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Neurodegeneration in equine grass sickness is not attributable to niacin deficiency

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Keywords: horse; grass sickness; dysautonomia; B vitamins; niacin; nicotinic acid

Summary

Background: The aetiology of equine grass sickness (EGS) is currently unknown. We hypothesised that acute deficiency of niacin (vitamin B3), which has a key role in neural homeostasis, may contribute to neurodegeneration in EGS. Niacin deficiency could potentially result from ingestion of niacin antagonists produced by pasture mycotoxigenic fungi.

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Objectives: To compare the niacin status of EGS and control grazing horses. A secondary objective was to compare blood concentrations of vitamins B1, B2 and B6 in EGS and control grazing horses to determine if the status of these vitamins was altered in EGS.

Study design: Case-control study.

Methods: Indices of niacin status, namely the erythrocyte nicotinamide adenine dinucleotide:nicotinamide adenine dinucleotide phosphate ratio (NAD:NADP ratio) and erythrocyte concentrations of NAD and NADP, were compared in blood collected from EGS and healthy control grazing horses. Blood concentrations of vitamins B1, B2 and B6 were also compared.

Results: There was no significant inter-group difference in the NAD:NADP ratio, the main index of functional niacin status (control median 2.1, interquartile range 1.8-2.6; EGS 2.1, 1.9-2.6). EGS horses had significantly higher (median value increased by 25%) concentrations of NADP. There were no inter-group differences in blood concentrations of vitamins B1, B2 and B6.

Main limitations: Interpretation of data was limited by lack of previously defined equine reference ranges for many of the analytes. Sample size was low.

Conclusions: Niacin deficiency does not contribute to EGS neurodegeneration.

Introduction

The aetiology of equine grass sickness (EGS), a multi-system neuropathy typically affecting grazing horses [1], is unknown. We hypothesised that EGS represents an *acute* deficiency of niacin (also termed vitamin B3 and nicotinic acid) and its derivatives, the coenzymes NAD and NADP. Niacin deficiency, termed pellagra, causes neurodegeneration because NAD and

NADP have essential roles in redox reactions and as cofactors for many enzymes key to neural homeostasis [2,3]. Consequently, niacin and its cofactors have important roles in treating neurodegeneration [4,5] and could be of potential use for treatment of EGS.

We hypothesised that acute niacin deficiency could be induced by ingestion of niacin antagonists such as 6-aminonicotinamide, 3-acetylpyridine, 3-aminopyridine, indole, quinolines and picolinic acid. Some of these antagonists are produced by *Fusarium* spp. [6] which are present in increased numbers on grasses in EGS fields [7] and which are currently being investigated as a potential cause of EGS (R.S. Pirie, *personal communication*). Consistent with this hypothesis, human acute neurologic pellagra may be caused by ingestion of mycotoxin contaminated maize [8], and administration of niacin antagonists produces acute neurologic disease in experimental animals [9-11]. Establishing a definitive causal link between mycotoxin ingestion and disease can be difficult; consequently, the identification of specific biomarkers of mycotoxin activity was considered a potential indirect means of supporting such a link.

EGS, human acute neurologic pellagra and experimental neurologic pellagra share many similarities, including some common neuro-anatomic targets (including enteric neurons, dorsal root ganglia, brainstem nuclei), nature of neurodegeneration (central chromatolysis), and some key clinical signs (weight loss, anorexia, muscle weakness, ptosis, abdominal discomfort, abdominal distension, constipation, tremors, dysphagia and hypotension) [12-18]. Human pellagra also induces alterations in the serum amino acid profile [19,20] resembling those reported for EGS [21]. It should be noted that this *acute* niacin deficiency hypothesis contrasts with *chronic* niacin deficiency which results from longer term dietary inadequacy and which causes a different range of symptoms.

In this study, indices of niacin status including erythrocyte NAD:NADP ratio and concentrations of NAD and NADP were compared in blood collected from EGS horses and healthy control horses which were grazing fields where EGS had not occurred. In niacin deficiency levels of the niacin coenzymes NAD and NADP are disproportionately maintained, with NAD levels falling while NADP is maintained at a stable level [3]. Consequently a reduced ratio of erythrocyte NAD to NADP suggests niacin deficiency. Blood concentrations of vitamins B1, B2 and B6 were also compared to determine if the status of these vitamins was altered in EGS.

Materials and Methods

Collection of blood

Heparinised venous blood samples were collected from 10 mixed-breed horses with EGS (8 acute and 2 sub-acute cases; median age 7 years, IQR 5-8.3; 4 geldings, 6 females). EGS was categorised as previously described [21] and confirmed by post mortem examination including histopathological examination of autonomic ganglia [22]. Blood samples were also obtained from 12 control mixed-breed clinically healthy horses (median age 12 years, IQR 9-20; 8 geldings, 4 females) from 3 premises (with 8, 3 and one horse on each premise) that were grazing fields where EGS had not occurred. Heparinised whole blood was posted to arrive at the laboratory within 24 h of collection.

Vitamin assays

A method for measuring erythrocyte NAD and NADP levels, based on the enzymatic cycling method of Nisselbaum and Green (1969) [23] was used to assess vitamin B3 status in blood samples. Vitamin B1 (thiamine), vitamin B2 (riboflavin) and vitamin B6 (pyridoxine) were

measured using coenzyme stimulation assays [24]. Results were expressed as the ratio of activated to basal enzyme activity corrected for haemoglobin concentration.

Data analysis

The Mann-Whitney U test was used to compare age and concentrations of analytes in EGS and control groups, assuming significance at $P < 0.05$. Sample size estimates with 80% power and 95% confidence were conducted using effect sizes and pooled standard deviations estimated from the data. All statistical analyses were performed using Minitab®^a.

Results

There was no statistically significant inter-group age difference. There was no significant inter-group difference in the NAD:NADP ratio, the main index of niacin status (Table 1). A sample size estimate indicated that 68,496 horses would be required in each group to identify significant inter-group differences in this index. EGS horses had significantly ($P = 0.02$) higher concentrations of NADP. There was no significant inter-group difference in NAD concentration and in the sum of NAD and NADP concentrations. There were no significant inter-group differences in the blood concentrations of vitamins B1, B2 and B6 (Table 1). Sample size estimates indicated that 35, 81 and 36 horses would be required in each group to identify significant inter-group differences in vitamin B1, B2 and B6 concentrations, respectively.

Discussion

In this study, the main index of functional niacin status, the NAD:NADP ratio, was almost identical in EGS and control horses, indicating that neurodegeneration in EGS is not a consequence of deficiency of niacin and its derivatives NAD and NADP.

There are few published data regarding niacin biology in the horse. Niacin is synthesised in the liver from tryptophan [25] and by intestinal microbes [26,27]. Niacin deficiency has not been reported in the horse, there are no National Research Council recommendations for dietary requirements of niacin in horses [28] and there are no published reference ranges for indices of niacin status in the horse. While Parker *et al.* (1997) [29] reported that horses would be considered niacin deficient based on human reference ranges for NADP:NADP ratio, actual data were not presented. In the current study, the NAD:NADP ratio of control grazing horses (median 2.1, IQR 1.8-2.6) was considerably lower than the mean ratio for humans (3.3) [3,30]. Applying criteria used to interpret human NAD:NADP ratios, many horses in the current study would be considered to have mild (ratio of 2.1–2.5; 3 EGS and 4 control horses) or marked (ratio <2.1; 5 EGS and 4 control horses) niacin deficiency. Similarly, applying criteria used to interpret human blood NAD (reference range 200-300 nmol/mL RBC) and NADP (reference range 60-100 μ mol/L RBC) concentrations, all horses except one EGS horse would be considered to have low concentrations of NAD, and all horses except 5 EGS horses would be considered to have low concentrations of NADP. However, rather than indicating that healthy horses are niacin deficient, it is more likely that these data and those of Parker *et al.* (1997) [29] indicate that the reference ranges for NAD:NADP ratios and for blood concentrations of NAD and NADP for healthy horses are lower than those for humans.

Despite EGS and control horses having almost identical NAD:NADP ratios, EGS horses had significantly higher (median value increased by 25%) concentrations of NADP. This may reflect upregulation of NAD synthetic pathways in EGS in an attempt to promote vital and/or regulatory functions [2,4].

EGS was not associated with significant alterations in blood concentrations of vitamins B1, B2 and B6. Comparison of data for these vitamins with published data is not possible because of absence of published reference ranges for some analytes and differences in the units in which some analytes are expressed (IU/gHb in the present study). The current data, in combination with those derived from a previous study demonstrating adequate vitamin B12 status in EGS [31], indicate that the overall vitamin B status is maintained, at least in the early stages of EGS, despite the intestinal dysbiosis which occurs in EGS [32,33] potentially reducing the synthesis of B vitamins by intestinal microbes.

While sample sizes were low, a significant inter-group difference was observed in NADP concentrations. Sample size estimates indicated that lack of significant inter-group differences in concentrations of NAD and vitamins B1, B2 and B3 may have been the result of low sample size. However a very large sample size (68,496 horses per group) would have been required to identify a significant inter-group difference in NAD:NADP ratio, suggesting that there is unlikely to be an association between EGS and a low index of functional niacin status. However because of lack of reference range data for the analytes, sample size estimations were calculated using the study data rather than using clinically relevant effect sizes.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the University of Edinburgh Research Ethics Committee. Owners gave informed consent for their horses' inclusion in the study.

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Authorship

B.C. McGorum, R.C. Jago, E. Cillan-Garcia, R.S. Pirie, J.A. Keen and R.J.M. Reardon were responsible for study design, sample collection, data interpretation and manuscript preparation. P.Y. Saffu and N.J. Miller performed assays and interpreted data. All authors reviewed the final manuscript.

Manufacturer's address

^aMinitab statistical software, version 6.1.1. Minitab Ltd., Coventry, UK.

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Table 1: Indices of niacin status, including erythrocyte NAD:NADP ratio, erythrocyte concentrations of NAD and NADP and sum of erythrocyte concentrations of NAD and NADP ($\mu\text{mol/L}$ RBC), and concentrations of vitamins B1, B2 and B6 (IU/gHb) in blood from control (n = 12) and EGS (n = 10) horses. Median and Q1-Q3.

Group	NAD: NADP Ratio	NAD	NADP	NAD+NADP	Vitamin B1	Vitamin B2	Vitamin B6
Control	2.1	96.5	48.0*	144	1.17	1.85	1.19
	1.8-2.6	87.5-131.0	42.3-52.3	133-178	1.01-1.30	1.33-1.98	1.08-1.43
EGS	2.1	122.5	60.0*	180	1.07	1.44	1.46
	1.9-2.6	101.7-143.3	49.4-62.3	148-200	1.00-1.18	1.17-1.99	1.20-1.69

*Significant inter-group difference $P = 0.02$