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# 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 *Sapindacea* 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, methylenecyclopropylformate; 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.[5] 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.[6] 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.[7,8] 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 butylated13C214N 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. Glycine 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

toxin metabolites in milk proves the ingestion of maple toxins by the mare.

The concentrations detected in the milk were low compared to the known toxin content of the maple seeds or seedlings. It should be stressed that in our case, the milk was taken 2 days after the last possibility to ingest maple components, and the purchased samples were mixtures of milk from several mares. This means that in the latter, the toxins were probably diluted accordingly. We therefore speculate that the colostrum ingested by the foal described earlier[5] might have contained much higher concentrations of toxins.

Unfortunately only 1 milk sample was taken from the sick mare and the time of milk collection was very late. One would have wished for several samples collected over a longer period of time. For the detection of the maple toxins themselves, the late collection is certainly a disadvantage, because they are quickly metabolized. On the other hand, it is possible to derive metabolic information from the sample taken late. While MCPG was no longer found in the milk and HGA only in a very low concentration, the associated metabolites were still present in considerable quantities. Also impressive are the very high levels of acylcarnitines, which, 2 days after the last ingestion of the toxins, prove the persistence of specific metabolic inhibitions despite clinical recovery of the mare.

Our results provide a new perspective to the previous report[5] . The authors had emphasized that the newborn foal developed symptoms within hours after parturition, that is, during a period in which the prenatal and predominantly glycolytic metabolism is converted to oxidative energy production accompanied by increased postpartum energy requirements bringing into effect functional defects in enzymes involved in energy metabolism. The foal had been fed milk from its dam before the onset of symptoms and continued to be fed while its physical state was deteriorating. Considering our results, it is conceivable that toxins contained in the milk of the mare, contributed to the disease of the newborn. We believe that the possibility of vertical transmission of maple toxins should be considered when pregnant or lactating mares are grazing in pastures where maple seed or seedlings are present.

Mare's milk is traditionally used for human nutrition in various Asian nations. Its consumption is now increasingly popular in western industrialized countries as well.[9,10]

Considering the fact that horse milk is often advertized as a product that is beneficial to human health, it is important to exclude any risks that might be caused by toxic components contained therein. The analysis of further samples of horse milk could help to evaluate whether contamination of horse milk with maple toxins is of relevance to the health of consumers. In this context one should be aware that HGA and MCPG interfere with essential metabolic pathways. Individuals with congenital functional disorders of the involved enzymes could be at a particular risk. The toxins inhibit several enzymes involved in the β-oxidation of fatty acids and disrupt the metabolic use of branched amino acids. Various congenital genetic defects that affect these metabolic pathways are known.[11] They can be clinically symptomatic or asymptomatic. In some cases, even the addition of minor functional impairments, caused by the ingestion of maple toxins, might lead to a breakdown of an organism's energy supply.

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 MCPG 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.[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.

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## 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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