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Investigation of manganese homeostasis in dogs with anaemia and chronic enteropathy

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Abstract
Lethargy is a frequent and important clinical feature of anaemia; however, it does not absolutely correlate with the severity of anaemia. Manganese is efficiently absorbed through the gastrointestinal tract via divalent metal transporter 1 (DMT1), which is also responsible for iron transport. DMT1 is upregulated in iron deficiency (ID). Increased manganese concentrations are reported in ID anaemia (IDA) in various species. Manganese is neurotoxic and therefore may contribute to lethargy observed in some anaemic patients. In addition, anaemia and ID are common in human inflammatory bowel disease. Little is known about how anaemia influences manganese metabolism in veterinary patients and how common is anaemia in dogs with chronic enteropathy (CE). If elevated manganese concentrations are found, then potentially neurotoxicity may be contributing to morbidity in these cases. The objectives of this study were to investigate the hypothesis that whole blood manganese concentrations would be increased in dogs with anaemia, particularly in dogs with confirmed IDA, and that anaemia would be common in canine CE. Medical records from 2012-2016 were reviewed for dogs with CE that were anaemic, as well as dogs with confirmed IDA, where a sample suitable for manganese analysis was held in an archive. Manganese concentration was measured in whole blood from: 11 anaemic dogs with CE, 6 dogs with IDA, 9 non-anaemic ill controls, and 12 healthy controls. Mann-Whitney U and Kruskal-Wallis tests with post-test Dunn's multiple comparisons tests were performed, with $P<0.05$ considered significant. The prevalence of anaemia in canine CE was 20.6% (33/160). Manganese concentrations were significantly different between all groups ($P=0.0001$) and higher in non-anaemic than anaemic dogs ($P=0.0078$). Manganese concentrations were also higher in healthy compared to ill controls ($P<0.0001$), anaemic dogs with CE ($P=0.0056$) and to dogs with IDA ($P=0.0001$). No differences were observed between anaemic dogs with CE, IDA and ill controls. Although anaemia was frequently observed in canine CE, the hypothesis that dogs with anaemia would have increased manganese concentrations, possibly contributing to a lethargic state was not supported. Further research is warranted to understand the influence of anaemia on whole blood manganese.
Keywords: Inflammatory bowel disease, Iron, Trace element.

Introduction

Lethargy is a common clinical sign in anaemia. Although the reduction in haemoglobin concentration results in decreased delivery of oxygen to tissues, lethargy does not always correlate with anaemia severity (Chervier *et al*., 2012; Bager, 2014). Therefore, it is possible that other factors might be contributing to lethargy. In humans with iron deficiency anaemia (IDA), increased intestinal iron absorption also results in increased absorption of other trace elements including manganese (Mn) (Meltzer *et al*., 2010). Manganese is an essential trace element and a necessary cofactor for a few enzymes and metabolic pathways. Manganese is efficiently absorbed from the gastrointestinal tract via divalent metal transporter 1 (DMT1), which is concurrently responsible for iron transport (Au *et al*., 2008).

Iron deficiency (ID) causes upregulation of DMT1 and has been associated with increased Mn concentrations in many species (Kim and Lee, 2011; Smith *et al*., 2013).

Manganese is neurotoxic when present in excessive concentrations (Racette *et al*., 2017). The mechanism of neurotoxicity appears multifactorial. Astrocytic dysfunction occurs due to their high affinity transport system accumulating high intracellular concentrations of Mn (Yin *et al*., 2008). Manganese also causes microglial activation by induction of cytokines and reactive oxygen species (Dodd and Filipov, 2011), as well as causing disruption of several neurotransmitters and mitochondrial dysfunction, culminating in neuronal apoptosis (Smith *et al*., 2017).

Hyperintensity of the basal ganglia on magnetic resonance imaging (MRI) is pathognomonic for Mn deposition in humans, correlating with neurological signs and fatigue in human cirrhosis as well as blood Mn concentrations (Burkhard *et al*., 2003; Forton *et al*., 2004). Hyperintensity in focal areas of the basal ganglia

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has also been shown in canine and feline congenital portosystemic shunts, which contained increased concentrations of Mn on post-mortem examination (Torisu *et al*., 2008).

Iron deficiency has been linked with Mn accumulation within the brain, and increased uptake by astrocytes, mediated by DMT1 upregulation, in the absence of Mn overexposure (Erikson and Aschner, 2006). The presence of clinical Mn neurotoxicity with concurrent ID, and without Mn exposure, has been suggested in developing mice and confirmed in one child (Kwik-Uribe *et al*., 1999; Brna *et al*., 2011).

Anaemia is one of the most common extra-intestinal clinicopathological abnormalities in human inflammatory bowel disease (IBD), associated with a multifactorial aetiology, however over half of the cases demonstrated ID (Filmann *et al*., 2014). Notably, ID is suggested to affect quality of life in human IBD, even in the absence of anaemia (Herrera-deGuise *et al*., 2016).

There are only a few studies examining Mn homeostasis in human IBD. One report demonstrated higher whole blood Mn levels in 5/55 patients with ulcerative colitis, not associated with clinical or MRI evidence of toxicity, with 2/5 cases confirming low ferritin levels (El Muhtaseb *et al*., 2007). The remaining studies have shown no differences, though all reports were based in serum or plasma Mn (Whineray *et al*., 2000; Ma *et al*., 2013). More importantly, none have concentrated specifically on anaemic, or iron deficient subjects.

Chronic enteropathies (CE) are a common cause of morbidity and mortality in dogs (Allenspach *et al*., 2016). Metabolic complications are well described in dogs with CE, notably hypoalbuminaemia, hypocobalaminaemia and altered vitamin D status (Allenspach *et al*., 2007; Gow *et al*., 2011). Canine CE appears to share similarities to human IBD (Cerquetella *et al*., 2010). Surprisingly, studies characterising anaemia in canine CE are scant, although a prevalence is reported of between 12-18% in studies with relatively small numbers (Craven *et al*., 2004; Marchetti *et al*., 2010). Essential trace elements in canine CE are even less well studied, with only one abstract reporting plasma Mn concentrations, showing no difference compared to laboratory beagle controls (Yokoyama *et al*., 2016).

Given the high prevalence of anaemia in human IBD, the aim of this study was to investigate the prevalence of anaemia in a larger number of cases of canine CE, and to then establish whether anaemia is associated with increased Mn concentrations. An additional aim was to assess if confirmed absolute IDA, regardless of underlying aetiology, is associated with higher Mn concentrations. Non-anaemic controls, both ill and healthy, served as cohorts for comparison.

Materials and Methods

Samples

The small animal teaching hospital archive, at the authors' institution, was used to retrieve the samples for the study. The archive database of -80ºC stored residual samples previously taken for clinical diagnostic purposes, with informed consent from the owners, was searched for ethylenediaminetetraacetic acid (EDTA) samples from January 2012 to January 2016.

Inclusion criteria

Using the corresponding patients' case numbers, a retrospective search using the hospital's electronic record system was undertaken to retrieve clinical information that would match inclusion criteria for each group: anaemic dogs with a clinical diagnosis of CE, either food responsive, antibiotic responsive or idiopathic, confirmed histologically from endoscopic or full thickness surgical gastric and/or intestinal biopsies; dogs with absolute IDA; ill dogs with normal haematology; and healthy dogs based on history and physical examination, with a normal packed cell volume (PCV). Mn analysis was performed on small cohorts of the first and last two groups, and on all patients with confirmed IDA.

Analyses

Gastrointestinal histology and haematology analyses were performed by the institution's pathology laboratory, with results confirmed by board-certified veterinary pathologists and clinical pathologists, respectively. Histological results confirming a diagnosis of CE would need to include mild, moderate or marked mucosal infiltration by inflammatory cells (lymphocytes, plasma cells, eosinophils). Anaemia was defined by a PCV below the lower limit of the laboratory reference range (0.39 L/L). Iron status analyses were performed by an external laboratory and diagnosis of absolute ID was defined by serum iron below the reference interval, combined with transferrin saturation below the reference interval and a normal total iron binding capacity (TIBC).

Manganese concentrations were prospectively determined at an external laboratory, by graphite furnace atomic absorption spectrometry (1100 Spectrometer, PerkinElmer Life and Analytical Sciences, Milan, Italy), in whole blood anticoagulated with EDTA, after dilution with Triton X-100 solution (Sigma-Aldrich, St. Louis, MO). All samples had been frozen within 4 hours of collection, archived as abovementioned, and subsequently shipped in dry ice to the reference laboratory. The assay had a limit of quantitation of 16nmol/L, an inter-assay coefficient of variation (CV) of 3.1% and an intra-assay CV of 5.4%.

Statistical analysis

Assessment for normality was performed with the Kolmogorov-Smirnov test for each group and the overall population regarding Mn concentrations, PCV

and age. As not all groups were normally distributed in each of the three categories, nonparametric tests were therefore used for statistical analysis throughout, with data expressed as median (minimum/maximum ranges). Mann-Whitney U and Kruskal-Wallis tests with post-test Dunn's multiple comparisons tests were performed for assessment of differences in between groups. The correlation between Mn concentration and PCV was assessed by the Spearman's rank correlation coefficient (r_s). Statistical significance level was set at $P < 0.05$. Statistical analysis was performed with two commercial software packages (GraphPad InStat, version 3.10, GraphPad Software Inc.; and Minitab®, version 17.1.0, Minitab Inc.).

Results

A total of 1847 whole blood EDTA samples were available, from which 160 (8.7%) corresponded to dogs with histologically confirmed CE. Of these, anaemia was present in 33 (20.6%), with one patient being microcytic and hypochromic, and four normocytic and hypochromic. A cohort of the most anaemic dogs with CE (n = 11) was selected for Mn analysis, of which four had iron status results available, confirming absolute IDA in two, and anaemia of chronic disease (ACD) with relative IDA (low serum iron with normal transferrin saturation and low TIBC) in another two.

Within the archived available whole blood EDTA samples, six corresponded to cases from which an iron status confirmed absolute ID. Of these, two dogs were in the previously selected CE group, and the remaining had an underlying diagnosis each of: intestinal lymphoma, immune-mediated haemolytic anaemia, urinary bladder mass, or suspected disseminated histiocytic neoplasia.

Including the non-anaemic ill (n = 9, Table 1) and healthy (n = 12) cohort controls, a total of 36 samples were then prospectively analysed for Mn concentration. No differences were seen between groups with regard to age ($P = 0.2025$) or gender ($P = 0.1025$). Eight individuals were crossbreeds and the most commonly represented breeds included: Labrador Retriever (n = 6), Cocker Spaniel (n = 4), followed by Boxer (n = 2), German Shepherd (n = 2), Hungarian Vizla (n = 2) and Springer Spaniel (n = 2). Mn concentrations and PCVs in each group are summarised in Figure 1 and Table 2. Manganese concentrations were significantly different between the four groups ($P = 0.0001$), being higher in healthy dogs compared to: anaemic dogs with CE ($P = 0.0056$), dogs with IDA ($P = 0.0001$), and non-anaemic ill controls ($P < 0.0001$). No differences were observed between Mn concentrations in anaemic dogs with CE, dogs with IDA and non-anaemic ill controls. Mn concentrations were also higher in overall non-anaemic compared to anaemic dogs ($P = 0.0078$), with an overall moderate positive correlation between PCV and Mn concentration ($r_s = 0.6252$, $P < 0.0001$).

Table 1. Problem list/diagnoses of a cohort of non-anaemic ill dogs (n = 9).

Diagnosis	Patients (n)
Chronic cystitis	2
Chronic intermittent diarrhoea	2
Acute gastroenteritis	1
Cervical carcinoma, prostatic mass	1
Hepatosplenic nodules, gastric mass, chronic kidney disease	1
Septic peritonitis of unknown origin	1
Sinonasal aspergillosis	1

Table 2. Descriptive statistics of manganese concentrations and packed cell values distribution within four cohorts.

	Mn (nmol/L)		PCV (l/l)	
Cohort	Median	Range	Median	Range
Anaemic CE	366	219-1817	0.27	0.13-0.32
IDA	292	170-710	0.20	0.13-0.30
Non-anaemic ill	608	303-839	0.46	0.42-0.51
Healthy	1006	893-1240	0.53	0.45-0.64

Footnote: CE – chronic enteropathy; IDA – iron-deficiency anaemia; Mn – manganese; PCV – packed cell value.

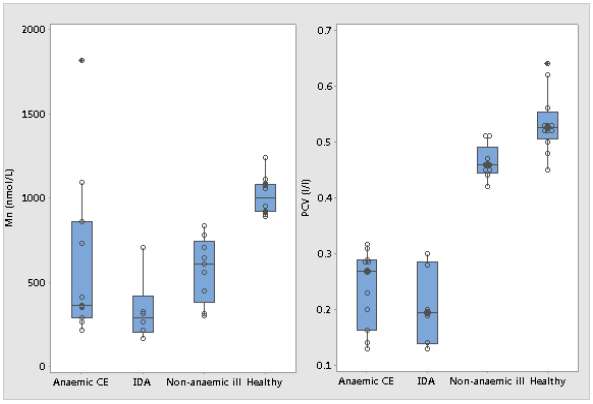

Fig. 1. Box and whisker plots and individual values depicting manganese (Mn) concentrations (left) and packed cell values (PCV, right) distribution within four cohorts. (CE): Chronic enteropathy; (IDA): Iron-deficiency anaemia.

However, when analysing healthy dogs alone, there was no correlation between PCV and Mn ($r_s = -11.35$, $P = 0.7329$). Moreover, when comparing PCV overall amongst groups, the difference obtained ($P < 0.0001$) was attributed only to the comparison between each of the anaemic groups (dogs with CE or ID), with each of the non-anaemic ill or healthy control groups, and no difference was seen in between the latter two cohorts on post-test Dunn's multiple comparisons test.

Discussion

Anaemia in canine CE was frequent within the population studied. The obtained prevalence of 20.6% was higher than previously reported, as was the overall number of patients assessed, totalling 160, compared with 22 and 77 in other studies (Craven *et al*., 2004; Marchetti *et al*., 2010). This prevalence is similar to that of 24% obtained in a meta-analysis in human IBD (Filmann *et al*., 2014). This confirms that anaemia is a common clinicopathological abnormality in canine CE and may be contributing towards overall morbidity.

The results obtained in this study showed no evidence that ID is associated with increased whole blood Mn concentrations. Moreover, anaemic dogs with CE or IDA, as well as non-anaemic ill dogs, had lower Mn concentrations when compared to healthy controls. This does not support the hypothesis that Mn neurotoxicity contributes to the clinical sign of lethargy in these cases.

There are many potential factors which may explain this result. As the majority of circulating Mn is within erythrocytes, it is possible that anaemia may be a confounding factor, although no correlation between Mn and PCV was found in the healthy controls (Pleban and Pearson, 1979).

Measurement of whole blood Mn is recommended to reflect whole body manganese (Clegg *et al*., 1986). Whole blood Mn is less variable within an individual, when compared to plasma, better reflecting Mn in a single sample (Baker *et al*., 2015). A higher risk of inaccuracy is seen with serum measurements, combined with wider variation of reference ranges (Baruthio *et al*., 1988). Even if no macroscopic haemolysis is present, there can be a considerable amount of Mn that is released from the red blood cells into the serum or plasma if not separated, leading to potential erroneous interpretations. In addition, to be reliable, serum or plasma analysis requires very sensitive assays, since Mn concentration is approximately 10-30 times lower than within erythrocytes (Versieck *et al*., 1974).

Moreover, other studies have used whole blood samples to assess Mn status in ID and were able to confirm increased Mn concentrations in anaemic samples (Meltzer *et al*., 2010; Kim and Lee, 2011; Smith *et al*., 2013).

Whole blood manganese is obtained from dietary intake. These clinical cases were on a range of diets, which would likely have widely different Mn content, as previously documented on a comparison of several commercial diets (Gagne *et al*., 2013). Hyporexia is a common finding in any illness, and one of the main clinical signs of canine CE (Gianella *et al*., 2017), therefore reduced dietary intake is a potential confounding factor for both the non-anaemic ill and CE groups. The healthy control group dogs were all fed a standard complete diet with a Mn content of 25.1mg/kg on a dry matter basis, which is within the minimum and maximum amounts (5.8mg/kg and 170mg/kg, respectively) recommended for adult dogs, by the European Pet Food Industry Federation (Zentek, 2016). It is possible that this standardised diet may explain the higher concentrations seen in the healthy group in this study.

Inflammation seen in canine CE might stimulate hepcidin, a peptide produced in the liver, controlling iron homeostasis by inhibiting iron transport (Brasse-Lagnel *et al*., 2011). This hormone downregulates DMT1 and also binds to the iron and Mn exporter ferroportin 1, leading to its internalisation and degradation, resulting in iron (and likely Mn) entrapment and decreased transport across the basolateral membrane (Seo and Wessling-Resnick, 2015). Hepcidin has been shown to be upregulated in human IBD, resulting in ACD due to relative ID (Bergamaschi *et al*., 2013). As a further variable, Vitamin D is an important suppressor of hepcidin, and dogs with severe CE have been shown to have reduced vitamin D concentrations (Gow *et al*., 2011; Bacchetta *et al*., 2014). Moreover, in inflammatory conditions, the action of metal transporters can be disrupted, independently of hepcidin (Guida *et al*., 2015). In human IBD, active disease was seen to correlate with decreased mucosal DMT1 expression (Wu *et al*., 2015). Conversely, in a study of human IBD, where 14/19 patients were anaemic while in histological remission, upregulation of DMT1 was confirmed, and negatively correlated with haemoglobin, possibly explained by absolute ID overriding the effects of ACD (Sukumaran *et al*., 2014). Investigation of DMT1 expression in enterocytes in canine CE would help define this effect. In addition, is unknown how the presence of other iron and Mn transporters, namely ZIP8 and ZIP14, might influence overall canine Mn homeostasis (Shawki *et al*., 2015). Given that dogs with gastrointestinal disease (CE, lymphoma, and non-specified) were present within the absolute IDA and non-anaemic ill groups (n = 3 in each group), is possible that impaired Mn absorption in these patients has accounted for the lower concentrations observed, alongside the anaemic CE group.

Finally, the liver is responsible for removing most of the Mn absorbed through the gastrointestinal tract from the portal circulation, excreting it via the biliary system and allowing only around 2% of the absorbed Mn to reach the systemic circulation (Papavasiliou *et al*., 1966; Klaassen, 1974). This system is very efficient and dogs fed large quantities of Mn over many weeks demonstrated no significant increase in whole blood manganese concentrations, yet had increased hepatic concentrations, thought to be due to trafficking through the liver for excretion in bile (Reiman and Minot,

1920). Therefore, it is possible that despite increased Mn delivery in the portal vasculature, the canine liver is more efficient at Mn extraction, leading to no systemic increase. Dogs with hepatic dysfunction and portosystemic shunting have altered Mn homeostasis, leading to Mn accumulation (Kilpatrick *et al*., 2014). Consequently, further research would be needed to assess if ID could play a role in exacerbating increased Mn concentrations in dogs with established liver pathology, as reported in human chronic liver disease (Malecki *et al*., 1999).

This study carries several limitations. Although Mn levels were prospectively measured, the population was retrospectively assessed, thereby leading to lack of standardisation within and between groups. The number of dogs in each group was also small, which could have led to subsequent type II error regarding the absence of difference between anaemic and non-anaemic ill dogs. Iron status analysis was only available for eight subjects, precluding further conclusions. In addition, diet was not standardised, which could have affected results (Lopez-Alonso *et al*., 2007).

This study demonstrates that anaemia is common in dogs with chronic enteropathy. However, anaemic dogs with chronic enteropathy and/or iron-deficiency anaemia do not have increased whole blood manganese concentrations, compared to non-anaemic ill or healthy cohorts. More investigation is warranted to understand the complex interplay of canine anaemia, iron deficiency and inflammatory disease, namely chronic enteropathy, in manganese concentrations.

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Conflict of interest

The authors declare that there is no conflict of interest.

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