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Hypoglycin A Concentrations in Seeds of *Acer Pseudoplatanus* Trees Growing on Atypical Myopathy-Affected and Control Pastures

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Background: Hypoglycin A, found in seeds of *Acer negundo*, appears to cause seasonal pasture myopathy (SPM) in North America and is implicated in atypical myopathy (AM) in Europe. *Acer negundo* is uncommon in Europe. Thus, the potential source of hypoglycin A in Europe is unknown.

Hypothesis and Objectives: We hypothesized that seeds of *Acer pseudoplatanus* were the source of hypoglycin A in Europe. Our objective was to determine the concentration of hypoglycin A in seeds of *A. pseudoplatanus* trees located in pastures where previous cases of AM had occurred.

Animals: None.

Methods: University of Berne records were searched to retrospectively identify 6 farms with 10 AM cases and 11 suspected AM deaths between 2007 and 2011. During October 2012, *A. pseudoplatanus* seeds were collected from 2 to 6 trees per pasture on 6 AM farms (7 pastures) from trees in or close to 2 pastures on 2 control farms where AM had not been previously reported. Hypoglycin A in seeds was analyzed by GC–MS.

Results: *Acer pseudoplatanus* trees were identified on all AM pastures. Hypoglycin A was detected in all *A. pseudoplatanus* seeds in highly variable concentrations ranging from 0.04 to 2.81 μg/mg (mean 0.69) on AM farms and 0.10 to 9.12 μg/mg (mean 1.59) on control farms.

Conclusion and Clinical Importance: Preventing horses from grazing pastures containing *A. pseudoplatanus* seeds during late fall and early spring might be the best means to prevent AM.

Key words: Lipid; Multiple acyl-CoA dehydrogenase deficiency; Rhabdomyolysis.

Two highly fatal pasture myopathies have been reported in North America and Europe termed seasonal pasture myopathy (SPM) and atypical myopathy (AM), respectively.[1,2] The 1st cases of AM date back to 1939 where several outbreaks were reported in the north of Wales in the United Kingdom.[3] Since the 1980s, several outbreaks have occurred at irregular intervals in Northern and Western Europe.[4,5] A similar disease, seasonal pasture myopathy (SPM), exists in North America.[6] Both AM and SPM affect horses grazing fall and occasionally spring pastures for more than 12 hours a day. Leaves and dead wood are often present on affected pastures and inclement weather or high winds have been reported to precede clinical signs of AM.[1,2,6] Cases of SPM and AM cease in December/January after several days of heavy frost. In North America, small numbers of horses are affected on a given pasture, whereas in Europe many horses on the same farm are frequently affected.[2,4] The annual incidence of SPM and AM varies widely from no cases up to hundreds of cases reported in certain years.[4,5] The incidence appears to be increasing in Europe over the last few decades.[7] These facts suggest that SPM and AM are caused by a toxin whose abundance is highest in the fall, varies from year to year, and reaches lethal levels with prolonged exposure to certain pastures.

Abbreviations:

AM	atypical myopathy
CK	serum creatine kinase
GC–MS	gas chromatography–mass spectrometry
MADD	multiple acyl-CoA dehydrogenases
SPM	seasonal pasture myopathy

Both SPM and AM are because of an acquired enzymatic deficiency of multiple acyl-CoA dehydrogenases (MADD), which disrupts fatty acid ß-oxidation and amino acid metabolism, leading to excessive myofiber lipid storage and elevations in serum acylcarnitine concentrations.[1,8] For decades, the cause of AM remained unclear, although ingestion of toxins from *Clostridium* species in the soil or tar spot fungus on *Acer* species trees were proposed etiologies.[9,10] Recently, ingestion of the nonproteogenic amino acid hypoglycin A contained in the seeds of *Acer negundo* (box elder) was found to cause SPM in North America.[7] *A. negundo* is also present in Europe although it is not a native species and less abundant than other *Acer* species trees.[11] One study from 1973 suggests that hypoglycin A is also present in seeds from certain other *Acer* species present in Europe based on semiquantitative methods.[12] A previous study of AM

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identified *Acer pseudoplatanus* (sycamore maple) on all pastures (n = 14) on which horses affected with AM had been housed; however, analysis of hypoglycin A has not been performed on the seeds of sycamore trees.[10]

The purpose of this study was to determine the concentration of hypoglycin A in the seeds of *A. pseudoplatanus* trees obtained in the fall from trees in pastures where AM had occurred previously.

Materials and Methods

AM Farms

Six AM farms (farms 1–6) with 7 AM pastures in which previous cases of AM occurred (AM pastures) were retrospectively identified from records of the Equine Clinic of the Swiss Institute for Equine Medicine at the University of Berne (2007–2011). Inclusion criteria for confirmed cases were history of rhabdomyolysis in unexercised horses on pasture in the fall, serum creatine kinase (CK) activity ≥10,000 U/L, or post mortem examination confirming severe myofiber necrosis including involvement of postural, respiratory, and cardiac musculature (Table 1). Serum samples were available from 1 fatal AM case on farm 3 and from a surviving AM case from farm 5 for analysis of acylcarnitines and carnitine conjugates of the toxic metabolite of hypoglycin A, methylenecyclopropyl acetic acid (MCPA-carnitine). Deaths suspected to be because of AM based on history and their contemporaneous occurrences on the same pastures with other AM cases were recorded. A diagnostic workup was not possible because of either rapid death or financial constraints (Table 1).

Farms were located from 800 to 1,200 m above sea level (mean 1025 ± 163 m) either in the Swiss Jura or the foothills of the Swiss Freiburg Alps. Site visits of farms where cases of AM occurred were performed by 2 investigators (LU, SV) during the last week of October 2012. Two farms near the University of Berne (farm 7, 8) well known to one of the authors were selected as unaffected controls because they had no previously reported cases of AM. One pasture (Farm 7) contained 1 *A. pseudoplatanus* tree and 1 pasture had 1 tree within about 10 m of the pasture. The 2nd control farm (Farm 8) had 2–6 *A. pseudoplatanus* trees within about 5 m of 2 equine pastures.

Acer pseudoplatanus

Each pasture was inspected for the presence of *Acer* species trees containing samaras (the outer husk containing a seed). The goal was to obtain at least 10 samaras each from branches of up to 6 *A. pseudoplatanus* trees per pature if present. Photographs taken of each *Acer* tree sampled were reviewed by a botanist to confirm the species as *A. pseudoplatanus*. The abundance of samaras per tree was subjectively scored from 0 (none) to 3 (abundant) and presence of European tar spot on leaves noted. *Acer pseudoplatanus* leaves with European tar spot fungus were picked from at least 1 tree per pasture. Samaras and leaves were placed in labeled paper bags, allowed to dry, and shipped in sealed plastic bags at room temperature to the laboratory in Minnesota (according to USDA permits). Upon arrival in the laboratory, samples were freeze-dried and stored at room temperature until seeds were dissected from the samaras and analyzed for hypoglycin A in the Plant Metabolomics Laboratory at the University of Minnesota.

Hypoglycin A Analysis

Five well-preserved freeze-dried seeds per tree were dissected from the samara husk, pooled, pulverized with a mortar and pestle, and divided into triplicate samples which were weighed. Amino acid analysis was performed on (1) pooled seeds, (2) pulverized samara husks without seeds from farm 3, and (3) two 1-cm square portions of pulverized leaves from farm 6 with and without tar spot. Amino acids were purified and derivatized with methyl chloroformate as previously described.[13] An annotated GC–MS fragmentation spectrum was determined for methyl chloroformate derivatized hypoglycin as well as an annotated GC–MS chromatogram showing the hypoglycin peak and other amino acids. The mass spectral fragmentation pattern for derivatized hypoglycin A was identical to that which was observed from hypoglycin A extracted from *A. negundo* seed tissue.[1] After our previous publication, we purified several hundred milligrams of hypoglycin A from *A. negundo* samaras by using the protocol

Table 1. The location and characteristics of atypical myopathy (AM) and control farms, abundance of *Acer pseudoplatanus* samaras (1 = mild, 3 = marked), number of AM cases confirmed at postmortem and maximum serum creatine kinase (CK) activity measured per farm. nd, not determined.

Farm	Location	Samara Abundance Score (1–3)	AM Pastures	Horses per Pasture	AM; Suspected Cases/Year	No. of Histologically Confirmed AM	Maximum Serum CK (U/L)
Affected farms							
1	Foothills Freiburg Alps	2	1	15	3; 2/2007, 1; 2/2011	4	918,700
2	Foothills Freiburg Alps	2	1	4	1; 1/2009, 0; 1/2011	1	nd
3	Swiss Jura	2	1	20	1; 1/2009	1	498,300
4	Swiss Jura	2	1	10	1; 1/2009	1	10,000
5	Swiss Jura	3	1	40	1; 1/2009	1	372,100
6	Swiss Jura	2	2	9	1; 1/2009, 0; 1/2010	0	326,500
Control farms							
7	5 km from Berne	2	0	20	0	nd	nd
8	5 km from Berne	2	0	3	0	nd	nd

published for purification from ackee (*Blighia sapida*) by Kean.[14] The structure and stereochemistry (diastereomeric mixture of 2S, 4R and 2S, 4S forms) were confirmed by ^{1}H-NMR spectroscopy by comparison with the published spectral data to be identical to those observed for hypoglycin A from ackee fruit. The mean concentration of hypoglycin A per tree was determined by averaging the triplicate concentrations. To estimate the amount of hypoglycin A per seed, a representative seed weight was obtained by averaging the weights of 4 randomly selected freeze-dried seeds. The estimated amount of hypoglycin A per seed was calculated as the measured concentration in pulverized seeds multiplied by the average seed weight.

Results

AM and Control Farms

Ten AM affected horses and 10 contemporaneous deaths suspected to be because of AM were identified among horses grazing 7 pastures on 6 farms (Table 1). Up to 5 AM-afflicted horses and 3 contemporaneous deaths from suspected AM were reported per farm among 4–40 horses on the pastures. Three AM outbreaks had occurred (outbreak 1: October 25–26/2007, outbreak 2: October 03–26, 2009 and April 30, 2010, outbreak 3: October 13–November 07, 2011). All AM horses spent 24 hours/d on overgrazed pastures and were not fed supplemental hay when AM occurred except on 1 AM farm (farm 3) where all horses were fed hay overnight in a stable. Horses were fed supplemental hay on both control farms.

Horses

All AM horses had a history of rhabdomyolysis on pasture in the fall, and serum creatine kinase (CK) activity ≥10,000 U/L was identified in 9 horses and was above the limit of detection in 1 horse but no dilution of serum was performed. A muscle biopsy was evaluated in 1 surviving horse and postmortem examination was performed in 8 horses confirming severe myofiber necrosis including involvement of postural, respiratory, and cardiac musculature (Table 1). Electron microscopy was performed on 3 of the included cases and in these 3 cases, excessive myofiber lipid was identified.[9] In 1 fatal AM case where serum was available, short-, medium-, and long-chain acylcarnitines were well above the reference range; C4 8.49 reference <1.06 μmol/L; C5 10.23 reference <0.46 μmol/L; C6 1.86 reference <0.12 μmol/L; C18:1 0.46 reference <0.02 μmol/L. Serum available from 1 surviving AM case had slight elevations of short-chain acylcarnitines (C4 1.22 μmol/L, C5 0.68 μmol/L) were evident. Carnitine conjugates of the toxic metabolite of hypoglycin A, methylenecyclopropyl acetic acid (MCPA-carnitine) were detected in serum in both fatal and surviving samples at 16.3 and 0.2 nmol/L, respectively (reference <0.01 nmol/L).

Acer pseudoplatanus in Pastures

Pastures where previous AM cases had occurred contained at least 2 *A. pseudoplatanus* trees (Fig 1A, Table 1). Most trees had an abundance of samaras (Fig 2A and B, Table 1). One *A. pseudoplatanus* tree was located within a control farm pasture and several outside the pasture on the 2nd control farm (farm 8) (Fig 1B). There was a moderate to marked abundance of tar spot fungus on the leaves of all trees on both AM and control farms.

Hypoglycin A Concentrations

Hypoglycin A concentrations in seeds were highly variable both among trees on the same farm and among farms (Fig 3). Hypoglycin A concentrations in pulverized seeds from AM pastures ranged from 0.04 to 2.81 μg/mg (mean 0.69 μg/mg) (Fig 3). Pulverized seeds from the control farms also showed extreme variation in hypoglycin A concentrations among trees (Fig 1). In fact, the highest recorded concentration of 9.12 μg/mg was measured in pulverized seeds from a tree from a control farm with a mean for control farms of 1.59 μg/mg farm. Samara husks (undetectable to 0.01 μg/mg) and leaves with (undetectable to

Fig 1. Distribution of *Acer pseudoplatanus* trees on an affected (**A**) and near a control pasture (**B**). Arrows indicate the presence of numerous *A. psuedoplatanus* trees on the affected pasture that were only present along the perimeter of the control pasture.

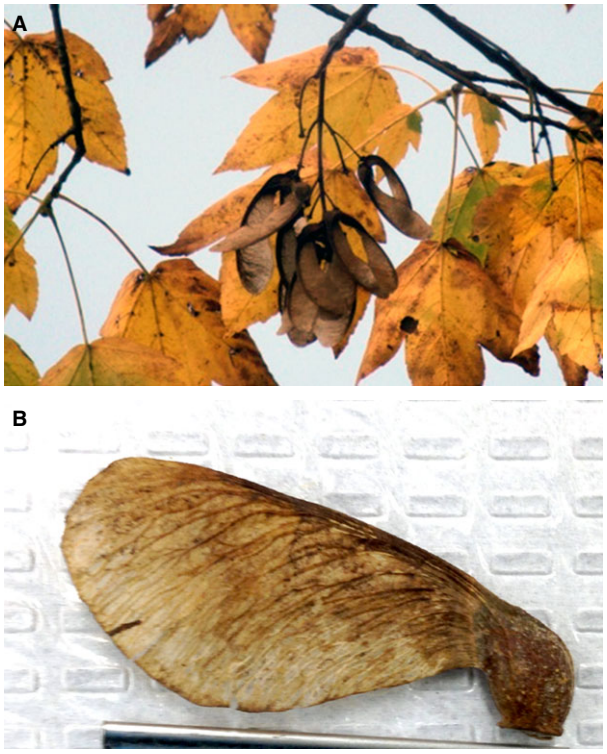

Fig 2. (**A**) *Acer pseudoplatanus* leaves and seeds on a tree in an atypical myopathy-affected pasture. (**B**) Samara with the seed contained in the globular end and size indicated relative to a scale bar of 1 cm.

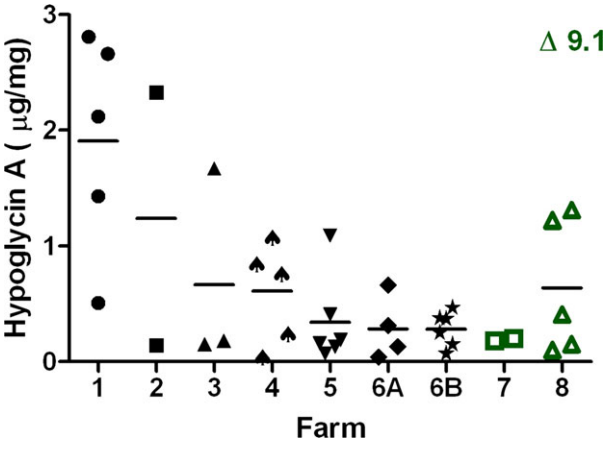

Fig 3. Average concentration of hypoglycin A in 5 pooled seeds run in triplicate from individual trees growing in atypical myopathy (black solid symbols), or near unaffected (open squares, triangles) farm pastures. Numbers on *X* axis indicate individual farms, letters indicate different pastures on the same farm.

0.01 μg/mg) or without tar spot fungus (undetectable) contained very little hypoglycin A. The average weight of *A. pseudoplatanus* seeds was 90 mg. The estimated mean content of hypoglycin A per individual seed ranged from 3.6 to 252.9 μg for seeds from the AM pastures and 9.0 to 820.8 μg for the control farms.

Discussion

Acer pseudoplatanus is the 3rd most common broadleaf tree in Switzerland after ash and beech trees. Its predominant geographic location in the Swiss Jura and the foothills of the Alps perfectly correlates with the regions of occurrence of AM in Switzerland.[15] Results of this study confirm that hypoglycin A is present in large amounts in seeds of *A. pseudoplatanus* or "sycamore maple." Hypoglycin A has also been found in the seeds of other *Acer* species trees such as the Japanese maple (*Acer palmatum*), silver maple (*Acer saccharinum*), mountain maple (*Acer spicatum*), and sugar maple (*Acer sacharum*), but was reported in lesser amounts than in *A. pseudoplatanus* or *A. negundo*.[12] It is of note, however, that the methodology used in the study of other *Acer* species was semiquantitative and amounts are only reported as weak, moderate, and strong. As the toxic metabolite of hypoglycin A, MCPA, has previously been found in conjugated form in the bloodstream of horses with SPM and AM, and *A. negundo* or *A. pseudoplatanus* are present on all AM pastures reported to date, it seems highly likely that hypoglycin A is the cause of both pasture-associated myopathies.[1,7] Although the tar spot fungus was previously suggested to cause AM,[10] the concentration of hypoglycin A was negligible both in regions of leaves that contained the fungus as well as in regions that did not contain tar spot.

Seed samples were obtained at the same time of year as previous AM outbreaks; however, they were not obtained at the same time as a concurrent outbreak. As such it is not known whether hypoglycin A concentrations during an outbreak would be higher than those in this study during an AM outbreak. AM outbreaks vary from year to year which could reflect variations in hypoglycin A concentrations or variation in the numbers of seeds available to the horse for ingestion. The interval between large seed crops (maple mast years) is usually 1–3 years and mast years appear to be increasing possibly because of forest dieback and climate warming.[16] Interestingly forest dieback was first reported in Europe in the mid-1970s and an increasing incidence of AM reported since the early 1980s.[16] A possible link between mast years and outbreaks of AM or hypoglycin A concentrations in seeds and pasture myopathies requires further research.

It is of note that the highest hypoglycin A concentration was found in seeds from a tree outside of an unaffected pasture. This would suggest that not only is the concentration of toxin per seed important, but the number of seeds available for ingestion is also of importance. Seeds of *A. pseudoplatanus* fall to the ground in autumn and usually germinate the following spring. This time frame makes them readily accessible to horses before snow cover in the fall and before germination in the spring, which corresponds to the timing of AM outbreaks. The authors noted that horses congregated under the trees not only for shade and a windbreak, but also because the large fallen sycamore maple leaves appeared to insulate the ground under

the trees from October frost and allowed the grass to continue growing more than in areas away from the trees. We speculate that horses may selectively graze under the *A. pseudoplatanus* trees, resulting in a higher risk of inadvertent ingestion of fallen samaras or seeds. For trees outside pastures, strong winds would be necessary to distribute large numbers of heavy sycamore maple seeds into pastures. It is of note, therefore, that heavy winds often precede outbreaks of AM.[1,2]

In conclusion, seeds of the European sycamore maple tree contain hypoglycin A, an acquired cause of MADD and the likely cause of AM based on the consistent presence of sycamore trees on AM pastures,[1] the presence of hypoglycin A in sycamore seeds, and the presence of its toxic metabolite MCPA-carnitine in serum of AM affected horses.

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Conflict of Interest: Authors disclose no conflict of interest.

References

1. Valberg SJ, Sponseller BT, Hegeman AD, et al. Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree. Equine Vet J 2013;45:419–426.

2. Votion DM, Linden A, Saegerman C, et al. History and clinical features of atypical myopathy in horses in Belgium (2000-2005). J Vet Intern Med 2007;21:1380–1391.

3. Bowen JN, Craig JF. Myoglobinuria in horses. Vet Rec 1942;35:354.

4. van Galen G, Marcillaud Pitel C, Saegerman C, et al. European outbreaks of atypical myopathy in grazing equids (2006-2009): Spatiotemporal distribution, history and clinical features. Equine Vet J 2012;44:614–620.

5. van Galen G, Saegerman C, Marcillaud Pitel C, et al. European outbreaks of atypical myopathy in grazing horses (2006-2009): Determination of indicators for risk and prognostic factors. Equine Vet J 2012;44:621–625.

6. Finno CJ, Valberg SJ, Wunschmann A, et al. Seasonal pasture myopathy in horses in the midwestern United States: 14 cases (1998-2005). J Am Vet Med Assoc 2006;229:1134–1141.

7. Votion DM, van Galen G, Sweetman L, et al. Identification of methylenecyclopropyl acetic acid in serum of European horses with atypical myopathy. Equine Vet J 2014;2:146–149.

8. Westermann CM, Dorland L, Votion DM, et al. Acquired multiple Acyl-CoA dehydrogenase deficiency in 10 horses with atypical myopathy. Neuromuscul Disord 2008;18:355–364.

9. Unger-Torroledo L, Straub R, Lehmann AD, et al. Lethal toxin of *Clostridium sordellii* is associated with fatal equine atypical myopathy. Vet Microbiol 2010;144:487–492.

10. van der Kolk JH, Wijnberg ID, Westermann CM, et al. Equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in 14 horses associated with ingestion of Maple leaves (*Acer pseudoplatanus*) covered with European tar spot (*Rhytisma acerinum*). Mol Genet Metab 2010;101:289–291.

11. Porte AJ, Lamarque LJ, Lortie CJ, et al. Invasive *Acer negundo* outperforms native species in non-limiting resource environments due to its higher phenotypic plasticity. BMC Ecol 2011;11:28.

12. Fowden L, Pratt HM. Cyclopropylamino acids of genus acer – Distribution and biosynthesis. Phytochem 1973;12:1677–1681.

13. Chen WP, Yang XY, Hegeman AD, et al. Microscale analysis of amino acids using gas chromatography-mass spectrometry after methyl chloroformate derivatization. J Chromatogr B 2010;878:2199–2208.

14. Kean EA. Improved method for isolation of hypoglycins A and B from fruit of *Blighia sapida*. J Pharm Pharmacol 1974;26:639–640.

15. Egli BWB. Ergebnisse einer Studentenübung "Portraits einheimischer Waldgehölzarten". Available at: http://www.gehoelze.ch/Bergahorn.pdf. Accessed November 15, 2012.

16. Pitelka LF, Raynal DJ. Forest decline and acidic deposition. Ecology 1989;70:2–10.