

Serial alterations in digital hemodynamics and endothelin-1 immunoreactivity, platelet-neutrophil aggregation, and concentrations of nitric oxide, insulin, and glucose in blood obtained from horses following carbohydrate overload

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Objective—To quantify changes in endothelium-derived factors and relate those changes to various aspects of digital hemodynamics during the prodromal stages of carbohydrate overload (CHO)-induced laminitis in horses.

Animals—20 adult horses without abnormalities of the digit.

Procedures—Digital and jugular venous blood samples were collected at 1-hour intervals (for assessment of endothelin-1 [ET-1] immunoreactivity and measurement of glucose, insulin, and nitric oxide [NO] concentrations) or 4-hour intervals (CBC and platelet-neutrophil aggregate assessment) for 8 hours or 16 hours after induction of CHO-associated laminitis in horses treated with an ET-1 antagonist. Effects of treatment, collection site, and time and the random effects of horse on each variable were analyzed by use of a repeated-measures model. Where treatment and collection site had no significant effect, data were combined.

Results—Compared with baseline values, CHO resulted in changes in several variables, including a significant increase from baseline in digital blood ET-like immunoreactivity at 11 hours; digital blood ET-like immunoreactivity was significantly greater than that in jugular venous blood at 8, 9, 11, and 12 hours. Digital and jugular venous blood concentrations of glucose increased from baseline significantly at 3, 4, and 5 hours; insulin concentration increased significantly at 5 hours; and the number of platelet-neutrophil aggregates increased significantly at 12 hours.

Conclusions and Clinical Relevance—In horses, concurrent increases in venous blood ET-1 immunoreactivity, insulin and glucose concentrations, and platelet-neutrophil aggregates support a role of endothelial dysfunction in the pathogenesis of CHO-induced laminitis. (*Am J Vet Res* 2007;68:87–94)

One hypothesis regarding the vascular theory of laminitis suggests that laminar cell damage results from ischemia of laminar tissues. However, results of studies^{1–10} of laminar and digital blood flow have been conflicting, with increases in blood flow in some studies^{6,7,9} and decreases in others.^{1–6,8,10} In attempts to resolve the controversy of increased versus decreased digital blood flow, recent investigations^{3–6,9,10} have included techniques such as scintigraphy, hoof-wall surface-temperature assessment, and Doppler ultrasonography to evaluate digital blood flow indirectly and

ABBREVIATIONS

CHO	Carbohydrate overload
ET	Endothelin

directly. Although those studies revealed that at different time points in different models of laminitis, blood flow in limbs may increase, decrease, or remain unchanged, they have failed to refute or prove that laminar ischemia develops during the early stages of laminitis in horses.⁷

Experimental models of laminitis have revealed that quantities of vasoconstrictor substances are increased in affected horses, compared with quantities in healthy horses.⁷ Endothelin-1, a potent vasoconstrictor peptide produced by the endothelium, is present in increased quantities in laminar tissue of horses with CHO-induced laminitis.¹¹ Furthermore, endothelium-dependent relaxation is decreased in digital vessels of horses with CHO-induced laminitis, suggesting that the NO-producing capacity of the digital vascular endothelium is reduced, thereby rendering the vessels more responsive to the effects of vasoconstrictive agents.¹² In addition, Katwa et al¹¹ identified increased quantities of ET-1 in laminar tissues of horses with experimentally induced and naturally occurring laminitis.

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In other studies,^{13,14} a significant increase in the number of platelet-neutrophil aggregates was detected in ponies that were administered CHO. Furthermore, pretreatment with a platelet aggregation inhibitor prevented development of laminitis in ponies administered CHO, highlighting the importance of these activated cell aggregates in the pathogenesis of laminitis.¹⁴ Activated leukocytes and platelets are of critical importance in the pathogenesis of atherosclerosis and intestinal, myocardial, and cerebral ischemia. Increased synthesis or release of ET-1 favors platelet aggregation and neutrophil adhesion and promotes vasoconstriction with sludging of blood in the microvasculature.¹⁵

Endothelial cell secretion of ET-1 is also increased in direct response to hyperinsulinemia.^{16–20} Horses with insulin resistance associated with obesity and pituitary pars intermedia dysfunction have an increased risk of laminitis attributable to insulin-resistance-associated hyperinsulinemia.^{21,22} During insulin-resistant states, endothelial cells are generally exposed to slightly supraphysiologic concentrations of glucose.^{23,24} Endothelial cell dysfunction resulting from chronic hyperglycemia is characterized by increased production of ET-1, decreased production of NO, and adoption of a relatively prothrombotic phenotype.²⁵ In addition, it is known that for maintenance of the hoof-lamellar attachment interface, keratinocytes have a high requirement for glucose.²⁶ Mobasher et al²⁷ reported that compared with findings in healthy horses, the expression of the insulin-dependent glucose transporter-4 was decreased in the hoof keratinocytes of horses with laminitis. Insulin resistance would further impair the ability of the lamellar epithelium to use glucose.

From these mechanisms, it is evident that alterations in the synthesis or release of ET-1, hyperinsulinemia, insulin resistance, and platelet activation with adherence to neutrophils could develop during the prodromal stages of CHO-induced laminitis. Subsequently, ischemia, reduced glucose utilization by epithelial cells, and activation of inflammatory and endothelial cells may all be initiating processes that ultimately weaken the lamellar attachment interface. The objective of the study reported here was to evaluate serial changes in digital hemodynamics (ie, digital arterial blood flow, pressure, and resistance) and ET-1 immunoreactivity, number of platelet-neutrophil aggregates, and concentrations of NO, glucose, and insulin in digital and jugular venous blood collected from horses during induction of CHO-associated laminitis. Blood samples from horses used in another study² of the prodromal stages of CHO-induced laminitis were further evaluated in this investigation.

Materials and Methods

Animals—This study was part of an investigation to evaluate the effects of CHO on digital Starling forces in horses, and all procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee.² The number of horses used was dictated by significant differences in responses of Starling forces to an ET receptor antagonist over time.² Twenty adult horses (5 to 15 years old) of various breeds were determined to be free of laminitis on the basis of results of thorough physical and lameness examinations and

radiographic evaluation. A CBC, assessment of plasma fibrinogen concentration, and serum biochemical analyses were performed to rule out inflammatory or systemic disease. Horses were conditioned to stand in the research area daily for 2 weeks prior to the implantation of ultrasonic flow probes.

Instrumentation—A perivascular transit-time ultrasonic Doppler flow probe^a was calibrated and placed around the lateral palmar digital artery in each anesthetized horse as previously described.^{2,28} The medial palmar artery (at the level of the midmetacarpal region) was isolated and elevated, and the fascia was sutured beneath it to hold the vessel in a more accessible subcutaneous position as previously described.² A 14-gauge, 13.3-cm polytetrafluoroethylene catheter^b was inserted into the left jugular vein for collection of blood samples. One 20-gauge, 5.1-cm polytetrafluoroethylene catheter^c was placed in the medial palmar artery for measurement of digital arterial blood pressure and administration of an ET receptor antagonist or physiologic saline (0.9% NaCl) solution (control treatment), and a second catheter was placed in the lateral palmar digital vein for measurement of digital venous pressure and collection of digital venous blood, as previously described.²

Experimental and temporal design—Monitoring sessions began 14 days after surgical implantation of ultrasonic flow probes and medial palmar artery elevation. Twenty horses were randomly divided into 2 groups of 10 horses each with 1 group receiving an ET receptor antagonist^d and a second group administered an equivalent volume of saline solution at similar times to evaluate the effects of the ET receptor antagonist on Starling force measurements.² Because the ET receptor antagonist had no effect on the data collected in the present study, the data from both groups of horses were pooled. Horses were anesthetized at either 8 hours (10 horses) or 16 hours (10 horses) for digital microvascular assessments in a previous study.²

Digital arterial blood flow, digital arterial pressure, and digital venous pressure were recorded. Digital and jugular venous blood samples were collected at baseline (ie, immediately before CHO administration; designated time 0) for a CBC; assessment of ET-like immunoreactivity; measurement of NO, glucose, and insulin concentrations; and quantification of platelet-neutrophil aggregates. Each horse was then administered a laminitis-inducing ration (85% corn starch and 15% wood flour; 17.6 g of starch/kg of body weight) via a nasogastric tube.^{11,14,29} Digital arterial blood flow, digital arterial pressure, and digital venous pressure were recorded subsequently at 1-hour intervals; digital and jugular venous blood samples were collected at 1-hour intervals (for assessment of ET-like immunoreactivity and measurement of NO, glucose, and insulin concentrations) or 4-hour intervals (for CBC and quantification of platelet-neutrophil aggregates) for 8 hours or 16 hours after CHO administration. Horses were euthanized at the end of experimentation.^e

Assay of endothelinlike immunoreactivity—Blood samples (7 mL) were collected from both a jugular vein and digital vein into separate polypropylene tubes each

containing 105 μL of EDTA and 370 μL of aprotinin. Samples were centrifuged at $1,500 \times g$ for 10 minutes. The plasma was placed in cryovials and stored at -70°C until analyzed for ET-like immunoreactivity by use of a commercial human ELISA kit^f that has been validated previously for use with equine plasma in our laboratory.³⁰ Samples were thawed and 1 mL of each sample was mixed with 1.5 mL of precipitating agent. Each sample was cooled to 4°C and centrifuged for 20 minutes at $3,000 \times g$; the supernatant was transferred to another polypropylene tube and dried under a stream of nitrogen gas at 37°C . Dried samples were reconstituted in 500 μL of assay buffer solution. Serial dilutions of the ET stock solution were prepared as standards; buffer solution was used as the zero standard.

Two hundred microliters of each standard, control sample, and unknown sample was pipetted into wells. Detection antibody (50 μL) was added to all wells except the blank well and mixed appropriately. Wells were covered with plastic film and incubated for 16 to 24 hours at 20° to 22°C . Contents of each well were discarded, and wells were washed 5 times with washing buffer solution. Conjugate (200 μL) was then added to all wells, and the wells were covered with plastic film and incubated for 3 hours at 37°C . Contents of the wells were discarded, and the wells were washed 5 times with washing buffer solution. Substrate (200 μL) was added to each well, and the wells were incubated in darkness for 30 minutes at 20°C . Fifty microliters of stop solution was then added to each well, and contents were mixed thoroughly. Absorption was determined immediately at 405 nm by use of an ELISA reader, with values at 620 nm used as a reference. All samples were analyzed in duplicate. Sensitivity of the assay was approximately 1.5 pg/mL. The intra- and interassay variability for equine plasma in our laboratory is 6.4% and 15.4%, respectively.

Assay of plasma NO concentration—Digital and jugular venous blood samples (8 mL) were collected into tubes containing heparin. Samples were centrifuged at $1,500 \times g$ for 10 minutes. The plasma was immediately placed in cryovials and stored at -70°C until thawed and analyzed; NO concentration was measured by use of an amperometric NO sensor.^g All samples were incubated with copperized cadmium to convert any nitrate present in the samples into nitrite. An amperometric sensor covered by a selective membrane was placed in the sample. Nitric oxide that diffused through the selective membrane was oxidized at a working electrode. The resulting redox current was proportional to the concentration of NO in the sample. The sensor was interfaced with a computer software package; all data were recorded and compared with a standard curve.

Assay of serum insulin concentration—Serum concentrations of insulin were measured by use of a commercial radioimmunoassay kit^h validated for use with equine plasma by Freestone et al.³¹ Serial dilutions of equine plasma were parallel to the human insulin standard curve. The intra- and interassay coefficients of variation were $< 10\%$.

Assay of plasma glucose concentration—Digital and jugular venous blood samples (10 mL) were col-

lected into tubes containing heparin. Samples were centrifuged at $1,500 \times g$ for 10 minutes. The plasma was immediately separated, placed in cryovials, and stored at -70°C until thawed and analyzed for glucose concentration.ⁱ

Surrogate estimates of insulin sensitivity—At each time point, estimates of insulin sensitivity³² were calculated including G:I ratio, HOMA-IS ($22.5/G \times I$), QUICKI ($1/\log G + \log I$), and the logarithm of the reciprocal of I ($\log 1/I$), where G represents the plasma glucose concentration and I represents the plasma insulin concentration.

Quantification of platelet-neutrophil aggregates—Within 30 minutes of blood sample collection, 1 mL of citrate-anticoagulated blood was placed in a 10-mL plastic tube, and RBCs were allowed to settle for 20 minutes. Thereafter, the platelet and leukocyte-rich plasma layer was removed, combined with 2 mL of autologous platelet-poor plasma, and centrifuged at $57 \times g$ for 5 minutes. The platelet-rich plasma layer was removed, and leukocyte-rich sediment was resuspended in 150 μL of autologous plasma. Wedge-type smears were prepared and stained with modified Wright stain. For each sample, 200 neutrophils were counted on each of 2 smears, and the percentage of neutrophils with platelets attached was enumerated.

Hematologic evaluations—Jugular and digital venous blood samples (8 mL) were collected into tubes containing EDTA. Complete blood counts were performed during which total WBC count, neutrophil count, PCV, and total plasma protein concentration were determined.

Digital vascular hemodynamic measurements—Digital arterial and venous pressures were recorded hourly via pressure transducers^k connected to the digital arterial and venous catheters.^{3,33} Digital vascular resistance was determined by dividing the arterial-to-venous pressure gradients by the rate of blood flow.³

Data analysis—Values for digital blood flow; WBC count; PCV; total plasma protein concentration; ET-like immunoreactivity; plasma NO, insulin, and glucose concentrations; surrogate estimates of insulin sensitivity; and the arcsine transformation of the percentage of neutrophils with platelet aggregates were evaluated for normality by use of the Shapiro-Wilk method with the null hypothesis rejected at $\alpha = 0.05$. All normally distributed data were analyzed by use of a mixed-effect general linear model (with repeated measures and horse considered as a random variable). Predetermined post hoc comparisons were made by use of a least square mean with a Bonferroni correction. A value of $P < 0.05$ was considered significant for all tests. All statistical analyses were performed by use of a statistical software package.¹

Results

Studies were performed in the prodromal stages of laminitis, and all horses were euthanatized before the onset of lameness, bounding digital pulses, and heat in the hoof and coronary band. The ET receptor antagonist

had no effect on the data collected in the present study; therefore, data from both groups of horses were pooled. Collection site (digital vs jugular vein) affected plasma ET-like immunoreactivity and plasma NO concentration; all other data were pooled for analysis.

Plasma endothelinlike immunoreactivity—Carbohydrate overload caused a significant increase from baseline in the concentration of ET-like immunoreactivity in digital venous blood at 11 hours after CHO administration (Figure 1). The concentration of ET-like immunoreactivity in digital venous blood was significantly greater than corresponding values in jugular venous blood at 8, 9, 11, and 12 hours after CHO administration.

Plasma NO concentration—Compared with their respective baseline values, there were no significant differences in jugular or digital venous plasma NO concentration over time; however, the digital venous value was significantly higher than the jugular venous value at baseline and 1, 2, 3, 4, and 5 hours after CHO administration (Figure 1).

Serum insulin and plasma glucose concentration—The serum insulin concentration increased ($P = 0.047$) gradually from a baseline value of $30.6 \pm 4.6 \mu\text{U/mL}$ to a peak value of $46.8 \pm 6.3 \mu\text{U/mL}$ at 5 hours

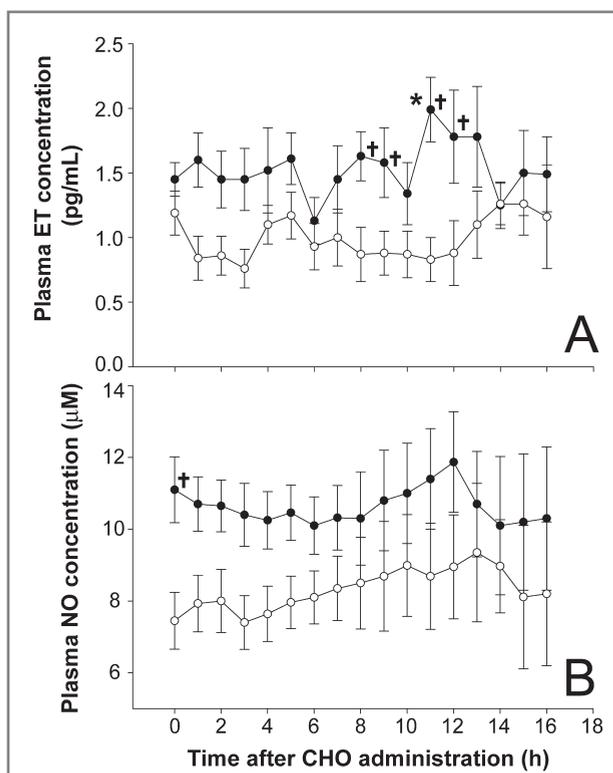


Figure 1—Mean + SEM endothelin-1 immunoreactivity ($\mu\text{U/mL}$; A) and nitric oxide concentration (μM ; B) in digital (closed circles) and jugular (open circles) venous blood samples collected from 20 horses administered CHO (corn starch gruel, 176 mg/kg of body weight). *Value significantly ($P < 0.05$) different from value at time 0. †Value in digital venous blood significantly ($P < 0.05$) different from that in jugular venous blood at this time point. Plasma NO concentration was not significantly different from baseline (time 0) value at any time point in either digital or jugular venous samples; however, jugular venous NO concentrations were significantly ($P < 0.05$) less than digital venous values beginning at time 0.

after CHO administration (Figure 2). Insulin concentration then returned to a value that was not significantly different from the baseline value. The plasma concentration of glucose in digital and jugular venous plasma samples increased significantly at 3, 4, and 5 hours after CHO administration to a peak value of $124 \pm 4 \text{ mg/dL}$ at 4 hours. All surrogate estimates of insulin sensitivity gradually decreased to values that were significantly different from baseline at 5 or 6 hours after CHO administration. Compared with baseline values, the G:I ratio was significantly different at 5 hours, the logarithm of the reciprocal of I ($\log 1/I$) was significantly different at 5 hours, the HOMA-IS value was significantly different at 5 hours and 6 hours, and the QUICKI value was significantly different at 5 hours.

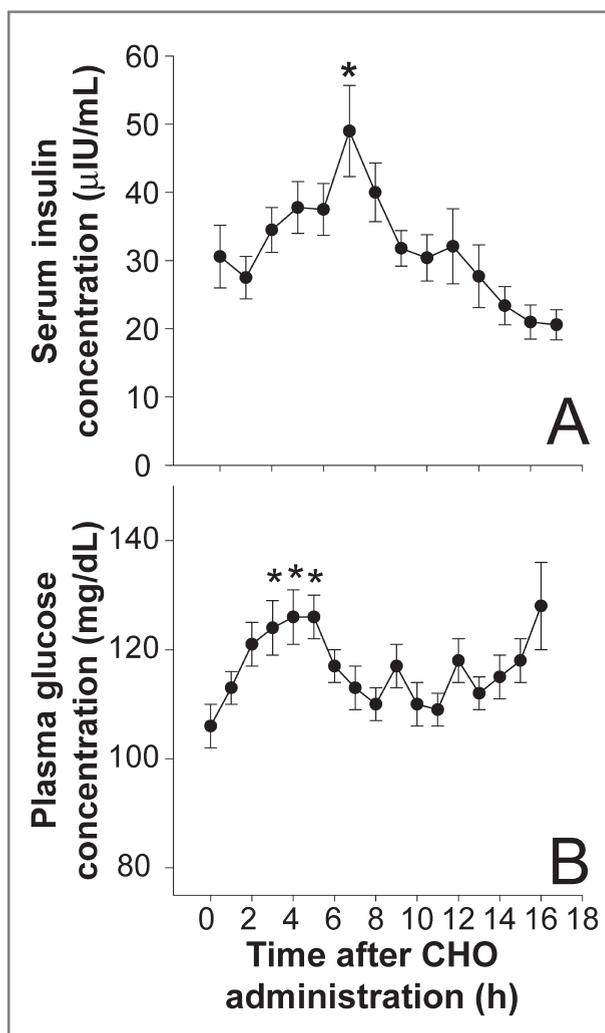


Figure 2—Mean + SEM insulin concentration ($\mu\text{U/mL}$; A) and glucose concentration (B) in digital and jugular venous blood samples collected from 20 horses administered CHO (corn starch gruel, 176 mg/kg of body weight). Because digital and jugular venous blood concentrations were not significantly different, values were combined. *Value significantly ($P < 0.05$) different from time 0 value. Values for surrogate estimates of insulin sensitivity were calculated at each time point including G:I ratio (baseline, 7.2 ± 0.9), HOMA-IS ($22.5/G \times I$; baseline, 1.7 ± 0.2), QUICKI ($1/\log G + \log I$; baseline, 0.30 ± 0.01), and the logarithm of the reciprocal of I ($\log 1/I$; baseline, -1.3 ± 0.1); values were significantly different from baseline at 5 hours after CHO administration (G:I ratio, 3.9 ± 0.3 ; HOMA-IS, 0.8 ± 0.1 ; QUICKI, 0.27 ± 0.01 ; and $\log(1/I)$, -1.6 ± 0.1).

Platelet-neutrophil aggregates—The percentage of neutrophils aggregated with platelets in digital and jugular venous blood samples increased significantly from baseline values at 12 hours after CHO administration, but there were no differences between the 2 sample types (Figure 3).

Hematologic data and digital vascular hemodynamic measurements—Among different time points, there were no significant differences in digital blood flow, digital arterial pressure, digital venous pressure, or digital vascular resistance in horses that were admin-

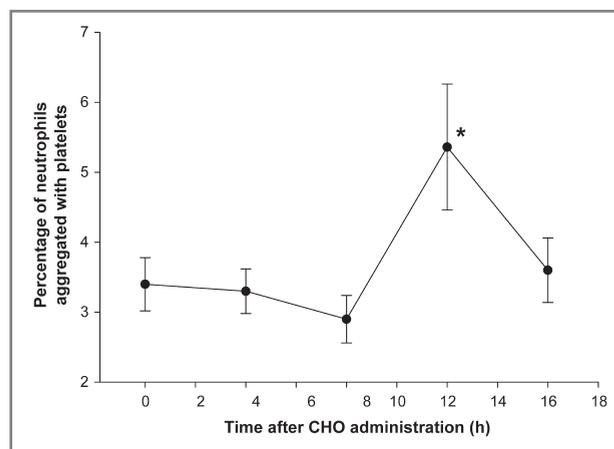


Figure 3—Percentage of neutrophils aggregated with platelets in jugular and digital venous blood samples collected from 20 horses administered CHO (corn starch gruel, 17.6 mg/kg of body weight). Because digital and jugular venous blood concentrations were not significantly different, values were combined. *Value significantly ($P < 0.05$) different from time 0 value.

istered CHO (Table 1). White blood cell count, neutrophil count, PCV, and total plasma protein concentration did not change significantly from baseline values over time (Table 2).

Discussion

Results of the present study have indicated that significant increases in digital venous ET-like immunoreactivity are detectable after CHO administration in horses. Digital venous ET-like immunoreactivity at baseline in the horses of the present study was similar to the value in jugular venous samples collected from healthy horses that were used as control horses in a study³⁴ of horses with naturally acquired colic; in that study, median plasma concentration of ET-like immunoreactivity was 1.80 pg/mL (range, 1.09 to 3.2 pg/mL) in healthy horses, whereas median jugular venous ET-like immunoreactivity was 10.02 pg/mL in horses with strangulating obstructions of the large intestine. In the present study, digital venous ET-like immunoreactivity increased to 2 pg/mL at 11 hours after CHO administration. Similarly, by use of an immunohistochemical technique, Katwa et al¹¹ detected increased ET-1 expression in the digital lamina of horses administered CHO, compared with findings in tissues obtained from horses without laminitis. In other species, most ET-1 produced by endothelial cells is released locally toward the vascular smooth muscle, resulting in higher concentrations abuminally than in the vascular lumen.³⁵ Therefore, the authors believe the increased palmar digital venous concentrations of ET-like immunoreactivity detected after CHO administration in the horses of the present study are likely indicative of much greater release of ET

Table 1—Digital arterial blood flow (measured by use of a long-term implanted extravascular Doppler ultrasonic flow probe), digital arterial pressure, digital venous pressure, and digital vascular resistance in 20 horses administered CHO (corn starch gruel, 17.6 mg/kg of body weight). For horses treated with either an ET receptor antagonist or saline (0.9% NaCl) solution, values for each variable did not differ from baseline (time 0) at any time point.

Time after CHO administration (h)	Treatment group	Digital arterial blood flow (mL/min)	Digital arterial pressure (mm Hg)	Digital venous pressure (mm Hg)	Digital vascular resistance (mm Hg/mL/min)
0 (baseline)	Antagonist	45.4 ± 8.0	94.2 ± 3.7	3.8 ± 1.4	2.3 ± 0.3
	Saline	47.1 ± 9.1	92.4 ± 6.4	1.6 ± 2.1	2.8 ± 0.3
1	Antagonist	49.5 ± 8.1	97.3 ± 4.8	6.4 ± 0.9	2.8 ± 0.8
	Saline	50.2 ± 11.4	92.7 ± 7.8	5.2 ± 2.2	2.0 ± 0.5
2	Antagonist	49.7 ± 9.1	93.3 ± 4.5	6.8 ± 1.9	3.0 ± 1.2
	Saline	61.5 ± 13.4	85.2 ± 5.1	2.6 ± 1.7	1.6 ± 0.3
3	Antagonist	59.1 ± 11.4	94.1 ± 5.4	6.3 ± 1.6	2.7 ± 0.8
	Saline	59.5 ± 15.1	90.1 ± 6.9	2.0 ± 1.3	1.8 ± 0.6
4	Antagonist	67.1 ± 11.6	97.6 ± 4.6	7.0 ± 0.7	2.9 ± 1.5
	Saline	53.9 ± 12.1	90.1 ± 6.8	4.3 ± 2.1	2.1 ± 0.7
5	Antagonist	63.6 ± 10.8	95.5 ± 6.9	5.5 ± 1.7	2.7 ± 1.3
	Saline	53.9 ± 12.1	90.1 ± 5.1	4.3 ± 2.1	1.8 ± 0.5
6	Antagonist	65.2 ± 12.1	98.4 ± 5.4	5.0 ± 1.4	2.0 ± 0.7
	Saline	62.7 ± 13.7	90.1 ± 6.1	4.1 ± 2.1	1.5 ± 0.3
8	Antagonist	66.6 ± 15.5	88.8 ± 5.5	4.3 ± 2.5	1.6 ± 0.4
	Saline	64.2 ± 14.8	92.5 ± 6.1	5.0 ± 2.3	1.4 ± 0.2
10	Antagonist	65.0 ± 19.6	86.3 ± 7.3	3.5 ± 2.4	1.6 ± 0.8
	Saline	61.7 ± 18.1	92.4 ± 12.7	3.4 ± 2.2	1.8 ± 0.4
12	Antagonist	71.4 ± 18.6	100.3 ± 4.1	5.2 ± 1.8	3.0 ± 1.1
	Saline	65.9 ± 16.4	92.8 ± 6.9	3.3 ± 3.1	1.6 ± 0.6
14	Antagonist	59.3 ± 13.2	103.0 ± 4.0	1.8 ± 2.7	1.8 ± 1.1
	Saline	62.9 ± 19.1	103.6 ± 7.1	0.9 ± 1.1	2.1 ± 1.1
16	Antagonist	52.8 ± 11.4	97.7 ± 3.7	0.75 ± 2.2	2.4 ± 0.6
	Saline	61.4 ± 15.6	101.1 ± 1.9	0.4 ± 2.1	3.7 ± 2.1

Table 2—Mean \pm SEM digital and jugular venous total WBC count, neutrophil count, PCV, and total plasma protein concentration in 20 horses administered CHO (corn starch gruel, 17.6 mg/kg of body weight). There were no differences between values for jugular and digital venous blood samples or values for different treatment groups (ET receptor antagonist or saline solution), and thus data were pooled for statistical analyses. Values for each variable did not differ from baseline (time 0) at any time point.

Time after CHO administration (h)	Total WBC count (cells/mL)	Total neutrophil count (cells/mL)	PCV (%)	Total plasma protein concentration (g/dL)
0	10,740 \pm 573	8,370 \pm 509	32 \pm 1.2	6.5 \pm 0.04
4	11,155 \pm 780	8,765 \pm 664	32 \pm 1.4	6.6 \pm 0.08
8	10,950 \pm 768	8,750 \pm 681	33 \pm 1.2	6.6 \pm 0.08
12	10,620 \pm 1,025	8,880 \pm 839	32 \pm 1.9	6.7 \pm 0.11

toward the abuminally located smooth muscle. There is the potential that the increased synthesis or release of ET-1 results in digital venoconstriction in the developmental stages of acute laminitis^{2,3}; this is supported by the finding that the ET antagonist improved digital resistance and digital blood flow in horses with CHO-induced laminitis in a previous study.²

Short-term administration of the ET receptor antagonist PD145065 would not be expected to alter ET-1 synthesis or plasma ET-1 concentrations because that antagonist does not alter resting vascular tone.^{36,37} The lack of an effect of the ET antagonist in the present study suggests that ET-like factors are not important markers of endothelial dysfunction and contribute little to the development of laminitis. However, because our experiments were terminated in the early stages of the disease, greater long-term influences of the antagonist on endothelial dysfunction cannot be ruled out. Furthermore, the short duration of these experiments would not allow the full influence of transcription of various genes involved in endothelial function or dysfunction to be realized. Furthermore, the duration of our study was not sufficient for endothelial dysfunction to decrease constitutive NO synthase activity and digital venous NO concentration. Plasma NO concentration was higher in digital venous blood than jugular venous blood, reflecting a need for greater vasodilatory substances in the digital circulation under basal conditions.

In the study of this report, there was no significant alteration in digital arterial blood flow, digital arterial or venous pressure, or digital vascular resistance throughout the recording period. In contrast, Pollitt et al⁶ detected increases and Hood et al⁴ detected decreases in hoof wall surface temperature after CHO administration to horses. In the latter study,⁴ the onset of the decrease in hoof wall surface temperature was 8 to 12 hours before the onset of lameness (at least 48 to 56 hours after CHO administration); therefore, the duration of the recording period in the present study was not sufficient to detect similar changes in blood flow. In the former study,¹ the increased hoof wall surface temperature was detected 12 to 16 hours after CHO administration; this temperature change was not evident in the present study, and the difference in the carbohydrate used for induction of laminitis could be a factor. Decreased blood flow in the hoof² despite blood flow in the distal portion of the limb could be a result of increased blood

flow through arteriovenous anastomoses.^{38–40} Furthermore, increased flow in the larger vessels of the distal portion of the limb could mask decreases in flow in the microvasculature of the hoof when total flow in the distal portion of the limb is measured as performed in the present study.

The results of the study of this report indicated that concentrations of insulin and glucose in jugular and digital blood increase after CHO administration in horses. Administration of starch would be expected to cause postprandial hyperglycemia (and secondary, delayed hyperinsulinemia) through simple digestion of starch, absorption of glucose, and secondary hormone release.⁴¹ Moreover, stress associated with CHO-induced intestinal disease or nasogastric intubation could increase plasma glucose and insulin concentrations.⁴² Hyperglycemia (and hyperinsulinemia) could also be explained by the development of insulin resistance as a consequence of endocrinologic effects of stress (eg, hypercortisolism).^{43,44} The surrogate estimates of insulin sensitivity calculated in the present study indirectly suggest that the horses administered CHO were insulin insensitive.³² In that case, the development of insulin resistance could contribute to the risk of laminitis because hoof keratinocytes possess a high essential requirement for glucose.²⁶ Any endocrinologic change that might reduce the uptake of glucose by laminar epithelial cells could lead to development of laminitis. Regardless of the cause of the increased plasma glucose and insulin concentrations, it is possible that these factors could contribute to the pathogenesis of laminitis.⁴³ The importance of normal glucose regulation is underscored by the results of 1 study²⁶ that indicate that healthy maintenance of the hoof lamellar interface is critically dependent on the availability of glucose for hoof keratinocytes.

Increased serum insulin concentrations can lead to cardiovascular and endothelial dysfunction and result in increased ET-1 production and development of a relatively procoagulative state.⁴³ Furthermore, increased synthesis or release of ET-1 can lead to insulin resistance per se.⁴⁵ The design of the present study does not enable us to definitively prove a cause-and-effect relationship between CHO and increased plasma insulin concentration. However, our results do suggest that further investigation of these endocrine mechanisms in CHO-associated laminitis is warranted.

The percentage of neutrophils that were aggregated with platelets in the digital and jugular venous blood samples increased at 12 hours after CHO administration in horses. Activation of platelet-platelet and especially platelet-neutrophil aggregation has been identified in a study⁴⁶ of CHO-associated laminitis in ponies. Furthermore, treatment of ponies with an antagonist of platelet aggregation prior to administration of CHO prevented development of clinical signs of laminitis and blocked increases in numbers of platelet-neutrophil aggregates.¹⁴ These platelet-neutrophil aggregates may localize in the laminar circulation because of high venous resistance.³⁹ Activated cells that become lodged in the capillary bed reduce perfusion and nutrient delivery and thereby cause ischemia to develop.³⁹ Endothelin, platelet-activating factor, and several other platelet and inflammatory cell activators can increase platelet aggregation to neutrophils.⁴⁷ Administration of ET receptor antagonists improves perfusion and reduces platelet aggregation in rats with experimentally induced ischemia.⁴⁸ These data are particularly important to the present study in which increased synthesis or release of ET-1 was detected even though there were no differences in total limb blood flow between horses treated with saline solution or the ET receptor antagonist. That is, the antagonist of platelet aggregation may restore perfusion and prevent ischemia caused by release of ET-1. This ischemia is not detected during measurement of total blood flow, which can be maintained by flow through shunts at the expense of capillary perfusion.

In horses of the present study, WBC count, neutrophil count, PCV, and total plasma protein concentration did not change significantly from baseline values during the 12-hour period after CHO administration. In another study²⁹ of horses administered CHO, significant increases (compared with baseline values) in WBC count, PCV, and total plasma protein concentration were detected after 24 hours; these changes were attributed to the glucocorticoid response and also hemoconcentration from fluid compartment shifts. In contrast, Moore et al⁴² found no significant change in total WBC count but increased neutrophil count in horses administered CHO. The results of the present study suggest that changes in WBC and neutrophil numbers do not occur in the earlier prodromal stages of laminitis.

After CHO administration to horses in the present study, concentrations of digital venous ET-like immunoreactivity increased from baseline value with no change in jugular venous ET-like immunoreactivity; therefore, ET-1 could be responsible for digital vasoconstriction, increased digital vascular resistance, and increased postcapillary resistance following CHO administration. Furthermore, increases in digital and jugular venous insulin and glucose concentrations detected after CHO administration suggest that further study of endocrine factors during CHO is of value. Although there was no decrease in digital venous NO concentration following CHO administration, the concurrent increases in ET-1 immunoreactivity, plasma insulin and glucose concentrations, and number of platelet-neutrophil aggregates support a role of endothelial dysfunction in the pathogenesis of laminitis in horses.

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- a. Transonics Systems Inc, Ithaca, NY.
 - b. Angiocath vascular access, Becton Dickinson & Co, Sandy, Utah.
 - c. RA-04020, Arrow International, Reading, Pa.
 - d. PD145065, American Peptide Co, Sunnyvale, Calif.
 - e. Socumb, Butler Co, Columbus, Ohio.
 - f. ET Kit, Biomedica, American Research Products Inc, Belmont, Mass.
 - g. ISO-NO Mark II, World Precision Instruments Inc, Sarasota, Fla.
 - h. Diagnostic Products Corp, Los Angeles, Calif.
 - i. Stat Profile CCX, Nova Biomedical Corp, Waltham, Mass.
 - j. 7PY, Grass Medical Instruments, Quincy, Mass.
 - k. P231D, Grass Medical Instruments, Quincy, Mass.
 - l. Proc Mixed, SAS, version 9.1, SAS Institute Inc, Cary, NC.
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References

1. Garner HE, Hahn AW, Salem C, et al. Cardiac output, left ventricular ejection rate, plasma volume, and heart rate changes in equine laminitis-hypertension. *Am J Vet Res* 1977;38:725-729.
2. Eades SC, Holm AS, Moore RM. Effects of an endothelin receptor antagonist and nitroglycerine on digital microvascular function in horses during the prodromal stages of carbohydrate overload laminitis. *Am J Vet Res* 2006;67:1204-1211.
3. Allen D Jr, Clark ES, Moore JN, et al. Evaluation of equine digital Starling forces and hemodynamics during early laminitis. *Am J Vet Res* 1990;51:1930-1934.
4. Hood DM, Wagner IP, Brumbaugh GW. Evaluation of hoof wall surface temperature as an index of digital vascular perfusion during the prodromal and acute phases of carbohydrate-induced laminitis in horses. *Am J Vet Res* 2001;62:1167-1172.
5. Eaton SA, Allen D, Eades SC, et al. Digital Starling forces and hemodynamics during early laminitis induced by an aqueous extract of black walnut (*Juglans nigra*) in horses. *Am J Vet Res* 1995;56:1338-1344.
6. Pollitt CC, Davies CT. Equine laminitis: its development coincides with increased sublamellar blood flow. *Equine Vet J Suppl* 1998;(26):125-132.
7. Robinson NE, Scott JB, Dabney JM, et al. Digital vascular responses and permeability in equine alimentary laminitis. *Am J Vet Res* 1976;37:1171-1176.
8. Kirker-Head CA, Stephens KA, Toal RL, et al. Circulatory and blood gas changes accompanying the development and treatment of induced laminitis. *Equine Vet Sci* 1986;6:293-301.
9. Trout DR, Hornof WJ, Linford RL, et al. Scintigraphic evaluation of digital circulation during the developmental and acute phases of equine laminitis. *Equine Vet J* 1990;22:416-421.
10. Adair HS III, Goble DO, Schmidhammer JL, et al. Laminar microvascular flow, measured by means of laser Doppler flowmetry, during the prodromal stages of black walnut-induced laminitis in horses. *Am J Vet Res* 2000;61:862-868.
11. Katwa LC, Johnson PJ, Ganjam VK, et al. Expression of endothelin in equine laminitis. *Equine Vet J* 1999;31:243-247.
12. Schneider DA, Parks AH, Eades SC, et al. Palmar digital vessel relaxation in healthy horses and in horses given carbohydrate. *Am J Vet Res* 1999;60:233-239.
13. Weiss DJ, Geor RJ, Johnston G, et al. Microvascular thrombosis associated with onset of acute laminitis in ponies. *Am J Vet Res* 1994;55:606-612.
14. Weiss DJ, Evanson OA, McClenahan D, et al. Effect of a competitive inhibitor of platelet aggregation on experimentally induced laminitis in ponies. *Am J Vet Res* 1998;59:814-817.
15. Snyder F. Platelet-activating factor and related acetylated lipids as potent biologically active cellular mediators. *Am J Physiol* 1990;259:C697-V708.
16. Anfossi G, Cavalot F, Massucco P, et al. Insulin influences immunoreactive endothelin release by human vascular smooth muscle cells. *Metabolism* 1993;42:1081-1083.
17. Ferri C, Pittoni V, Piccoli A, et al. Insulin stimulates endothelin-1 secretion from human endothelial cells and modulates its circulating levels in vivo. *J Clin Endocrinol Metab* 1995;80:829-835.

18. Piatti P, Monti LD, Conti M, et al. Hypertriglyceridemia and hyperinsulinemia are potent inducers of endothelin-1 release in humans. *Diabetes* 1996;45:316–321.
19. Wolpert H, Steen SN, Istfan NW, et al. Insulin modulates circulating endothelin-1 levels in humans. *Metabolism* 1993;42:1027–1030.
20. Juan C, Shen Y, Chien Y, et al. Insulin infusion induces endothelin-1-dependent hypertension in rats. *Am J Physiol* 2004;287:E948–E954.
21. Schott H. Equine Cushing's syndrome associated with pars intermedia dysfunction. *Vet Clin North Am Equine Pract* 2002;18:237–270.
22. Field JR, Jeffcott LB. Equine laminitis—another hypothesis for pathogenesis. *Med Hypotheses* 1989;30:203–210.
23. Consentino F, Luscher TF. Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* 1998;32(suppl 3):S54–S61.
24. Luscher TF, Dohi Y, Tanner FC, et al. Endothelium-dependent control of vascular tone: effects of age, hypertension and lipids. *Basic Res Cardiol* 1991;86(suppl 2):143–158.
25. Scheikofer S, Balletshofer B, Andrassy M. Endothelial dysfunction in diabetes mellitus. *Semin Thromb Hemost* 2000;26:503–511.
26. Pass MA, Pollitt S, Pollitt CC. Decreased glucose metabolism causes separation of hoof lamellae in vitro: a trigger for laminitis? *Equine Vet J Suppl* 1998;(26):133–138.
27. Mobasheri A, Critchlow K, Clegg PD, et al. Chronic equine laminitis is characterized by loss of GLUT-1, GLUT-4, and ENaC positive laminar keratinocytes. *Equine Vet J* 2004;36:248–254.
28. Ingle-Fehr JE, Baxter GM. Evaluation of digital and laminar blood flow in horses given a low dose of endotoxin. *Am J Vet Res* 1998;59:192–196.
29. Garner HE, Coffman JR, Hahn AW, et al. Equine laminitis of alimentary origin: an experimental model. *Am J Vet Res* 1975;36:441–444.
30. Tetens J, Moore RM, Hosgood GL, et al. Effects of infusion of adenosine triphosphate-magnesium chloride on cardiopulmonary and clinicopathologic variables, cytokine activity and endothelin concentrations in horses administered a low dose of endotoxin. *Am J Vet Res* 2004;65:225–237.
31. Freestone JF, Wolfsheimer KJ, Kamerling SG, et al. Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. *Equine Vet J* 1991;23:219–223.
32. Kronfeld DS, Treiber KH, Geor RJ. Comparison of nonspecific indications and quantitative methods for the assessment of insulin resistance in horses and ponies. *J Am Vet Med Assoc* 2005;226:712–719.
33. Korthuis RJ, Granger DN, Taylor AE. A new method for estimating skeletal muscle capillary pressure. *Am J Physiol* 1984;246:H880–H885.
34. Ramaswamy CM, Eades SC, Venugopal CS, et al. Plasma concentrations of endothelin-like immunoreactivity in healthy horses and horses with naturally acquired gastrointestinal tract disorders. *Am J Vet Res* 2002;63:454–458.
35. Wagner OF, Christ G, Wojta J, et al. Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem* 1992;267:16066–16068.
36. Ekelund U, Adner M, Edvinsson L, et al. Effects of the combined ETA and ETB receptor antagonist PD145065 on arteries, arterioles, and veins in the cat hindlimb. *J Cardiovasc Pharmacol* 1995;26:S211–S213.
37. Tanus-Santos JE, Gordo WM, Udelsmann A, et al. Nonselective endothelin-receptor antagonism attenuates hemodynamic changes after massive pulmonary air embolism in dogs. *Chest* 2000;118:175–179.
38. Robinson NE. Digital blood flow, arteriovenous anastomoses and laminitis. *Equine Vet J* 1990;22:381–383.
39. Weiss DJ. Equine laminitis: a review of recent research. *Equine Pract* 1997;19:16–20.
40. Hood DM, Amoss MS, Hightower D. Equine laminitis I: radioisotopic analysis of the hemodynamics of the foot during the acute disease. *J Equine Med Surg* 1978;2:439–444.
41. Schmidt O, Deegen E, Fuhrmann H, et al. Effects of fat feeding and energy level on plasma metabolites and hormones in Shetland ponies. *J Vet Med A Physiol Pathol Clin Med* 2001;48:39–49.
42. Moore JN, Garner HE, Coffman JR. Haematological changes during development of acute laminitis hypertension. *Equine Vet J* 1981;13:240–242.
43. Johnson PJ, Messer NT, Ganjam VK. Cushing's syndromes, insulin resistance, and endocrinopathic laminitis. *Equine Vet J* 2004;36:194–198.
44. Keen JA, McLaren M, Chandler KJ, et al. Biochemical indices of vascular function, glucose metabolism, and oxidative stress in horses with equine Cushing's disease. *Equine Vet J* 2004;36:226–229.
45. Said SA, Ammar el SM, Suddek GM. Effects of bosentan (ETA/ETB receptor antagonist) on metabolic changes during stress and diabetes. *Pharmacol Res* 2005;51:107–115.
46. Weiss DJ, Evanson OA, McClenahan D, et al. Evaluation of platelet activation and platelet-neutrophil aggregates in ponies with alimentary laminitis. *Am J Vet Res* 1997;58:1376–1380.
47. Uhlmann D, Glasser S, Gaebel G, et al. Improvement of post-ischemic hepatic microcirculation after endothelin A receptor blockade-endothelin antagonism influences platelet-endothelium interactions. *J Gastrointest Surg* 2005;9:187–197.
48. Uhlmann D, Glasser S, Lauer H, et al. Endothelin-a receptor blockade improves postischemic hepatic microhemodynamics. *J Cardiovasc Pharmacol* 2004;44(suppl 1):S103–S104.