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Ammonium tetrathiomolybdate treatment of copper-associated hepatopathy in dogs

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Abstract

Background: Copper-associated hepatopathy (CAH) is a common cause of liver disease in dogs. Although D-penicillamine can be an effective treatment, some dogs fail treatment or develop adverse effects. Ammonium tetrathiomolybdate (TTM) has been used to treat pathologic copper accumulation in other species, but its therapeutic potential for CAH is unknown.

Objectives: To investigate short-term safety and efficacy of TTM for treatment of CAH.

Animals: Ten dogs with CAH.

Methods: Prospective study. All dogs were treated with TTM PO for 6 weeks, and hepatic biopsies were performed after the treatment course. Dog experiencing initial decreases in hepatic copper concentrations ($[Cu]_H$) received 6 additional weeks of TTM treatment and underwent 1 additional biopsy. Physical and laboratory examinations were performed every 2 weeks for study duration.

Results: Eight of 10 dogs had decreases in $[Cu]_H$. Compared to baseline (median, 1606 μg/g; range, 572-5158 μg/g), $[Cu]_H$ were decreased at 6 weeks (1033 μg/g, 450-2975 μg/g; $P = .04$) and 12 weeks (931 μg/g, 218-1677 μg/g; $P = .02$). Hepatic molybdenum concentrations increased >50-fold ($P < 0.001$). Changes in histologic scores and hematologic and biochemical test results were variable and not significantly different from baseline. One dog developed presumed immune-mediated anemia and thrombocytopenia, but it was unclear if this was related to TTM administration.

Conclusions and Clinical Importance: Results suggest that TTM can effectively decrease $[Cu]_H$ in some dogs with CAH. Larger studies are needed to determine the overall safety and efficacy of TTM for treating CAH and how it compares with current treatments.

KEYWORDS

chelation, copper toxicosis, hepatitis, Labrador Retrievers, molybdenum

1 | INTRODUCTION

Copper-associated hepatopathy (CAH) is characterized by progressive hepatic copper accumulation leading to hepatitis, cirrhosis, and eventual

Abbreviations: CAH, copper-associated hepatopathy; $[Cu]_H$, hepatic copper concentrations; TTM, ammonium tetrathiomolybdate.

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death if left untreated.[1,2] The disease has garnered considerable attention from the veterinary community in recent years.[3–5] Once thought to be a rare condition affecting only Bedlington Terriers, CAH is widely recognized in Labrador Retrievers, Doberman Pinschers, West Highland White Terriers, and Dalmatians.[6–11] More recently, CAH has been recognized in various breeds and crosses not typically considered predisposed to CAH.[4,12,13] The genetic basis for disease in Bedlington Terriers is well described, but the etiology in other breeds is complex and multifactorial.[4,14–16]

The primary goals of treatment are to decrease hepatic copper concentrations ($[Cu]_H$) and prevent subsequent re-accumulation. Both dietary copper restriction and PO administration of zinc salts can prevent further copper accumulation in some dogs, but these treatments do not quickly affect the copper load already present in the liver.[17,18] Some dogs develop gastrointestinal upset when treated with high doses of zinc salts, and some dogs continue to accumulate copper despite feeding of a copper-restricted diet.[2,19] Consequently, these treatments are indicated for dogs with subclinical disease or for maintenance treatment after more aggressive treatment.[1] D-penicillamine is considered the initial treatment of choice for most dogs with CAH including those with clinical illness and those with moderate to severe hepatic histopathologic abnormalities.[1,2] It is a potent copper chelator that can rapidly induce urinary copper excretion and negative copper balance.[20] However, it is not uniformly effective, and some dogs experience minimal or no decrease in $[Cu]_H$ despite months of treatment.[21] Even in dogs that respond to treatment, the duration of treatment necessary to restore normal $[Cu]_H$ often exceeds 6-10 months.[22] Anorexia and vomiting are common adverse effects, and in some instances, these can preclude D-penicillamine usage.[2,23] Although D-penicillamine remains an effective drug for many dogs with CAH, these limitations warrant investigation of alternative treatments.

Ammonium tetrathiomolybdate (TTM) is a copper chelator that has proven to be an effective treatment for Wilson's disease in humans, copper toxicosis in sheep, and pathologic copper accumulation in rodents.[24–26] This molybdenum salt forms a tight tripartite complex with copper and albumin, most of which then undergoes hepatobiliary excretion.[24] It is unique because of its additive actions in the intestines, plasma, and liver.[27] Administration of TTM to healthy dogs increases serum copper concentrations, which suggests that TTM mobilizes tissue copper.[28] However, the use of TTM to treat dogs with CAH has not been reported. Our objectives were to determine if TTM altered $[Cu]_H$ or the extent of liver injury in dogs with CAH. The clinical, hematologic, and biochemical effects of TTM administration also were investigated. Our primary hypothesis was that TTM administration would decrease $[Cu]_H$ in dogs with CAH.

2 | MATERIALS AND METHODS

2.1 | Animals

A prospective single-arm clinical study was conducted to investigate the therapeutic potential of TTM for CAH in dogs. A sample size calculation was performed using archived data from CAH-affected dogs that underwent pre- and post-chelation biopsies in our hospital. The analysis suggested that 8 dogs would be needed to detect an approximate 350 μg/g difference in $[Cu]_H$ after chelation treatment with power of 0.8 and alpha of 0.05. Most dogs recruited to the study were expected to have mild to moderate disease based on the inclusion and exclusion criteria described below. The magnitude of decrease in $[Cu]_H$ considered to represent therapeutic improvement likely varies among clinicians and depends upon initial disease severity, but a value of 350 μg/g was thought to represent a clinically relevant decrease for this population of dogs.

Client-owned and clinically stable dogs with increased ALT activity on at least 2 occasions in the preceding 6 months and ALT activity > ALP activity were recruited from the Michigan State University Veterinary Medical Center (MSU-VMC) and surrounding general practice clinics. An initial evaluation was performed including history, physical examination, CBC (Advia 120 Hematology System; Siemens Healthcare Inc, Deerfield, Illinois), serum biochemical profile (Olympus AU640e; Olympus America Inc, Center Valley, Pennsylvania), urinalysis, and abdominal ultrasound examination. Dogs were excluded from further participation if any of the following were present: (1) vomiting >3 times per week, (2) consumption of <50% of estimated daily resting energy requirements, (3) consumption of a prescription low-copper diet, (4) increased serum bilirubin concentrations (>0.5 mg/dL), (5) increased serum creatinine concentrations (>1.6 mg/dL), or (6) sonographic evidence of neoplastic disease or gall bladder disease.[29] Dogs not meeting exclusion criteria underwent hepatic biopsy as described below. Dogs with centrilobular to panlobular copper accumulation (rhodanine score ≥2), $[Cu]_H$ > 400 μg/g, histologic evidence of chronic hepatitis, and negative microbial cultures were considered to have CAH and enrolled in the study.[1,30,31]

2.2 | Experimental protocol

Capsules containing powdered TTM (purity, 99.9%; Sigma-Aldrich Inc, St Louis, Missouri) were compounded by the MSU-VMC pharmacy. Dogs received TTM according to the following schedule based on body weight:

Dogs >60 kg: 40 mg PO 4 times daily.
Dogs 50-59.9 kg: 25 mg PO 4 times daily.
Dogs 30-49.9 kg: 20 mg PO 4 times daily.
Dogs 20-29.9 kg: 12.5 mg PO 4 times daily.
Dogs 13-19.9 kg: 8 mg PO 4 times daily.
Dogs 7-12.9 kg: 5 mg PO 4 times daily.

Capsules were prepared for each dog upon entry into the trial and again at the time of 6-week reevaluation. Analysis of random capsules by the use of inductively coupled plasma—mass spectrometry established that capsules were prepared to mean ± SD of 95.6% ± 8.8% of target dose. The TTM dosage of approximately 0.5 mg/kg was extrapolated from previous studies of humans and dogs.[28,32,33] Owners were instructed to administer 1 dose with morning and evening meals, whereas the other 2 doses were administered on an empty stomach 1 hour before or 2 hours after meals. This dosing schedule, which is similar to those used in the treatment of humans with Wilson's disease, was anticipated to result in both copper chelation and decreased dietary copper absorption.[24,32] Owners maintained medication logs

and recorded the time of TTM administration, amount of TTM administered, and whether TTM was administered fasted or with food. In addition, owners recorded any vomiting episodes.

A reevaluation physical examination, CBC, and serum biochemical profile were performed every 2 weeks for study duration. Hepatic biopsy was repeated in all dogs at 6 weeks. Treatment was extended for an additional 6 weeks in dogs experiencing either a >15% decrease or a >150 μg/g decrease in $[Cu]_H$ as compared to baseline, and a final biopsy was performed in these dogs at 12 weeks. Dietary changes were not allowed, and dogs were not permitted to receive ursodeoxycholic acid treatment, zinc salts, other copper chelating agents, or dietary supplements containing trace minerals for the duration of the trial. After study conclusion, dogs were transitioned to standard of care treatments at the discretion of their attending veterinarian.

2.3 | Hepatic histopathology and quantitative mineral determinations

Limited assessments of coagulation, including determination of prothrombin and activated partial thromboplastin times, were normal in all dogs before each hepatic biopsy procedure. Ultrasound-guided hepatic biopsies were performed while dogs were under general anesthesia. For each procedure, 6-7 tissue specimens were collected using a 14-gauge single action needle biopsy device (EZ Core; Products Group International Inc, Lyons, Colorado). Four specimens were fixed in 10% neutral buffered formalin, 2 specimens were placed in a mineral-free container and submitted for determination of trace mineral concentrations, and 1 specimen from the initial biopsy procedure was submitted for microbial culture. Paraffin-embedded sections of liver tissue were routinely sectioned and stained with hematoxylin and eosin (H&E) and rhodanine. The needle biopsy specimens provided approximately 20-30 portal triads for evaluation at each time point. All slides were evaluated by a single board-certified veterinary pathologist (R.C.S.) who was blinded to clinical data, timing of the sample, and quantitative mineral concentrations. The H&E-stained slides were scored for necroinflammatory activity and fibrosis (Table 1). A validated scoring system for hepatopathology in dogs does not exist, and the scoring system used herein was derived from the same sources used in previous veterinary publications.[22,34–36] It closely resembles a scoring system proposed by the World Small Animal Veterinary Association Liver Standardization Group with the exception that centrilobular inflammation was treated as a distinct category, given the classic pattern of copper accumulation and inflammation in CAH.[31] Briefly, scores were assigned for the severity of fibrosis (range, F0-F4), centrilobular inflammation (range, C0-C3), portal inflammation (range, P0-P3), lobular necrosis (range, LN0-LN2), and piecemeal necrosis (range, PMN0-PMN3). An overall necroinflammatory activity score (NI) was obtained by adding the scores for centrilobular inflammation, portal inflammation, lobular necrosis, and piecemeal necrosis. Necroinflammatory activity scores could range from 0 to 11. Rhodanine-stained slides were assessed for copper accumulation using a previously described system with potential scores ranging from 0 (no detected copper granules) to 5 (panlobular presence of large numbers of copper granules in hepatocytes, usually associated with copper-containing macrophages).[30] Quantitative hepatic copper and molybdenum concentrations (μg/g, dry matter basis) were determined on liver specimens using inductively coupled plasma-mass spectroscopy as described elsewhere.[4] Hepatic molybdenum concentrations were measured because they are a potential marker for TTM.[27,28] The reference intervals in our laboratory for hepatic copper and molybdenum concentrations in dogs are 137-400 μg/g and 0.8-2.3 μg/g, respectively. These reference intervals were established by evaluating the concentrations in all hepatic tissue submissions over a several-year time period and utilizing the 10%-90% range of obtained values as the normal reference interval. However, the reference interval for copper was modified to be consistent with other laboratories that have adopted 400 μg/g as the upper-end of normal given that rhodanine staining often is positive at this threshold.[1–3,7,10]

2.4 | Data and statistical analysis

Data sets were assessed for normality by Shapiro–Wilk testing and box plot analysis. Data that approximated normal distributions were reported as means ± SDs, whereas ordinal data and data not approximating normal distributions were reported as medians and ranges. Statistical analyses were performed to investigate potential changes in hepatic mineral concentrations, histologic scores, and hematologic and biochemical variables after TTM treatment. The effect of treatment over time on routine hematologic and biochemical variables was evaluated using a repeated-measures analysis of variance with Tukey's post hoc testing. The effect of treatment over time on hepatic mineral concentrations and histologic scores was evaluated using Friedman testing with Dunn's post hoc testing. Statistical analyses were performed with commercially available software (GraphPad Prism Version 6.0; GraphPad Software Inc, La Jolla, California), and for all analyses, $P \leq .05$ was considered significant.

3 | RESULTS

3.1 | Dogs

Eleven dogs met initial inclusion criteria and were enrolled in the clinical trial, but 1 dog was removed from participation after 1 week of TTM treatment because of noncompliance with dietary guidelines. Data from this dog were not included in the study. The 10 remaining dogs included 5 Labrador Retrievers, 2 Pit Bull Terrier crosses, 1 Doberman Pinscher, 1 West Highland White Terrier, and 1 Mastiff. The median (range) age and weight of the dogs were 6 years (3-10 years) and 34.0 kg (8.7-99.0 kg), respectively. The mean ± SD initial TTM dosage for these 10 dogs was 0.56 ± 0.09 mg/kg PO 4 times daily.

Dog 1, a 3-year-old neutered male Mastiff, had been receiving prednisolone (0.3 mg/kg PO q24h) for 2.5 months for treatment of known CAH. Liver biopsy specimens from 2.5 months before enrollment and again at the time of enrollment featured centrilobular inflammation and increasing $[Cu]_H$ (1764 μg/g and 2125 μg/g, respectively). Laboratory data and histology scores from this dog were included in all analyses reported herein. The inclusion or exclusion of

TABLE 1 Scoring system used to assess hepatic pathology

	Centrilobular inflammation	Portal inflammation	Lobular necrosis	Piecemeal necrosis	Fibrosis
Score Criteria	C0 Absent	P0 Absent	LN0 <1 necroinflammatory focus per lobule	PN0 Absent	F0 Absent
Score Criteria	C1 Mononuclear aggregates in some centrilobular areas	P1 Mononuclear aggregates in some portal tracts	LN1 At least 1 necroinflammatory focus per lobule	PN1 Focal alteration of the periportal plate in some portal tracts	F1 Portal fibrosis without septae
Score Criteria	C2 Mononuclear aggregates in all centrilobular areas	P2 Mononuclear aggregates in all portal tracts	LN2 Several necroinflammatory foci per lobule or confluent or bridging necrosis	PN2 Diffuse alteration of periportal plate in some portal tracts or focal lesions around all portal tracts	F2 Portal fibrosis with rare septae
Score Criteria	C3 Large and dense mononuclear aggregates in all centrilobular areas	P3 Large and dense mononuclear aggregates in all portal tracts		PN3 Diffuse alteration of the periportal plate in all portal tracts	F3 Numerous septae without cirrhosis
Score Criteria					F4 Cirrhosis

All slides were scored for necroinflammatory activity and fibrosis using the above guidelines. A composite score for necroinflammatory was created by summating the scores for centrilobular inflammation, portal inflammation, lobular necrosis, and piecemeal necrosis with potential scores ranging from 0 to 11.

this data did not affect statistical significance. Dog 6, an 8-year-old neutered male Labrador Retriever, had been receiving phenobarbital (1.8 mg/kg PO q12h) for 7 years for management of presumed idiopathic epilepsy. Hepatic biopsy specimens in this dog indicated classic CAH characterized by $[Cu]_H$ of 2087 μg/g and a rhodanine score of 4. No evidence of phenobarbital induced hepatotoxicosis was observed. Dog 7, a 10-year-old spayed female Doberman Pinscher, had been receiving phenylpropanolamine (1.5 mg/kg PO q8h) and conjugated estrogen (0.625 mg PO q 4 days) for 8 years for urinary incontinence. Medications for these 3 dogs were continued throughout the study. No other dogs were receiving concurrent medications.

3.2 | Clinical monitoring

Oral TTM treatment was well tolerated at the initial dosage in 8 dogs (dogs 1, 2, 3, 4, 6, 7, 8, and 9). Lethargy and anorexia were not observed in any of these dogs. Only 17 episodes of vomiting were observed out of 579 total study days for these 8 dogs which equates to an average of <1 vomiting episode per dog per month. Dog 10, a 7-year-old neutered male Labrador Retriever, vomited 5 times during the first week of treatment and had an approximate 60% decrease in food intake. The daily TTM dosage was decreased by 25%, and the dog was treated with ondansetron (0.25 mg/kg PO q12h) for the remainder of the study. No additional vomiting was observed in this dog. Dog 5, a 4-year-old spayed female Pit Bull Terrier cross, vomited on 4 occasions during the first 2 weeks of treatment. Drug administration was stopped for 2 days and restarted at the same dosage. Only 1 additional vomiting episode was observed in this dog, and appetite and energy were normal throughout the first 10 weeks of the study. This dog developed presumed immune-mediated anemia and thrombocytopenia during the 11th week of TTM treatment at which time the dog became anorexic and lethargic. Laboratory evaluation disclosed poorly regenerative anemia (hematocrit, 30%; reference interval, 41%-55%) and thrombocytopenia ($19 \times 10^3/\mu L$; reference interval, 160-401 $\times 10^3/\mu L$). Spherocytosis was not observed, but cytologic evaluation of a bone marrow aspirate disclosed increased macrophages with phagocytized erythrocytes and erythrocyte precursors. Treatment with TTM was discontinued, and the dog was placed on a tapering course of prednisone (initial dosage, 1 mg/kg PO q12h). The dog achieved complete remission of anemia and thrombocytopenia, and the final evaluation and hepatic biopsy were performed at week 16 instead of week 12. Blood results and histologic scores for necroinflammatory activity and fibrosis from the final evaluation were not included in statistical analyses because of the recently initiated prednisone treatment. Other possible adverse events were not reported in any dogs. Long-term follow-up was not performed as part of the study, but all dogs were alive >6 months after study completion.

3.3 | Clinicopathologic evaluations

Hematologic and serum biochemical evaluations were performed every 2 weeks for study duration (Table 2). Five of 10 dogs (dogs 3, 5, 8, 9, and 10) had decreases in serum ALT activity at week 6 as compared to baseline, but overall changes were not significant ($P = .34$). Serum ALT activity had increased >2-fold in 2 dogs (dogs 6 and 7) at the time of the week 6 reevaluation. These same 2 dogs did not have decreases in

TABLE 2 Selected hematologic and biochemical results from the dogs with copper-associated hepatopathy that participated in the ammonium tetrathiomolybdate trial

	Baseline	Week 6	Week 12
HCT (41%-55%)	50 ± 6.2	49 ± 5.6	46 ± 6.5
WBC (6.1-12.0 × 10^3/μL)	7.8 ± 2.3	8.3 ± 4.0	10.3 ± 4.7
ALT (21-68 U/L)	351 ± 171	446 ± 281	339 ± 300
ALP (10-92 U/L)	227 ± 165	227 ± 127	348 ± 340
Alb (2.8-4.0 g/dL)	3.3 ± 0.2	3.2 ± 0.3	3.3 ± 0.3
Bili (0.1-0.4 mg/dL)	0.29 ± 0.06	0.26 ± 0.07	0.26 ± 0.08
Chol (124-343 mg/dL)	278 ± 76	267 ± 70	269 ± 54
SUN (12-27 mg/dL)	14.7 ± 3.7	16.0 ± 4.2	14.8 ± 3.8

Values represent the mean ± SD for 10 dogs (baseline and week 6), and 7 dogs (week 12). The reference intervals are included in parentheses. Variable associated with liver function, including albumin, cholesterol, and bilirubin, remained normal throughout study duration. There were no significant changes in any of the above variables over the course of the study.
Abbreviations: Alb, albumin; Bili, bilirubin; Chol, cholesterol; HCT, hematocrit; SUN, serum urea nitrogen; WBC, white blood cell count.

$[Cu]_H$ at week 6, and the study was concluded at this point in both dogs. Mean serum concentrations of bilirubin, cholesterol, albumin, and urea nitrogen, which are potential biochemical markers of liver function, remained within reference intervals for study duration.

3.4 | Hepatic mineral concentrations and histologic scores

Treatment with TTM decreased $[Cu]_H$ (Figure 1). Hepatic copper concentrations (median, range) at week 6 (1033 μg/g, 450-2975 μg/g) and week 12 (931 μg/g, 218-1677 μg/g) were less than $[Cu]_H$ at baseline (1606 μg/g, 572-5158 μg/g; P = .04 and P = .02, respectively). At week 6, $[Cu]_H$ was decreased by >15% of baseline or >150 μg/g in 8 of 10 dogs (dogs 1, 2, 3, 4, 5, 8, 9, and 10). Dog 6, which experienced an increase in $[Cu]_H$ of 137 μg/g, and dog 7, which experienced a decrease of 110 μg/g, were considered to be nonresponders based on study design. Treatment with TTM was continued for an additional 6 weeks in the 8 responders. Five of these 8 dogs (dogs 1, 2, 3, 5, and 10) had further decreases in $[Cu]_H$ at week 12, but the overall change between weeks 6 and 12 was not significant (P = .99). In these 8 dogs, median $[Cu]_H$ at week 12 was 675 μg/g less than median $[Cu]_H$ at baseline. Median hepatic molybdenum concentrations increased from 1.4 μg/g (range, 1.0-2.5 μg/g) at baseline to 73.9 μg/g (range, 17.2-263.2 μg/g) at week 12 (P < .001). Hepatic molybdenum concentrations at week 6 (102.2 μg/g, 10.3-225.3 μg/g) were not different from concentrations at baseline or week 12 (P = .14 for both comparisons).

Rhodanine and H&E stained slides were evaluated and scored for each dog. At study conclusion (week 6 for 2 dogs and week 12 for 8 dogs), rhodanine scores were decreased in 6 dogs (dogs 1, 2, 3, 7, 9, and 10) and were unchanged in 4 dogs. Median fibrosis scores were unchanged throughout the study, and no dog had worse scores for fibrosis at study conclusion as compared to baseline. Five dogs (dogs 4, 6, 7, 8, and 9) had worse composite scores for necroinflammatory activity, whereas activity scores were improved (dogs 1 and 3) or unchanged (dogs 3, 5, and 10) in the remaining dogs. A summary of histologic scores for rhodanine, necroinflammatory activity, and fibrosis is presented in Table 3.

4 | DISCUSSION

Our results indicate that PO administration of TTM can lower $[Cu]_H$ in most dogs with CAH, thus providing support for continued investigations

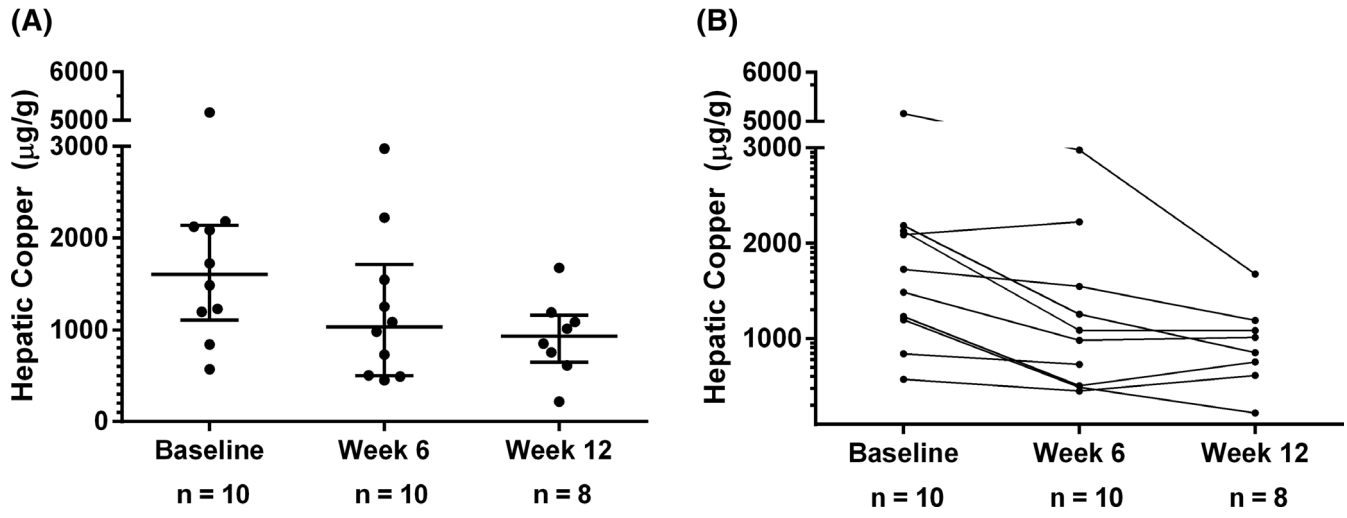

FIGURE 1 Hepatic copper concentrations in dogs with copper-associated hepatopathy that were treated with ammonium tetrathiomolybdate. Results are presented as a scatterplot (A) to depict central tendency and a line graph (B) to depict individual responses. The center and outer horizontal lines in (A) represent medians and interquartile ranges, respectively. Black circles represent individual data points. Median $[Cu]_H$ were decreased by 573 μg/g (P = .04) and 675 μg/g (P = .02) in weeks 6 and 12, respectively, as compared to baseline. The change in $[Cu]_H$ between weeks 6 and 12 was not significant (P = .99)

TABLE 3 Histologic scores for the dogs with copper-associated hepatopathy that participated in the ammonium tetrathiomolybdate trial

	Baseline	Week 6	Week 12	*P*-value
Rhodanine	3.5 (2-5) n = 10	2.5 (2-5) n = 10	2 (2-4) n = 8	.10
Necroinflammatory activity	5 (2-10) n = 10	5.5 (2-11) n = 10	6 (2-10) n = 7	.99
Fibrosis	2 (1-3) n = 10	2 (0-3) n = 10	2 (1-3) n = 7	.17

Values represent the medians, and ranges are provided in parentheses. Rhodanine-stained slides were scored to further assess copper accumulation with potential scores ranging from 0 to 5. Hematoxylin and eosin–stained slides were scored for necroinflammatory activity and fibrosis with potential scores ranging from 0 to 11 and 0 to 4, respectively.

of TTM in the clinical setting. Median $[Cu]_H$ was decreased by >650 μg/g after 12 weeks of TTM treatment. Results of rhodanine scoring also supported the changes in $[Cu]_H$ because median rhodanine scores decreased from 3.5 at baseline to 2.0 at week 12. Although this decrease was not significant (P = .10), the scores of 3.5, 2.5, and 2.0 at baseline, week 6, and week 12, respectively, are in the expected range of the observed $[Cu]_H$ at each time point.[2,30] The decreases in $[Cu]_H$ compare favorably to some current treatments for CAH including PO zinc salt administration and dietary copper restriction. Clinical effects of zinc salt administration can be delayed for several months.[17] Over 6 months of dietary copper restriction typically are required to achieve 300-400 μg/g reductions in $[Cu]_H$ in dogs with CAH.[18,19] Furthermore, some Labrador Retrievers will progressively accumulate copper despite feeding of a copper-restricted diet.[19] Speculative comparisons of TTM with D-penicillamine, the most frequently used copper chelating agent for treatment of CAH, are more difficult. An average detoxification rate of 900 μg/g per year has been reported anecdotally in D-penicillamine-treated Bedlington Terriers with copper toxicosis.[37] However, detoxification in this breed is more difficult than in other breeds, and lifelong treatment often is necessary.[2,37] Labrador Retrievers with $[Cu]_H$ > 1500 μg/g are thought to require at least 10 months of D-penicillamine treatment to normalize $[Cu]_H$,[22] but D-penicillamine may not have been administered under ideal conditions in that study.[23] Our unpublished clinical observations suggest that 4 months of D-penicillamine treatment usually decreases $[Cu]_H$ by >1000 μg/g in dogs in which initial $[Cu]_H$ was >2000 μg/g, although these dogs often are concomitantly fed copper-restricted diets.

Clinically affected dogs with advanced CAH are in greatest need of alternative treatment options. The dogs enrolled in our study typically had mild to moderate disease, and most had no overt clinical abnormalities. The inclusion criteria were designed to select this population of dogs given the uncertainty of TTM efficacy. Treatment with TTM can be effective for humans with advanced neurologic manifestations of Wilson's disease, but efficacy is based on clinical improvements and alterations in serum copper pools as opposed to improvements in $[Cu]_H$.[24,32] Administration of TTM can preserve life and decrease $[Cu]_H$ in rodents with severe hepatitis and copper accumulation.[27] These findings in other species suggest that TTM could have a similar role for treating dogs with more advanced CAH.

Ammonium tetrathiomolybdate treatment of Wilson's disease is well-tolerated in most people.[24,32] Conversely, D-penicillamine treatment of Wilson's disease has been associated with numerous adverse effects, which can contribute to poor patient compliance.[38] Many of the immunologic reactions and adverse neurologic effects described in humans have not been recognized in dogs, but adverse gastrointestinal effects do occur in 20%-50% of dogs treated with D-penicillamine.[2,22,23] Ammonium tetrathiomolybdate treatment was well tolerated in the majority of dogs with CAH in our study. Adverse gastrointestinal effects were sporadic and did not preclude treatment in any dog. Only 1 of 10 dogs required dosage modifications because of gastrointestinal upset. The most concerning potential adverse event was the development of immune-mediated anemia and thrombocytopenia in 1 dog. Tetrathiomolybdate can induce various hematologic abnormalities in humans including anemia and leukopenia.[24,32,39] These abnormalities are a consequence of copper deficiency in the bone marrow as opposed to immune-mediated destruction of erythroid or myeloid cells.[39–41] Consequently, it is unknown if the anemia and thrombocytopenia observed in the dog were caused by TTM or whether abnormalities occurred independent of TTM treatment.

Liver enzyme activities and histologic scores remained static despite decreases in $[Cu]_H$. These results are similar to a study of D-penicillamine-treated Labrador Retrievers with CAH in which liver enzyme activities and fibrosis scores were unchanged, and although median necroinflammatory activity scores decreased, some individual dogs had worsening necroinflammatory scores.[22] Reasons for the lack of changes in our study are likely complex and multifactorial. The decreases in $[Cu]_H$ were of clinical relevance in most dogs, but they were unlikely to be of the magnitude necessary to completely ameliorate copper-induced hepatocyte injury.[1,2] Many patients with Wilson's disease receiving TTM also experience increased ALT activity during initial treatment, which is thought to be a result of increased copper mobilization during the initial phases of treatment.[39,40] Similar to our study, serum markers of liver function usually remain normal in these patients.[24,39,40] The lack of decrease in histology scores and liver enzyme activities could be perceived as a discouraging finding, but it is important to note that these variables did not worsen. Treatment with TTM may have stabilized disease in some dogs and prevented further progression of liver injury, but this conclusion is speculative pending additional investigation.

The overall decreases in $[Cu]_H$ after TTM treatment are a promising finding, but reasons for the lack of response in 2 dogs are unknown as are the reasons for the lack of continued decrease in $[Cu]_H$ at week 12. Therapeutic failures in dogs with CAH are observed with both dietary copper restriction and D-penicillamine chelation treatment.[19,22] Most current treatments for CAH are utilized for longer time periods than 6-12 weeks, and a longer TTM treatment course might be necessary to further decrease $[Cu]_H$.[8,22] Interestingly, the duration of TTM treatment for Wilson's disease is only 8-16 weeks, and other copper-lowering treatments are initiated after this initial course.[24,38,39] It is possible that maximal TTM effects also are achieved in the initial phases of treatment

in dogs, and continued treatment may not confer additional benefit. More detailed studies of TTM are needed to address these possibilities and clarify potential roles of TTM for treatment of CAH.

The antagonistic and inverse relationships between molybdenum and copper are well described, and it is possible that TTM efficacy could correlate with tissue molybdenum concentrations.[24,42] However, the limited sample size and variable results precluded meaningful assessment of potential relationships between hepatic copper and molybdenum. The increases in hepatic molybdenum concentrations were pronounced, and some dogs experienced >50-fold increases after TTM treatment. The effects of this degree of accumulation, if any, are unknown. Some of the hepatic molybdenum likely was complexed with copper in an inactive form.[26,27,39] There may be benefit of TTM treatment beyond solely decreasing $[Cu]_H$ because TTM bound copper, even if still present within hepatic tissue, would not be expected to result in tissue injury. However, excess tissue molybdenum could be pathologic. Molybdenum toxicosis typically results in abnormalities consistent with copper deficiency such as hyporegenerative anemia and leukopenia.[42] Infrequently, molybdenum can have a direct hepatotoxic effect.[26,42] Furthermore, the ultimate consequences of accumulated molybdenum within liver or other tissues of dogs with CAH are unknown. Hepatic molybdenum concentrations decrease several months after experimental TTM treatment of sheep with variable hepatic copper status.[43] However, molybdenum was retained for months to years in organs such as brain, pituitary gland, and adrenal glands.[43,44] This accumulation was speculated to result in endocrine dysfunction, generalized ill thrift, and infertility.[43,44] Similar abnormalities have not been reported in patients with Wilson's disease treated with TTM.[39,41]

The etiology of CAH in most breeds of dogs is complex and poorly characterized, but dietary copper likely is involved in the pathogenesis.[2–4,15] Dietary copper concentrations were not investigated as part of our study, and it is possible that TTM responsiveness was influenced by dietary copper. Feeding of a standardized diet before and during the study may have resulted in more uniform and consistent responses. Although this factor should be considered in future studies, dietary changes were not permitted during the course of the study to prevent changing exposure levels within individual dogs. A limitation hampering clinical use of TTM in companion animal medicine is that TTM is not commercially available as a pharmaceutical grade product. Copper chelation with TTM is an active area of research for treatment of various oncologic and neurologic disorders in humans, and commercial products could become available pending continued evaluations.[45,46] Also, high purity analytical grade TTM is inexpensive and available from several chemical supply companies (Sigma-Aldrich Inc). Practitioners have used such formulations for decades to treat copper poisoning in sheep.[25] The dosing schedule for TTM may not be practical for some dog owners. This protocol was chosen because it was comparable to those used in humans with Wilson's disease, and it would be most likely to result in a detectable clinical effect.[24,40] Alternate dosing strategies might lessen compliance concerns and still result in efficacy. Indeed, an experimental formulation of tetrathiomolybdate that requires once daily dosing is currently in development for humans with Wilson's disease.[47]

In conclusion, TTM treatment resulted in decreases in $[Cu]_H$ in most dogs with CAH. The decreases in copper load after TTM treatment might be of greater magnitude than the decreases observed with other commonly used treatments. The lack of changes in histology scores and liver enzyme activities require further study as does the possibility for immunologic reactions such as may have occurred in 1 dog. Adverse gastrointestinal effects were mild in most dogs, and only 1 dog required a dose reduction because of gastrointestinal upset. In the aggregate, these results provide support for continued investigation of TTM as a treatment for CAH in dogs.

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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

This study was approved by the Michigan State University IACUC, and informed consent was obtained from all dog owners.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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