Detection of maple toxins in mare's milk

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Abstract

Background: Plants from the Sapindaceae family that are consumed by horses (maple) and humans (ackee and litchi) are known to contain the toxins hypoglycin A and methylenecyclopropylglycine which cause seasonally occurring myopathy in horses and entero-encephalopathic sickness in humans. Vertical transmission of these toxins from a mare to her foal has been described once. However the mare's milk was not available for analysis in this case. We investigated mare's milk in a similar case.

Objective: We hypothesized that hypoglycin A and methylenecyclopropylglycine, like other amino acids' are secreted into the milk.

Animals: Mare with atypical myopathy.

Methods: A sample of the mare's milk and 6 commercial horse milk samples were extracted with a methanolic standard solution and analyzed for hypoglycin A, methylenecyclopropylglycine, and metabolites using tandem mass spectrometry after column chromatographic separation.

Results: There were hypoglycin A (0.4 μg/L) and the associated metabolites methylenecyclopropylacetyl glycine and carnitine (18.5 and 24.6 μg/L) plus increased concentrations of several acylcarnitines in the milk. The milk also contained methylenecyclopropylformyl glycine and carnitine (0.8 and 60 μg/L). The latter substances were also detected in 1 of 6 commercial horse milk samples.

Conclusions and Clinical Importance: Transmission of the maple toxins can occur through mare's milk. Vertical transmission of Sapindaceae toxins might also have importance for human medicine, for example, after consumption of ackee or litchi.

KEYWORDS
atypical myopathy, horse milk, hypoglycin A, methylenecyclopropylglycine

1 | INTRODUCTION

There have been numerous cases of atypical myopathy (AM) in adult horses that have been caused by the maple (Acer species) toxins hypoglycin A (HGA) and methylenecyclopropylglycine (MCPG).¹⁻⁴ The intoxication of newborn foals by maple components however, has, to our knowledge, only once been reported.⁵ The foal identified showed typical clinical signs of AM, and the disease caused high activity of creatine kinase (CK) and elevated concentrations of a spectrum of acylcarnitines. Maple poisoning was proven by the detection of methylenecyclopropylacetyl-carnitine. While the biochemical findings were unambiguous, the question remains as to whether the toxins

Abbreviations: AM, atypical myopathy; HGA, hypoglycin A; MCPA, methylenecyclopropylacetate; MCPF, methylenecyclopropyiformate; MCPG, methylenecyclopropylglycine; UPLC-MS/MS, ultrahigh-performance liquid chromatography-tandem mass spectrometry.
and metabolites thereof were transferred to the foal via the placenta or with the dam’s colostrum, or both. Milk of the mare in this case, however, was not available for analysis. The question of vertical transmission of HGA and MCPG and their metabolites is of interest not only for veterinary medicine but also for human medicine. Severe human poisoning is observed especially in children. Maple toxin in mare’s milk is of further interest because it is also consumed by humans.

HGA and MCPG are naturally occurring amino acids. We hypothesized that, after ingestion of HGA and MCPG containing plant material, they are secreted into the milk, like other amino acids. To test this hypothesis, we examined various samples of mares’ milk.

2 | MATERIALS AND METHODS

Milk was collected from a mare (sample A), who with her foal was housed in a pasture near *Acer pseudoplatanus* where several cases of AM had previously been confirmed. With the appearance of depression and weakness in the mare and, more pronounced, in the foal, the animals were seen by a veterinarian on the 8th day after birth. Because of the severity of the symptoms the foal was euthanized.

The urine of the mare as well as of the foal was described as dark brown suggesting myoglobinuria which is a typical sign of AM. An analysis of the mare’s serum by the veterinarian showed highly elevated activities of creatine kinase and lactate dehydrogenase, also typical markers of AM. Beginning with the consultation by the veterinarian the mare was fed oats and hay from a maple-free pasture only. The veterinarian collected a milk sample 10 days postpartum, that is, 2 days after starting the maple-free diet and sent it to our laboratory for diagnostic purposes. The data obtained were used here with owner informed consent.

Samples were also collected from commercially available horse milk (samples B) offered for human dietary purposes. The milk was purchased from 6 suppliers based in different regions of Germany. The commercially packaged milk was deep frozen when sold.

3 | METHODS

The methods followed here were originally described for investigations into serum and urine. For analysis of the milk, 25 µL samples were extracted with 300 µL of methanolic standard solution. The extract was centrifuged for 10 minutes at RCF 17000. Of the clear supernatant 200 µL were transferred to a microtiter plate and dried at 65°C under a gentle stream of nitrogen. The residue was treated with 50 µL 3 N butanolic HCl for 15 minutes at 65°C and dried again at 65°C. The dried material was resolved in 200 µL methanol/water (80 : 20 vol/vol). This solution was further diluted 1 : 5 with water. Ninety microliters were transferred to a 384 microtiter plate, centrifuged at RCF 17000. The solutions were analyzed using ultrahigh-performance chromatography-tandem mass spectrometry (UPLC-MS/MS). The column was an ACQUITY UPLC BEH C18 (1.7 µm, 2.1 × 50 mm, Waters, Eschborn, Germany) operated at 40°C for gradient chromatography (gradient A: water plus 0.1% formic acid and 0.01% trifluoro acetic acid, gradient B: acetonitrile plus the same additives, starting with 80% A, reaching 1% A after 10 minutes). The analysis was done on a Xevo UPLC-MS/MS system (Waters).

The analyzed transitions [m/z] were 198.1 > 73.9 for the butyl ester of HGA and 191.0 > 89.0 for butylated d3 leucine used as internal standard. Further transitions [m/z] were 184.0 > 110.7 for the butyl ester of MCPG and 187.0 > 113.7 for butylated [13C215N]-MCPG standard, for butylated MCPF-glycine: 212 > 80.93, for butylatedd13C214N MCPF-glycine standard: 215.07 > 80.93, butylated MCPF carnitine: 298.15 > 84.98, butylated MCPA-glycine: 226 > 73.95, butylated 13C214N MCPA-glycine standard: 229.1 > 75.92, and butylated MCPA-carnitine: 312.2 > 84.98. As no specific standards were available for MCPA and MCPF carnitines we used d3 octanoylcarnitine and d3 butyrylcarnitine respectively as reference material. The butyl esters were detected in ESI positive mode by multiple reaction monitoring (MRM). A ratio was calculated from the signals that were obtained for the substances to be quantified and the corresponding internal standards. With this method, C4 to C6 and C16 acylcarnitines were also quantitatively determined using corresponding labeled internal standards.

4 | RESULTS

A clear chromatographic separation of the sought-after substances and the relevant internal standards, without any overlapping with other compounds, was obtained for the respective mass traces.

Sample A had 0.4 µg/L (3 nmol/L) HGA. Furthermore, the concentrations of the associated metabolites methylenecyclopropylacetyl glycine and carnitine were 18.5 and 24.6 µg/L (82 and 79 nmol/L), respectively. The related toxin MCPG was not detected, but the associated metabolites methylenecyclopropylformyl glycine and carnitine were present at concentrations of 0.8 and 60 µg/L (4 and 201 nmol/L), respectively. In addition, in the milk of this mare we found a strong increase in the concentrations of C4 to C6 and C16 acylcarnitines. The levels exceeded those of the negative milk samples by the following factors: Isobutyrylcarnitine 32, butyrylcarnitine 10, isovalerylcarnitine 130, hexanoylcarnitine 34, palmitoylcarnitine 10.

Samples B: One of the 6 commercial varieties was found to have 2.4 µg/L (17 nmol/L) HGA and 1.3 µg/L (10 nmol/L) MCPG. Glicine derivatives were not detected but the carnitine conjugates of methylenecyclopropyl acetic and formic acids were 0.4 and 2.7 µg/L (1.3 and 9 nmol/L), respectively. The other 5 commercial samples showed no traces of the toxins or their derivatives. Concentrations of acylcarnitines were not elevated.

5 | DISCUSSION

This report demonstrates that HGA, MCPG, and their characteristic metabolites are secreted into mares’ milk. The detection of HGA and
With the detection of these toxins in mare's milk, questions will arise as to whether they can also be found in the milk of other mammals. HGA and MCPP are, as mentioned, also components of the fruits ackee and litchi, which are enjoyed by humans, and are known to have previously caused serious poisonings, especially in children.\textsuperscript{6,12–14} Whether vertical transfer of Sapindaceae toxins could play a role in pregnant women or lactating mothers therefore is an interesting question that requires further investigation.

ACKNOWLEDGMENT
No funding was received for this study.

CONFLICT OF INTEREST DECLARATION
Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

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How to cite this article: Sander J, Terhardt M, Janzen N. Detection of maple toxins in mare’s milk. J Vet Intern Med. 2021;35:606–609. https://doi.org/10.1111/jvim.16004