_3) via passage through a 27-gauge needle several times. The BBMVs were divided into aliquots and stored in liquid nitrogen until use. All steps in the procedure were carried out at 4°C.

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Enzyme assays in cellular homogenates and brush-border membrane fractions—Sucrase and maltase activities were measured at 38°C in the original cellular homogenates and isolated brush-border membrane fractions, as described previously.[16] Concentration of D-glucose released as a result of hydrolysis of disaccharide substrates (final concentration of sucrose and maltose, 28 mmol/L) was measured by use of a commercially available kit according to the manufacturer's instructions. Alkaline phosphatase activity was measured at pH 10 in the presence of 5 mmol/L $MgCl_2$ with *p*-nitrophenyl phosphate as the substrate.[18]

The presence of any basolateral and organelle membranes was determined via assessment of the activities of marker enzymes, such as K^+-activated phosphatase (basolateral membrane), α-mannosidase (Golgi membranes), Tris-resistant α-glucosidase (membranes of the endoplasmic reticulum), and succinate dehydrogenase (mitochondrial membranes), as described previously.[16]

Estimation of protein in cellular homogenates and brush-border membrane fractions—Protein concentration was determined by its ability to bind Coomassie Blue by use of a colorimetric technique.[j,16] Bovine γ-globulin (1 to 100 µg of protein) was used as the standard.

Assay of Na^+-dependent D-glucose transport in cellular homogenates and brush-border membrane fractions—The initial rate of D-glucose transport into the BBMVs isolated from intestinal tissues from both control horses and horses with EMND was measured at 38°C in a solution containing either 100mM NaSCN or 100mM KSCN and 100mM mannitol, 20mM HEPES/Tris (pH, 7.4), 0.1mM $MgSO_4$, and 0.02% (wt/vol) NaN_3. Incubation was stopped after 3 seconds by the addition of 1 mL of an ice-cold buffer containing 0.1 mM phlorizin.[16] Aliquots of this solution were then filtered rapidly through 0.2-µm cellulose acetate-nitrate filters[k] by use of the filtration stop technique, as described previously.[17] Uptake values were measured in triplicate.

The abundance of SGLT1 protein in the equine intestinal BBMVs and the respective cellular homogenates was determined via western blotting analysis, as described previously.[17,19] The protein contents of BBMVs and cellular homogenates were separated on 8% polyacrylamide gels containing 0.1% (wt/vol) SDS and were electrotransferred to the polyvinylidene difluoride membrane.[k] Samples were blotted with an antibody against a synthetic nonodecapeptide (STLFTMDIYTKIRKKASEK) that corresponded to amino acids 402 through 420 of the SGLT1 sequence, which is a highly conserved region of SGLT1 in horses and other species.[17,19,20] The 75-kd immunoreactive band was blocked when antibodies were preincubated with the immunizing peptide, thereby indicating the specificity of the immunoreaction. The membranes were developed by use of the enhanced chemiluminescence system[l] and exposed to photographic film.[m] The intensities of the immunoreactive bands detected in the BBMVs were quantified by use of scanning densitometry[n] and reported as arbitrary units.

Statistical analyses—An independent *t* test was performed on data obtained from horses with EMND and control horses by use of analytical software[o] to compare the increase in plasma glucose concentration during both GTTs. With respect to EMG analysis, data were assessed for normality by use of histograms and the Kolmogorov-Smirnov test. When data were not normally distributed, transformation of the data into the **natural logarithm (ln)** was performed. To facilitate the interpretation, the ln-transformed data are represented as **geometric mean (gmean)** values that were derived from back transformation of the ln-transformed mean data. A 2.5-percentile analysis was calculated because of its independence to the distribution of data. Correlations between variables were assessed by use of partial correlation coefficients. A 1-way ANOVA with post hoc Bonferroni testing was performed on transformed data to compare values among muscles. Differences were considered significant when the value of P was < 0.05 (2-tailed). The values are reported as mean ± SD unless otherwise indicated.

Results

Blood sample analyses—Among the horses with EMND, results of serum biochemical analyses indicated high mean activities of lactate dehydrogenase (1160 ± 787 U/L; upper reference limit, < 420 U/L), aspartate aminotransferase (397 ± 169 U/L; upper reference limit, < 275 U/L), and creatine kinase (301 ± 365 U/L; upper reference limit, < 200 U/L). In addition, mean serum vitamin E concentration was extremely low (0.73 ± 0.6 µmol/L; reference range, 2 to 10 µmol/L), whereas the plasma total protein concentration (60 to 85 g/L), serum selenium concentration (> 70 µg/L), serum glutathione peroxidase activity (120 to 300 U/g of Hb), leukocyte count (7 to 10 $\times$ 10^9 WBCs/L), and serum creatinine concentration (118 ± 11 µmol/L; n = 4) were within their reference ranges. All other clinicopathologic variables were also within reference limits.

Semiquantitative needle EMG examination—The EMG examination revealed the presence of pathologic spontaneous activity and broad, large, polyphasic or complex MUPs in all EMND-affected horses. Among the spontaneous activities detected, fibrillation potentials and positive sharp waves were most frequently observed in all muscles in all horses; doublet or triplet MUPs, complex repetitive discharges, and neuromyotonia were observed to a lesser extent.

Quantitative EMG analysis of spontaneous activity—In the EMND-affected horses, insertional activity was recorded in the subclavian, triceps, and vastus lateralis muscles (480 ± 47.0 milliseconds, 533 ± 41.6 milliseconds, and 521 ± 207 milliseconds, respectively), which did not differ significantly from published data[11,12] for control horses. In addition, the fibrillation potentials that were recorded had a mean duration of 4.2 ± 1.5 ms, mean amplitude of 58 ± 29.9 µV, and mean numbers of phases and turns of 2.48 ± 0.51 and 2.48 ± 0.51, respectively. The positive sharp waves had a mean duration of 5.8 ±1.9 milliseconds, mean amplitude of 64 ± 38 µV, and mean numbers of phases and turns of 1.71 ± 0.31 and 1.76 ± 0.62, respectively. Doublet MUPs were detected in 2 of the 4 horses analyzed, whereas complex repetitive discharges were observed in 1 horse (firing frequency of 100 Hz) and neuromyotonia in another (firing frequency of 158 ± 11.3 Hz).

Quantitative EMG analysis of MUPs—In the subclavian, triceps, and vastus lateralis muscles of the EMND-affected horses, the gmean ± SD of MUP duration was 10.2 ± 1.9 milliseconds, 12.1 ± 2.9 milliseconds, and 10.6 ± 2.4 milliseconds, respectively; the gmean MUP amplitude was 868 ± 2.5 µV, 1,373 ± 4.8 µV, and 655 ± 5.8 µV, respectively. In those muscles, the mean MUP numbers of phases and turns was 3.34 ± 1.45 and 4.11 ± 1.54, 3.08 ± 1.43 and 4.38 ± 1.77, and 3.34 ± 1.57 and 4.31 ± 1.74, respectively.

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In the subclavian muscles of the EMND-affected horses, all gmean MUP variables were significantly (P = 0.00) increased, compared with those values in the subclavian muscles of the control horses. In the triceps muscles of the EMND-affected horses, the gmean MUP numbers of phases and turns was significantly ($P < 0.001$) increased, compared with those values in the triceps muscles of the control horses; in 1 horse, the gmean MUP duration and amplitude in the triceps muscle were significantly ($P < 0.001$) greater than that of the control horses. In the vastus lateralis muscles of the EMND-affected horses, the gmean MUP numbers of phases and turns was significantly ($P < 0.001$) increased as well as the MUP duration and amplitude ($P < 0.001$ and < 0.001 to 0.03, respectively), compared with those values in the vastus lateralis muscles of the control horses.

For the MUP duration, amplitude, and numbers of phases and turns of the subclavian muscle in the EMND-affected horses, the 95% **confidence intervals (CIs)** of nontransformed data were 6.6 to 12.1 milliseconds, 678 to 1,111 µV, 3.02 to 3.69, and 3.65 to 4.63, respectively. In the control horses, these values were 6.0 to 6.6 milliseconds, 282 to 321 µV, 2.7 to 2.9, and 2.7 to 2.9, respectively. For the MUP duration, amplitude, and numbers of phases and turns of the triceps muscle in the EMND-affected horses, the 95% CIs of nontransformed data were 8.9 to 16.5 milliseconds, 874 to 2,157 µV, 2.77 to 3.4, and 3.72 to 5.16, respectively. In the reports of control horses, these values were 5.9 to 7.0 milliseconds, 367 to 497 µV, 2.3 to 2.5, and 2.6 to 2.8, respectively. For the MUP duration, amplitude, and numbers of phases and turns of the vastus lateralis muscle in the EMND-affected horses, the 95% CIs of nontransformed data were 8.7 to 13.0 milliseconds, 436 to 984 µV, 3.01 to 3.71, and 3.79 to 4.90. In the reports of control horses, these values were 5.6 to 6.8 milliseconds, 313 to 406 µV, 2.5 to 2.7, and 2.9 to 3.2, respectively.

In the subclavian, triceps, and vastus lateralis muscles of the EMND-affected horses, the prevalence of polyphasic MUPs was 15.6 ± 2.1%, 18.7 ± 4.3%, and 31.2 ± 18.0%, respectively; the prevalence of complex MUPs was 31.4 ± 0.6%, 38 ± 11.2%, and 37.4 ± 15.2%, respectively. In the subclavian, triceps, and vastus lateralis muscles of the control horses, the reported prevalence of polyphasic MUPs was 4.0 ± 2.2%, 9.2 ± 3.6%, and 6.9 ± 2.9%, respectively; the reported prevalence of complex MUPs was 5.9 ± 2.8%, 4.0 ± 7.8%, and 9.8 ± 2.7%, respectively. The values in the subclavian and triceps muscles of the EMND-affected horses were significantly ($P < 0.001$ to 0.02) higher than the corresponding values in the control horses in the present study.

Results of the GTTs—In 6 of the 8 horses with EMND, the plasma glucose curve obtained during the OGTT was decreased, compared with that of control horses. The mean peak plasma glucose concentration in the horses with EMND (43 ± 19%; range, 21% to 79%) was significantly lower than that of the control horses (83 ± 40%; range, 44% to 174%; **Figure 1**). In the EMND group, the mean increase in plasma glucose concentration from the baseline (0 minutes) value during the IVGTT was 172 ± 55% (range, 95% to 226%) after 15 minutes; the increase returned to baseline value within 90 minutes after administration of glucose. This increase in plasma glucose concentration from the baseline value in EMND-affected horses was significantly ($P < 0.05$) lower than the increase detected in the control horses (385 ± 65%; **Figure 2**). In 2 of 6 horses with EMND, the peak plasma glucose concentration during the IVGTT was less than the lower limit (mean minus 2SD) of the reference range (12.8 mmol/L). In the horses with EMND, the mean baseline plasma insulin concentration was less than the upper limit of the reference range (< 408 pmol/L); the peak plasma insulin concentration was reached 15 minutes after administration of glucose. The mean peak plasma insulin concentration in EMND-affected horses was not significantly ($P > 0.05$) lower than that of the control group (245 ± 15.2 pmol/L vs 432 ± 214 pmol/L; **Figure 3**).

Mean plasma glucose half-life during the IVGTT in control horses (25.6 ± 11.8 minutes) was not significantly ($P > 0.05$) different from the value in horses with EMND (19.0 ± 12.5 minutes), despite a plasma glucose half-life during the IVGTT of only 4.5 minutes in a 7-year-old Warmblood mare with EMND.

Postmortem examination—Mean weight of the horses with EMND was significantly lower ($P < 0.05$) than the weight of control horses (510 ± 42 kg vs 584 ± 55 kg). The tentative clinical diagnosis was confirmed at necropsy in all horses with EMND; histologically, the cell bodies of the motor neurons in the ventral horns of the

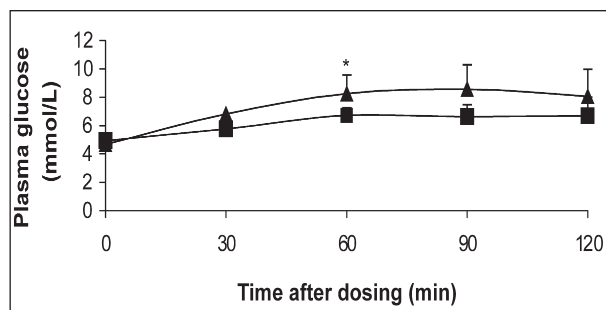

Figure 1—Mean + SD plasma glucose concentration during an oral glucose tolerance test in clinically normal horses (n = 10; triangles) and horses with equine motor neuron disease (EMND [8; squares]) following administration of 1 g of glucose/kg (dissolved in 2 L of water) via a nasogastric tube immediately after blood samples were obtained at 0 minutes. *Values for the 2 groups were significantly (P < 0.05) different at this time point.

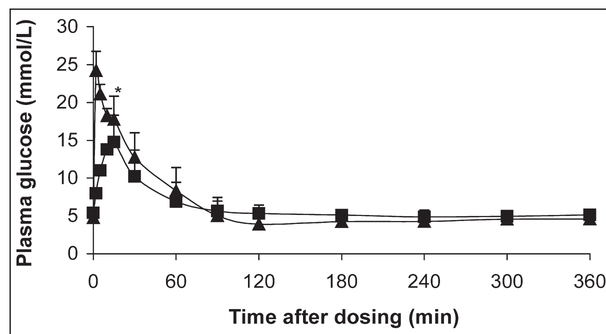

Figure 2—Mean + SD plasma glucose concentration during an IV glucose tolerance test in clinically normal horses (n = 11; triangles) and horses with EMND (6; squares) following IV administration of 50% glucose solution (0.5 g/kg) immediately after blood samples were obtained at 0 minutes. *See* Figure 1 for key.

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spinal cord and in the medulla oblongata consistently had chromatolysis and acidophilic necrosis. No notable lesions were detected in samples of the wall of the intestinal tract; however, moderate numbers of lymphocytes and plasma cells and some eosinophils had infiltrated the lamina propria. There were no changes in submucosal and myenteric ganglia. To rule out equine grass sickness, histologic examination of the autonomic ganglia was performed in 6 of the 8 horses with EMND, but lesions consistent with equine grass sickness were not found in any of these horses. Application of additional stains to sections of vastus lateralis muscle tissues did not reveal the presence of **polysaccharide storage myopathy (PSSM)**. None of the 8 EMND-affected horses had type grouping (muscle fiber types are normally distributed in a checkerboard pattern and type grouping is the complete encircling of a fiber of the least predominant fiber type by fibers of the same type), whereas 4 horses had elongation of atrophic muscle fibers, which is a characteristic of neuropathy. The other horses had grouped atrophy, which is a less specific characteristic of neuropathy.

Enzyme assays—Compared with values in the original cellular homogenates, the specific activities of the brush-border membrane marker enzymes (sucrase, maltase, and alkaline phosphatase) were 10 to 18 times as great in the BBMVs isolated from either EMND-affected or control horses. These data indicated that the membrane vesicles originated from the brush-border membrane; furthermore, the degree of purification of the membrane vesicles was similar in the 2 groups of horses. In the BBMVs isolated from the intestinal tracts of EMND-affected and control horses, the activities (or abundance) of marker proteins characteristic of organelle or basolateral membranes were negligible, which indicated that these preparations of BBMVs were highly purified.

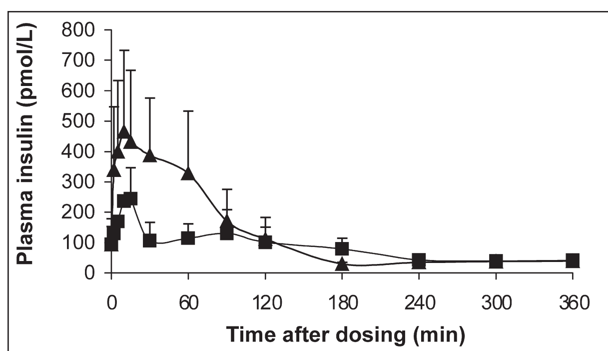

Figure 3—Mean + SD plasma insulin concentration during an IV glucose tolerance test in clinically normal horses (n = 6; triangles) and horses with EMND (6; squares) following IV administration of 50% glucose solution (0.5 g/kg) immediately after blood samples were obtained at 0 minutes.

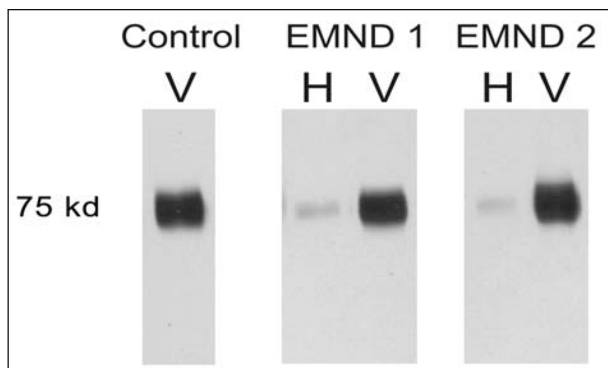

Figure 4—Results of the assessment of the abundance of the Na+/glucose cotransporter isoform 1 (SGLT1) protein in brush-border membrane vesicles isolated from jejunal mucosal scrapings of 2 horses with EMND (EMND 1 and 2) and 23 clinically normal horses (control). Cellular homogenates (H) and brush-border membrane vesicles (V) were separated on 8% polyacrylamide gels (20 µg of protein/lane), electrotransferred to polyvinylidene difluoride membranes, and western blotted with an anti-SGLT1 polyclonal antibody. Results indicated the presence of the 75-kd SGLT1 protein.

Determination of SGLT1 protein abundance via western blotting analysis—The SGLT1 antibody recognized a single protein (75 kd) in BBMV samples from both control and EMND-affected horses; this protein is characteristic of SGLT1 in horses[17] and other species[21] (**Figure 4**). In the BBMV preparation from EMND-affected horses, the abundance of SGLT1 was 11 to 16 times as great as that of the original cellular homogenates. Densitometric analysis of the SGLT1 signal intensity indicated that the abundance of SGLT1 protein in the BBMVs isolated from the intestinal tracts of healthy controls and the EMND-affected horses were similar.

Determination of SGLT1 activity via glucose transport studies—The initial rates of 0.1mM D-glucose transport (± SE) into BBMVs isolated from the jejuna of control (24.7 ± 1.2 $pmol \cdot s^{-1} \cdot mg^{-1}$ protein) and 2 EMND-affected horses (21.2 ± 1.8 and 24.1 ± 5.3 $pmol \cdot s^{-1} \cdot mg^{-1}$ protein) indicated that glucose is transported into the membrane vesicles in the presence of NaSCN but not KSCN. This finding indicated that glucose transport is Na^+-dependent and confirmed the presence of functional SGLT1 in these preparations. The initial rates of glucose transport in the EMND-affected and control horses were similar. The results indicated that the level of SGLT1 protein in the jejuna of horses with EMND is similar to that of clinically normal horses and that the protein appears to be functioning normally in EMND-affected horses.

Discussion

The decreased plasma glucose concentration curve obtained during the OGTT performed in horses with EMND (compared with that obtained in clinically normal horses) in the present study has been detected in other studies,[5,8,10,b] but the underlying mechanism of this phenomenon has yet to be fully elucidated. Our OGTT data are in agreement with those previously reported results.[5,8-10,b]

The plasma glucose concentration curve obtained during the IVGTT in horses with EMND was also different from that of the control group. In horses with EMND, the mean increase in plasma glucose concentration during the IVGTT was significantly lower than that of the control horses (172% vs 385%) but was associated with a somewhat similar insulin response. In contrast, the plasma glucose concentration curve obtained during an IVGTT in a horse with EMND in a

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study by Kyles et al[10] was not different from that expected in clinically normal horses. This difference in findings might be attributed to breed or age differences between the study populations. However, in the latter study,[10] no control values were available. In our study, the control horses also had an acute insulin response to the IVGTT, as reported earlier.[14]

Interestingly, in a study[22] of horses with PSSM, similar peak plasma glucose concentrations were detected in control and PSSM-affected horses during an IVGTT, but the latter group had significantly lower mean blood glucose concentrations from 0 to 180 minutes after IV administration of glucose. However, mean plasma glucose half-life was significantly lower in horses with PSSM than it was in control horses, which is in contrast with the findings of the present study involving horses with EMND. In combination, these data suggest that myopathic and neuropathic equine muscle may each function in a stereotypical manner with regard to glucose clearance and insulin response, reflecting the large role that skeletal muscle plays in whole-body glucose metabolism. With regard to both EMND and PSSM in horses, further research of glucose metabolism is needed in which the euglycemic hyperinsulinemic clamp is used as the gold standard.

In the study of this report, the EMG analysis in horses with EMND revealed neurogenic abnormalities consistent with motor neuron or axon loss. The neurogenic abnormalities that resulted from denervation and reinnervation processes included both myogenic and neurogenic pathologic spontaneous activity; the myogenic pathologic spontaneous activity was observed as fibrillation potentials, positive waves, and complex repetitive discharges, and the neurogenic pathologic spontaneous activity was observed as doublet, triplet, and multiplet MUPs and neuromyotonia. Results of the quantitative MUP analysis indicated increases in MUP duration, number of phases, number of turns, and prevalence of polyphasic and complex MUPs in horses with EMND, compared with those values in clinically normal horses, and these data are considered consistent with the presence of a neurogenic disorder. In our laboratory, morphometric evaluation of muscle tissue in horses with EMND has revealed that reinnervation is associated with a decrease of the mean group size of the least predominant fiber type and reduction in the distances between type 1 fibers.[a] Compared with the histologic evaluation of tissues postmortem in the present study, EMG examination including MUP analysis proved to be very sensitive for early antemortem detection of neurogenic changes in horses via a comparatively noninvasive technique.

The decreased plasma glucose concentration curve obtained during OGTTs in horses with EMND in our and other studies, compared with that obtained in the control horses, may be attributed to intestinal dysfunction. In mammals, the absorption of glucose in the small intestine is accomplished with 2 types of glucose carriers: SGLT1 on the luminal membrane and the facilitated glucose transporter (GLUT2) on the basolateral membrane.[21] The presence of SGLT1 has been detected in both intestinal epithelial cells and kidney proximal epithelial cells. Variations in transporter activity could result in decreased or total absence of glucose uptake from the intestinal lumen. Historically, experimental techniques to quantitatively assess mechanistic changes that result in a decreased rate of intestinal glucose absorption in horses were limited because of the lack of equine-specific molecular probes. However, with the development of both DNA probes and specific antibodies, the presence of SGLT1 and its role in the transport of glucose and galactose in the equine intestine has been demonstrated.[17] In the present study, we have determined that there was no change in the activity and level of luminal membrane SGLT1 in 2 horses with EMND, compared with values in clinically normal horses. Interestingly, in humans, mutations in the gene encoding GLUT2 have been detected and result in Fanconi-Bickle syndrome.[23]

In addition, histologic examination of samples of jejunum, ileum, and colon obtained from the horses of our study revealed no notable abnormalities and the plasma total protein concentration in all EMND-affected horses was within reference limits. This lack of abnormalities in enterocyte absorption is in agreement with the findings of Divers et al[5] who investigated abnormalities in the mitochondria of the intestinal epithelium as the probable cause of the decreased plasma glucose concentration curve obtained during OGTT in horses with EMND. Overall, those authors also failed to find convincing evidence to support the theory that abnormalities in enterocyte absorption occurred.

On the basis of our data, 2 possible explanations for the decreased plasma glucose concentration curve obtained during OGTTs in horses with EMND are suggested; compared with clinically normal horses, EMND-affected horses may have enhanced glucose metabolism (rather than a defect in intestinal transport) or there may be abnormalities in the activity or amounts of the facilitated glucose transporter GLUT2. To ascertain whether the latter is the cause of the altered response to the OGTT in horses with EMND would require further investigation of any potential mutations in the GLUT2 gene in those horses and the corresponding activity and the expression of GLUT2 in their intestinal tracts. Such data may be useful in the development of methods for antemortem diagnosis of EMND in horses and aid in supportive treatment.

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i. Potter-Elvejhem, Vineland, NJ.
j. Bio-Rad Laboratories, Hercules, Calif.
k. Millipore, Billerica, Mass.
l. Amersham-Pharmacia Biotechnology Ltd, Little Chalfont, Buckinghamshire, UK.
m. Biomax ML, Kodak Ltd, Hemel Hempstead, Hertfordshire, UK.

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