Seasonal changes in plasma concentrations of cecum-derived amines in clinically normal ponies and ponies predisposed to laminitis

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Objective—To measure concentrations of amines formed in the cecum of clinically normal ponies, determine amine concentrations in plasma samples collected in spring and winter, and compare concentrations of amines and serotonin in plasma samples obtained from clinically normal ponies and ponies predisposed to laminitis.

Sample Population—Cecal contents obtained from 10 ponies euthanatized at an abattoir and blood samples obtained from 42 adult ponies.

Procedure—Cecal contents were assayed for amines by high-performance liquid chromatography (HPLC). Blood samples were collected at various times of the year from 20 ponies predisposed to acute laminitis and 22 clinically normal ponies. Plasma serotonin concentration was measured by HPLC, and tryptamine (TRP), tyramine (TYR), phenylethylamine (PEA), and isoamylamine (IAA) were measured by liquid chromatography-mass spectrometry.

Results—15 amines were identified in cecal contents. Plasma TRP, TYR, PEA, and IAA concentrations ranged from 10pM to 100nM in both groups of ponies. Plasma concentrations of serotonin or other amines did not differ between clinically normal ponies and those predisposed to laminitis; however, significantly higher concentrations of TRP, PEA, and IAA were found in samples obtained in the spring, compared with winter samples.

Conclusions and Clinical Relevance—Various amines are found in the cecum of ponies, several of which can be detected in the plasma. Concentrations increase significantly in the spring and may reach concentrations close to the threshold for causing vasoconstriction. Release of amines from the cecum into the systemic circulation may contribute to hemodynamic disturbances in horses and ponies with acute laminitis. (*Am J Vet Res* 2003;64:1132–1138)

Acute laminitis is most frequently recognized as a sequel to gastrointestinal disturbances. Fermentation of excess carbohydrate in the equine cecum and colon that leads to overgrowth of gram-positive bacteria and a marked decrease in pH experimentally causes acute laminitis[1] and is associated with reduced digital perfusion in the prodromal stages.[2,3] The trigger factor or factors released from the cecum that bring about disturbances in digital hemodynamics or other changes associated with the pathogenesis of this condition remain to be elucidated, although a number of candidates have been proposed.

Amines have been found in fermented foodstuffs[4] and in intestinal contents of ruminant and monogastric animals.[5,6] These amines are formed by bacterial decarboxylation of amino acids. This process may be part of the response of bacteria to acid conditions following carbohydrate fermentation.[7] Various amine compounds are capable of exerting vasopressor effects in the circulation as a result of structural similarities with endogenous amines such as the catecholamines and serotonin (ie, **5-hydroxytryptamine [5-HT]**).[8] Thus, they may directly stimulate 5-HT or α-adrenoreceptors on digital arteries and veins[9,10] or displace endogenous amines from nerve endings or platelets, bringing about vasoconstriction.[11]

The study reported here was designed to measure amine compounds in cecal contents of clinically normal ponies by use of a **high-performance liquid chromatography (HPLC)** method. By adapting this method for **liquid chromatography-mass spectrometry (LC-MS)**, 4 of these amines were measured in plasma of clinically normal ponies maintained on grass pastures. Plasma concentrations of cecum-derived amines and serotonin were compared at various times of the year that corresponded to various stages of grass growth. Plasma concentrations were compared between a population of ponies identified as being predisposed to laminitis and a clinically normal (control) population.

Materials and Methods

Measurement of amines in cecal contents obtained from ponies—Cecal contents (50 mL) were obtained from 10 clinically normal adult native-breed ponies (height at shoulders, < 1.45 m) that were euthanatized at an abattoir during the summer (June to August). Cecal contents were centrifuged at 1,500 × *g* for 15 minutes at 4°C. Supernatant was filtered,[a] and aliquots were frozen at –80°C until required for assay.

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An HPLC technique was used to measure amine concentrations in cecal contents in accordance with a method described elsewhere.[12] Briefly, 1 mL of cecal contents was diluted 1:4 (vol:vol) with acetone:water (2:1), and an internal standard (heptylamine, 5 µg/mL) was then added. Following the addition of 1 mL of borax buffer (pH, 10.5), amines were derivatized with 1% dansyl chloride in acetone at 65°C for 25 minutes and then extracted from the matrix by use of solid-phase extraction cartridges.[b]

Separation and detection of amines were performed with HPLC[c] by use of a guard column[d] and reverse-phase C18 column[e] and ultraviolet detection.[f] The mobile-phase gradient was gradually changed from 25 to 80% acetonitrile in water during the 45-minute period, and compounds were detected by measuring their absorbance at 250 nm. In another study[12] that examined cecal contents of horses, this assay was found to be reproducible (interassay coefficient of variation, < 16%), precise (intra-assay coefficient of variation, < 8%), accurate (spiking recovery of amines between 87 and 100%), and sensitive (limit of detection between 0.03 and 0.22 µg/mL).

Sample populations of ponies predisposed to laminitis and clinically normal ponies—Examination of seasonal changes in plasma concentrations of 5-HT and cecum-derived amines in clinically normal ponies and ponies predisposed to laminitis was conducted at a facility that maintained approximately 1,200 horses and ponies on similar grazing and management conditions. The facility had an on-site veterinary staff and good computerized records. Ponies predisposed to laminitis were included in the study if they were diagnosed with acute laminitis by the veterinary staff 3 or more times during the preceding 3 years and examination of lateromedial radiographs of both forefeet did not reveal evidence of displacement of the pedal bone (angle of rotation, < 5°) or other foot abnormalities. In addition, all ponies included in the study were examined to ensure that they did not have signs of other diseases. Ponies were screened for abnormalities by use of serum biochemical analyses and hematologic tests. Furthermore, all ponies were subjected to a 19-hour (overnight) dexamethasone-supression test in an attempt to exclude the possibility of dysfunction of the pituitary pars intermedia.[13] On the basis of these criteria, 20 adult ponies (14 geldings and 6 mares) were selected for use in the study.

For comparison, a group of 22 clinically normal (control) ponies (16 geldings and 6 mares) was selected. These poniess were healthy, as determined on the basis of clinical history, examination of radiographs of the forefeet, and results of serum biochemical analyses and dexamethasone-suppression tests.

Collection of samples for analysis of plasma concentrations of amines—Blood samples were collected from the 22 clinically normal ponies and 20 ponies predisposed to laminitis 4 times during the year (mid-June [summer], early November [autumn], beginning of March [winter], and early May [spring]). None of these had signs of laminitis at the time blood samples were collected. Average monthly climatologic data for the region were obtained[g] to quantify seasonal changes.

Samples were collected into heparinized tubes that contained clomipramine (an amount adequate to yield a blood concentration of 1µM) and phenelzine (10µM) to inhibit uptake and metabolism, respectively, of 5-HT and other amines by platelets.[14] Samples from each pony were centrifuged (300 × *g* for 10 minutes) within 1 to 2 hours after collection to provide platelet-rich plasma, aliquots of which were then further centrifuged at 10,000 × *g* for 10 minutes to provide platelet-poor plasma, which was stored at –80°C until assayed.

Meaurement of plasma concentrations of 5-HT—Plasma concentrations of 5-HT were measured by use of an HPLC method with electrochemical detection, as described elsewhere.[15] Briefly, plasma proteins from 200-µL aliquots of platelet-poor plasma were precipitated with 10M trichloroacetic acid, and an internal standard (*N*-methyl 5-HT) was added to provide a final concentration of 25 ng/mL. Twenty microliters was injected onto the column (reverse-phase C_{18}), and the mobile phase flow rate was set at 1 mL/min. Peaks were detected at 450 mV electrode potential.[h] Plasma concentration of 5-HT was calculated from a standard curve (concentrations of 1nM to 100nM 5-HT) constructed from the ratio of the area under the curve for the 5-HT peak to that of the internal standard peak. Results were expressed in nanomolar concentrations. Other studies[15,i] have documented an intra-assay coefficient of variation of 4.1%, interassay coefficient of variation of 7.0%, and limit of detection of 6nM.

Meaurement of plasma concentrations of cecum-derived amines—Ten microliters of heptylamine (5 µg/mL) was added to 1 mL of plasma to provide an internal-standard concentration of 50 ng/mL, which was followed by the addition of 4 mL of acetone:water (2:1); the mixture was then centrifuged to remove precipitated plasma proteins. Amines were derivatized by the addition of dansyl chloride and extracted from the sample, as described previously. Following extraction of the amines, the eluate was evaporat-

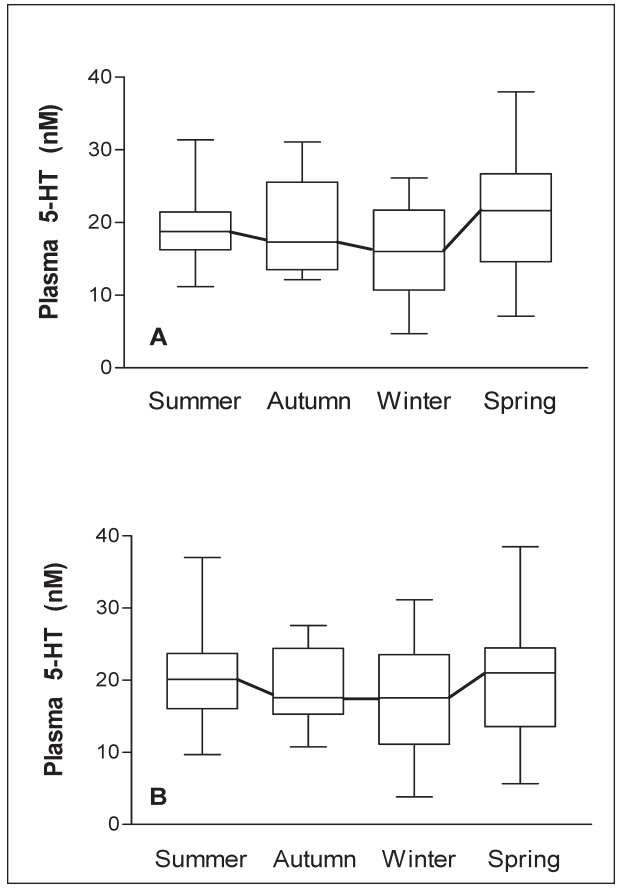

Figure 1—Box-and-whisker plots of plasma concentrations of 5-hydroxytryptamine (5-HT) measured in platelet-poor plasma obtained from a group of 22 clinically normal ponies (A) and 20 ponies predisposed to laminitis (B) at various times of the year. Boxes define the interquartile range (25 to 75%), horizontal lines in the center of each box represent median values, and outer bars define the 95% confidence intervals.

ed to dryness under a stream of nitrogen at 70°C and then reconstituted in 100 µL of acetonitrile:0.1% formic acid (50:50) in HPLC-grade water.

Separation and detection of the dansylamines by liquid chromatography combined with mass spectrometry were performed at the Horseracing Forensic Laboratories, Newmarket, Suffolk. Separation was performed by use of a C_{18} column as described previously but with the following mobile-phase gradient: initial conditions consisted of 25% acetonitrile, 74.9% water, and 0.1% formic acid, increasing to 70% acetonitrile at 5 minutes and returning to initial conditions from 10 to 15 minutes. The flow rate was set at 0.7 mL/min.

The compounds were detected on the basis of their retention time and molecular mass by use of the atmospheric pressure chemical ionization method of mass spectrometry.[j,k] Vaporizer temperature was 400°C, source temperature was 120°C, and flow rate for the drying gas was 447.5 L/h. Four amines were measured (ie, tryptamine, phenylethylamine, isoamylamine, and tyramine), and the respective molecular masses of the dansylated amines were 394.3, 355.3, 321.3, and 604.3. It should be mentioned that tyramine forms a dimer when derivatized. A standard curve of known concentrations of each of the amines (range, 10 pg/mL to 100 ng/mL) was constructed, and amine concentrations in the samples were calculated from the area under the curve for the amine divided by the area under the curve for the internal standard (molecular mass, 349.3; 50 ng/mL) multiplied by a factor determined from the calibration curve and calculated by the machine software.[l] Values were then converted into molar concentrations.

Intra-assay coefficient of variation for the amines was 16.5, 12.5, 15.4, and 11.9% for tryptamine, phenylethylamine, tyramine, and isoamylamine, respectively. Interassay coefficient of variation for heptylamine was 17.2%. Limit of detection was 1 pg/mL for phenylethylamine and tyramine, 2 pg/mL for isoamylamine, and 5 pg/mL for tryptamine.

Statistical analysis—Comparisons were performed by use of computer software packages.[m,n] Cecal and plasma amine concentrations and plasma concentrations of 5-HT were expressed as the median value with interquartile range. Concentrations of 5-HT and the 4 cecum-derived amines measured in plasma of the 2 groups of ponies were analyzed to detect differences between the groups and seasonal changes within groups by use of a 3-way ANOVA followed by the Dunn multiple-comparison post-hoc test.

Results

Concentrations of amines in cecal contents—Fifteen amines were identified in cecal contents obtained from clinically normal ponies. Amines were identified on the basis of their chromatographic retention times, and all were at concentrations > 1 µmol/L. Median (interquartile range) concentrations were determined for the aliphatic monoamines (methylamine,

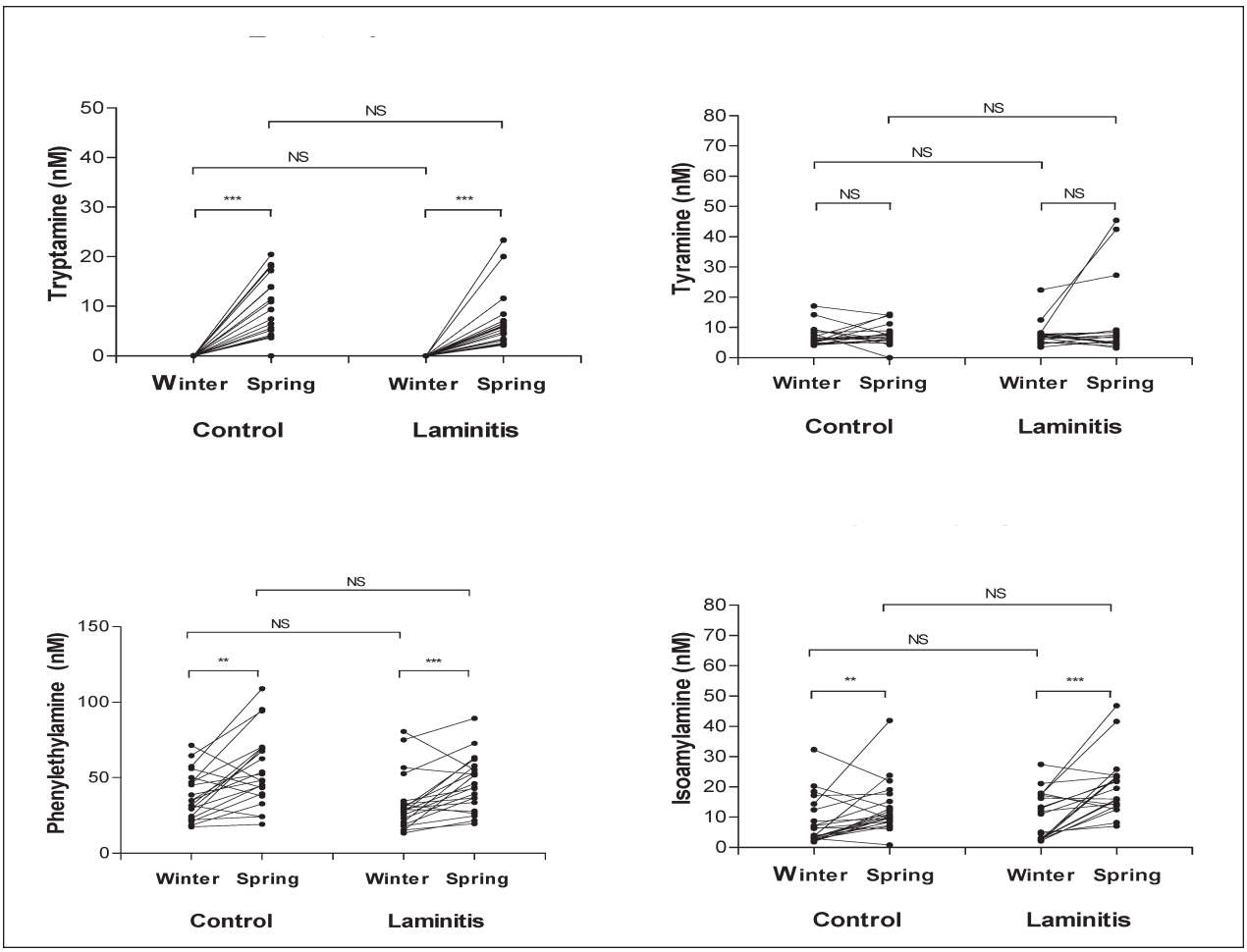

Figure 2—Concentrations of 4 cecum-derived amines measured in platelet-poor plasma obtained from a group of 22 clinically normal ponies (Control) and 20 ponies predisposed to laminitis (Laminitis) in winter and spring. **Values within a group differ significantly (P = 0.01) between seasons. ***Values within a group differ significantly ($P < 0.001$) between seasons. NS = Not significant.

88.8μM [65.7 to 99.1μM]; ethylamine, 17.7μM [13.4 to 25.4μM]; propylamine, 16.0μM [5.3 to 25.9μM]; isoamylamine, 67.4μM [47.1 to 94.9μM]; and isobutylamine, 5.2μM [0.2 to 26.1μM]), the aromatic monoamines (tryptamine, 11.5μM [7.4 to 17.9μM]; tyramine, 6.1μM [4.9 to 8.4μM]; kynuramine, 5.1μM [0.3 to 7.7μM]; and phenylethylamine, 34.8μM [11.9 to 46.2μM]), and the diamines (putrescine, 32.8μM [29.5 to 39.9μM]; cadaverine, 35.1μM [28.3 to 48.6μM]; histamine, 8.1μM [2.9 to 10.2μM]; diaminoheptane, 79.5μM [46.4 to 98.3μM]; spermidine, 106.8μM [81.4 to 156.5μM]; and spermine, 4.4μM [2.1 to 9.4μM]).

Plasma concentrations of 5-HT—We did not detect significant differences in plasma concentrations of 5-HT between the group of ponies predisposed to laminitis and the clinically normal ponies, nor were there significant seasonal changes within the 2 groups (**Fig 1**). Median (interquartile range) concentrations for control ponies were 1.6×10^{-8}M (1.1×10^{-8}M to 2.2×10^{-8}M) and 2.2×10^{-8}M (1.5×10^{-8}M to 2.7×10^{-8}M) in winter and spring, respectively, whereas corresponding values for ponies predisposed to laminitis were 1.8×10^{-8}M (1.1×10^{-8}M to 2.3×10^{-8}M) and 2.1×10^{-8}M (1.4×10^{-8}M to 2.4×10^{-8}M).

Plasma concentrations of cecum-derived amines—All 4 amines were detected in plasma samples of clinically normal ponies and ponies predisposed to laminitis. Concentrations of amines were within the range of 10pM to 100nM. Although we did not detect significant differences in concentrations between clinically normal ponies and those predisposed to laminitis, significantly higher concentrations were seen in 3 of the amines in samples obtained in the spring, compared with concentrations for samples obtained in the winter (**Fig 2**). Median (interquartile range) concentrations of phenylethylamine increased from 3.5×10^{-8}M (2.4×10^{-8}M to 4.8×10^{-8}M) and 2.9×10^{-8}M (2.0×10^{-8}M to 3.4×10^{-8}M) for clinically normal ponies and those predisposed to laminitis, respectively, in winter to values of 6.9×10^{-8}M (3.9×10^{-8}M to 15.6×10^{-8}M) and 6.0×10^{-8}M (3.3×10^{-8}M to 7.8×10^{-8}M), respectively, in spring. Concentrations of isoamylamine increased from 0.39×10^{-8}M (0.30×10^{-8}M to 1.34×10^{-8}M) and 1.1×10^{-8}M (0.3×10^{-8}M to 1.7×10^{-8}M) for clinically normal ponies and those predisposed to laminitis, respectively, in winter to values of 1.0×10^{-8}M (0.8×10^{-8}M to 1.4×10^{-8}M) and 1.6×10^{-8}M (1.3×10^{-8}M to 2.3×10^{-8}M), respectively, in spring. Most strikingly, median (interquartile range) concentrations of tryptamine increased from 6.2×10^{-11}M (6.0×10^{-11}M to 9.4×10^{-11}M) and 6.2×10^{-11}M (6.0×10^{-11}M to 24.9×10^{-11}M) for clinically normal ponies and ponies predisposed to laminitis, respectively, in winter to values of 690×10^{-11}M (390×10^{-11}M to $1,560 \times 10^{-11}$M) and 600×10^{-11}M (330×10^{-11}M to 780×10^{-11}M) for clinically normal ponies and those predisposed to laminitis, respectively, in spring.

Regional average of the minimum grass temperature for the month during which winter samples were obtained was –0.7°C, and there were 2.95 hours of sunshine/d. At the time the spring samples were obtained, the average of the minimum grass temperature was 6.0°C, and the mean number of daily hours of sunshine was 8.12.

Discussion

Analysis of these data reveals that a range of amine compounds is found in the cecum of clinically normal ponies. Furthermore, several of these compounds can be detected in the plasma, and they attain higher concentrations in the spring. Compounds or toxins that have the potential to cause peripheral vasoconstriction may be important in the pathophysiologic mechanisms of laminitis, because the clinical signs and pathologic changes are considered consistent with ischemia of the digital tissues followed by reperfusion injury.[16] The compounds or toxins released from the cecum following carbohydrate fermentation that may induce vascular disturbances have not yet been identified; however, the study reported here describes some compounds measureable in plasma that may be capable of such effects.

Several putative trigger factors for laminitis are currently the subject of study. Endotoxin has been measured in the plasma of horses fed a carbohydrate overload,[17] and endotoxin may reduce digital blood flow when infused IV for short periods.[18] However, endotoxin itself does not induce laminitis, and its role as a direct trigger factor is now questioned.[19] Exotoxins such as thermolysin from *Streptococcus bovis* have also been proposed as trigger factors by virtue of their ability to activate matrix metalloproteinase enzymes in lamellar explant preparations in vitro,[20] although they have not yet been detected in peripheral plasma, and the precise mechanism by which these enzymes in the lamellae become selectively activated in naturally occurring laminitis is not known.

Amine compounds in the equine cecum are largely attributable to production by bacteria. Bacterial decarboxylase enzymes convert the corresponding amino acid into the amine.[21] Many of the amines that are described in the cecum of ponies have been found in fermented foods and beverages.[22,23] This metabolic process leads to the production of carbon dioxide as well as the basic amine compounds; thus, it is believed to be part of the means by which bacteria modulate intracellular pH in response to acid conditions.[7] Some of these compounds have been assayed in the rumen and large intestine of other species,[6,24] and their concentrations can increase in response to carbohydrate fermentation.[12]

The amines described in the study reported here may mimic many effects of endogenous amines, such as the catecholamines (epinephrine, norepinephrine, and dopamine) and 5-HT, if allowed access to the systemic circulation. Therefore, they may stimulate α-adrenoreceptors and 5-HT receptors to cause vasoconstriction, displace endogenous amines from intracellular stores, and impede clearance of amines from the plasma, which causes vascular disturbances. In humans, aromatic amines, such as tyramine and phenylethylamine, have been implicated as pressor agents in the pathogenesis of migraines following ingestion of foods such as cheese or wine.[25,26] Tryptamine, tyramine, and phenylethylamine can cause vasoconstriction of isolated equine digital arteries and veins in vitro as well as displacing 5-HT from platelets and inhibiting 5-HT reuptake.[27,11]

Until now, it has not been clear whether amine compounds are absorbed into the systemic circulation from the cecum, nor the extent to which amines would be cleared from plasma through uptake into platelets and endothelial cells or through oxidative deamination by monoamine oxidase. However, it has been speculated that amines may play a role in diseases such as laminitis.[28] The kinetics of uptake and metabolism processes in equine tissues suggest a high capacity but low affinity for clearance of amines[14,o]; therefore, it was not surprising to detect low concentrations of tyramine, tryptamine, phenylethylamine, and isoamylamine in the range of 10pM to 100nM. Other amine compounds may also be detectable in equine plasma, but for practical reasons, only these 4 amines were measured because of the fact they are of particular relevance as a result of their potencies for causing digital vasoconstriction.[11] Furthermore, the production of phenylethylamine and isoamylamine increases when inulin or starch is added to equine cecal contents in vitro.[12]

In the study reported here, it was clearly documented that plasma concentrations of tryptamine, phenylethylamine, and isoamylamine were significantly higher during spring, compared with concentrations during winter. In both the clinically normal group of ponies and those with a predisposition to acute laminitis, plasma concentrations of phenylethylamine increased approximately 2-fold, isoamylamine increased 1.5- to 2-fold, and tryptamine concentrations increased nearly 100-fold to approximately 6 nmol/L. In contrast, plasma 5-HT concentrations did not change significantly throughout the year; as an endogenous mediator, it is clear that concentrations of 5-HT are more tightly controlled, and plasma concentrations of the cecum-derived amines measured in this study were not sufficient to cause substantial displacement of 5-HT from platelets or to interfere with its clearance from plasma.

The cause of the higher circulating concentrations of tryptamine, phenylethylamine, and isoamylamine in the spring versus the winter remains to be determined. It is possible that increased production of these amines in the cecum or enhanced absorption of the amines from the gastrointestinal tract explains these observations. Alternatively, reduced clearance of the amines from the plasma may also explain the higher concentrations found in the spring.

In another study[29] in which our laboratory group examined cecal contents from horses fed various diets, we documented higher cecal concentrations of phenylethylamine and isoamylamine when horses were fed spring grass, compared with those fed winter hay. This finding supports the suggestion that the higher plasma concentrations of phenylethylamine and isoamylamine found in the study reported here possibly resulted from increased production of cecal amines related to increased amounts of fermentable carbohydrate and proteins found in lush spring or summer grass.[30] The fact that the addition of fructans to an in vitro model of cecal fermentation results in increased production of phenylethylamine and isoamylamine also supports this suggestion.[12] Fructans, the storage carbohydrates of grasses that are not digested in the equine small intestine, are found in increased amounts in the spring and summer months,[31] coinciding with the main risk periods for laminitis in pastured horses in the United Kingdom.[32] Another factor that may be a limiting step in the production of individual amines is the amino acid composition of the forage. In particular, tryptophan concentrations may be increased during periods of grass growth.[33]

Plasma concentrations of phenylethylamine, tyramine, and isoamylamine in winter and spring and tryptamine in winter that were measured in this study were less than the threshold at which individual amines cause vasoconstriction of digital blood vessels in vitro.[11] However, there is evidence that the acidic conditions that accompany carbohydrate overload (attributable to production of lactic acid) cause ultrastructural damage to the mucosa of the cecum[34] and may increase the permeability of the mucosa to polar and large molecular weight substances.[35] Therefore, damage to the cecal mucosa may result in much higher plasma concentrations of amines and other toxins. However, the effect of fructans or carbohydrate overload on plasma amine concentrations in vivo remains to be determined.

The concentration of tryptamine found in plasma samples (6 nmol/L) obtained in the spring was close to the threshold concentration required to cause vasoconstriction of isolated digital blood vessels (7 and 29 nmol/L for veins and arteries, respectively).[11] Analysis of these data would suggest that small increases in absorption of tryptamine from the gastrointestinal tract would lead to substantial digital vasoconstriction, particularly on the venous side of the circulation. Caution should always be exercised when extrapolating between in vitro and in vivo data, however, because factors such as plasma protein binding and accessibility of receptors to circulating factors may confound such extrapolations. Nevertheless, infusion of tryptamine at a rate of 1.6 μg/kg/min during a 30-minute period causes significant decreases in digital perfusion, documenting that low doses of tryptamine delivered from the circulation will affect digital vascular tone.[p]

We did not detect significant differences between the cecum-derived amine concentrations measured in plasma obtained from clinically normal ponies, compared with concentrations in those predisposed to laminitis, nor were there differences in the plasma concentration of the endogenous amine mediator 5-HT. However, none of the ponies selected for use in the study had signs of acute laminitis at the time of sample collection. The ponies classified as predisposed to laminitis had a history of repeated bouts of acute laminitis during the past 3 years. It would have been interesting to obtain plasma samples from ponies during the prodromal stages of a naturally occurring bout of laminitis to determine plasma amine concentrations at that time. Because of the steps required in sample handling to ensure accurate results and the difficulties of identifying ponies in the prodromal stages of laminitis, it was not possible to collect samples from these ponies when they were in the developmental stages of the disease. Such a study would be problematic to con-

duct in ponies with naturally occurring laminitis, because it would be extremely difficult to time collection of the samples to coincide with disturbances in digital blood flow.[36]

The clinically normal ponies and the ponies predisposed to laminitis were maintained under the same management conditions and on similar pasture types on 2 nearby premises as part of a herd of approximately 1,200 animals. Using data from the computerized veterinary records during the previous 3 years, it was established that the peak incidence of laminitis in the herd was during the months of May to July, which correlated well with the hours of sunshine experienced in those months.[36] The times of sample collection for measurement of plasma amines in the study were chosen accordingly to examine the concentrations before and during peak grass growth; local weather data indicated a minimum grass temperature of 6°C and a substantial increase in the number of hours of sunshine during the spring.

Analysis of these data revealed that many amine compounds are found in the cecum of clinically normal ponies. Furthermore, several of these compounds can be detected in plasma, with concentrations increasing significantly during periods of grass growth. Vasoactive properties of some of the amines studied in vitro (tryptamine in particular) suggest that should there be an increase in release of amines from the cecum into the systemic circulation following damage to the mucosa, these compounds would contribute to hemodynamic disturbances observed in ponies and horses with acute laminitis. Additional studies are warranted to investigate the role of these amines in the pathophysiologic mechanisms of laminitis in horses and ponies.

[a]Watman No. 1 filter paper, Sigma-Aldrich Ltd, Gillingham, UK.
[b]Waters SEP-PAK C18 cartridges, 500 mg, Waters Ltd, Watford, UK.
[c]Waters liquid chromatograph model 600E gradient controller and pump, Waters Ltd, Watford, UK.
[d]Symmetry Shield RP_{18} guard column (internal diameter, 20 × 3.9 mm; particle size, 5 μm), Waters Ltd, Watford, UK.
[e]Symmetry Shield RP_{18} column (internal diameter, 150 × 3.9 mm; particle size, 5 μm), Waters Ltd, Watford, UK.
[f]Model 484 UV absorbance detector, Waters Ltd, Watford, UK.
[g]UK Government Meterological Office, Bracknell, Berkshire, UK.
[h]Intro electrochemical detector, Antec Ltd, Amsterdam, The Netherlands.
[i]Bailey SR. *Study of the vascular effects and factors regulating the concentration of 5-hydroxytryptamine in the equine digital circulation*. PhD dissertation, Department of Veterinary Basic Sciences, Royal Veterinary College, University of London, London, UK, 1998.
[j]Model HP1050 liquid chromatograph, Hewlett Packard Ltd, UK.
[k]Micromass Platform LC API mass spectrometer system, Micromass Ltd, Wythenshawe, UK.
[l]Masslynx software, version 3.0 for Windows, Micromass Ltd, Wythenshawe, Manchester, UK.
[m]GraphPad Prism, version 3.00 for Windows, GraphPad Software Inc, San Diego, Calif.
[n]SPSS 9.0 for Windows, SPSS Inc, Chicago, Ill.
[o]Williams RB. *Aspects of amine oxidase activity in some tissues of the horse*. Masters thesis, Department of Pharmacology, University of Cambridge, Cambridge, UK, 1986.
[p]Bailey SR, Marr CM, Menzies-Gow N, et al. The effects of vasoactive amines from the equine hindgut on digital blood flow in the normal horse (abstr). *J Vet Intern Med* 2002;16:335.

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